Cytotechnology (2018) 70:1–11
https://doi.org/10.1007/s10616-017-0106-3

METHODS PAPER

Development of an 8-color antibody panel for functional phenotyping of human CD8+ cytotoxic T cells from peripheral blood mononuclear cells

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Received: 20 March 2017 / Accepted: 9 May 2017 / Published online: 27 May 2017

Abstract The study of CD8 positive cells in peripheral blood has become an essential part of research in the field of cancer immunotherapies, vaccine development, inflammation, autoimmune disease, etc. In this study, an 8-color flow cytometry panel, containing lineage and functional markers, was developed for the identification of CD8+ cytotoxic T cells in previously cryopreserved peripheral blood mononuclear cells from healthy human donors. By studying functional markers in naïve and CD3/CD28 activated T cells we demonstrate that the panel is capable of detecting protein markers corresponding to different T cell activation statuses. Data generated by flow cytometry were corroborated by different antibody based assay technologies to detect soluble cytokines. Our findings suggest that there is an inter donor variability in both baseline and activation responses. We have also successfully developed an antibody panel for flow cytometry that could be used to study cytotoxic function of CD8 T cells in clinical immunology research areas.

Keywords CD8+ cytotoxic · PBMC · Flow cytometry · Antibody panel

Background

CD8+ T cells are lymphocytes that comprise 5–25% of total peripheral blood mononuclear cells (PBMCs). They employ a variety of effector mechanisms and are capable of mounting immunogen-specific responses, potentiating immune responses through cytokine release, and enacting cell-mediated killing of virally-infected and tumor cells through a perforin and granzyme-dependent cytotoxic pathway (Harty et al. 2000; Salti et al. 2011; Pender et al. 2014). The evaluation of specific markers involved in such responses is important to understanding immune response in pathological conditions.

In immune-oncology research, immune phenotyping is critical for evaluating patient responses to clinical immunotherapies and the ability of T cell based therapies to effectively target tumors (Kalos 2011; Malyguine et al. 2012). Polychromatic flow cytometry assays of CD8+ T cells compliment currently available immunologic monitoring technologies by enabling more detailed evaluation of

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cytotoxic T lymphocytes and the relationship between effector function and clinical outcomes (Macchia et al. 2013; Zaritskaya et al. 2010). Applications of these assays go beyond the scope of cancer immunotherapies as CD8+ T cells also play a role in the human immune response to HIV and other viral infections (Kutscher et al. 2008; Speiser et al. 2005).

Using an array of lineage (CD3, CD4, CD8 and CD56), viability dye and functional markers (IFNγ, MIP1β, perforin, and granzyme B) we developed an 8-color flow cytometry antibody panel for the characterization of CD8+ T cells within cryopreserved human PBMC populations isolated from whole blood. This panel allows for the simultaneous evaluation of multiple parameters and offers insight into both the activation status and the cytotoxic function of CD8+ T cells.

Materials and methods

Collection and isolation of healthy donor PBMCs

Leukophoresis samples were obtained from six healthy donors, per the blood collection protocol approved by the Institutional Review Board of Brigham and Women's Hospital. All participants gave written informed consent prior to blood draw. Aphaeresed peripheral blood mononuclear cells (PBMCs) were isolated within 6 h of blood collection from six healthy donors by density gradient separation with Ficoll-Paque PLUS (GE Healthcare Biosciences, Uppsala, Sweden). Blood was diluted 1:1 with phosphate buffered saline (PBS) and slowly layered on 12 ml of Ficoll-Paque PLUS in a 50 ml conical tube by pipetting down the side of tube with a transfer pipette. Tubes were centrifuged at 436RCFs for 20 min. PBMCs were aspirated from density separation using a transfer pipette, taking precaution not to aspirate Ficoll layers. Cells were washed by adding PBS, followed by centrifugation at 272RCFs for 5 min and resuspended in fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA); + 15% dimethylsulfoxide (DMSO; Fisher Scientific, Pittsburgh PA, USA) at a concentration of $4-10 \times 10^7$ cells/ml. Cells were frozen overnight at −80 °C in 0.5 ml aliquots at a controlled rate of cooling of −1 °C/min and transferred to liquid nitrogen for cryogenic storage until assay.

T cell activation

Frozen PBMCs were thawed in a 37 °C water bath and resuspended in warmed Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1× antibacterial-antimycotic (Life Technologies). For each stimulation condition, 10×10^6 cells were cultured for 48 h in six well plates at a concentration of 5×10^6 cells/ml. Stimulated cells were cultured for 24 or 48 h with 2.6×10^6 CD3/CD28 Dynabeads/ml (Life Technologies; Trickett and Kwan 2003; Schade et al. 2008). Before staining, Dynabeads were pulled out of the culture using a magnet. Untreated cells (naïve) were used as negative control, and cultured in DMEM + 10% FBS + antibacterial-antimycotic for 48 h with out stimulation. No golgi plug, monensin, or brafeldin was added to better mimic PBMCs isolated from patients, as this panel was developed for ex vivo conditions using clinical samples. After culture, cells were harvested and washed by centrifugation at 391RCFs for 5 min and incubated on ice for the duration of staining procedure. Supernatant from each culture condition was collected by centrifugation and stored undiluted at −80 °C until later use in soluble cytokine assays as described below.

Multicolor flow cytometry

All antibodies and the viability dye were titrated at 0.5, 1 and 2X the recommended concentrations. Both naïve and activated cells were tested, and optimal concentrations were determined by choosing the lowest concentration where positive populations were clearly visible in FlowJo. The concentration of Zombie NIR is undisclosed by vendor for proprietary reasons. Instead, the volume of dye was resuspended in 100 µl DMSO (stock solution).

Cells were resuspended at 6.7×10^6 cells/ml in PBS and aliquoted in a 96 well plate at 1×10^6 cells/well. After washing twice with PBS to remove medium, cells were stained with Zombie NIR fixable viability dye at a 1:2500 dilution of the stock solution into PBS in a total volume of 150 µl. Cells were incubated for 15 min on ice and then centrifuged at 272RCFs for 5 min at 4 °C (all centrifugations for the staining procedure were performed under these conditions). Cells were washed once with 150 µl PBS and then incubated on ice for 18 min in Fc Block (Miltenyi Biotec, Cambridge, MA, USA), diluted 1:625 in FACS

buffer (PBS, 2.5% FBS). After Fc block incubation, cells were washed once with FACS buffer and stained for surface markers CD3-PeCy7, CD8-vioBright FITC and CD56-PerCPcy5.5 in 150 µl total volume for 45 min, diluted per titration results (Table 2). Cells were then washed three times in 150 µl FACS buffer and immediately fixed and permeabilized with BD Cytofix/Cytoperm per manufacturer's protocol (BD Biosciences, San Jose, CA, USA). After fixation, cells were stained for intracellular markers perforin-PE, MIP1β-BV421, IFNγ BV510, and granzyme B-AF647 in 150 µl of BD perm/wash buffer, following manufacturer's instructions, at the concentrations reported in Table 2. Antibody clones, fluorophores, vendors and catalog numbers are provided in Table 1.

Cells were acquired using an LSRII analyzer (BD Biosciences) with the laser configuration described in Table 2. Daily maintenance was run on LSRII in which freshly prepared ($\sim$2 drops/0.5 ml in H_2O) eight peak rainbow beads were acquired and recorded for 10,000 scatter gated events every morning after thorough cleaning (5–10 min) the instruments with 10% bleach and distilled water. Bead peak 7 was standardized to be centered at a 50,000-peak channel number for all detectors and the relative median fluorescence intensity on the all detectors excited by each laser was recorded

Table 1 Information about antibodies and viability dye used for the development of the CD8+ multicolor flow cytometry panel

Specificity	Clone	Fluorophore	Vendor	Catalog number	Purpose
CD3	UCHT1	PE-Cy7	eBioscience (San Diego, CA, USA)	25-0038-42	Lineage
CD8	BW135/80	vioBright FITC	Myltenyi Biotec	130-104-519	Lineage
CD56	B159	PerCPcy5.5	BD Biosciences	560842	Lineage (negative gate)
Perforin	BD48	PE	Biolegend (San Diego, CA, USA)	353303	Cytotoxic function
MIP1β	D21-1351	BV421	BD Biosciences	562900	Activator
IFNγ	b27	BV510	BD Biosciences	563287	Cytotoxic function
Granzyme B	GB11	AF647	BD Biosciences	560212	Cytotoxic function
Viability dye	–	Zombie NIR	Biolegend	423105	Dead cell exclusion

Table 2 LSRII flow cytometer bandpass and specific emission filters utilized for each fluorophore for the configuration of LSRII laser lines

Laser line	488 nm				633 nm		407 nm	
Emission filters	530/30	575/26	710/50	780/60	660/20	780/60	450/50	525/50
Bandpass	505	550		755		755		505
Fluorochrome	AF488	PE	PerCPcy5.5	PEcy7	AF647	Zombie NIR	BV421	BV510

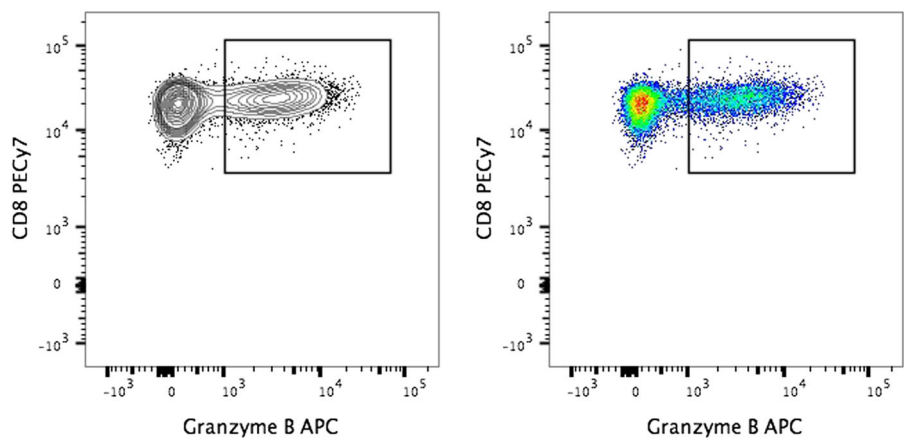

Fig. 1 Sample gating strategy. FlowJo generated *Contour plots* (left) and *dot plots* (right) were used to determine gates. FMO and unstimulated cells were used as baseline

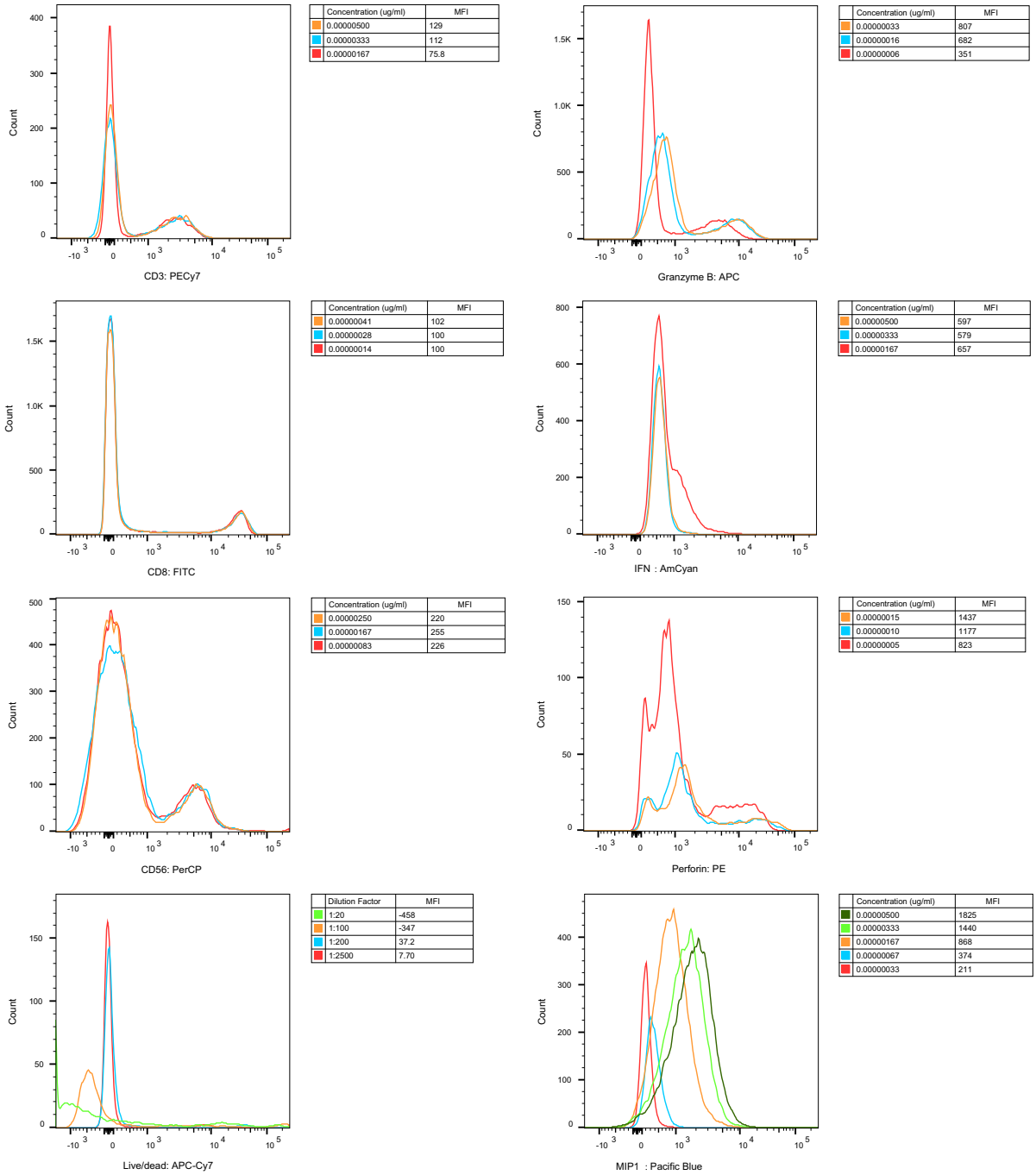

Fig. 2 Titration of CD3-PECy7, CD8-FITC, CD56-PerCP, granzyme B-APC, IFNγ-BV510, perforin-PE, MIP1β-BV421 antibodies and Live/Dead-APC-Cy7 Zombie NIR viability dye. *Histograms* representing positive and negative populations of each marker at different concentrations and their median fluorescent intensities (MFIs). *Keys* represent antibody and viability dye concentrations tested and corresponding MFIs

and documented. Results were then put into a spreadsheet and monitored over time to note any signal drift or major discrepancies from previous acquisitions.

Compensation controls were established using pooled cells from all donors. A mean of 80,000 events was collected for each control and experimental population.

Table 3 Final concentration of antibody (Ab) conjugates added to the panel and stock concentration from the manufacturers

Specificity	Fluorophore	Ab amount per well (μg)	Stock Ab concentration (μg/ml)
CD3	PeCy7	0.5	100
CD8	vioBright FITC	0.0206	8.25
CD56	PerCPCy5.5	0.25	50
Perforin	PE	0.0075	3
MIP1β	BV421	0.1	100
IFNγ	BV510	0.5	100
Granzyme B	AF647	0.025	10

Table 4 Voltages set on LSRII for each parameter applied to the antibody panel to keep cell populations within scale and obtain the best separation of negative and positive cell populations

Parameter	Voltage
FSC-A	423
FSC-H	423
FSC-W	423
SSC-A	374
SSC-H	374
SSC-W	374
FITC-A	551
PE-A	545
PerCPCy5.5-A	769
PE-Cy7-A	621
APC-A	467
APC-Cy7-A	687
AmCyan-A	368
Pacific Blue-A	382

Electronic FCS files were analyzed and gated using FlowJo software (Treestar, Ashland, OR, USA). Gating strategy was determined by fluorescence minus one (FMO) controls for granzyme B, perforin, MIP1β, and IFNγ and contour plots (Fig. 1).

Natural Killers (CD3−CD56+) and Natural Killer T cells (CD3+CD56+) were excluded from intracellular cytokine analysis (Grossman et al. 2004).

Bar graphics were generated in Graphpad Prism version 7 (GraphPad Software, La Jolla, CA, USA).

Soluble biomarker detection

Cell culture supernatant was diluted 1:4 and used for perforin ELISA (Abcam, Cambridge, MA, USA), and for a 3-plex cytokine assay of IFNγ, granzyme B, and MIP1β (Affymetrix, Santa Clara, CA, USA) following manufacturers protocol and read on a Luminex MAGPIX Analyzer (Bio-Rad, Hercules, CA, USA).

Results

All antibodies and viability dye were titrated and the concentrations that gave the best separation between negative and positive populations were selected to use in the panel as shown in MFIs representing histograms (Fig. 2).

Table 5 Average compensation of three technical replicates of each fluorophore applied to the antibody panel

	FITC-A	PE-A	PerCPCy5.5-A	PE-Cy7-A	APC-A	APC-Cy7-A	AmCyan-A	Pacific blue-A
FITC-A	1.00	0.30	0.03	0.00	0.00	0.00	0.01	0.00
PE-A	0.01	1.00	0.13	0.01	0.00	0.00	0.00	0.00
PerCP-Cy5-5-A	0.01	0.02	1.00	0.11	0.00	0.03	0.01	0.01
PE-Cy7	0.00	0.04	0.03	1.00	0.00	0.05	0.00	0.00
APC-A	0.01	0.00	0.07	0.00	1.00	1.05	0.01	0.01
APC-Cy7-A	0.01	0.00	0.13	0.35	0.00	1.00	0.01	0.01
AmCyan-A	0.05	0.05	0.05	0.01	0.00	0.01	1.00	0.47
Pacific blue-A	0.00	0.00	0.00	0.00	0.00	0.00	0.04	1.00

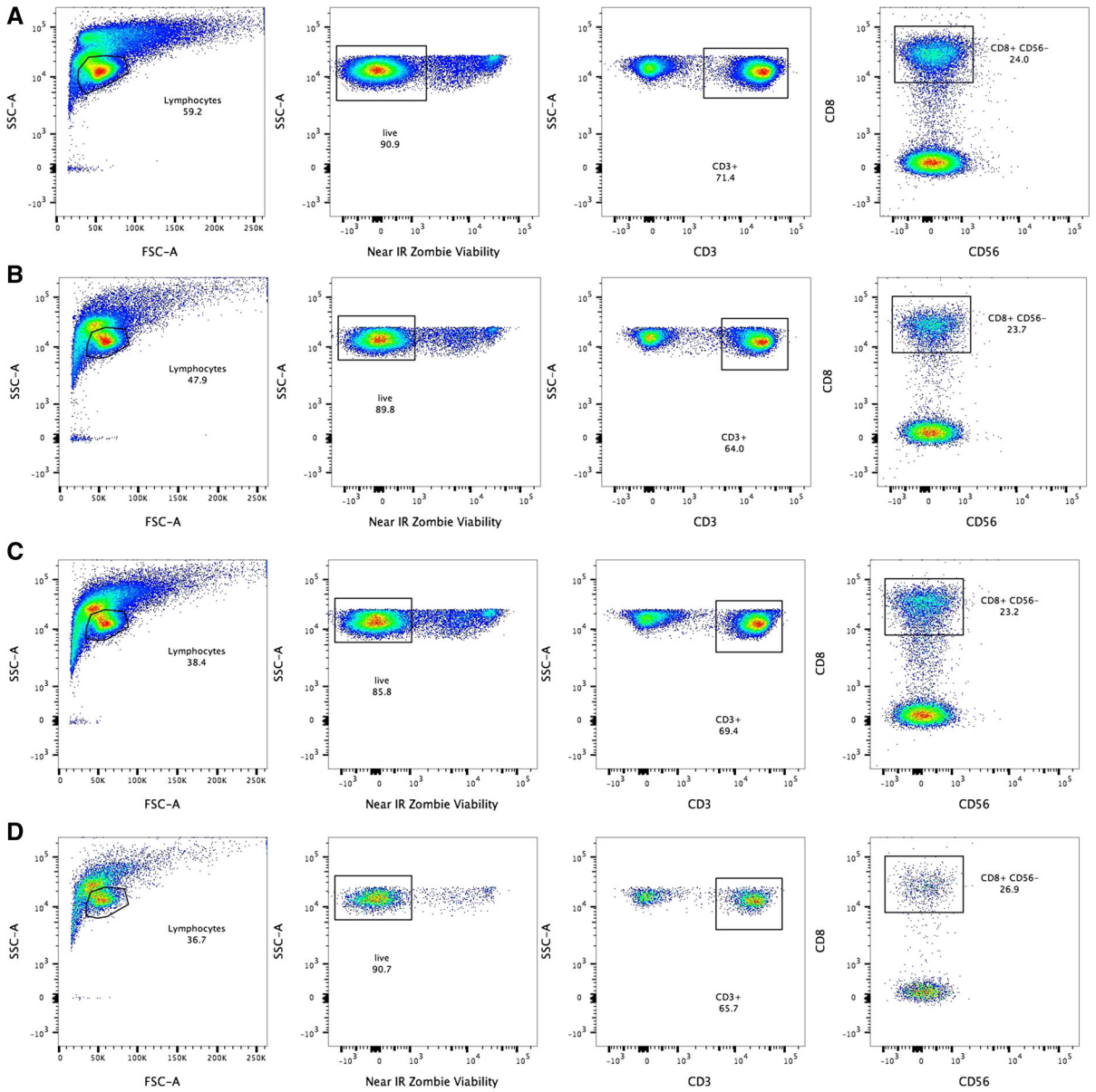

Fig. 3 FlowJo dot plots demonstrating gating strategy for the lineage markers in untreated, cultured (**A**), 24 h stimulation (**B**), 48 h stimulation (**C**) and the FMO control for functional markers (**D**). All cells were gated for Lymphocytes/Live Cells/CD3+/CD8+/CD56

After the appropriate concentration of antibodies was established, the same amounts of antibodies and viability dye were used in the panel consistently across all donors (Table 3).

Voltages were set such that the negative population fell at approximately 10^3 MFIs and the positive population was on scale, and voltages were held constant between samples with different concentrations of the same antibody to ensure that the changes in MFIs were due to antibody concentration and not voltage (Table 4).

Compensation was applied to the panel to account for spillover between the channels (Table 5).

Gating plots for Lymphocytes/live/CD3+/CD8+, CD56− cells were done based on FMOs and unstained controls where a good separation of the parent populations was observed (Fig. 3). The gating strategy to detect functional markers was performed as shown

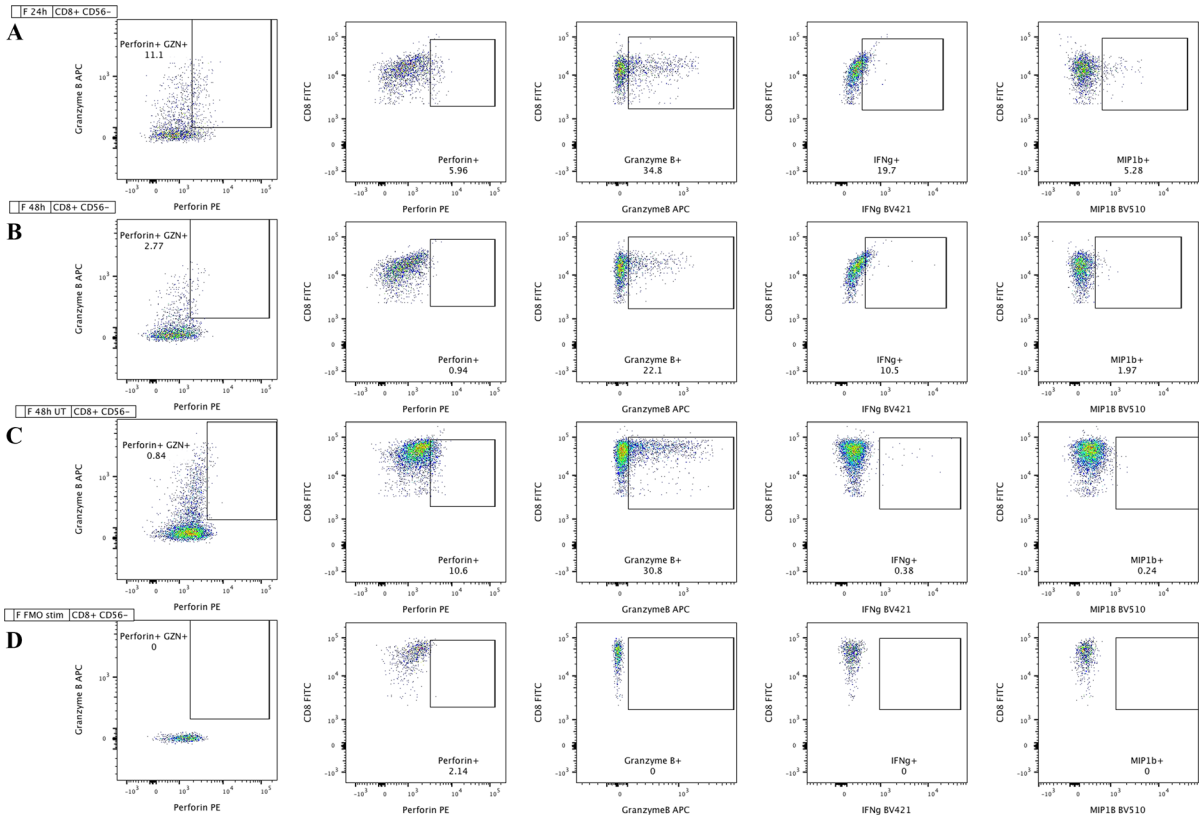

Fig. 4 Gating strategy for CD8+ cells expressing functional markers. *Dot plots* show lymphocytes/single cells/live/CD3+/CD8+/CD56− population of cells from untreated, cultured (**A**), 24 h stimulation (**B**), 48 h stimulation (**C**) and the FMO control (**D**). Shown here are representative data, using Donor A

in Fig. 4 where the double positive populations (CD8+/MIP1β+, CD8+/perforin+, CD8+/granzyme B+ and CD8+/IFNγ+) were defined per FMOs and unstained controls. All gatings were performed individually by sample.

Figure 5 represents averages of percent cellular populations of technical replicates of all PBMCs donors. CD3+ and CD8+ populations do not seem to change within the same donor depending on stimulation status (Fig. 4A, B). The functional markers show an increasing trend in percent of double positive populations (Fig. 4C–F). We observed an inter donor variation in unstimulated parent populations that did not necessarily translate to stimulated populations (e.g. Donor E). Fold changes were calculated for each single donor, marker and stimulation treatment (Table 6). We show increases in functional markers between 1.2- and 100-fold in stimulated cells depending on each donor and stimulation time point.

The concentrations of secreted proteins perforin, granzyme B, IFNγ and MIP1β increased from 24 to 48 h across the donor population, except for MIP1β in Donor B. IFNγ was notdetected in the untreated cell culture supernatant of Donors C, D, and F (Fig. 6). Increased levels of intracellular markers were also observed by soluble protein detection assays for the same proteins.

Discussion

It is advantageous to use a flow cytometry antibody panel that encompasses multiple functional markers simultaneously to more completely understand the activity of cytotoxic cells. Multiparametric flow allows for the same cells to be evaluated in a multi-dimensional capacity and gives insight into the activity of the cells of interest. Unlike past studies

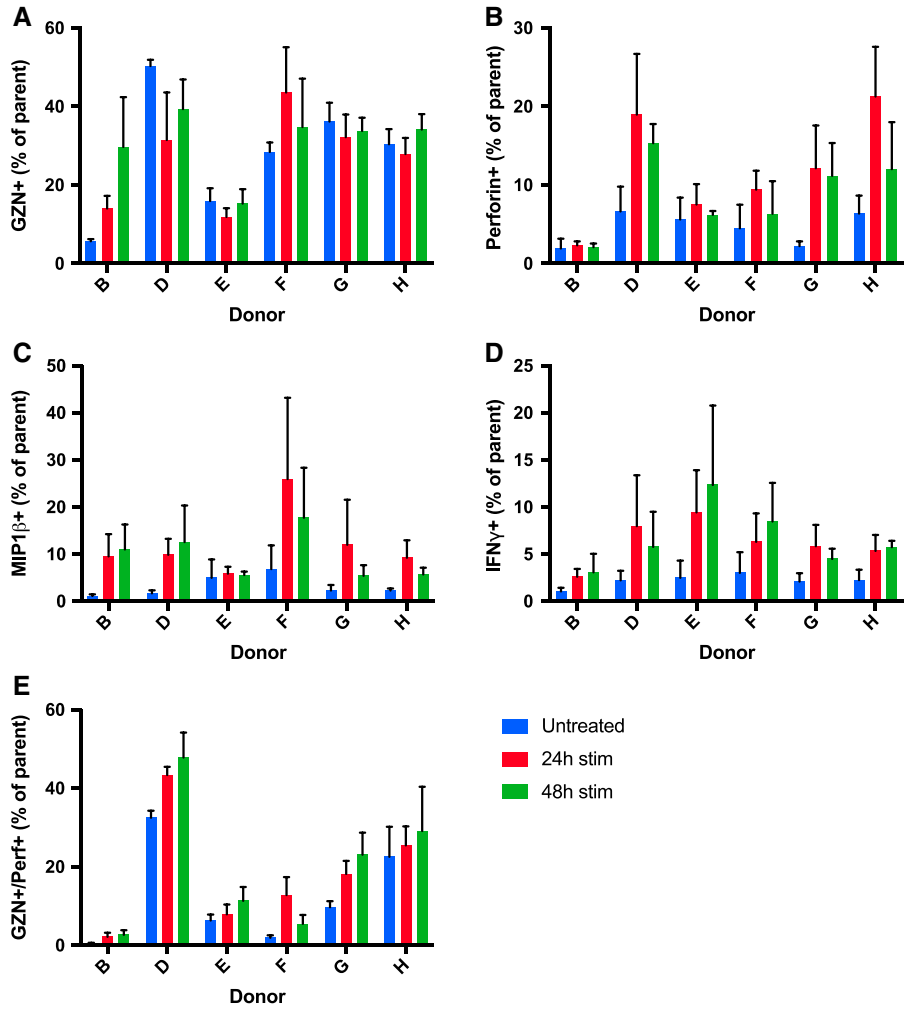

Fig. 5 Summary data from three iterations of the flow cytometry panel. All data are percent of parent population. All cells were gated lymphocytes/live/singlets/CD3+. Averaged granzyme B+ (**A**), perforin+ (**B**), MIP1β+ (**C**), IFNγ+ (**D**) and granzyme+/perforin+ (**E**), data with SEM. Data were analyzed for general patterns of change and no statistical tests were run

Table 6 Fold differences from percent populations of 24 and 48 h stimulated PBMCs (24 and 48 h) from six healthy donors when compared to unstimulated cells

	CD3+	CD8+	Perforin+	Granzyme B+	IFNγ	MIP1β+
Donor A 24 h	0.75	0.79	2.88	6.42	9.93	140.02
Donor A 48 h	0.80	0.80	1.98	12.16	6.88	139.58
Donor B 24 h	0.95	0.67	1.98	1.62	1.33	94.38
Donor B 48 h	1.27	0.77	1.97	1.68	0.80	36.06
Donor C 24 h	1.04	1.06	1.99	1.20	13.52	50.67
Donor C 48 h	1.29	0.85	1.58	2.39	10.22	23.75
Donor D 24 h	0.93	0.75	5.28	1.83	2.21	155.51
Donor D 48 h	0.77	0.89	4.48	0.96	2.57	51.02
Donor E 24 h	0.76	1.00	20.15	1.80	5.76	43.69
Donor E 48 h	0.88	1.02	17.94	1.50	4.28	19.33
Donor F 24 h	0.92	0.83	1.84	1.01	3.37	27.45
Donor F 48 h	0.98	0.83	1.18	0.86	3.47	19.33

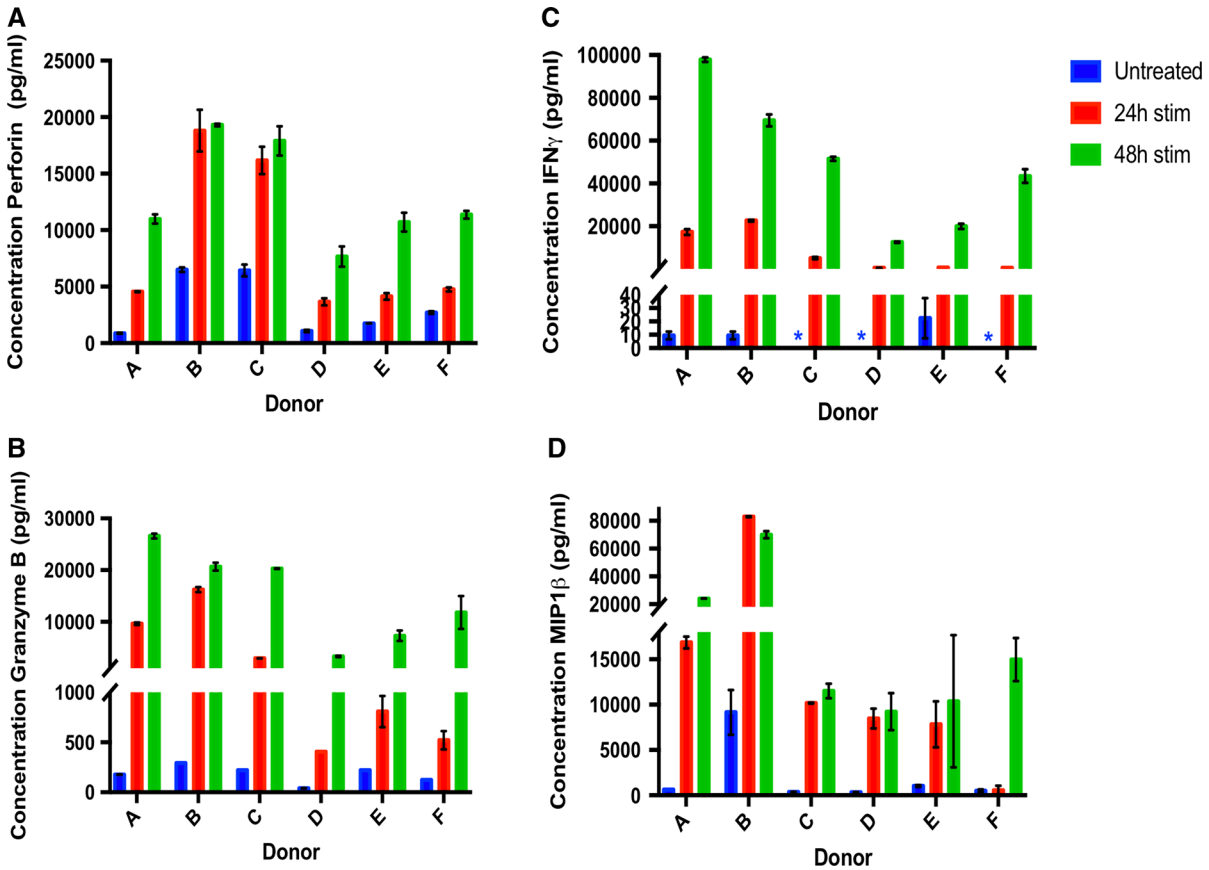

Fig. 6 Soluble protein markers in healthy donors. *Bar graphs* reflect the mean observed concentrations, measured by ELISA, of perforin (**A**), and by multiplex assay of granzyme B (**B**), IFNγ (**C**), and MIP1β (**D**) from two technical replicates from unstimulated (untreated), and stimulated cells (24 and 48 h stimulation with CD3/CD28 Dynabeads). *Designates samples at undetectable concentrations

(Horton et al. 2007), this panel development uses T-cell specific activation and analyzes cells in a context specific to immune oncology by including perforin and granzyme in the analysis.

Before the panel development, it is essential that all antibodies and viability dyes are titrated to the right concentration where positive cell populations can be distinguished from negative ones. As previously described, CD3/CD28 Dynabeads can activate human T cells (Trickett and Kwan 2003; Schade et al. 2008). Here, in the in vitro stimulation with Dynabeads, we have demonstrated the ability of the panel to distinguish between activated and non-activated subsets of CD8+ cells by measuring cell populations expressing IFNγ, MIP1β, perforin, and granzyme B. By studying functional markers in unstimulated and activated T cells populations, we demonstrate that the panel can detect various levels of protein expression that may occur in vivo. Cytokine expression levels and degree of response to Dynabead activation can vary as seen in these results due to many uncontrolable variables. These data may differ based on the race, gender, age and other factors, as seen previously (Swee et al. 2016; Koide and Engleman 1990; Bernin et al. 2016; Bouman et al. 2004; Kee et al. 2012; Laux et al. 2000; Nociari et al. 1999). Thus, inter-donor variation in baseline expression levels, health status and individual response to CD3/CD28 activation is normal, and due to the anonymity of the donors was not controlled for.

The soluble biomarker data shown here is a functional validation of cytotoxic T cells ability to release intracellular cytokines and enzymes assessed in this panel. Since supernatant was collected from total PBMC culture, it is impossible to determine the specific cell subtypes responsible for generating the

cytokines and enzymes detected in the multiplex and ELISA results from cell culture supernatants. Whereas supernatants contained accumulated soluble proteins released, proteins detected in flow cytometry were expressed by the cells at the time when the assay was performed. Thus, these data should not be used in place of one another but rather as complimentary technologies to better understand the functional status of CD8+ T cells in an in vitro system.

CD8+ cytotoxic T cells play a role in immune response to cancers, autoimmune diseases, inflammation and viral infections including HIV (Di Meglio and Duarte 2013; Salti et al. 2011; Speiser et al. 2005). For example, in the context of lung and melanoma cancer research, exploration of activated CD8+ T cells is of interest in studying tumor evasion of perforin and granzyme-mediated killing (Hodge et al. 2014; Daud et al. 2016). It is of further interest to study CD8+ T cells within tumor-infiltrating lymphocyte populations to explore effector molecules released by activated CD8+ T cells that are recruited to the tumor microenvironment in the context of various cancer therapies (Oelkrug and Ramage 2014).

Conclusions

In this study, we show the development of an 8-parameter flow cytometry antibody panel that identifies cytotoxic CD8+ T cells, and their functional status, using cryopreserved PBMCs from healthy donors, and could potentially be applied to any area of clinical research where CD8+ cells play a role as a cellular biomarker.

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