Development of a T Cell Activation Assay

Comparison of Commercial and In-House Methods

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Abstract

T cells are a specific type of immune cell that plays an important role in the immune system. When activated, they produce different types of cytokines to, for instance, destroy tumour cells. The aim of this study was to develop a T cell activation assay, which can be applied in the immunotherapy field. This was performed by comparing the T cell production of the cytokines interferon-γ (IFN-γ) and interleukin 2 (IL-2) using two different methods. T cells from two healthy individuals were activated according to both methods and, thereafter, analyzed in multicolor flow cytometry. The results indicated that the in-house method activates T cells more effectively, as a higher percentage of IFN-γ was produced compared to the commercial method. Regarding IL-2 production, it was no or an extremely small difference between the two methods. Additionally, the in-house method is more than 50 times less expensive per experiment and is, therefore, an economically beneficial T cell activation assay, which can be applied within the cancer research field. The in-house method will effectively activate T cells in order to investigate future immunotherapy treatment possibilities.

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List of Abbreviations

APC	Antigen-presenting cell
BFA	Brefeldin A
CD3$^+$	Cluster of differentiation 3 positive
CD4$^+$	Cluster of differentiation 4 positive
CD8$^+$	Cluster of differentiation 8 positive
DMSO	Dimethyl sulfoxide
FACS	Fluorescence-activated cell sorting
HIV	Human immunodeficiency virus
IFN-γ	Interferon-γ
IL-2	Interleukin 2
mAb	Monoclonal antibody
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
RPMI	Roswell Park Memorial Institute
TCR	T cell receptor

1 Introduction

1.1 The Immune System

The human body is continuously exposed to various microorganisms. Some of these may cause disease as a result of the virulence factors at its disposal [1]. The immune system is an organisation of biological structures combating these microbial invasions. There are two constitutive types of responses to encroaching microbes; i) the first line of defense, called *innate immunity*, which responds to the same extent irrespective of how many times the infectious agent is encountered; and the ii) *adaptive immunity*, which improves on repeated exposure to pathogens of the same species [2].

The innate immune response is a series of mechanisms encompassing phagocytic cells (neutrophils, monocytes and macrophages), cells that release inflammatory mediators (basophils, mast cells and eosinophils) and natural killer (NK) cells [3]. However, these cells might not be powerful enough to fully eliminate the infection nor do the mechanisms lead to long-term protection.

Adaptive immunity constitutes primarily of antigen-specific B and T cells (these are also referred to as lymphocytes) as well as the proliferation of them, which occurs when their surface receptors bind to an appropriate antigen. B cells secrete antigen-specific antibodies responsible for eliminating invading microorganisms. T cells help B cells to produce antibodies, but can also kill virally infected cells. The innate and adaptive immunities collaborate to eradicate pathogens [2].

The adaptive immune response is initiated in organs called secondary lymphoid tissues, which are parts of the lymphatic system. The lymphatic system is an extensive system that collects extracellular fluid from the tissues and returns it to the blood. Lymph nodes are one type of secondary lymphoid tissues found where the lymphatic vessels converge. They contain large numbers of lymphocytes. Antigen from sites of infection encounter naive lymphocytes that constantly recirculate through peripheral blood tissue in the lymph nodes. Therefore, it is in the lymph nodes the immune response commences.

1.1.1 Adaptive Immunity

The adaptive immune system is precise, but usually takes several days or weeks to develop. The adaptive response has memory and focuses specifically on long-lived protection against pathogens [4]. The adaptive immunity is additionally divided into two sections: humoral and cell-mediated immunity. The former is built up by the antibody-producing B cells while the latter comprises a variety of T cells [5], which both are types of leukocytes. During an infection, B cells produce antibodies that achieve a markedly higher level of response to the pathogen over time, particularly in secondary and subsequent immunizations. This phenomenon is called affinity maturation. When binding to antigen, the cells are activated to divide and produce many identical progenies, in a process known as clonal expansion. Both clonal expansion and affinity maturation are functions of the adaptive immunity that effectively help the cells combat the pathogen that elicited the response [3]. This study focuses on the efficiency of different methods to activate T lymphocytes, which are commonly defined by expression of the CD3 glycoprotein. T cells can also express other glycoproteins, which divides them into one of several subsections.

1.1.2 Helper T Lymphocytes

In addition to CD3 glycoprotein expression, helper T cells are defined by expression of the CD4 glycoprotein. Helper T cells have two different functions; boosting the ability of macrophages to attack pathogens and helping B cells to produce antibodies. Cells that have been infected by bacteria, parasites or other extracellular antigen display peptides (protein fragments) of these on the surface of the cell. They do this via major histocompatibility complexes class II (MHC-II), which are molecular platforms on the cell surface of antigen-presenting cells (APCs). T cell receptors (TCRs) on the surface of helper T cells bind to MHC-II, which causes the T cell to activate in order to eradicate the invader by performing its designated part of the adaptive immunity.

1.1.3 Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes are defined by expression of the CD8 glycoprotein in addition to CD3. They kill infected cells by programming them to undergo apoptosis (programmed cell death). Peptides of intracellular antigen, such as viruses, are displayed on the surface of the infected cells by major histocompatibility complex class I (MHC-I) molecules, which are present on the surface of all nucleated cells as they have a protein production. The cytotoxic T cells bind through their TCR to the MHC-I of infected cells [4].

1.1.4 Activation of T lymphocytes

As mentioned previously, naive $CD4^+$ and $CD8^+$ T cells are activated when encountering antigen specific to their TCR. After activation, the cells differentiate into effector T cells that perform their part of the immune system in order to eliminate the certain pathogen that caused infection. When activated, T cells start to produce cytokines; proteins that affect the behaviour of other cells. Cytokines made by lymphocytes are often called interleukins. There are several interleukins, but two of the most common ones are the cytokines interferon-γ (IFN-γ) and interleukin 2 (IL-2). The former is a cytokine of the interferon structural family secreted by effector T cells. The primary function of IFN-γ is to activate macrophages. The IL-2 protein is secreted by naive T cells and is essential for their differentiation into effector T cells. Due to this it is one of the key cytokines in the development of an adaptive immune response [3].

1.2 Background to Methods

Many methods have been developed in order to study T cells and investigate immunological mechanisms. For this specific study, methods for isolation of peripheral blood mononuclear cells (PBMC) and T cell activation will be used as well as multicolor flow cytometry.

1.2.1 Isolation of Peripheral Blood Mononuclear Cells

In order to study T cells *in vitro*, outside of their normal biological context, it is necessary to isolate them from other hematocytes (blood cells). The density gradient centrifugation method that was used is based on the density of different hematocytes. The blood colloid must be depleted of erythrocytes and granulocytes, which leaves a modified population of PBMC constituting primarily of lymphocytes, monocytes and macrophages. As erythrocytes and granulocytes have different density than lymphocytes, they will descend to the bottom of the test tube when centrifuged [6].

1.2.2 T Cell Activation Assays

In laboratory activation processes, T cells are activated by encountering monoclonal antibodies (mAbs) instead of antigens. This investigation focuses on the detection of the cytokines IFN-γ and IL-2. Ideal T cell activation assays can be used to effectively activate T cells from cancer tissue in order to study their cytokine production. Another application is to test T cell activation properties with different compounds (inter alia potential drugs and chemotherapeutic agents) in order to acquire knowledge about how the protein expression of T cells is affected. This might be useful for future treatment possibilities within the immunotherapy field [7].

1.2.3 Multicolor Flow Cytometry

Flow cytometry is a laser-based, biophysical technology employed in both qualitative and quantitative analysis of multiple characteristics of single cells such as size, production of certain proteins and internal complexity [8]. Individual cells are treated with fluorochrome labeled mAbs, whereafter they individually pass through a laser beam. The laser light will be scattered and any dyed molecules bound to the cell will be excited and therefore fluoresce. Sensitive photomultiplier tubes detect both the scattered light and the fluorescence emissions with different wavelengths. The former gives information about inter alia, the size and granularity of the cell, while fluorescence emissions give information

about the expression of cell surface proteins on cell membranes [3]. After the execution, the retrieved data can be exported from the device into a computer analysis software.

1.3 Aim of the Study

The aim of this study was to develop a T cell activation assay investigating two different methods, one in-house and one commercial. T lymphocytes from two healthy individuals were used to compare IFN-γ and IL-2 production in different T cell populations ($CD3^+$, $CD4^+$ and $CD8^+$). This was performed in order to create an assay applied in the field of immunotheraphy and specifically cancer.

2 Materials and Methods

For an overview of the experimental procedure, see the flowchart presented in Figure 1.

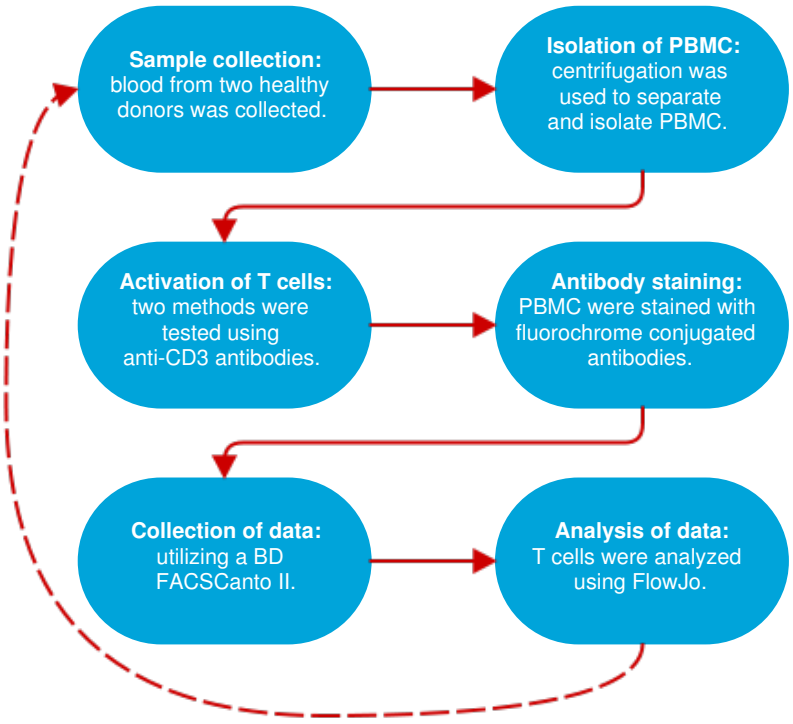

Figure 1: A flowchart to summarize the method.

2.1 Patients and Sample Collection

Blood samples were obtained from two anonymous, healthy patients at Karolinska University Hospital in Huddinge, Sweden, June 9 and June 24, 2014. A total volume of 40 mL blood was collected. PBMC from both individuals were prepared and cryopreserved the date of arrival.

2.2 Experimental Procedures

2.2.1 Isolation of Peripheral Blood Mononuclear Cells

The patient blood was transferred to four 50 mL Falcon tubes, which were each filled with 10 mL blood respectively. The blood in each tube was diluted with 10 mL phosphate buffered saline (PBS, pH7.4), an isotonic and to cells non-toxic buffer solution, to facilitate storing of PBMC. A volume of 30 mL of the density gradient Lymphoprep (Fresenius Kabi AS, Oslo, Norway) was aliquoted (distributed evenly) to four new 50 mL Falcon tubes and the blood colloid was slowly added on top of the Lymphoprep utilizing a Pipette Boy. The tubes were then carefully transported to a centrifuge (Rotina 420, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) where they were centrifuged for 20 min in a centrifuge at $800g$ with 0 braking and 1 acceleration.

After the centrifugation, the tubes were carefully taken out from the centrifuge without mixing the layers of fluid that had formed. The white band of PBMC was collected with a pipette and transferred into a new tube. In order to wash the cell population, the suspension was centrifuged for 10 min at $500g$ with 9 braking and 9 acceleration. The supernatant (excessive liquid) was discarded and PBS was added to the 45 mL mark to wash the cell pellet. The washing procedure was repeated twice in order to remove possible Lymphoprep leftovers.

2.2.2 Cryopreservation of Peripheral Blood Mononuclear Cells

The collected cells were diluted with 5 mL Roswell Park Memorial Institute (RPMI-1640) medium to facilitate cooling and thereafter aliquoted in 10 cryo tubes. The cryo tubes were transferred to a fridge in order to cool the cells to sub-zero temperatures. A mixture of 1 mL dimethyl sulfoxide (DMSO), a toxic compound that prevents cell death during cryopreservation, and 4 mL PBS was prepared. Initially, they were cooled in a normally temperatured refrigerator. After 15 min, the DMSO and medium solution was aliquoted to the cryo tubes. The cells were quickly moved to a temperature of -80 °C in which they were left overnight.

When thawing, the cells were heated using body temperature until the suspension had liquefied. It was carefully diluted with 10 mL of RPMI-1640 medium to prevent cell death by DMSO, and then washed once using identical washing executions as previously described.

2.2.3 Cell Counting

A volume of 10 µL of the cell suspension was added to one well in a 96-well plate which already contained 10 µL Eosin, a fluorescent red dye that binds to dead cells. The substances were resuspended and 10 µL of the mixture was added to a hemocytometer (Bürker chamber) with a cover glass. The living cells were counted in three double lined squares of the hemocytometer using a microscope (Nikon, Tokyo, Japan), and the mean was used for calculations of the total number of live cells. A lens magnification of 40x was used for the counting.

2.2.4 Activation of T Cells

Two different T cell activation assays were used; one in-house and one commercial method. Commencing with the in-house method, the supplied concentration of anti-human CD3 mAb (1 mg mL^{-1}) in sterile filtered PBS (Mabtech AB, Nacka, Sweden), was diluted to the company's recommended concentration for optimal cytokine production (100 ng mL^{-1}),

which is a dilution of 1:10000 from the stock solution. A volume of 1 mL diluted mAb solution was added to two wells in a 6-well plate. Finally, 1 mL PBS was added to one well as a control, in order to subtract the background cytokine production, which is present.

The microwell plate was incubated in room temperature for three hours. Thereafter, an unspecified amount of PBS was carefully added to the plate using a pipette in order to wash the suspension. The PBS was removed and discarded. Approximately 5 million PBMC in a volume of 3 mL culture medium were added to each well. A proper concentration is in general 1-2 million cells per mL.

In this investigation, a T Cell Activation/Expansion Kit for human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was employed for the commercial method. It constituted of 2 mL Anti-Biotin MACSiBead Particles and 0.4 mL human antibody CD3-Biotin, with a concentration of 100 µg mL^{-1}. The Anti-Biotin MACSiBead Particles were used to mimic APCs in order to activate T cells [9].

A volume of 300 µL CD3-Biotin was pipetted into a 2 mL tube and mixed well. The Anti-Biotin MACSiBead Particles were vortexed to obtain a homogenous suspension, whereafter 500 µL of them were added to the CD3-Biotin in order to load the Anti-Biotin MACSiBead Particles. A volume of 200 µL PBS was added to adjust the total volume to 1 mL. Afterwards, the suspension was incubated for 2 h at approximately -8 °C under constant, gentle rotation using a rotator (Swelab Instruments AB, Stockholm, Sweden).

The loaded Anti-Biotin MACSiBead Particles were resuspended, after which 100 µL of them was transferred to a 15 mL Falcon tube. A volume of 200 µL culture medium was added to the loaded Anti-Biotin MACSiBead Particles and then centrifuged at 300 g for 5 min. The supernatant was discarded and the loaded Anti-Biotin MACSiBead Particles were resuspended in 12 mL PBMC with a concentration of approximately 5 million cells per 3 mL culture medium. A volume of 3 mL of the mix was added to four wells in a 6-well plate. Additionally, 3 mL of only PBMC was added to two wells respectively.

Afterwards, both 6-well plates were incubated overnight at 37 °C for 12 h. Brefeldin A

(BFA)($5\,\mathrm{mg\,mL^{-1}}$), a protein transport inhibitor that prevents the cell from releasing the produced cytokines, was added after 6 h.

2.2.5 Intracellular Antibody Staining

A total of five colors were used in the experiment. Four of them included fluorochromes conjugated to mouse anti-human antibodies and one of them to rat anti-human antibodies. The antibodies were used for extra- and intracellular staining.

Antibodies for extracellular staining were added according to titrated volumes (see Table 1). Afterwards, the cells were incubated for 15 min at 4 °C in darkness and then centrifuged for 4 min at 700*g*. The cells were washed with 100 µL PBS once, employing identical executions as described earlier in 2.2.4. The plate was centrifuged for 4 min at 700*g* again and then the supernatant was discarded.

The cells were resuspended in 50 µL Cytofix/Cytoperm (BD Biosciences, San Diego, CA) and incubated for 10 min at 4 °C in darkness. The cells were washed once again with 130 µL PBS, centrifuged for 4 min at 700*g*, after which the supernatant was discarded.

Thereafter, the cells were resuspended in 50 µL Perm/Wash (BD Biosciences), which was already diluted 10 times in distilled water, and then incubated 15 min at 4 °C in darkness.

Antibodies for intracellular staining were added according to the producer's recommended volumes (see Table 1). The cells were resuspended and incubated for 30 min at 4 °C in darkness. After, they were washed with 100 µL PBS and centrifuged for 4 min at 700*g*, after which the supernatant was decanted. Finally, 200 µL PBS was added to each sample and the cells were transferred to fluorescence-activated cell sorting (FACS) tubes.

Table 1: Antibodies used for Extra- and Intracellular Staining.

Color[1]	**Type**[2]	**Clone no.**[3]	**Volume (µL)**
Pacific Blue	anti-CD3	UCHT1	2
AmCyan	anti-CD4	RPA-T4	2
Peridinin chlorophyll	anti-CD8	SK1	5
Phycoerythrin-cyanine 7	anti-IFN-γ	B27	5
Alexa Flour 700	anti-IL-2	MQ1-17H12	1

1. The different colors that were used for the analysis.

2. The first three antibodies were stained extracellularly and the last two intracellularly. The first four were produced by BD Biosciences and the last one by BioLegend, San Diego, CA.

3. Clone numbers represent specific cell lines. Properties of different lines might differ.

2.3 Data Acquisition by Multicolor Flow Cytometry

The data was acquired on a BD FACSCanto II (BD Biosciences, San Jose, CA) with BD FACSDiva Software version 6.1.3 (Becton Dickinson Labware, Franklin Lakes, NJ). The data was analyzed and displayed using the flow cytometry analysis software FlowJo, version X.0.7 (Tree Star Inc, Ashland, OR). Single color stained samples and relevant fluorescence minus one samples were used for compensation and proper gating. Gates including singlets, lymphocytes, $CD3^+$ T cells and further subpopulations were defined.

3 Results

In-house and commercial methods were used to study T cell activation. PBMC from two healthy individuals were used for the experimental procedure. A comparison of the produced amounts of IFN-γ and IL-2 in $CD3^+$ T cells from the first patient was performed (see Figure 2). Figure 3 shows the utilized gating strategy and is found in Appendix A. The results from another individual were analyzed to confirm the first results (see Figure 2).

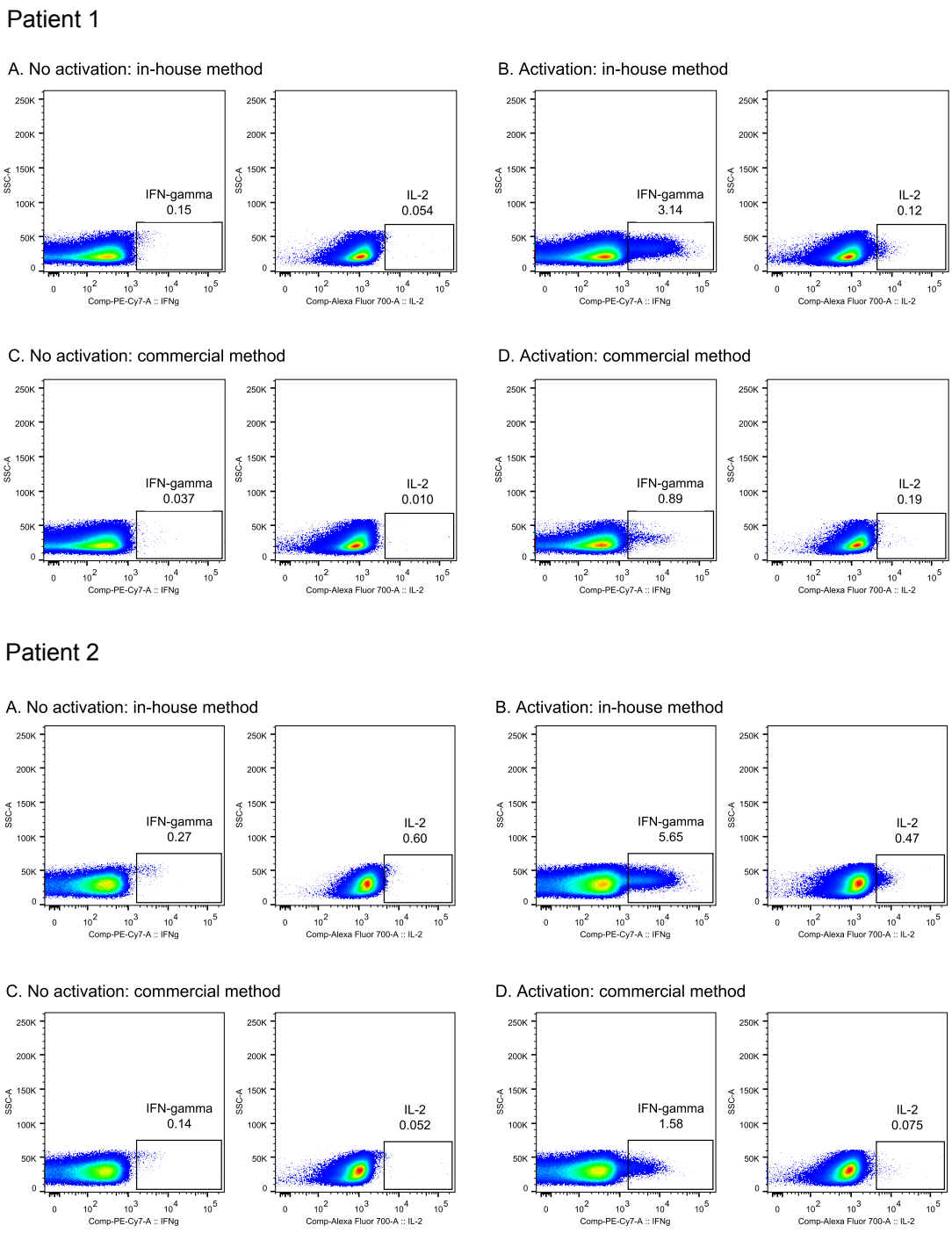

Figure 2: IFN-γ and IL-2 production in CD3$^+$ T cells. (A) Production of IFN-γ and IL-2 when the T cells are not activated, employing the in-house method. (B) Production of IFN-γ and IL-2 when the T cells are activated, employing the in-house method. (C) Production of IFN-γ and IL-2 when the T cells are not activated, employing the commercial method. (D) Production of IFN-γ and IL-2 when the T cells are activated, employing the commercial method.

The $CD4^+$ and $CD8^+$ T cell cytokine production was also investigated. The graphical results are shown in Figure 4 and 5 in Appendix B. IFN-γ and IL-2 production results for different T cell populations for both patients are summarized in Table 2.

Table 2: Cytokine production results.

Patient	**Method**	IFN-γ[1]			IL-2[1]		
		CD3	**CD4**	**CD8**	**CD3**	**CD4**	**CD8**
1	in-house	3.0	1.2	9.5	0.1	0.1	0.2
	commercial	0.9	0.4	2.0	0.2	0.3	0.1
2	in-house	5.4	7.2	3.0	-[2]	0.1	-[2]
	commercial	1.4	2.0	1.9	0.7	0.1	0.1

1. Total production in % with subtraction of the background activation.

2. Background cytokine production overpowered activated production.

The in-house method resulted in that all investigated T cell populations produced more IFN-γ than the commercial method (0.8-7.5 %). However, the commercial method resulted in a slightly higher IL-2 production compared to the in-house method (0.1-0.7 %). The largest increase in IL-2 production was found in the $CD3^+$ T cell population from patient 2, while the largest increase in IFN-γ production was found in the $CD8^+$ T cell population from patient 1.The IL-2 production was not affected significantly in any of the experiment.

4 Discussion

4.1 Comparison of the Methods

This study showed that the largest difference in produced cytokines was observed in IFN-γ when comparing methods is to be found when observing the IFN-γ production, since the in-house method provides a larger number of produced cytokines in all experiments. The commercial method resulted in slightly higher IL-2 production in 66% of the trials, however, the difference between in-house and commercial results was minimal in comparison with the IFN-γ production results. Due to this, the results indicate that the in-house method is more efficient for T cell activation compared to the commercial one in a majority of the experiments.

4.2 Economy

The commercial T Cell Activation/Expansion Kit for human (2 mL Anti-Biotin MACSiBead Particles) costs 6676 SEK[1] (approximately $980) and suffices for 4 experiments while the anti-human CD3 mAb (1 mg), utilized in the in-house method, costs 3200 SEK[2] (approximately $470) and suffices for 100 experiments. That makes the in-house method 52 times less expensive than the commercial method *per experiment*. The latter is also more effective, and research in the area of immunotherapy can utilize this economically beneficial T cell activation method to obtain more convenient results.

4.3 Future Perspectives

Dead cells are incapable of synthesizing proteins and can give rise to false positive results in flow cytometry. Therefore, identifying and removing them in the analysis gives more reliable and accurate results. This can be possible by including a live/dead marker in the antibody panel used for multicolor flow cytometry. It is also important to analyze other

[1] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany. 2014-07-09.
[2] Mabtech AB, Nacka, Sweden. 2014-07-09.

cytokines than IFN-γ and IL-2, which are important in the immunotheraphy field. The ultimate stage of this experiment is to isolate T cells from cancer tissue and apply the assay in order to receive activation results. This may result in similar T cell activation data as from healthy donors, or they may be activated differently due to the different properties of the tumour cells. Even though the in-house method has proven to give more satisfying results than the commercial one it is important to compare the two when applying the assay to cancer tissue. However, it is unlikely that the commercial method will provide better activation of T cells from cancer tissue since the properties of the T cells are unaltered.

An interesting investigation would be to determine whether it is possible to optimize the in-house method additionally. Different concentrations of the anti-human CD3 mAb may cause activations of different strength. It is desirable to obtain knowledge of which concentration that causes the as effective activation as possible. Due to this, the incubation time for coating of the antibody should be adapted for the mAb concentration, as more concentrated solutions may suffice with shorter incubation times whereas less concentrated solutions may demand longer incubation times.

5 Acknowledgements

I would like to thank my mentor, Dr. Melissa Norström, who has been an everlasting source of inspiration and aid during my time at Karolinska Institute. It is an honor to me to have worked with Hanna Peterson for two amazing weeks. I am grateful to Agnes Nordquist who has spent a lot of time giving me feedback, and of course my fantastic counsellor Mariam Andersson with a neverending belief in all of us.

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A Gating Strategy

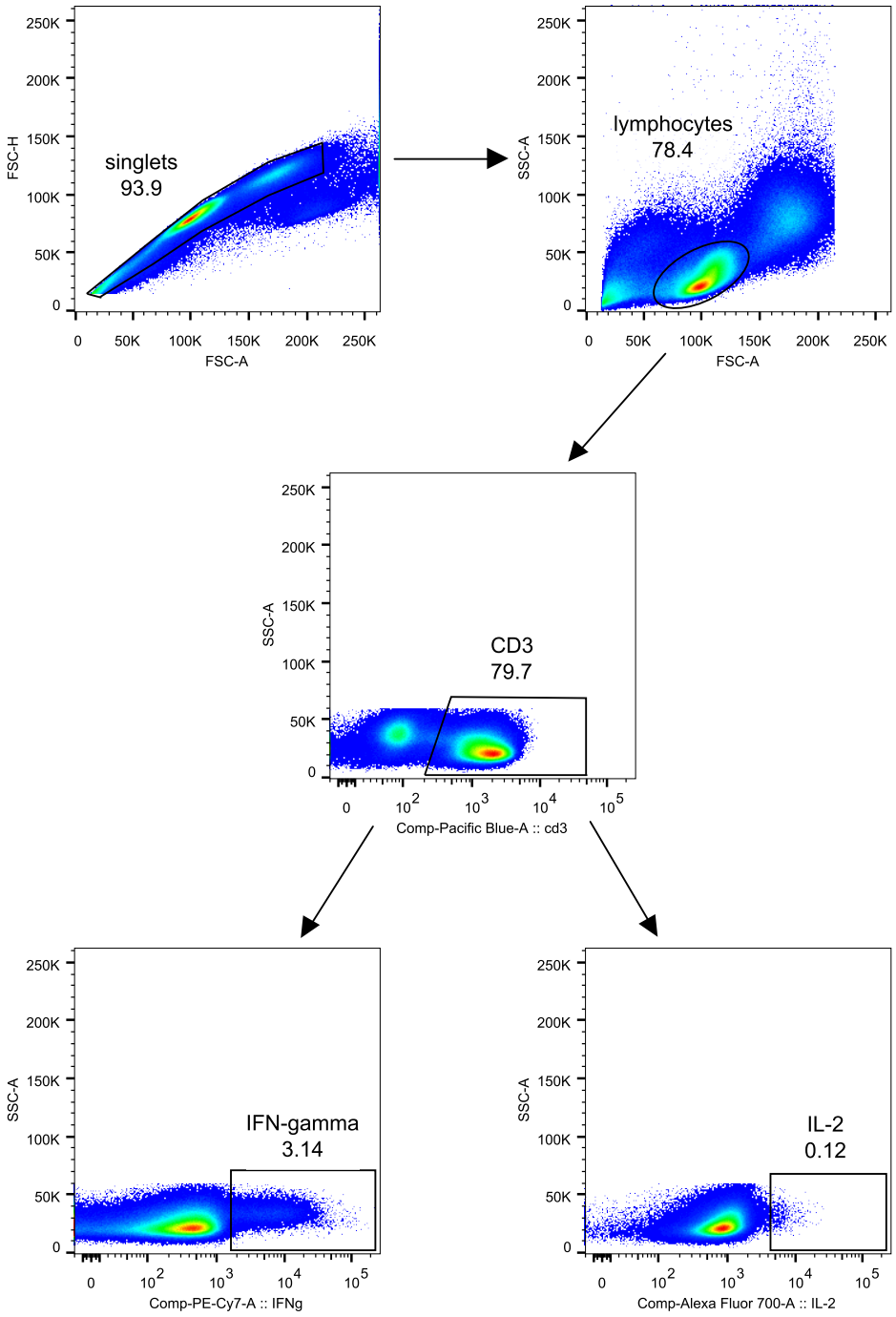

Figure 3: The results were analyzed using this gating strategy, but also by analyzing $CD4^+$ and $CD8^+$ T cells and their cytokine production gated from $CD3^+$ T cells.

B Flow Cytometry Results

B.1 Helper T Cell Data

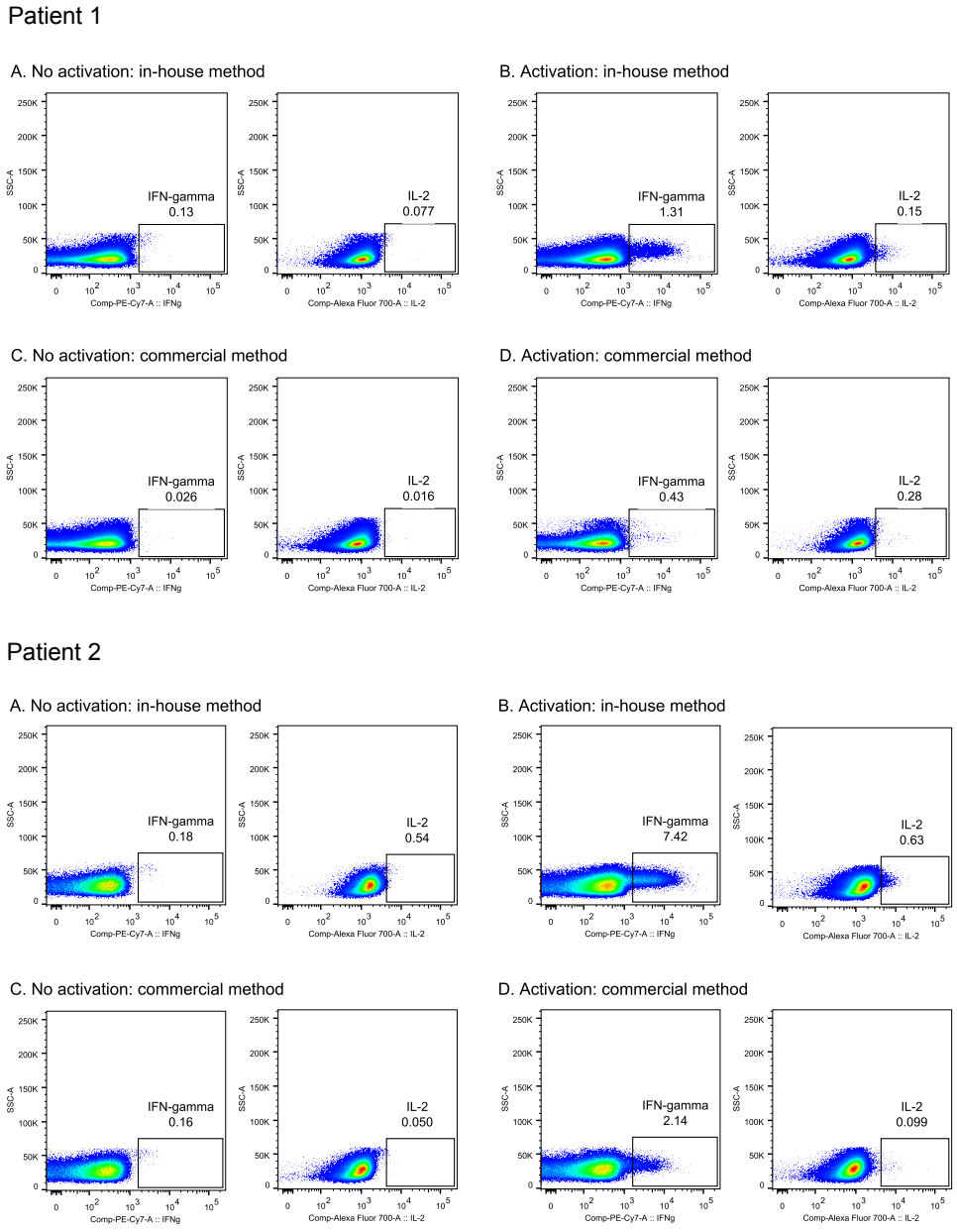

Figure 4: IFN-γ and IL-2 production in CD4$^+$ T cells. (A) Production of IFN-γ and IL-2 when the T cells are not activated, employing the in-house method. (B) Production of IFN-γ and IL-2 when the T cells are activated, employing the in-house method. (C) Production of IFN-γ and IL-2 when the T cells are not activated, employing the commercial method. (D) Production of IFN-γ and IL-2 when the T cells are activated, employing the commercial method.

B.2 Cytotoxic T Cell Data

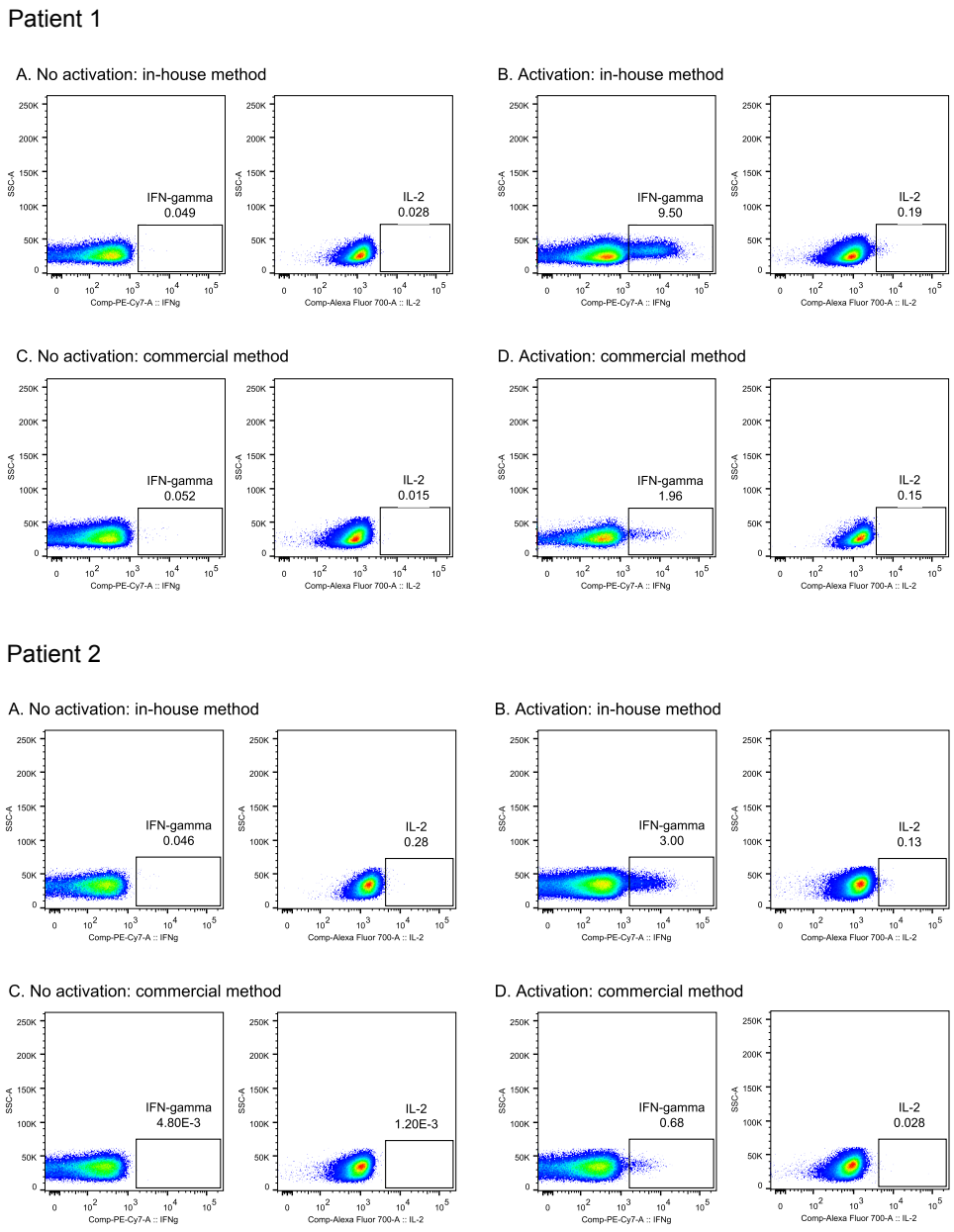

Figure 5: IFN-γ and IL-2 production in CD8$^+$ T cells. (A) Production of IFN-γ and IL-2 when the T cells are not activated, employing the in-house method. (B) Production of IFN-γ and IL-2 when the T cells are activated, employing the in-house method. (C) Production of IFN-γ and IL-2 when the T cells are not activated, employing the commercial method. (D) Production of IFN-γ and IL-2 when the T cells are activated, employing the commercial method.