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Exploring the in vitro expansion of CD4 T cells

For improved culturing of CD4 T cells linked to FNAIT

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Master's thesis in Biomedicine, May 2018

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Acknowledgements

The laboratory work resulting in this thesis was performed at the Immunology research group from January 2017 to May 2018.

First of all I would like to thank everyone in the Immunology research group for being open and friendly and welcoming me into the group.

I would especially like to thank both of my supervisors during this project. I want to thank Tor Stuge for teaching me how to work with cells and immunological methods, and for always coming up with new ideas. I want to thank Maria Therese Ahlén for her thoroughness and for keeping me grounded and focused.

Finally, I would like to thank everyone mentioned as well as my friends and family for supporting me through this project and encouraging me to keep going even when things were not working the way I wanted them to.

Susannah von Hofsten
May 2018

Abbreviations

APC	Antigen presenting cell
BCR	B cell receptor
B-LCL	B lymphoblastoid cell line
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTLA4	Cytotoxic T lymphocyte-associated protein 4
EBV	Epstein-Barr virus
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FcRn	Neonatal Fc receptor
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
FSC	Forward scattered light
GAL9	Galectin-9
HLA	Human leukocyte antigen
HPA	Human platelet antigen
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-15	Interleukin-15
IMDM	Iscove's modified Dulbecco's medium
LAG3	Lymphocyte-activation gene 3
MACS	Magnetic-activated cell sorting
MFI	Median fluorescence intensity
MHC II	Major histocompatibility complex class II

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSA 0.2 %	Phosphate buffered saline + 0.2 % bovine serum albumin
PD1	Programmed cell death protein 1
PD-L1	PD1 ligand
PHA	Phytohemagglutinin
PS	Proliferation score
SSC	Side scattered light
TCR	T cell receptor
TIM3	T cell immunoglobulin and mucin-domain containing-3
T_H1	T helper 1 cell

Summary

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a rare disease that may cause serious bleedings in the fetus or neonate of a woman who has developed antibodies against the fetus' platelets. Development of FNAIT has been linked to the presence of platelet reactive CD4 T cells that help B cells to develop into antibody producing plasma cells. To be able to conduct research on such T cells, the Immunology research group must be able to expand and keep them in long term cultures. Recent work revealed that several established T cell clones had started proliferating poorly. In an attempt to understand why and to possibly improve the culturing of future T cell clones, this study looked into some of the conditions that may influence the growth of these cells when expanded in vitro. The established anti-CD3 expansion culture protocol was compared to one that used PHA, but no advantage of using the latter was detected. It was demonstrated that different B-LCLs used as growth promoting feeder cells expressed varying levels of the surface molecules B7-1 and B7-2. This did however not seem to influence their feeder capacity despite the fact that expanding CD4 T cells were shown to express high levels of CD28, which costimulates growth when bound by B7. Expanding CD4 T cells also expressed the inhibitory molecule PD-1, and it was revealed that expression of its ligand, PD-L1, was induced in B-LCLs when used as feeder cells along with PBMCs. Whether this influences the efficiency of an expansion culture is yet to be determined.

1 Introduction

All living species are at risk of being infected by diseases, which may cause illness or even death. Over the course of evolution, most species have therefore developed advanced systems to protect against disease. Specialized cells, tissues and molecules together make up the immune system, whose role is to recognize infectious agents and then eradicate them, thereby creating resistance to disease (1). In humans and other jawed vertebrates, the immune system consists of two branches: innate and adaptive immunity (1, 2).

Innate immunity is based on the recognition of molecular structures that are present on microorganisms (1, 2). Detection of these structures leads to an immune response mediated by certain cell types, including different phagocytes, dendritic cells and natural killer cells, as well as by the enzymatic proteins of the complement system that are able to kill microbes. Physical barriers created by epithelial cells are also part of the innate immunity (1).

The adaptive immunity, also called acquired immunity, develops more slowly compared to the innate, but is more specialized (1). This branch of immunity is mediated by T and B lymphocytes, which are cells that express antigen receptors. The antigens can be various types of proteins or other molecules expressed by foreign microbes. Somatic recombination of the genes encoding the antigen receptors in T and B cells leads to a great repertoire of different receptors, and each individual T or B cell clone expresses its own unique receptor. The repertoire of different T and B cells increases with age and as an individual is affected by different diseases throughout their lifetime. This is why this branch of the immune system is called adaptive or acquired (1).

T and B cells play different roles in the immune response against a foreign microbe. Two main types of T cells exist: cytotoxic CD8 T cells that kill virus infected or damaged cells and CD4 helper T cells that help B cells to start secreting antibodies. Antibody is the name of a B cell receptor when it is released into circulation. Circulating antibodies recognize the antigen that they are specific for, which normally leads to destruction of the microbe expressing the antigen. This can for instance happen through phagocytosis by a macrophage (1).

Despite the fact that the immune system is a remarkable part of our bodies that protects us from an array of infectious diseases, it can also play against our advantage in some cases. Alloimmunity is an immune response to antigens from the same species, but a different

individual. In some rare cases this can happen during pregnancy, where the mother's immune system attacks an antigen expressed in the fetus. This is the case with fetal and neonatal alloimmune thrombocytopenia (FNAIT).

1.1 Fetal and neonatal alloimmune thrombocytopenia

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a condition that can affect the fetus or neonate of a woman who has developed antibodies specific for the platelets of the child (3).The antibodies cross the placenta during pregnancy and bind to the fetal platelets, causing their destruction, which then results in thrombocytopenia (deficiency in platelets) in the fetus (4). Since platelets play an essential role in blood clotting, thrombocytopenia can cause bleedings in the fetus, with the most severe and feared consequence being intracranial hemorrhage and possible death (4). Severe FNAIT is defined as a platelet count of $<20 \times 10^9$ per liter of blood in the infant, and this occurs at a frequency of 1 in 1000 to 2000 births (4).

In most cases of FNAIT, the maternal antibodies bind to an epitope on integrin β3 on the platelet surface, which is known as human platelet antigen 1 (HPA-1) (3). The epitope is defined as a single amino acid difference at residue 33 of integrin β3. The most common variant is to have a leucine at this position, which is called HPA-1a, and 98% of Caucasians carry this variant (4). Another variant, called HPA-1b, has a proline at position 33. About 2 % of women are homozygous for the proline variant (HPA-1bb) and if these women become pregnant with a fetus that has inherited the HPA-1a variant from their father, they are in danger of developing antibodies against the fetal platelets (4). This is because the maternal immune system perceives the fetal platelets as a foreign invader. There exists other HPA genes that can also lead to development of FNAIT, but the HPA-1 incompatibility is certainly the most common in western countries, accounting for approximately 80 % of cases in Caucasians (5). FNAIT caused by HPA-1a is therefore the main focus at the Immunology research group.

1.1.1 Cellular mechanisms involved in FNAIT

Since FNAIT is caused by antibodies, there are certain cellular mechanisms that are likely to be involved in the development of this disease. Antibodies are produced by plasma cells, which have developed from B cells that have recognized foreign antigens. Normally, B cells require help from activated CD4 helper T cells that have recognized the same antigen to be

activated and become an antibody producing plasma cell. T and B cells are activated separately and then come together in lymphoid follicles (1).

An immune response leading to production of antibodies starts when a foreign microbe, or in this case, a foreign platelet, enters the body. Exactly how this happens in FNAIT is not fully understood. Normally, fetal and maternal blood does not mix during pregnancy (6), so the mother's immune cells would not come in contact with the fetal platelets. However, some have suggested that due to the small size of platelets, they may be able to leak into the maternal circulation (7). Another possibility is that the immunization occurs as a consequence of fetal-maternal bleeding during birth, which would only lead to FNAIT in a subsequent pregnancy (7, 8). Yet another possible mechanism of immunization includes exposure of the antigen, HPA-1a on integrin β3, to the maternal immune system by other cell types than platelets. Integrin β3 has in fact been shown to also be expressed by trophoblast cells in the placenta, which have developed from fetal cells, but are in contact with the maternal blood circulation (6). Spermatozoa have also been shown to express integrin β3 and may cause antigen exposure before pregnancy (7, 8).

Regardless of how the mother's immune system comes in contact with the HPA-1a antigen, this contact starts an immune response. Dendritic cells are a type of immune cells that express various membrane receptors that they use to recognize foreign microbes, or platelets in this case, and when they do, they ingest the foreign agent by endocytosis (1). The dendritic cells are so-called professional antigen presenting cells (APC), which break down the ingested material to fragments that are presented on the surface of the cell. Antigens from extracellular microbes or cells that have been endocytosed are presented on major histocompatibility complex class II (MHC II) molecules. At this point the dendritic cells simultaneously start to migrate to nearby lymph nodes (1).

Naïve T cells continuously circulate through the peripheral lymphoid organs in our body, and when they enter a lymph node, they may encounter a dendritic cell that presents antigen on MHC II molecules. Helper T cells express the coreceptor CD4, which binds to MHC II. In addition, each T cell has its own T cell receptor (TCR) with a certain specificity that can recognize a specific foreign antigen. If a T cell recognizes antigen presented by a dendritic cell it can become activated (1).

Naïve B cells, like T cells, also express receptors that can recognize antigen. The receptors are molecules called immunoglobulins (Ig), which when bound to the surface of the cell function as B cell receptors (BCR). There exists different classes of immunoglobulins, but the ones

that function as receptors are of class M and D (IgM and IgD). The majority of B cells are follicular B cells, which circulate through the follicles of lymphoid organs. There, they may recognize antigens that have been transported to the follicles. Recognition of antigen, along with some other signals, initiates an activation process in the B cell, which leads to proliferation and differentiation. Protein antigens make B cells more able to interact with T lymphocytes. Protein antigens are also endocytosed and displayed on MHC II by the B cells, which migrate into the areas of the lymphoid organs where helper T cells reside. The helper T cells are then able to recognize the antigen presented by the B cell if they have previously been activated by a dendritic cell presenting the same antigen (Figure 1). The activated CD4 helper T cells express CD40 ligand, which can bind to CD40 on the B cells. This interaction stimulates proliferation and antibody production as well as isotype switching, which is a process where the B cells can start to produce other classes of immunoglobulins than IgM and IgD. IgG is the immunoglobulin class that is associated with phagocytosis of foreign agents by neutrophils and macrophages. IgG antibodies also have the special ability to bind to a receptor expressed in the placenta called the neonatal Fc receptor (FcRn), which makes it possible for IgG antibodies specific for HPA-1a to be transported from maternal blood to a fetus during pregnancy (1, 9). Production of IgG antibodies is stimulated by the cytokine interferon-γ (IFNγ), which is typically secreted by a subclass of helper T cells called T helper 1 (T_H1) cells. Antibody producing B cells can eventually mature into fully differentiated plasma cells that can survive for years and secrete antibodies while circulating through the body.

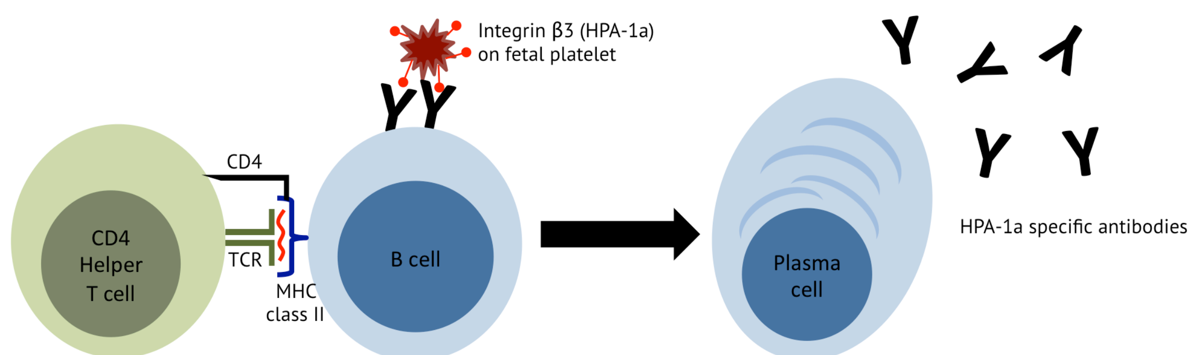


Figure 1 A CD4 helper T cell that has been activated by an APC presenting the HPA-1a antigen encounters a B cell that has recognized HPA-1a on a fetal platelet, endocytosed the platelet and is presenting the HPA-1a antigen on MHC class II. This leads to activation of the B cell, which differentiates into a plasma cell secreting HPA-1a specific antibodies that may cross the placenta, move into the fetal circulation and cause destruction of platelets.

1.2 Human leukocyte antigen

Interestingly, not all HPA-1bb women who become pregnant with HPA-1a positive children develop the platelet reactive antibodies. In most cases, other aspects also seem to play important roles, such as the MHC genes of the mother. The gene complex that contains the genes encoding MHC molecules is called the human leukocyte antigen (HLA) complex (1). The genes encoding MHC II molecules are called *HLA-DP, HLA-DQ*, and *HLA-DR*. Each individual carries one pair of HLA-DP genes (DPA1 and DPB1), one pair of HLA-DQ genes (DQA1 and DQB1), one HLA-DRα gene (DRA1) and one or two HLA-DRβ genes (DRB1 and DRB 3, 4 or 5) on each chromosome (1). The genes on both chromosomes, one inherited from the mother and one from the father, are expressed equally. There exists numerous different variants (alleles) of each of the HLA genes, which leads to great variability in the HLA gene complex among different individuals. The different alleles have numeric names that are used to describe the HLA complex of a certain individual (1).

It has been shown that over 90 % of the women who produce anti-HPA-1a antibodies carry the *HLA-DRB3*01:01* allele, whereas this allele is only present in less than 30 % of the general population (4, 10). The *HLA-DRB3*01:01* allele together with *HLA-DRA* encode the MHC II molecule HLA-DR52a (9). It is believed that the CD4 T cells involved in the production of antibodies in FNAIT are restricted to only recognizing peptides presented on the HLA-DR52a MHC molecule. Each MHC molecule has a peptide binding groove, where the peptide antigens that they present are attached and only peptides that fit in this groove can be presented (1). Since there is such a strong connection between the *HLA-DRB3*01:01* allele and the production of antibodies in FNAIT, the general belief is that the leucine present in position 33 in integrin β3 on the fetal platelets must be present in the peptide that is presented to the CD4 T cells (9).

In an article from 1997 it was shown that a peptide derived from integrin β3 containing the leucine at position 33 could bind to the HLA-DR52a MHC molecule, whereas a peptide with proline in this position that was otherwise identical was not able to bind (11). The same article suggested that the leucine in position 33 works as an anchor residue for binding to HLA-DR52a along with two other amino acids. When leucine is exchanged for a proline and the peptide is no longer able to bind HLA-DR52a, it is believed that this is because the hydrophobic residue of leucine can function as an anchor residue, but the polar proline residue cannot (9).

1.3 Immune checkpoint molecules

In addition to the recognition of an MHC-peptide complex, other interactions can occur between a T cell and an APC. These interactions can be either stimulatory or inhibitory, and a balanced expression of these molecules is needed to maintain self-tolerance and prevent autoimmunity (12). The receptors and ligands involved in these types of interactions are called immune checkpoint molecules.

1.3.1 Costimulation

During the activation of a naïve T cell the recognition of peptide presented on MHC is referred to as signal 1, but a second signal is also required for activation. Signal 2, also referred to as costimulation, usually involves interaction of the surface marker CD28, expressed by the vast majority of T cells, with the ligand B7-1 (CD80) or B7-2 (CD86), which is expressed by APCs (1). The expression of B7 is upregulated in APCs when they encounter microbes or other foreign cells. When activated by antigen and costimulators, the transcription of genes encoding interleukin-2 (IL-2) and IL-2-receptor components starts in T cells. The main function of IL-2 is to stimulate survival and proliferation of T cells (1).

1.3.2 Inhibitory checkpoint molecules

Interestingly, B7-1 and B7-2 are stronger ligands for a receptor called *cytotoxic T lymphocyte-associated protein 4* (CTLA4) than for CD28. CTLA4 negatively effects T cell responses, but is not constitutively expressed (1, 13, 14). CTLA4 is an inhibitory checkpoint molecule. Expression of CTLA4 on the surface of T cells is tightly regulated and it is practically only expressed by fully activated T cells, memory and regulatory cells (14).

Several other inhibitory checkpoint molecules exist and another example is *programmed cell death protein 1* (PD1) (12, 13). PD1 is structurally similar to CTLA4 (15), and when it is engaged, the cell cycle is arrested and production of IL-2 is significantly reduced, leading to inhibition of proliferation. Resting T cells do not express PD1, but activation of T cells through the TCR or with other stimulants results in PD1 expression (13, 15). PD1 has two ligands: PD-L1 and PD-L2, which both inhibit proliferation but have different expression patterns. PD-L1 is mainly expressed by various antigen presenting cells, but not constitutively. Resting B cells do not express PD-L1, but its expression can be induced by activation through the BCR (13, 15). Expression of PD-L2 on the other hand is quite restricted and is not associated with expression by B cells, but it can be expressed by dendritic cells (16, 17). Interestingly, both T and B cells are able to express both PD1 and PD-L1 (13).

Other examples of immune checkpoint molecules expressed by T cells include *lymphocyte-activation gene 3* (LAG3) and *T cell immunoglobulin and mucin-domain containing 3* (TIM3), which bind to MHC and *galectin-9* (GAL9) on APCs respectively (12). Figure 2 summarizes some of the most important immune checkpoint molecules.

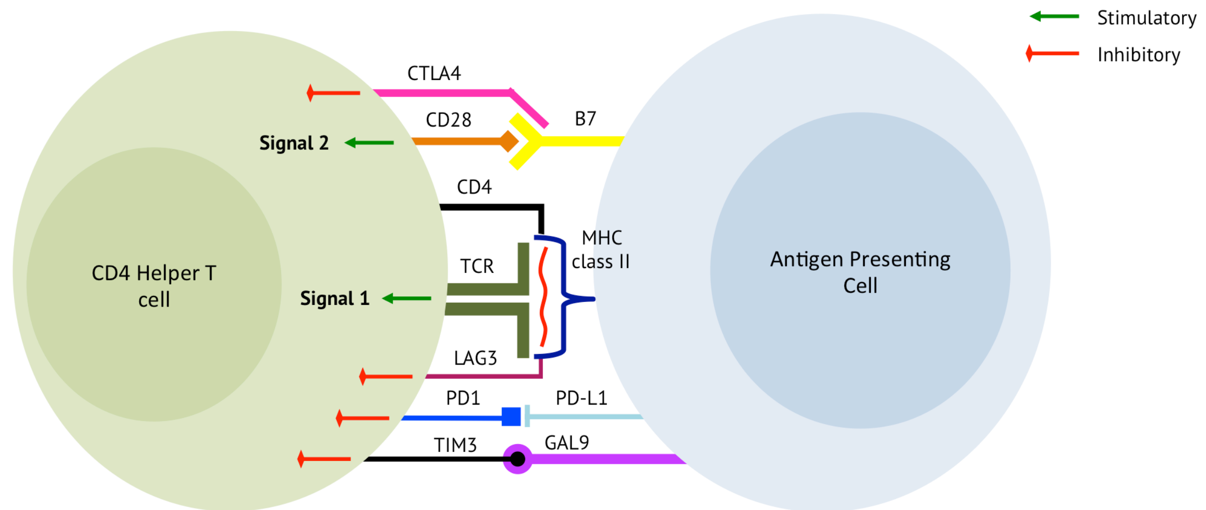


Figure 2 Recognition of a peptide antigen presented on MHC class II by an antigen presenting cell is the first signal needed for activation of a CD4 T cell. Interaction of B7 with CD28 can provide the second signal needed for activation. B7 can also be bound by CTLA4, which has an inhibitory effect on the T cell. LAG3, PD1 and TIM3 inhibit T cell proliferation if bound by their ligands MHC II, PD-L1 and GAL9 respectively.

1.3.3 Immune checkpoint molecules in cancer

Since the discovery of inhibitory checkpoint molecules and their ligands, it has been found that several cancer cells can express these ligands and engage the corresponding receptors on T cells. In this way, the cancer cells inhibit the proliferation of tumor infiltrating T cells and thereby resist their immune response (12). This knowledge has again led to the development of new cancer treatments that block these types of interactions between cancer cells and T cells. The blockade can either be accomplished by administering antibodies to the cancer that block the receptors on the T cells, so that they cannot be engaged, or the genes encoding the receptors can be knocked out for instance by the use of CRISPR/Cas gene-editing (12, 18).

The first immune checkpoint receptor to be clinically targeted was CTLA4 (12). Promising results were obtained with CTLA4 specific antibodies and one such drug has been approved by the US Food and Drug Administration for treatment of advanced melanoma (12). However, blockade of the interaction between PD1 and PD-L1 seems to have an even more

promising effects (12). Antibodies targeting both PD1 and PD-L1 are continuously being tested, and PD1 knockout T cells have been generated and proven to show increased T cell effector function (12, 19). Cancer immunotherapy directed towards PD1 or PD-L1 blockade is a research area that is currently receiving enormous amounts of attention.

1.3.4 Anergy

In the absence of a costimulatory signal, naïve T cells do not become activated. Interestingly, absence of this interaction is however not a neutral event, but can have a negative effect on T cell survival. A T cell that recognizes antigen presented on MHC, but does not simultaneously receive a costimulatory signal may enter a state of unresponsiveness called anergy (20). T cell anergy is a state where T cells maintain their morphology, but their ability to produce IL-2 is repressed, consequently preventing the cells from proliferating. Their ability to proliferate must however also be affected in another way as exogenous IL-2 can not prevent anergy (20). If anergic cells again are exposed to antigen as well as a costimulatory signal, they still do not respond. It is however important to note that the anergic state has been reversed in vitro by stimulating the cells with high concentrations of IL-2 (20).

1.3.5 Immunosenescence

Even if a cell does not become anergic during its lifetime, it will still eventually lose its proliferative capacities as it ages. Since lymphocytes have an extremely high replicative rate, it may be due to loss of telomerase activity, which is an enzyme that elongates the DNA to protect it from shortening during replication (21). Other signs of aging in CD4 T cells include low production of IL-2, low response to IL-2, high response to IL-15 and loss of expression of CD28. When T cells recognize antigen, CD28 is downregulated quickly, but then returns to normal. However, with sustained stimulation over time, the expression of CD28 is decreased and may be lost. These cells are called $CD28^{null}$ cells and they tend to accumulate in elderly individuals (21). Despite the fact that CD28 is lost, the cells remain functionally active and are resistant to apoptosis (21).

1.4 Culturing of T cells in vitro

All of the cellular interactions involving different immune cells that have been described until now are important to know about to be able to understand how the immune system functions inside the body, but they are just as important to consider when culturing immune cells in

vitro. Still, there are many mechanisms and interactions involved in the culturing of T cells that are being explored.

1.4.1 Restimulation and the role of CD28

As previously explained, the initial activation of a T cell happens when it encounters an APC presenting the antigen that it is specific for. This leads to a massive expansion of the T cell. After the immune response is over, most of the T cells involved in it die (1). Some of the T cells do however differentiate into long-lived memory cells which do not have any effector properties any more, but can be reactivated and again expand to high numbers of effector cells. This happens if the memory cell encounters the same antigen again. This process is called restimulation (1).

When culturing CD4 T cells in vitro, they can be expanded by subjecting them to a false restimulation. A frequently used way of doing this is not to expose the cells to antigen, but instead stimulate them with anti-CD3 antibodies. The antibodies bind to the TCR-CD3 complex and activate the signaling pathways that are normally initiated by antigen recognition by the TCR, which in turn leads to proliferation (22).

Whether or not the second signal, binding of CD28 to B7, which is required in the initial activation of a naïve T cell, is necessary for restimulation is unclear. For a long time, the general opinion was that CD28 plays no role in restimulation of T cells (23, 24). However, more recent research has shown that it probably does play a role after all (14, 25). A study from 2006 showed that when CD28 costimulation was blocked in memory T cells by an antibody binding to B7, their production of IL-2 and TNFα decreased (25). A study from 2009 by a different group, which looked at CD8 T cells, found that when CD28 costimulation was blocked, the re-expansion of memory cells was significantly reduced (14). The same study also showed that memory CD8 T cells that were generated in the absence of CD28 stimulation were only able to expand approximately 9 times, whereas memory cells generated with CD28 stimulation expanded more than 40 times (14). Because of this, many groups use anti-CD28 antibodies in addition to anti-CD3 when expanding T cells in vitro (22, 26).

1.4.2 Using PHA as stimulant in expansion cultures

An optional way of expanding T cell in vitro is to use stimulatory lectins, such as the plant lectin phytohemagglutinin (PHA) instead of anti-CD3. PHA does not specifically bind to only CD3, but is able to bind to many cell membrane glycoproteins, including the TCR-CD3 complex (27). This induces an activation response which leads to proliferation. PHA has been

reported as a more potent stimulant than soluble anti-CD3 (22, 27). It is however important to note that as anti-CD3 only leads to activation of the TCR this provides a more physiologically relevant response than PHA, which unspecifically activates several different pathways in T cells (22). PHA is not able to activate any other cell types than T cells (28).

1.4.3 Feeder cells

When expanding T cells, it is common to culture them along with so-called feeder cells. These can for instance consist of peripheral blood mononuclear cells (PBMCs) and B lymphoblastoid cell lines (B-LCLs) that have been exposed to irradiation and thereby have lost their ability to grow and proliferate, but remain viable and bioactive (29). This means that they are able to provide important survival signals for the T cells at the same time as they create a dense cell layer, which promotes growth of the T cells (29). Using feeder cells to expand another cell type is a widely used method within many different fields. Different cell types can be used as feeder cells and their proliferation can be inhibited using different methods (29).

The first use of feeder cells to promote the growth of single cell clones was reported in 1955 by Puck and Marcus (30). They used x-irradiated HeLa cells as feeder cells to grow other clones of HeLa cells. The irradiated HeLa cells did not seem to undergo any change during the first 48-72 hours, but then started to degenerate and form debris. They found that the non-irradiated cells grew steadily and formed colonies whereas the irradiated cells did not. Puck and Marcus concluded that this method could be used to form colonies from almost all single HeLa cells and that it could probably be used on other cell types as well. They did however not know exactly what the growth promoting factor was (30).

Using irradiated PBMCs and B-LCLs as feeder cells when expanding T cell clones is a method that is routinely used at the Immunology research group for the expansion of CD4 T cell clones and it has worked well for many years. However, the exact mechanisms by which these cells promote growth of the T cells are not known. Exactly what happens to the PBMCs and B-LCLs when they are irradiated is not known either. The general opinion is that irradiation creates double stranded breaks in the DNA, which inhibits replication, but still leaves the cell metabolically active for a while (30, 31). In this state, the feeder cells can release growth factors to the culture medium and express various ligands that help the T cells to proliferate (31, 32).

A population of PBMCs consists of several different cell types, including B cells. Knowing this, it would perhaps seem unnecessary to use B-LCLs in addition to PBMCs as feeder cells. However, in the early 1980's, a research group from the Netherlands performed some experiments to test various factors that could play a role in the efficiency of CD8 T cell clone expansion (33). They used irradiated PBMCs and B-LCLs as feeder cells. They found, among other things, that using PBMCs and B-LCLs together as feeder cells led to significantly better growth of the CD8 T cells than by the use of only one of the feeder cell types alone. The explanation for this observation was however not found. They also found that different B-LCLs had varying abilities to promote growth of the CD8 T cells. Some of their B-LCLs led to significantly better growth of the T cells and when these B-LCLs were combined in one expansion reaction, the growth was even better (33).

One way that feeder cells stimulate growth of other cells is that they express stimulatory ligands that can interact with the growing cells (32, 34). In the case of T cells cultured with B-LCL feeder cells, one such interaction could be between B7 on the B-LCL and CD28 on the T cells. However, it is important to note that if the B-LCLs express B7, this could also bind to CTLA4 and have an inhibitory effect on T cells if they are expressing CTLA4. There may also be several other negative interactions that can occur in such a co-culture system. Other inhibitory checkpoint molecules besides CTLA4 may also be involved.

1.5 Current research and aims of the study

The Immunology research group has been conducting research on FNAIT and the cellular processes involved in the disease for many years (4, 10, 35-38). In 2008, they managed to isolate the first HPA-1a-specific T cells from an HPA-1a-alloimmunized woman and prove that these cells became activated upon stimulation with HPA-1a (37). Several other HPA-1a-specific T cells were subsequently isolated and expanded in vitro to use in experiments (10).

To be able to conduct research on HPA-1a specific CD4 T cell clones, the Immunology research group is dependent on keeping these cells in long-term cultures. The T cell clones need to be expanded to high numbers so that the same cell line can be used in multiple experiments. This should be accomplished in a way that does not alter the characteristics of the specific T cell line. The current protocol for this type of expansion involves culturing the T cells with growth inhibited feeder cells and anti-CD3. In addition, the growth promoting cytokines IL-2 and IL-15 are added.

However, during the early work with this project, it was discovered that some of the established CD4 T cell lines would not grow as well as they previously had. It became harder to expand these cell lines using the protocol that previously had worked very well.

With this in mind, the primary objective of this study became to investigate the culturing conditions that the CD4 T cell lines normally are grown under to try to understand what may be the cause of the reduced proliferation.

Secondary objectives included:

- Comparison of the stimulatory effects of anti-CD3, PHA and different feeder cells
- Characterization of B-LCLs used as feeder cells and of CD4 T cells in expansion cultures
- Attempted isolation and establishment of new HPA-1a specific T cell lines for the possibility to study fresh clones

2 Methods and Materials

2.1 Materials and reagents

All mediums, buffers and reagents used during this study are listed in Table 1. Fluorophore-conjugated antibodies are listed in Table 2.

Table 1 Mediums, buffers and reagents used in this study

Name	Distributor	Usage
Phosphate Buffered Saline (PBS)	Medicago (Uppsala, Sweden)	Washing
PBS + 0.2% Bovine Serum Albumin (PBSA 0.2%)		Washing, Flow cytometry
Iscove's Modified Dulbecco Medium (IMDM)	Lonza (Basel, Switzerland)	Cell culture
Fetal Bovine Serum (FBS)	Thermo Fisher (Waltham, MA)	Cell culture
Penicillin-Streptomycin	Sigma (St. Louis, MO)	Cell culture
IL-2	PreproTech (London, UK)	Cell culture
IL-15	PreproTech	Cell culture
CFSE, CellTrace	Thermo Fisher	Flow cytometry, FACS
Dimethyl Sulfoxide (DMSO)	Wak-Chemie Medical GmbH (Steinbach, Germany)	Cryopreservation
Lymphoprep^{TM}	Axis-Shield, Dundee, Great Britain	PBMC isolation
Anti-CD3	Thermo Fisher	Cell culture
PHA (R30852801)	Thermo Fisher	Cell culture
UltraComp eBeads	Thermo Fisher	Flow cytometry

Table 2 Complete list of fluorescently labeled antibodies used in this study

Target	Conjugate	Distributor	Reference	Amount (µl) used in 100 µl reaction	Usage
CD4	V500	Becton Dickinson	860768	2.5	Flow cytometry, FACS
CD25	PE	Becton Dickinson	555432	10	FACS
HLA-DR	APC	BioLegend (San Diego, CA)	307610	1	Flow cytometry
PD1	PE	Biolegend	329906	2	Flow cytometry
CD274	eFluor 450	Thermo Fisher	48-5983-42	2	Flow cytometry
CD86	R-PE	Thermo Fisher	MHCD8604	0.75	Flow cytometry
CD80	FITC	Invitrogen	11-0809-42	2	Flow cytometry
CD28	PE-Cy7	Thermo Fisher	25-0289-42	2	Flow cytometry
CTLA4	APC	Becton Dickinson	555855	2	Flow cytometry

Some experiments used peptides for specific activation of T cells. These were obtained from Eurogentec (Liège, Belgium) and are listed in Table 3. The peptides were from integrin β3, with either a leucine or a proline in position 33 as is the case on HPA-1a and HPA-1b platelets respectively. LolP1 was used as a control peptide as it is known to bind to the *HLA-DRA/DRB3*01:01* molecule and has the same anchor residues as the HPA-1a peptide but is otherwise different (11). The peptides were dissolved in 60% ethanol and 40% water to 88 µM an stored at –20°C.

Table 3 Peptide antigens used in this study

Antigen	Peptide	Amino acid sequence*	Length
HPA-1a	L33 (integrin β3 19-38, Leu33)	VSPMCAWCSDEALPLGSPRC	20-mer
HPA-1b	P33 (integrin β3 19-38, Pro33)	VSPMCAWCSDEALPPGSPRC	20-mer
Rye Grass Pollen	LolP1 (191-210)	ESWGAVWRIDTPDKLTGPFT	20-mer

*Underlined residues represent anchor residues for the *HLA-DRA/DRB3*01:01* MHC II molecule

2.2 Cells

The study was approved by the Regional Committee for Medical Research Ethics, North-Norway (approval no. P REK NORD 66/2005).

Technical blood products from healthy volunteers consenting to their blood being used for research purposes were obtained from the Blood Bank at the University Hospital of North Norway (not requiring REK approval).

2.2.1 T cells

The HPA-1a specific CD4 T cell clone D8T106 that was used during this study had been previously isolated and cryopreserved at the Immunology research group (10).

Some experiments used a mix of CD4 T cells from PBMCs, isolated by positive selection magnetic-activated cell sorting (MACS). Previously isolated PBMCs from anonymous donors (method described later) were stained with CD4 microbeads (130-045-101, Miltenyi Biotec, Bergisch Gladbach, Germany) and isolation was carried out according to the manufacturer's protocol except that PBSA 0.2 % was used instead of the recommended buffer.

2.2.2 B LCLs

A panel of eight different B-LCLs were thawed for use during this project, both as feeder cells and as APCs (Table 4). These are B cell lines that are able to process and present antigen, and that have been transfected with Epstein-Barr virus (EBV), which makes them immortal (39).

The majority of the B-LCLs used are in-house cell lines, isolated from HPA-1a immunized women. This means that these cells are all HPA-1a negative, which makes them suited to use as APCs in experiments with HPA-1a specific T cells since HPA-1a positive APCs could lead to activation of sensitive clones (10). All the in-house cell lines also carry the *HLA DRB3*01:01* allele, which, as previously explained, is greatly associated with FNAIT (9). Two of the B-LCLs are obtained from the International Histocompatibility Working Group (IHWG, Seattle, WA). The STEINLIN cell line also carries the *HLA DRB3*01:01* allele, and is in fact homozygous for it. STEINLIN is the only B-LCL that is HPA-1a positive whereas EMJ is the only B-LCL that does not carry the *HLA DRB3*01:01* allele.

When B-LCLs were used as APCs they first had to be pulsed with peptide. This was performed by incubating the cells with the peptide at the desired concentration for approximately 3 hours and then irradiating them.

Table 4 All B-LCLs used during this project

Name	Origin	HLA-DRB3
D4BL4	In-house	01:01
D18BL	In-house	01:01
D8BL7	In-house	01:01
D8BL8	In-house	01:01
D48BL4	In-house	01:01
D48BL6	In-house	01:01
EMJ	IHW no. 9097	03:01
STEINLIN	IHW no. 9087	01:01

2.3 Cryopreservation

Cryopreservation is a method that enables the storing of cells for several years without having to keep them in culture. The cells are suspended in a protective medium and then cooled to very low temperatures and later stored in liquid nitrogen tanks holding –196°C. At any time the cells can be thawed again and used in experiments.

To prepare cells for cryopreservation, they were spun down and resuspended in freeze medium consisting of 90 % FBS and 10 % DMSO. The concentration of cells per ml varied, but was usually between 5 and 10 x 10^6. Cryo vials were then filled with 1.5 ml cell suspension and transferred to a pre-cooled Mr. Frosty freezing container (Sigma). The container was put in –70°C and the vials were later transferred to liquid nitrogen tanks.

Thawing of cryopreserved cells was done by immediately transferring the tubes from the nitrogen tank to a water bath holding approximately 50 °C and keeping them there until only a small clump of solid ice remained. This clump was thawed by finger warming the tubes and then the cells were immediately transferred to a 15 ml conical vial containing 10 ml cool culture medium. The cells were spun down once and then resuspended in fresh medium.

2.4 Cell culturing

All cells were cultured in IMDM completed with 10% FBS and 1% penicillin + streptomycin. The medium used for the T cells, when alone or in expansion cultures, was also provided with

3% human serum from HPA-1a negative donors. The cells were kept in culture flasks or plates at 37°C with 7.5% CO_2. Since the B-LCLs are EBV-transformed, they grow autonomously and do not need to be stimulated to grow, but they were regularly split and provided with fresh medium. T cell clones had to be stimulated in expansion cultures to make them proliferate.

The human serum was prepared from plasma from HPA-1a negative donors provided by the blood bank. $CaCl_2$ was added to the plasma at a 1:100 ratio and the plasma was put at 37°C over night. The next day, the liquid that had not formed clumps was transferred to tubes and centrifuged for 15 minutes at 3000 g. The supernatant contained the serum, which was filtered and transferred to a new tube. The serum was then heat-inactivated by putting it in a water bath holding 56°C for 25 minutes. The serum was kept at –70°C and thawed when needed for preparation of medium.

2.5 Expansion of T cell lines

T cells were expanded with feeder cells and soluble anti-CD3. The expansion cultures were normally kept in 24-well plates, starting with approximately 500 000 T cells per well. Each well was supplied with feeder cells consisting of approximately 4 x 10^6 irradiated PBMCs and 1 x 10^6 irradiated B-LCLs. The PBMCs had previously been isolated as described below. Each well was filled to 2 ml with medium containing anti-CD3 to make the final concentration in each well equal to 30 ng/ml. The following day IL-2 was added to the T cells at a concentration of 50 units/ml. This was repeated every other or every third day.

After 10-14 days, the dead feeder cells were removed by resuspending all cells in 5 ml complete medium and then layering the cell suspension over 5 ml Lymphoprep™ in a 15 ml conical tube. The tube was centrifuged at 800 g for 8 minutes with minimal acceleration and no brakes. The cell layer visible between the Lymphoprep™ and medium contained the live cells and was transferred to a new tube. They were washed two or three times in 0.2% PBSA or medium before they could be cultured further or used in experiments.

2.5.1 Expansion with PHA

In some expansion cultures PHA was used as stimulant instead of anti-CD3. The protocol for this was obtained from colleagues in Amsterdam. These expansion cultures were also kept in 24-well plates with 2 ml per well, and the feeder cells consisted of irradiated PBMCs and B-

LCLs, but at a concentration of 1 x 10^6 and 100 000 cells per well respectively. Between 200 000 and 300 000 T cells were added to each well. The medium used was the same as for the anti-CD3 expansion, but with added PHA at a concentration of 0.8 µg/ml instead of anti-CD3. IL-2 was added to the cultures the next day at a concentration of 120 units/ml and then again approximately every third day.

2.5.2 Expansion in smaller volumes

In some experiments 48- or 96-well plates were used for expansion of T cells, either following the anti-CD3 or the PHA protocol. If 48-well plates were used for expansion, all cell numbers were halved and the volume per well was 1 ml. In flat-bottom 96-well plates, the cell numbers were divided by 6.25 and the volume in each well was 200 µl.

2.6 Isolation of PBMCs

Blood buffy coats were obtained from the blood bank. 50 ml conical tubes were filled with 12 ml Lymphoprep™ and approximately 25 ml buffy coat, diluted 1:1 in PBS, was carefully layered on top. The tubes were then centrifuged for 25 minutes at 800 g with no brakes and minimal acceleration. The PBMCs, which were visible as a thin white layer on top of the Lymphoprep™ (see Figure 3), were transferred to a new 50 ml conical vial by the use of a Pasteur pipette. The PBMC suspension was then diluted approximately 1:4 with PBS and centrifuged at 250 g for 10 minutes. The supernatant was removed and the cells were washed twice in 0.2% PBSA, again centrifuging at 250 g for 10 minutes. The cells could then be resuspended in medium.

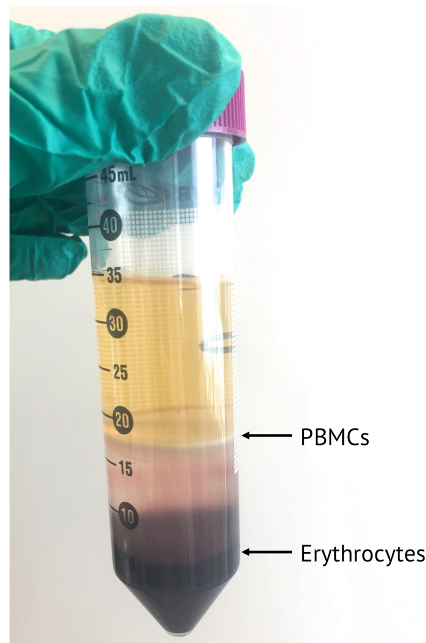


Figure 3: When diluted buffy coat is layered on top of Lymphoprep™ and then centrifuged at 800 g for 25 minutes, the erythrocytes concentrate at the bottom, below the Lymphoprep™, while the PBMCs are visible as a thin white layer on top of the Lymphoprep™ and can be transferred to a new tube.

2.7 Flow cytometry and fluorescent staining of cells

Flow cytometry is a method that allows for rapid analysis of a high number of cells. This is achieved by passing one cell at a time through one or more lasers. The amount of light that then scatters from the cells is detected and says something about the characteristics of the cells (40). The amount of forward scattered light (FSC) is proportional to the size of the cell, and side scattered light (SSC) is proportional to the complexity. In addition, staining cells with fluorescently labeled antibodies specific for surface markers of interest allows for detection of cells that have these markers. In these cases, the cells are passed through lasers that are able to excite the fluorescent dyes, and the emitted light is detected by specific detectors. The intensity of the fluorescence from a cell is proportional to the level of expression of the studied surface marker. The median fluorescence intensity (MFI) in a sample of cells can be used to compare different samples with each other. Some flow cytometers can be used to perform fluorescence-activated cell sorting (FACS), where cells can be sorted out into tubes based on the properties analyzed by the flow cytometer.

In this study, flow cytometric analyzes were performed on a BD LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA). Cell sorting was performed on a BD FACSAria III (Becton Dickinson). The data was analyzed in FlowJo® v10 (Becton Dickinson).

To stain cells with fluorescent antibodies, cells suspended in medium or 0.2% PBSA were mixed with one or more antibodies at the appropriate dilution (see Table 2). The cells were then put on ice in the dark for 25 minutes. The cells were washed once with 0.2% PBSA and then resuspended in 0.2% PBSA at an appropriate volume for flow cytometric analysis, normally 200-500 µl. To allow for compensation of fluorochromes with overlapping emission spectra where no positive control was available, UltraComp eBeads (Thermo Fisher) were used according to the manufacturers protocol.

2.8 CFSE staining and proliferation assays

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that was used to study cell proliferation. Cells stained with CFSE distribute their dye equally when they divide, which is something that can be measured with flow cytometry to monitor proliferation within a population of cells that have been stimulated in some way. CFSE staining can also be used to distinguish certain cells from others in a culture.

To prepare for staining, the cells were washed once in 0.2% PBSA and then resuspended in 500 µl 0.1% PBSA (prepared by mixing PBS and 0.2% PBSA 1:1). 2 µl CFSE (250 µg/ml, diluted in DMSO) was added and the cells were put in a water bath holding 37°C for 10 minutes. Approximately 5 ml cold medium was then added to the cells to stop the staining process and the cells were put on ice for a few minutes. The cells were washed twice with 0.2 % PBSA and once with medium.

Proliferation assays are a type of assay where cells are stained with CFSE before they are stimulated to proliferate. As the cells divide, the CFSE dye is equally distributed between the two daughter cells (41). When these cells are analyzed by flow cytometry, the cells that have divided once, twice or more times can be recognized as they appear at distinct points in the CFSE histogram (Figure 4). With careful dyeing and monitoring, up to 10 separate division cycles can be identified (42).

In this study, the results from proliferation assays were analyzed by calculating the proliferation score (PS). The PS is calculated from the MFI (CFSE) in the culture of stimulated cells as well as in a control well of unstimulated cells by using the following equation: $MFI_{stimulated} = MFI_{nonstimulated}/2^{PS}$. The PS was invented by members of the Immunology research group, and it is defined as the median number of division of stimulated T cells relative to a culture of unstimulated cells (37).

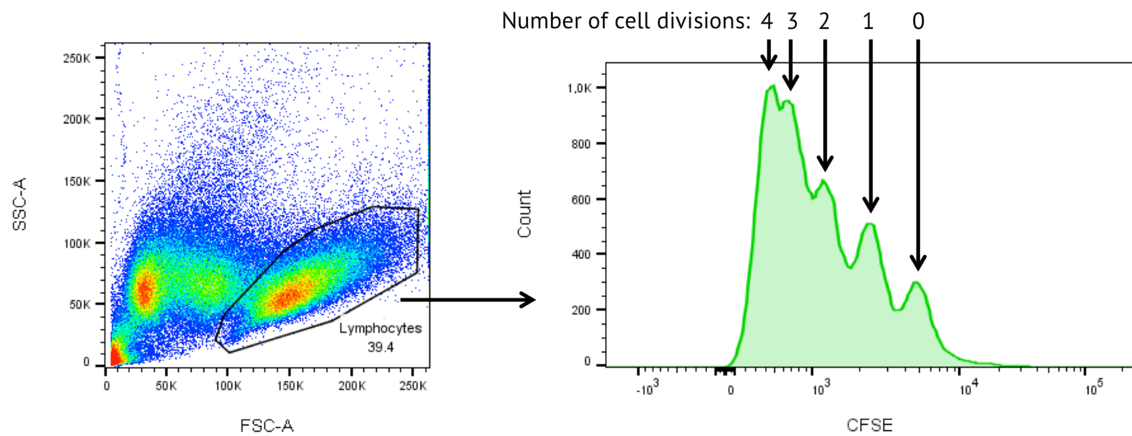


Figure 4 When cells are stained with CFSE and then stimulated to proliferate, it's possible to identify which cells have divided a certain amount of times by looking at the peaks in the histogram showing CFSE intensity. In this example, CD4 T cells were stained with CFSE and then expanded in culture with irradiated feeder cells. The CFSE histogram shows the cells in the lymphocyte gate, where one can easily distinguish the cells that have divided 1, 2, 3 or 4 times although the peak to the very left also includes unstained lymphocytes in the feeder population.

2.9 Isolation of HPA-1a specific T cell lines

Isolation of single HPA-1a specific T cell clones has successfully been achieved at the Immunology research group before (37). The cells are isolated from blood samples taken from HPA-1a immunized women. In this project, a sample of PBMCs were used that had been isolated from a blood sample taken from an immunized woman (Donor 9) one month before a planned cesarean section. The cells had been cryopreserved since then.

The PBMCs were thawed and rested over night. The next day, the cells were stained with anti-CD4 (V500) and anti-CD25 (PE) antibodies. Instead of 0.2% PBSA, they were washed and resuspended in a protective medium consisting of IMDM with 30% FBS and 1% Penicillin+Streptomycin. FACS was performed to sort all cells that were $CD4^+$ and $CD25^{intermediate}$ into a single tube. These represent CD4 cells that have become activated, as they express CD25, but are not regulatory (as these express higher levels of CD25). These cells were then stained with CFSE and stimulated with peptide antigen. 1/3 of the cells were given the P33 peptide and 2/3 of the cells were given L33, both at a concentration of 5 µM.

After 8 days, the cells were resuspended in the same protective medium to prepare them for sorting. The P33 stimulated cells were used as a control to compare with the L33 stimulated cells. Proliferating cells, as determined by decreased CFSE intensity, were single sorted out from the L33 stimulated cells into wells on a round-bottom 96-well plate. 240 cells in total were sorted. The wells had previously been filled with 100 µl culture medium. After sorting,

each well was also provided with 100 µl feeder cell mix with anti-CD3 so that each well received 10 000 irradiated PBMCs and 1000 irradiated B-LCLs. The concentration of anti-CD3 was 30 ng/ml. The following day, each well was given IL-2 at a concentration of 50 units/ml. This was repeated every 2-3 days. If the single sorted cells proceeded to forming colonies, these were moved to larger wells on a new culture plate.

3 Results

3.1 Established HPA-1a specific CD4 T cell clones demonstrated poor ability to proliferate

The preliminary experiments performed during this study used different HPA-1a specific CD4 T cell clones previously established at the Immunology research group. These cell lines had previously been expanded and cryopreserved in batches. Proliferation assays with peptide pulsed B-LCLs were performed to confirm that the cells had maintained their specificity. The cells did somewhat respond to stimulation with L33 pulsed B-LCLs, which triggered a higher degree of proliferation than did B-LCLs pulsed with the control peptide LolP1. However, during these first experiments, it was observed that the cells did not proliferate as much as they had done in previous experiments where they had been stimulated the same way. Furthermore, when expanding these cells with anti-CD3 stimulation, they did not grow very well.

Figure 5 shows the amount of proliferation in two batches of the same cell line, D8T106, stimulated with peptide pulsed B-LCLs; either L33 or LolP1. One batch (batch 1) had been expanded extensively and proliferated only slightly more when stimulated with L33 compared to LolP1. The other batch of D8T106 cells (batch 2) had not been expanded quite as many times and proliferated somewhat more, but not as much as would be expected upon this type of stimulation.

Several different established HPA-1a specific CD4 T cell clones showed the same poor proliferation and response to specific stimulation. It was clear that it would be difficult to keep performing experiments on these cell lines if they could not be expanded further. With these findings in mind it was decided that various elements that may affect the efficiency of an expansion culture were to be investigated further in hopes of improving the expansion protocol for future clones.

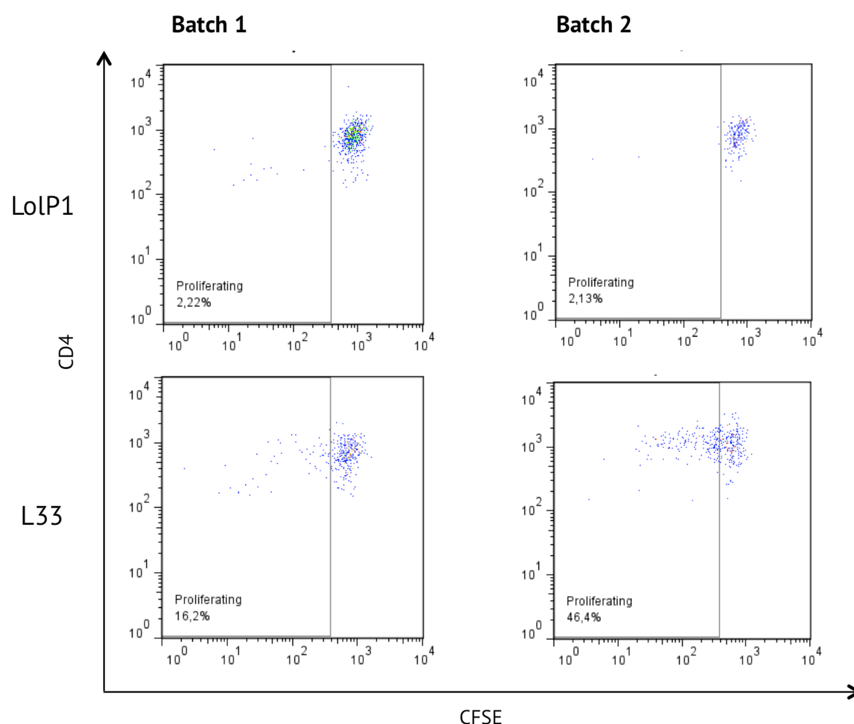
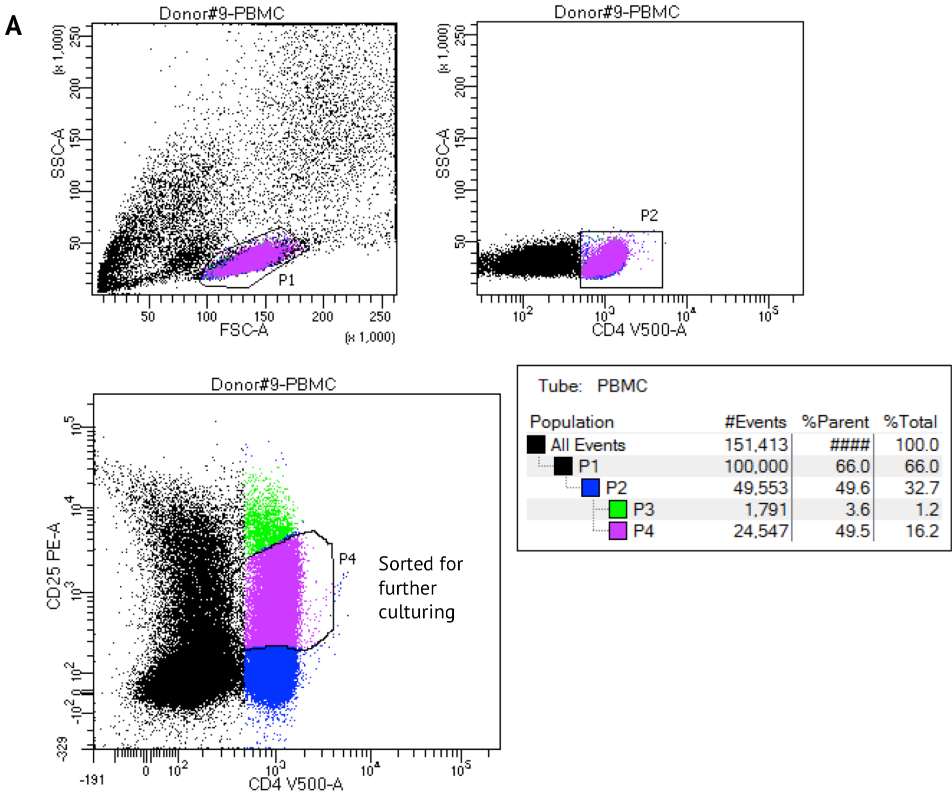


Figure 5 The HPA-1a specific CD4 T cell line D8T106 had been expanded several times since isolation and cryopreserved in different batches. CFSE dyed cells from two batches of these cell lines were stimulated with B-LCLs (D4BL4) pulsed with either L33 or P33 peptide (both at 5 µM). After 5 days, their degree of proliferation, as determined from CFSE intensity, was analyzed by flow cytometry. Batch 1 had been expanded numerous times and showed very little proliferation upon stimulation with L33 pulsed B-LCLs (**A**). Batch 2 had also been expanded several times, but not quite as many and proliferated somewhat more in response to the same stimulation (**B**).

3.2 Attempted isolation of HPA-1a specific CD4 T cell clones was not successful

To gain some insight into what happens to a clonal CD4 T cell line as it is cultured and expanded in vitro, one aim was to isolate fresh clones to compare with the established CD4 T cell clones. In an attempt to isolate new HPA-1a specific CD4 T cell clones, a cryopreserved PBMC sample taken from an HPA-1a immunized woman was thawed and used for sorting of potential new clones as shown in Figure 6. When the initially sorted CD4 T cells were stimulated with peptide there was a significantly higher proportion of proliferating cells among the L33 stimulated cells compared to the P33 stimulated cells, indicating the presence of HPA-1a specific cells. These were single sorted and stimulated to promote proliferation. After approximately 14 days in culture, some of the single sorted cells had started to form visibly larger colonies of cells. After another week, the seven T cell clones that seemed to have grown best were chosen to test for HPA-1a specificity. Unfortunately, none of the clones showed signs of being HPA-1a specific (data not shown). Nevertheless, two of the new cell

lines, named D9T5 and D9T10, which grew well were kept and cultured further so that they could be used as control cells. Such cells are also valuable for future experiments.



Tube: PBMC

Population	#Events	%Parent	%Total
All Events	151,413	####	100.0
P1	100,000	66.0	66.0
P2	49,553	49.6	32.7
P3	1,791	3.6	1.2
P4	24,547	49.5	16.2

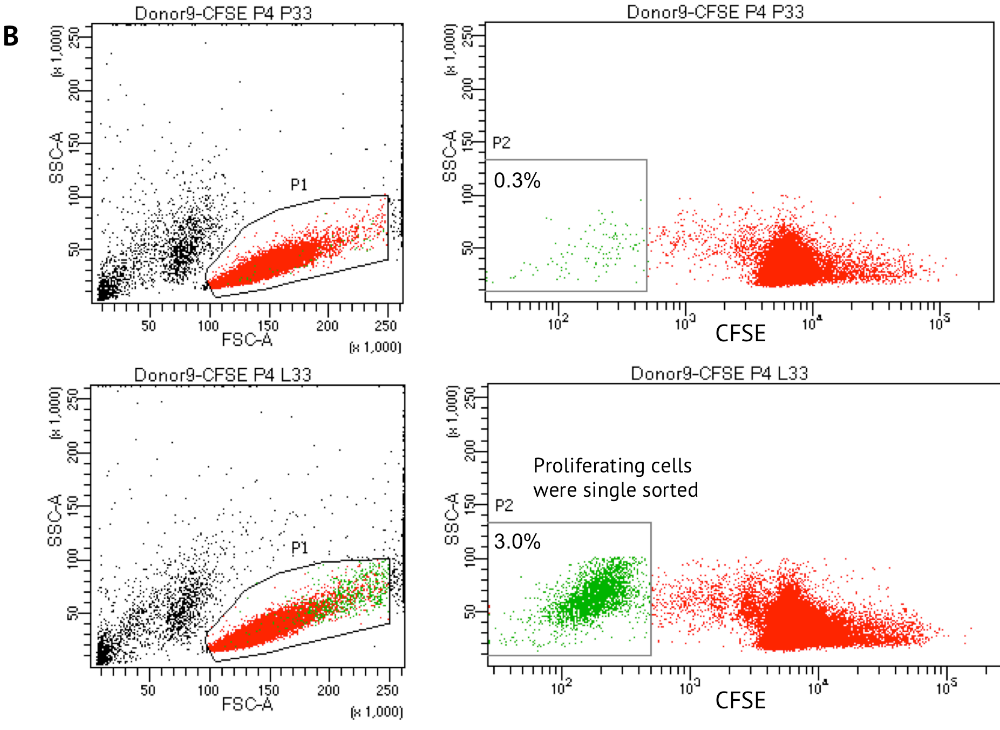


Figure 6 A) A PBMC sample from an HPA-1a immunized woman was stained with anti-CD4 (V500) and anti-CD25 (PE) antibodies. Activated non-regulatory CD4 T cells, as recognized by high CD4 and intermediate CD25 expression levels, were sorted from gate P4 into one tube before they were stained with CFSE, stimulated with peptide (P33 or L33 at 5 µM), and cultured further. **B)** After 8 days in culture, a significantly larger proportion of cells had proliferated (as determined by low CFSE fluorescence intensity) in the L33 stimulated batch compared to the P33 stimulated batch. From the proliferating cells in the L33 stimulated batch (gate P2), 240 cells were single sorted into round-bottom 96-well plates and provided with feeder cells and anti-CD3 to stimulate expansion.

3.3 Expression levels of CD28 vary significantly between different T cell clones

To decide whether the reduced proliferation of D8T106 may be due to loss of CD28, the expression of CD28 was measured in batch 1 of D8T106 used for the proliferation assay previously described. Unfortunately, due to the fact that this experiment was performed months after the previously described proliferation assay, the batch 2 cells were no longer available. CD28 expression was also measured in the newly isolated clones D9T5 and D9T10 in addition to three other already established HPA-1a specific T cell clones (D8T2, D8T104 and D48T1) (Figure 7). The heavily expanded D8T106 cells were indeed found to express none or very low levels of CD28, which indicates that loss of CD28 expression may in fact be the reason for the loss of proliferative abilities by D8T106. The remaining T cell clones expressed comparable levels of CD28 except for D48T1, which did not express CD28. Surprisingly, the newly isolated clone D9T10 expressed somewhat lower levels than the other clones.

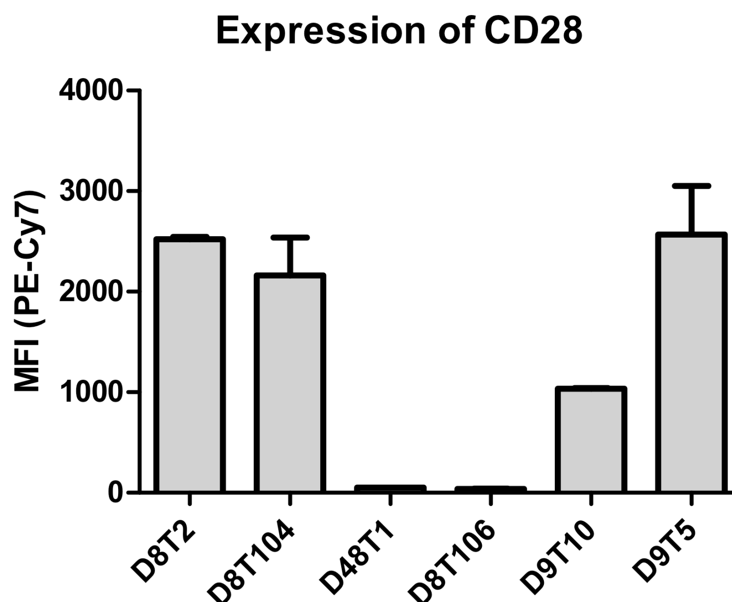


Figure 7 Expression of CD28 was measured in six different clonal CD4 T cell lines, including a heavily expanded batch of D8T106. CD28 expression was detected with a PE-Cy7 conjugated antibody and the figure shows the mean ± SEM MFI (PE-Cy7) of two parallel measurements for each cell line.

3.4 The resting expression levels of MHC II, B7-1 and B7-2 vary between different B-LCLs but are not fixed

To start to understand some of the interactions that may occur between T cells and feeder cells in an expansion culture, the expression levels of a selection of surface molecules present on B-LCLs routinely used as feeder cells were studied. The expression of MHC II (HLA-DR) and B7-2 was measured simultaneously in eight different resting B-LCLs in three separate experiments (Figure 8). Analysis of the results showed that the level of both MHC II and B7-2 expression varied between the different cell lines as well as within the same cell lines when samples were analyzed at different times. Some of the B-LCLs showed consistently high expression levels of both MHC II and B7-2 compared to some of the other cell lines. This included D4BL4, D8BL8 and D18BL. However, the expression levels were not fixed. On the third measurement, the level of B7-2 expression in D4BL4 had more than doubled from the two previous measurements and was more than twice as high as in any of the other cell lines. The first measurement of MHC II expression in D18BL also stood out as very high. D8BL7 consistently measured very low levels of B7-2.

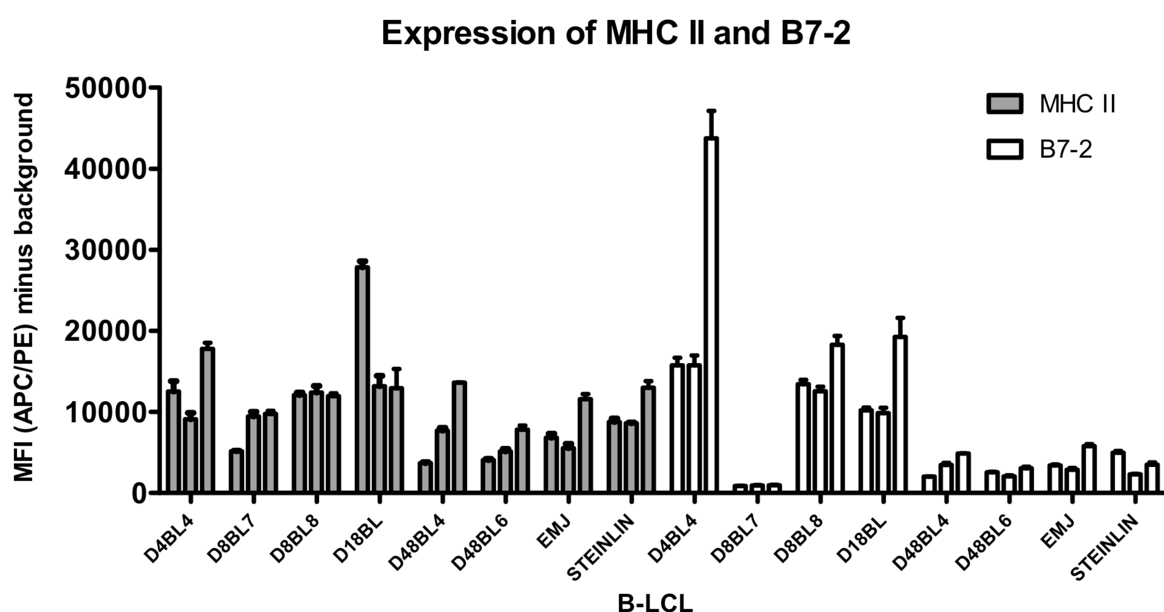


Figure 8 Expression of MHC II (HLA-DR) and B7-2 (CD86) was measured simultaneously by flow cytometry in eight resting B-LCLs. MHC II expression was detected with an APC conjugated antibody and B7-2 with a PE conjugated antibody. The figure shows the mean ± SEM MFI (APC or PE) of stained triplicates minus background (MFI measured in unstained sample).

The expression levels of B7-1 were measured separately for all B-LCLs except STEINLIN (Figure 9). Comparing these results with the B7-2 expression levels, there does seem to be even more variation in the expression of B7-1. It is difficult to point out one or more cell lines that stand out from the others in terms of expressing noticeably higher or lower levels of B7-1. D8BL7, D48BL4 and EMJ all expressed very low levels of B7-1 when the first measurement was performed, but then the expression increased for the subsequent measurements. D8BL8 was the only cell line that seemed to have a relatively stable expression level of B7-1, which it also had for B7-2 and MHC II.

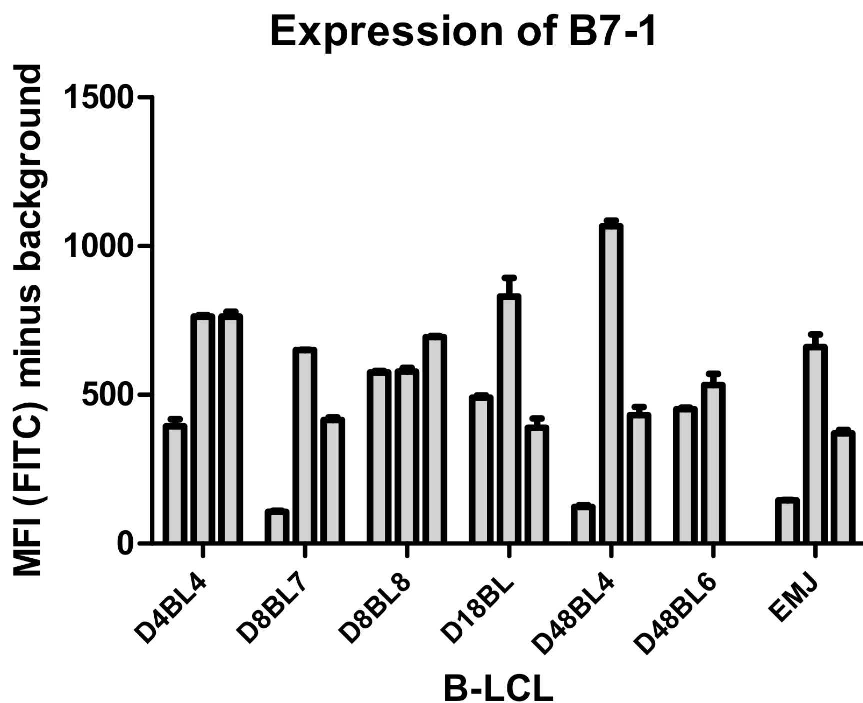


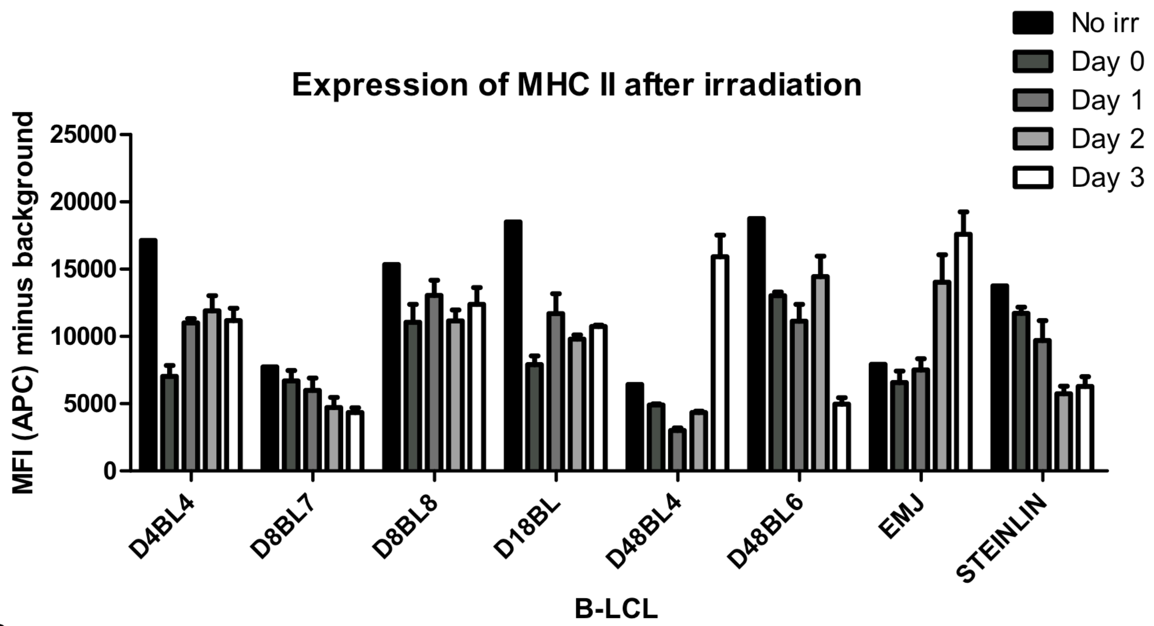
Figure 9 Expression of B7-1 (CD80) was measured by flow cytometry in seven resting B-LCLs. B7-1 expression was detected with a FITC conjugated antibody. Measurements were performed in three separate experiments (only two for D48BL6), and two parallel measurements were performed for each B-LCL during each experiment in addition to an unstained sample. The figure shows the mean ± SEM MFI (FITC) in each sample minus background (MFI measured in unstained sample).

3.5 Irradiation of B-LCLs does not significantly alter the expression of surface molecules

As the B-LCLs are irradiated before they are used as feeder cells, experiments were performed to evaluate the effect of irradiation on B-LCLs. Cells were irradiated and the expression of MHC II and B7-2 was measured again with flow cytometry for four consecutive days. The measurements were performed before irradiation, directly after irradiation, as well as on each of the following three days. Figure 10 shows that within the population of live cells, as determined from the forward versus side scatter plot, the expression levels of MHC II and B7-2 stayed relatively stable. There was some variation in expression levels from day to day, but it did not seem to be more than what was observed in the non-irradiated cells. These levels were also similar to the previously measured levels. Interestingly, for both B7-2 and MHC II and in all eight cell lines, the expression levels decreased between the first measurement before irradiation and the one directly after irradiation. In the following measurements the levels did however increase again in some cell lines, whereas they continued to decrease in others. It seems as though the irradiation may have an immediate effect on the cells, but that the cells that are still metabolically active return to normal and express fluctuating levels of both MHC II and B7-2.

When looking at the forward versus side scatter plots, the approximate percentage of live cells could be determined. Not surprisingly, as is shown in Figure 10C, the proportion of live cells decreased from day to day within all the different cell lines. By day 3, the majority of cells had died in most cell lines.

A



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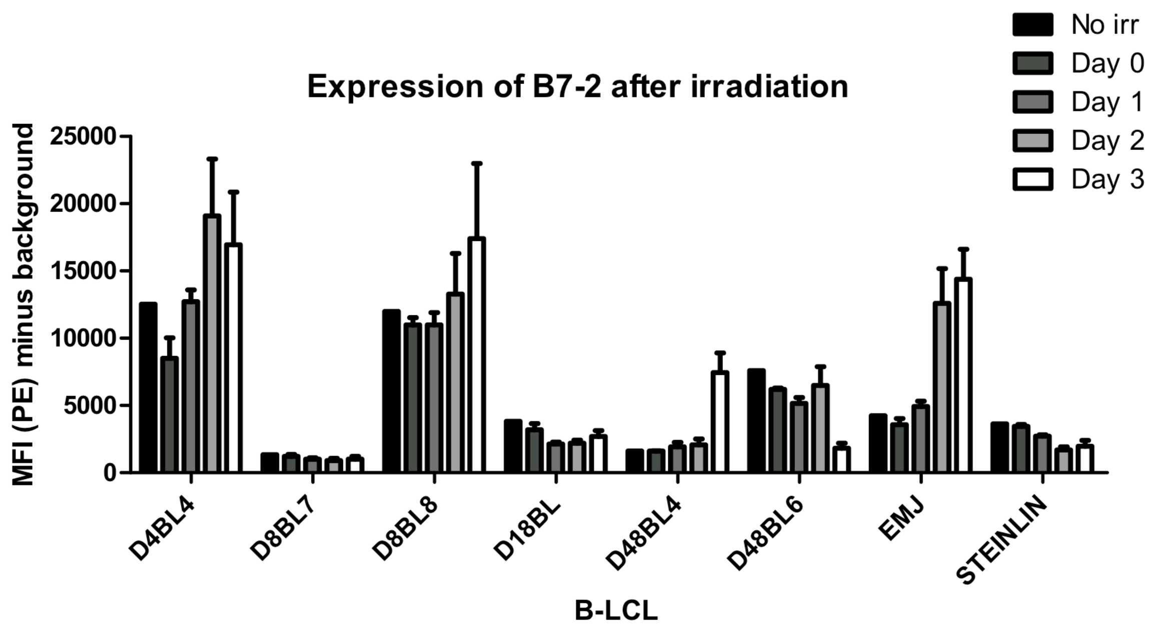


C

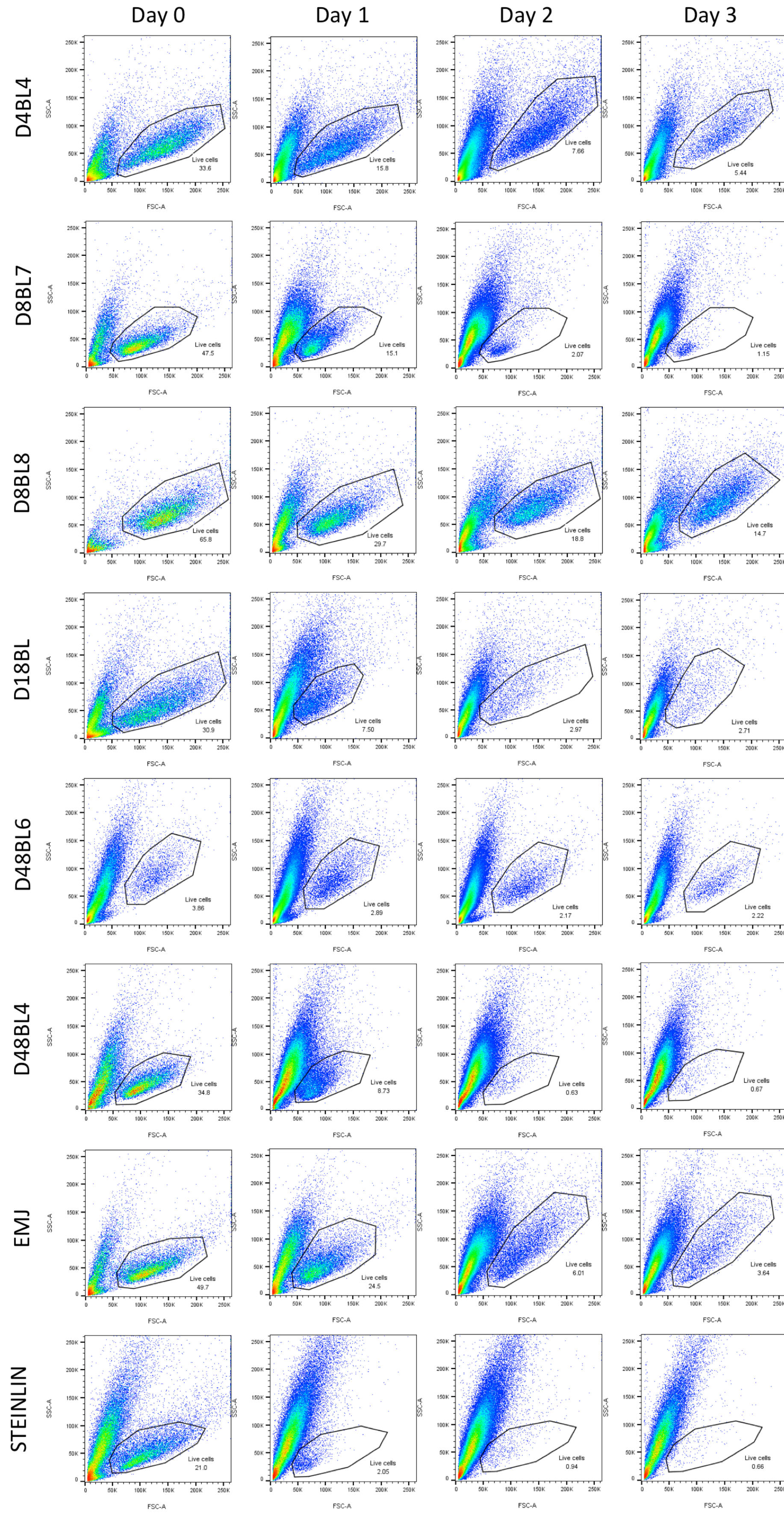


Figure 10 Expression of MHC II (**A**) and B7-2 (**B**) was measured by flow cytometry in eight different B-LCLs before irradiation (no irr), directly after irradiation, and once every day for the following three days. MHC II expression was detected with an APC conjugated antibody and B7-2 with a PE conjugated antibody. Two parallel measurements were performed for each sample and the figure shows the mean ± SEM MFI (APC or PE) in each sample minus background (MFI measured in unstained sample). The measurements used were from the cells in the live cells gate, as visualized in **C**.

3.6 No B-LCL stood out as a more capable feeder cell

Knowing that the expression levels of B7-1 and B7-2 vary between different B-LCLs, it was hypothesized that this could have an effect on their ability to stimulate growth of T cells in an expansion culture. This idea was also based on the fact that other groups had found differences in feeder capacity between different lines of B-LCLs (33).

Normal expansion cultures with CFSE stained CD4 T cells isolated with MACS were set up using all in-house B-LCLs as feeder cells in different wells together with PBMCs. The amount of proliferation was analyzed on days 4 and 8 after initiation of the culture. A shift towards the left in the CFSE histogram indicates that the cells have proliferated more. From Figure 11 A, the amount of proliferation seems to be relatively similar in all wells except for the one with D8BL8, where the cells seemed to have proliferated slightly less than in the others. This can also be seen in Figure 11 B, which shows the proliferation scores. This was observed on both day 4 and day 8. However, in a preliminary experiment that was performed in a similar way to this, D8BL8 did not stand out from the other B-LCLs as a less capable feeder cell (data not shown).

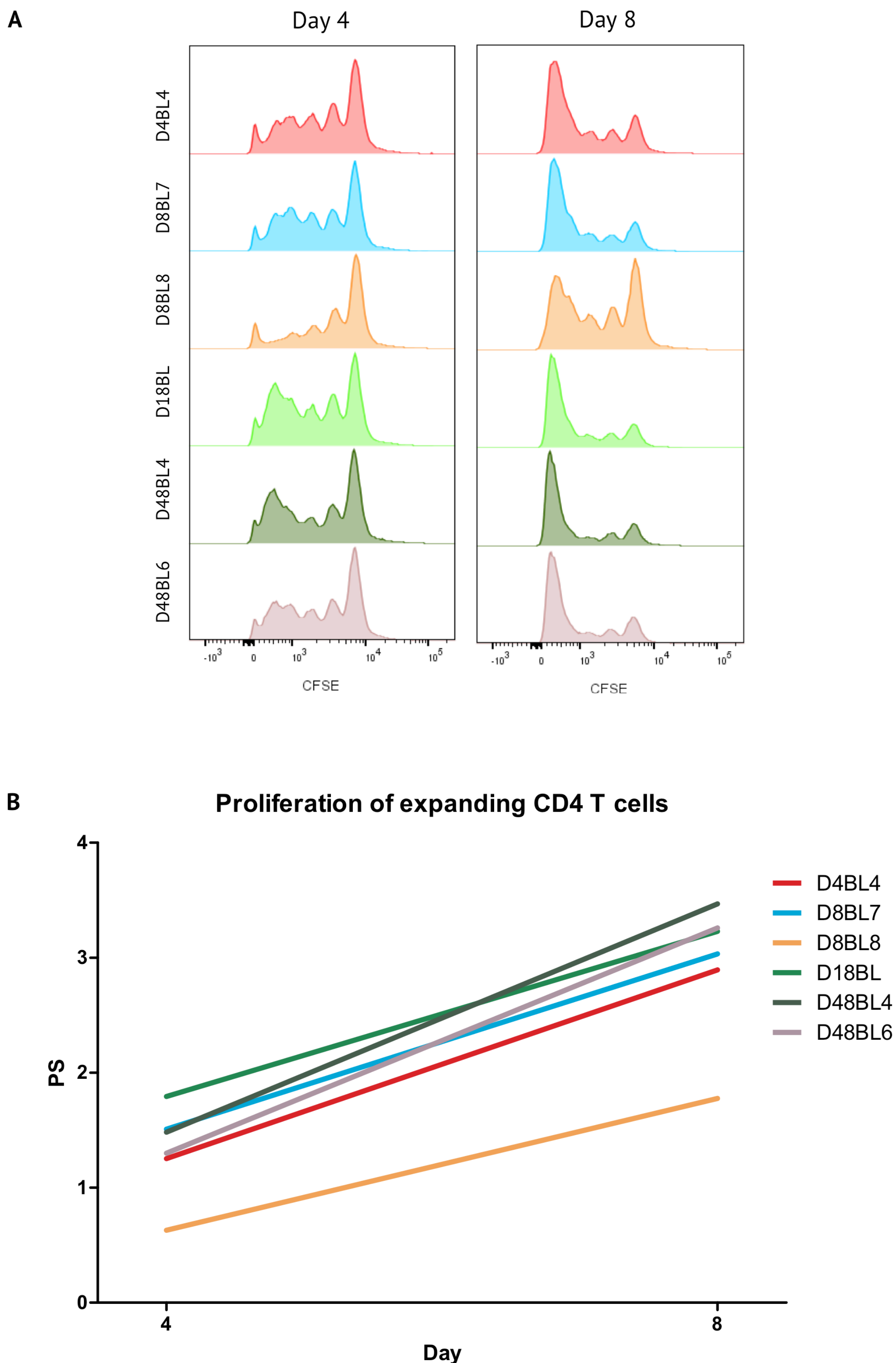
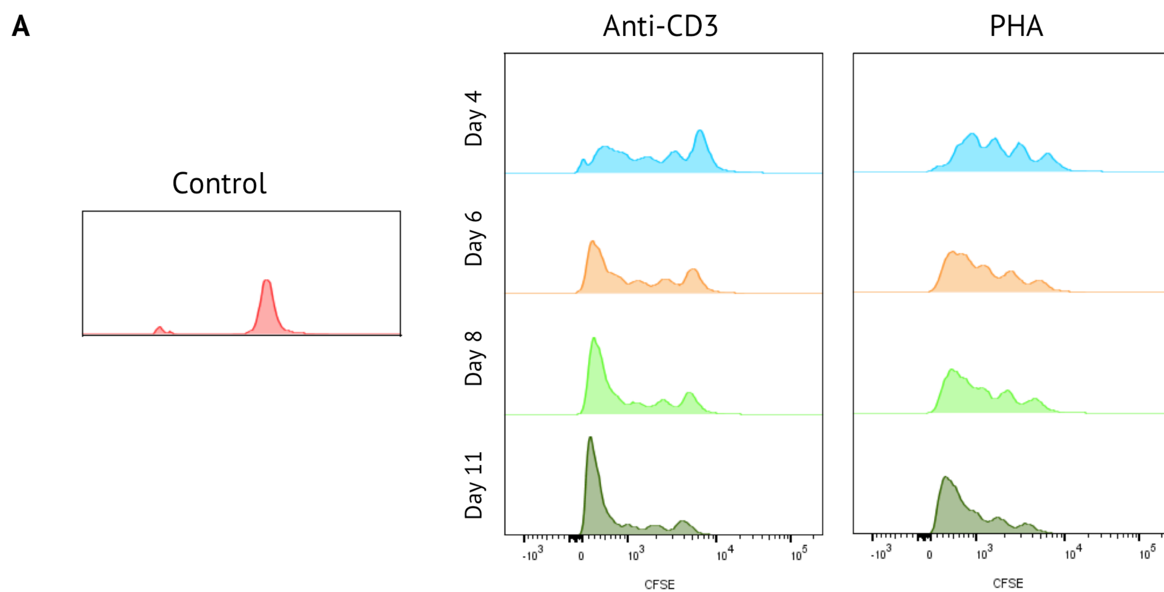


Figure 11 MACS separated CD4 T cells stained with CFSE were expanded in flat-bottom 96-well plates with anti-CD3, irradiated PBMCs and one out of six different irradiated B-LCLs to determine whether any of the B-LCLs stood out as a more capable feeder cell. On days 4 and 8 after initiation of the cultures, cells were taken out and analyzed for proliferation by flow cytometry. The degree of proliferation is visualized in CFSE histograms of the cells in the lymphocyte gate (**A**), and as proliferation scores (PS) calculated from the MFI (CFSE) of the cells in the lymphocyte gate and the MFI (CFSE) of cells that had not divided in each sample (to represent unstimulated cells) (**B**).

3.7 Expansion of CD4 T cells is more effective with anti-CD3 compared to PHA

While considering the factors that may influence the success of an expansion culture, the question was raised as to whether the expansion protocol could be improved by replacing anti-CD3 with another stimulant, such as PHA.

Parallel expansion cultures were set up following either the anti-CD3 or PHA expansion protocol explained in the methods section, but with the same number of CFSE stained CD4 T cells in each well. The CD4 T cells had been isolated with MACS. On days 4, 6, 8 and 11, cells were analyzed for proliferation by flow cytometry. The results are summarized in Figure 12 A and B. The results thus show that it seems like PHA initially has a greater effect on proliferation than anti-CD3, but then there is a shift to more proliferation with anti-CD3. The results from day 4 show that a larger proportion of cells had proliferated with PHA than with anti-CD3. By day 6 the results were more similar and by days 8 and 11 it was clear that in total there had been more proliferation among the anti-CD3 stimulated cells.



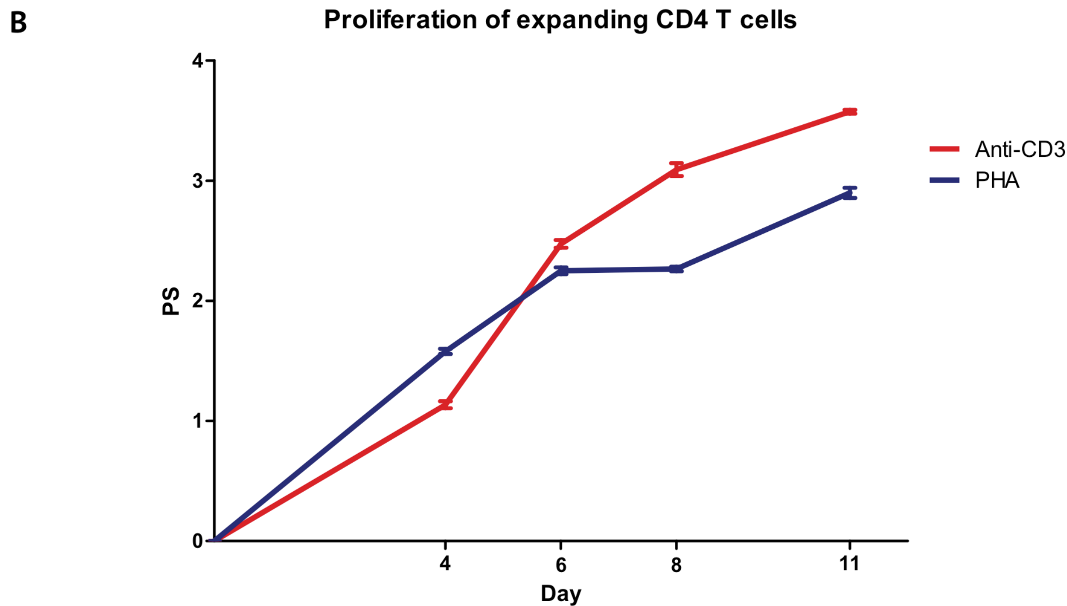


Figure 12 MACS separated CD4 T cells stained with CFSE were expanded in flat-bottom 96-well plates with irradiated feeder cells and either anti-CD3 or PHA. The starting number of CD4 T cells was 80 000 in each well when both the anti-CD3 and PHA protocols were followed. On days 4, 6, 8 and 11 after initiation of the cultures, cells were taken out and analyzed for proliferation by flow cytometry. Control cells consisting of CFSE stained resting CD4 T cells were analyzed on day 4. The degree of proliferation is visualized in CFSE histograms of the cells in the lymphocyte gate (**A**), ans as proliferation scores (PS) calculated from the MFI (CFSE) of the cells in the lymphocyte gate and the MFI (CFSE) of the control cells (**B**). The PS values are based on the mean ± SEM of values obtained from triplicate wells.

3.8 Expanding CD4 T cells express both stimulatory and inhibitory surface molecules

In order to gain some more insight about the interactions that may occur between cells in an expansion culture and to study the effects of stimulation, surface markers present on CD4 T cells were studied. The expression of CD28, CTLA4 and PD1 was measured by flow cytometry in the same CD4 T cells expanded either with the anti-CD3 protocol or with the PHA protocol as described in the previous section. Measurements were performed over several days to determine whether expression levels stayed stable or showed some variation.

Both anti-CD3 and PHA stimulated cells demonstrated a considerable increase in CD28 expression compared to resting cells, which also expressed some CD28. The effect was greatest with PHA, which also seemed to have a longer lasting effect on the cells (see Figure 13). While CD28 expression induced by anti-CD3 gradually decreased and was almost back to resting levels by day 11, expression in PHA stimulated cells stayed high for the entirety of the 11 days. The highest level of CD28 expression was in fact measured on day 11.

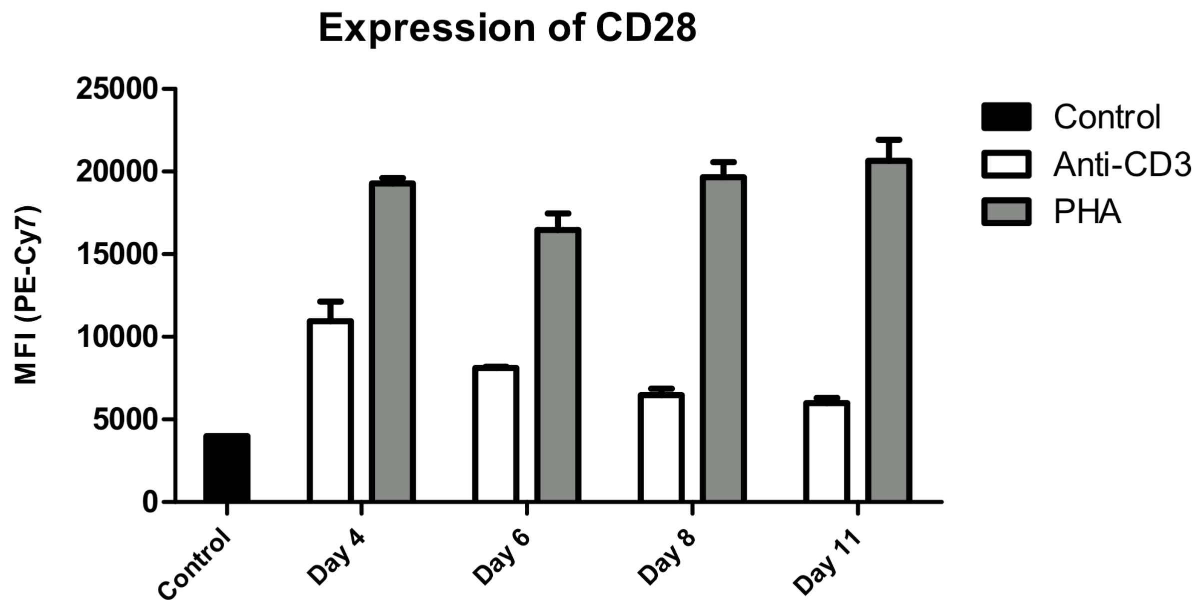


Figure 13 Expression of CD28 was measured by flow cytometry in MACS separated CD4 T cells when stimulated with either anti-CD3 or PHA, as well as irradiated feeder cells, at day 4, 6, 8 and 11 after the stimulation was started. Expression in a control sample consisting of unstimulated CD4 T cells was also measured on day 4. CD28 expression was detected with a PE-Cy7 conjugated antibody. Triplicate measurements were performed for each sample and the figure shows the mean ± SEM MFI (PE-Cy7) for each sample.

Like CD28, expression of PD1 was also increased by stimulation with both anti-CD3 and PHA (see Figure 14). However, in this case the expression in resting cells was close to zero. Again the effect of PHA was greater than that of anti-CD3, and as with CD28, the expression of PD1 gradually decreased after stimulation with anti-CD3. Expression in cells stimulated with PHA also decreased, but not as quickly as in the anti-CD3 stimulated cells. Only by day 11 was there a clear decrease in PD1 expression.

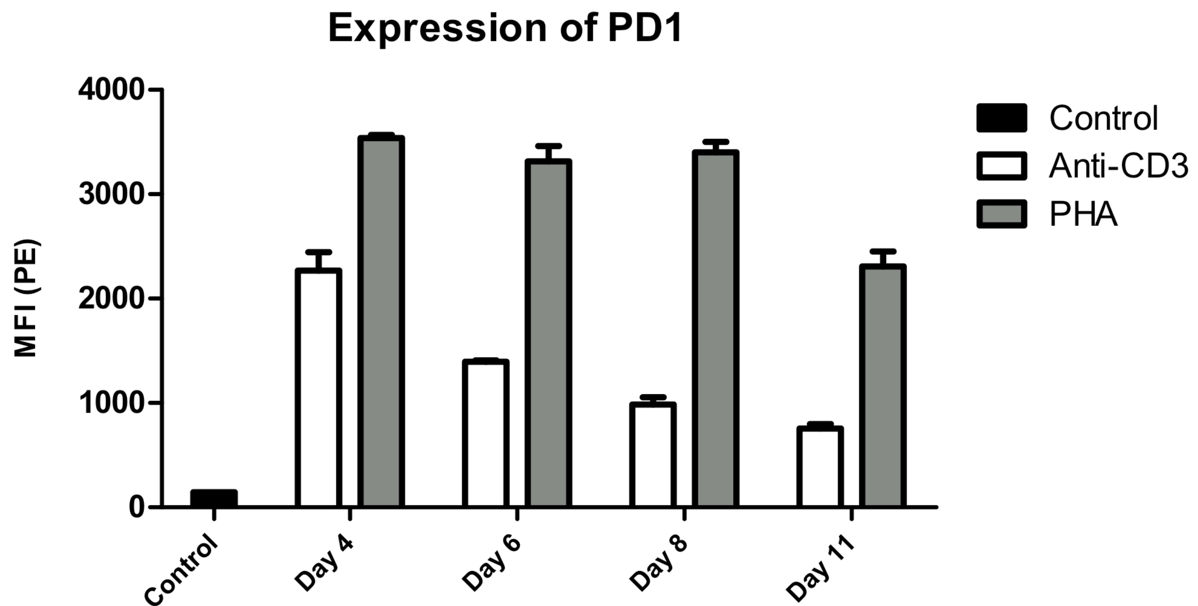


Figure 14 Expression of PD1 was measured by flow cytometry in MACS separated CD4 T cells when stimulated with either anti-CD3 or PHA, as well as irradiated feeder cells, at day 4, 6, 8 and 11 after the stimulation was started. Expression in a control sample consisting of unstimulated CD4 T cells was also measured on day 4. PD1 expression was detected with a PE conjugated antibody. Triplicate measurements were performed for each sample and the figure shows the mean ± SEM MFI (PE) for each sample.

The results from measurements of CTLA4 are not shown as neither anti-CD3 nor PHA stimulated cells seemed to express it at any time points.

3.9 Resting B-LCLs do not express PD-L1

Expression of PD-L1 was initially measured in all eight B-LCLs at the same time as MHC II and B7-2. When these measurements were performed, the cells had not been stimulated or otherwise treated in any way. The results are presented in Figure 15 and show that none of the eight cell lines seemed to express PD-L1 when resting as there was close to no difference in fluorescence intensity between stained and unstained cells. This result also showed that the used antibody produced very little background fluorescence caused by unspecific binding.

It was hypothesized that the presence of CD4 T cells might be able to induce expression of PD-L1 in the B-LCLs. To test this two of the cell lines were cultured with CD4 T cells in a 1:1 ratio for two days before new measurements were performed, but the results were negative (data not shown).

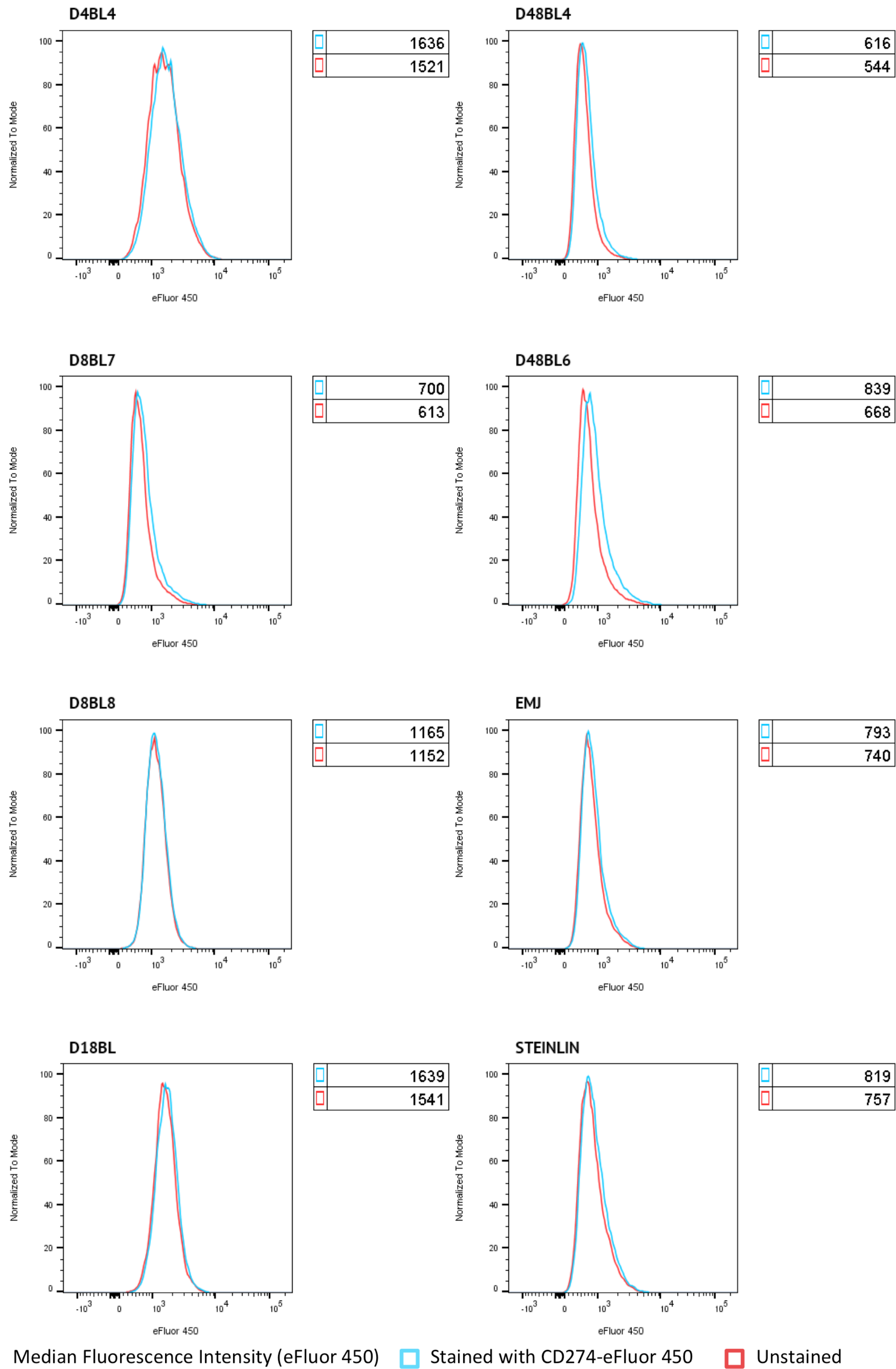


Figure 15 Expression of PD-L1 was measured by flow cytometry in eight different lines of resting B-LCLs stained with an antibody targeting PD-L1 (eFluor 450 conjugated anti-CD274). The figures show histograms of eFluor 450 fluorescence in the live cells gate for both stained and unstained cells. MFI (eFluor 450) values are included for both groups.

3.10 Expression of PD-L1 can be induced in B-LCLs

Despite the fact that the initial results indicated that B-LCLs do not express PD-L1, some more experiments were performed to confirm this result. Surprisingly it was found that expression of PD-L1 was actually induced in some B-LCLs when these were used as feeder cells to expand CD4 T cells. Staining the B-LCLs with CFSE before mixing them with the other cells in an expansion culture made it possible to later distinguish them from the others and measure PD-L1 expression. Figure 16 shows a case where this was performed in an expansion culture set up following the regular anti-CD3 protocol. After three days, both D18BL and D48BL6 seemed to have started expressing PD-L1 as the difference between stained and unstained samples now was noticeably higher than previously. It is worth mentioning that when this experiment was performed, the laser settings in the flow cytometer had been changed from the previous experiment resulting in higher autofluorescence.

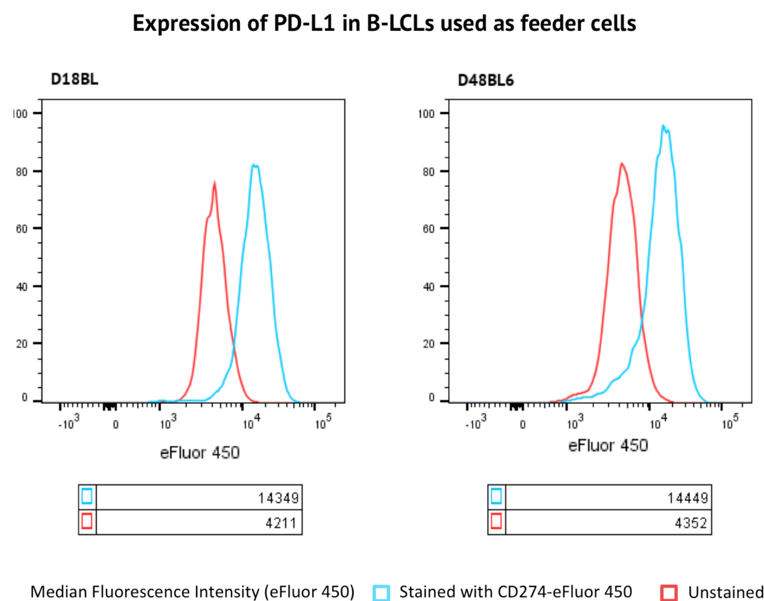


Figure 16 Expression of PD-L1 was measured by flow cytometry in the B-LCLs D18BL and D48BL6 when they were used as feeder cells in an expansion culture. The cells had been stained with CFSE and irradiated before they were mixed with CD4 T cells and irradiated PBMC. After 3 days in culture, cells were resuspended and some were stained with eFluor 450 conjugated anti-CD274 and some were left unstained. The figure shows histograms of eFluor intensity in stained versus unstained cells in the CFSE positive gate of the live cells. Median fluorescence intensity values are included for both groups.

To confirm that what looked like expression was real and not due to the changed flow cytometry settings or any unknown circumstantial factors, the experiment was repeated in a few variations. It was again postulated that it was the CD4 T cells that induced the expression of PD-L1, but that they had to be activated and not resting. To determine whether this was the case, some cultures were prepared with only B-LCLs, CD4 T cells and anti-CD3. Normal expansion cultures were also set up again. Additionally, to get an idea of how soon the B-LCLs started expressing PD-L1, measurements were performed at different time points after initiation of the culture.

Contrary to what was hypothesized, it did not look like it was the activated CD4 T cells that induced the expression of PD-L1. Only the culture that contained PBMCs in addition to B-LCLs and CD4 T cells resulted in a significant increase in PD-L1 expression. Interestingly, not all the B-LCLs present in the culture started expressing PD-L1. Two clear populations could be distinguished in the histogram; one negative and one positive for PD-L1 expression (Figure 17). When PBMCs were present, expression of PD-L1 was observed already after one day. Without PBMCs, no expression was detected even after 3 or 4 days, indicating that one or more cell types present in the PBMC population induced the expression.

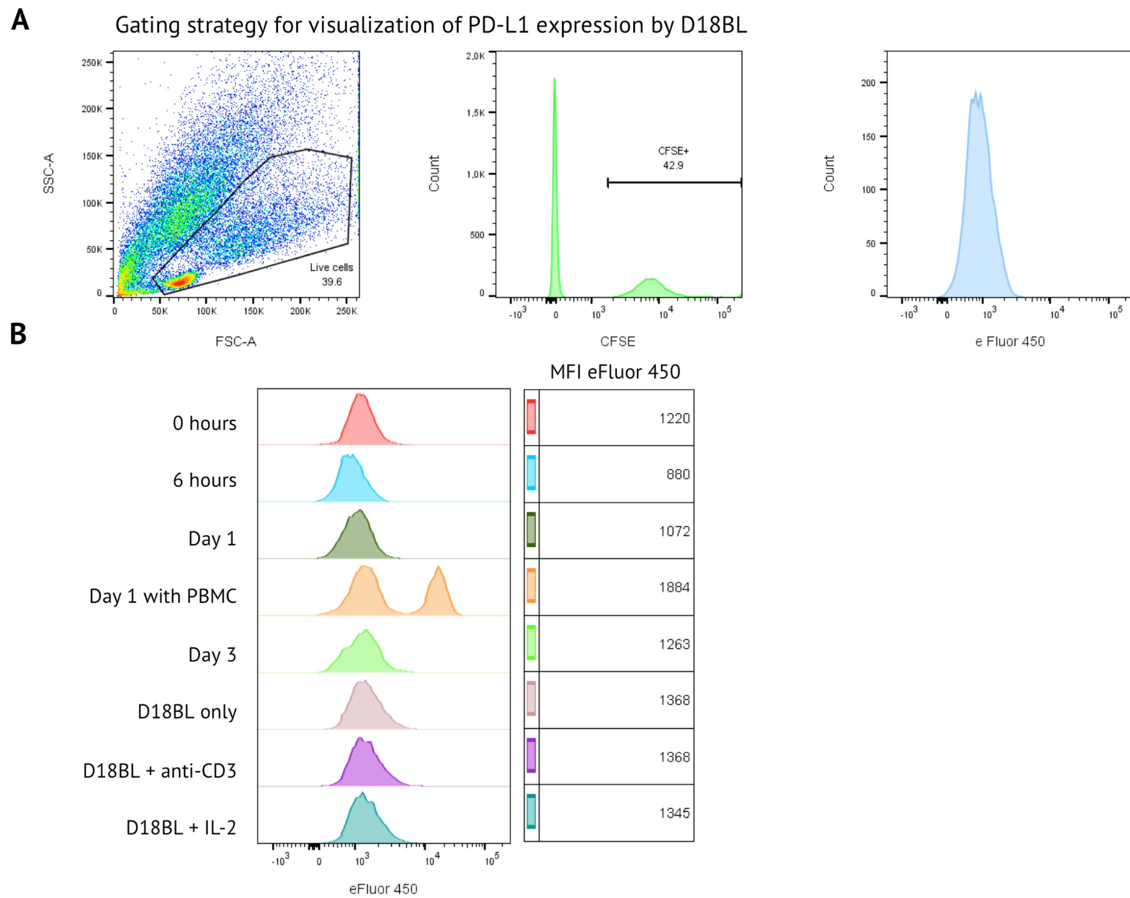


Figure 17 CFSE stained D18BL were irradiated and mixed with different combinations of CD4 T cells, anti-CD3 and IL-2. The cultures were stained with an anti-PD-L1 antibody (eFluor 450) and expression by D18BL was detected by first gating on all live cells and then on CFSE positive cells as shown in **A**. The PD-L1 expression by D18BL in the different culture combinations is visualized in **B** along with the MFI (eFluor 450). The cultures measured after 0 hours, 6 hours and on day 1 all contained CD4 T cells and anti-CD3 in addition to D18BL. The culture with PBMCs contained the same in addition to irradiated PBMCs. The control cultures without CD4 T cells were measured on day 4.

4 Discussion

By performing a variety of different experiments, some important insights about the conditions of a T cell expansion culture have been gained. The interactions that may occur between the T cells and the feeder cells have been studied and several questions have been answered at the same time as many new questions have appeared.

4.1 Optimal culturing and use of CD4 T cell lines

The early results from this project showed that HPA-1a specific T cell lines that had been expanded numerous times had more or less stopped responding to stimulation with specific antigen as well as stimulation with anti-CD3 and IL-2, which normally leads to massive proliferation of all CD4 T cells regardless of specificity. Likely, the reason for the decreased proliferation was that the cells had reached the stage of immunosenescence, which we know can affect T cells in vivo, but also in vitro when they have proliferated extensively (21, 43). As loss of expression of CD28 is one of the hallmarks of T cell immunosenescence (21), it was not surprising to find that the heavily expanded batch of D8T106 cells did not express detectable levels of CD28. As expected, the newly isolated clones did express CD28. Keeping track of CD28 expression by T cell clones could thus be important when culturing T cells in the future. However, it is worth mentioning that the established T8T2 clone, which also expressed close to zero CD28, has since been shown to grow well in expansion cultures. As mentioned in the introduction, immunosenescence could also be a result of for instance decreased telomerase activity.

These first results sparked an interest in studying and possibly improving the culturing methods currently used to expand CD4 T cells at the Immunology research group. Perhaps an improved expansion protocol could help in taking care of future HPA-1a specific CD4 T cell clones. However, it is important to keep in mind that if a single T cell clone is only capable of dividing a certain amount of times before it reaches the stage of immunosenescence, then optimizing the culture conditions for better growth will only lead to this stage sooner. Having a well thought out plan for the specific experiments one wants to perform on a certain T cell clone is essential.

Having experienced that isolation of new HPA-1a specific T cell clones is not easy, that stresses this point even more. The PBMC sample that was used for the attempted isolation of

new HPA-1a specific T cell clones during this project had been obtained from an HPA-1a immunized woman at the end of her pregnancy and should in theory have contained quite many HPA-1a specific CD4 T cells. This also seemed to be the case when comparing the P33 stimulated cells to the L33 stimulated cells, where a significantly higher proportion of cells had proliferated within the latter sample. Still, out of 240 single sorted proliferating cells, only a handful grew to larger colonies and out of these, none responded to stimulation with L33 peptide or L33 pulsed B cells. This makes it very clear that the HPA-1a specific CD4 T cell clones that successfully are isolated are very valuable, that they should be taken well care of, and that one should avoid "wasting" these cells.

The cells are clearly isolated so that one can use them in experimental assays, but many such assays lead to loss of the cells afterwards. When performing assays that for instance involve flow cytometry analysis, the cells cannot be reused after the assays are performed. Perhaps a strategy could be to only use cells that have expanded several rounds already in experiments so as to not "waste" cells that could still proliferate actively and give rise to many more cells. Ideally the cells used in experiments would be cells that are only capable of dividing a few more times, but still have not become immunosenescent. Understandably, it would be difficult to know exactly when the cells are at this stage, but keeping track of how many times a certain cell line has been expanded is certainly a start.

Unfortunately no new HPA-1a specific T cell clones were isolated in this study, but two control cell lines were obtained. We do not know which antigens these cell lines are specific for, but knowing that they do not recognize HPA-1a means that they can be used as negative control cells during future experiments to compare with cell lines that are HPA-1a specific.

4.2 Expression and effect of B7-1 and B7-2 in feeder cells

To start to understand the conditions that may affect the outcome of a CD4 T cell expansion culture, it seemed important to learn more about the cells used as feeder cells and how they may stimulate or possibly inhibit growth. In this study, we chose to focus on the B-LCLs. There is some uncertainty about the exact roles of B-LCLs when used as feeder cells together with PBMCs. As with all feeder cells, one of the main roles is certainly to create a more dense cell layer as we know that most cell types require this for optimized growth (29). Another suggested role of B-LCL feeder cells is that they can provide the T cells with the costimulation that they require for full activation. B7 present on the surface of the B-LCLs

may bind to CD28 on the T cells. There is reason to believe that costimulation can be of importance for expansion of T cells as experiments have been reported where cells stimulated with anti-CD3 and anti-CD28 demonstrated significantly better proliferation than cells treated with only anti-CD3 and IL-2 or PHA and IL-2 (34). Anti-CD3 stimulation alone has also been shown to induce anergy in some T cells (20).

In light of this, it was interesting to find that the levels of B7-1 and B7-2 expression varied quite a lot between different lines of B-LCLs. In particular, there were clear differences in the levels of B7-2 expression, whereas B7-1 expression seemed to fluctuate more. It was suggested that the levels of B7 expression could have an effect on the stimulatory effects of the B-LCLs when used as feeder cells. Whether this effect would be negative or positive was however not clear. As we know, B7-1 and B7-2 are stronger binders of the inhibitory T cell marker CTLA4 than of CD28, so it would be the expression of these on the T cells that would determine the effect of B7. As others have suggested, feeder cells expressing B7-1 and/or B7-2 may actually inhibit proliferation of T cells (34). A report from 1997 presented a method for expanding T cells without feeder cells for the sake of avoiding unwanted interactions (34). Instead of feeder cells, they only provided the T cells with beads coated with anti-CD3 and anti-CD28 antibodies. This method resulted in very efficient growth of the cells. In contrast, when the expression of CD28 and CTLA4 was measured on expanding CD4 T cells in this study it was found that CD28 was significantly up regulated, whereas CTLA4 expression could not be detected. If interaction with B7 on feeder cells plays a role in expansion cultures this would suggest that the B-LCLs expressing the highest levels of B7 would be the best suited feeder cells. One would perhaps expect that D8BL7, which consistently expressed very low levels of B7-2 and relatively low levels of B7-1 would be a worse suited feeder cell than for instance D8BL8 or D4BL4. However, as we saw, there was no clear difference in proliferation of CD4 T cells when expanded with use of different B-LCLs as feeder cells. D8BL8, which expressed high levels of both B7-1 and B7-2 did in fact lead to less proliferation than any of the other cell lines, although that was only observed once. D4BL4, which expressed similarly high levels of B7-1 and B7-2 compared to D8BL8 did not stand out as either a particularly good or bad feeder cell. This seems to indicate that the levels of B7 expression by feeder cells may not be important for their ability to stimulate proliferation. Whether this means that there is no interaction between B7 and CD28 is not certain. It could be that even if the B-LCLs express low levels of B7, they are still high enough to provide the

necessary costimulation. Perhaps because when an expansion culture is started, the number of B-LCLs is usually twice the number of T cells.

Another option is that the expression of B7-1 and/or B7-2 may in fact be changed when the cells are in the expansion culture. We have seen that irradiation does not significantly change the expression patterns, but now that we know that expression of PD-L1 by B-LCLs changes when they are in the expansion culture, this may also be the case for B7-1 and B7-2. In the report that first described the expression patterns of B7-2, it was in fact found that stimulated T cells could increase expression of B7-2 on B cells, although in this study the B and T cells had been stimulated with concanavalin A, and not anti-CD3 (44). In another study B cells were cocultured with CD4 T cells that had been pre-activated with anti-CD3, and this led to higher expression of B7-1 by the B cells (45). In hindsight, it may have been more beneficial to measure the expression of B7-1 and B7-2 on our B-LCLs after they had been in an expansion culture for a few days.

When discussing the expression of B7-1 and B7-2, another aspect that may be interesting to consider is the fact that although B7-1 and B7-2 often are thought of as two molecules that perform the same functions in a cell, they do in fact only share 25% homology (46), and their expression is regulated independently of each other (44). B7-2 expression is more rapidly upregulated in APCs than B7-1. In addition, it has also been shown that even though both B7-1 and B7-2 provide costimulation of T cells through CD28 and inhibition through CTLA4, the regulation of this may not work exactly the same way for these two molecules. In an article from 2003, Samson et al. argued that B7-2 may favor binding to CD28 over CTLA4, whereas B7-1 favors binding to CTLA4 (47). This argument was based on some of the structural differences that have been found between B7-1 and B7-2. They also presented the idea that B7-1 is the initial ligand, which maintains immune tolerance through binding to CTLA4, but then this interaction can be surpassed by upregulation of B7-2 expression by APCs when they encounter antigen. Since expression of B7-1 and B7-2 was measured with antibodies conjugated to different fluorochromes, it was not possible to say from the results obtained in this study if the expression of one of them was significantly higher than the other. Whether the relative expression levels of B7-1 and B7-2 mean anything for an expansion culture is hard to say. As already mentioned, expression of CTLA4 was not detected in our cultures, so both B7-1 and B7-2 would be expected to have stimulatory effects.

4.3 Expression levels of MCH II on B-LCLs vary greatly among different B-LCLs as well as within the same cell line

The B-LCLs studied in this project were cultured the same way, without addition of any cytokines and the measurements of MHC II expression that were performed should therefore represent their resting expression levels. The levels of expression varied within the same cell lines, although some kind of consistency was observed. This finding was more or less expected as we know from the literature that mature resting B cells constitutively express relatively high levels of MHC II, as well as class I, but the levels are not fixed and various factors can affect the expression (48, 49). The cytokine IFNγ as well as bacterial molecules like lipopolysaccharides can enhance the level of MHC II expression in B cells (49). This should however not have affected the B-LCLs in this study as they were cultured separate from other cells and stimuli.

There seemed to be less variation between the different cell lines in their expression of MHC II than of B7, but there was still some difference to be observed. While the expression levels of B7-1 and/or B7-2 were hypothesized to have an effect on activation when the T cells are unspecifically activated in an expansion culture, the levels of MHC II expression may affect the ability to activate T cells when presenting specific antigen. In vivo, the level of MHC II expression in different tissues directly influences the level of T cell activation at these sites (50). Knowing this, it is possible that some of the B-LCLs that were found to consistently express relatively high levels of MHC II could be more efficient at activating T cells when presenting the antigen that the T cells are specific for. This hypothesis was however not tested during this project, but the information about MHC expression levels among the different B-LCLs could be valuable for future experiments.

4.4 Effects of irradiation

When B-LCLs were used as feeder cells in expansion cultures they were exposed to irradiation to inhibit their proliferation. But as previously mentioned, very little in-depth information about what really happens to a cell after it has been irradiated is available in the literature. The metabolism eventually decreases, and as was seen from the results obtained in this project, the majority of cells seem to die within the first few days after irradiation. The fact that the cells eventually die from the irradiation was known, but exactly how fast it happens was not. After the initiation of an expansion culture, the T cells normally keep

growing for up to 14 days before they hit a plateau and proliferation stops. It was surprising to see that most of the B-LCLs die after only a few days. As could be seen from the results, the cells analyzed here did not consist of 100 % live cells to begin with, so exactly how long it takes before a population of 100 % live cells turns into 100 % dead cells could not be determined. Naturally, dead cells cannot release any stimulatory cytokines or interact with the expanding T cells, so the fact that the T cells keep growing even after the majority of feeder cells are dead must be a result of some other stimulation. It is possible that the increased cell density resulting from the proliferation of T cells stimulates them to keep proliferating. The IL-2 produced by the T cells as well as the added IL-2 also stimulates proliferation. When the proliferation stops, this may be because the anti-CD3 is used up in addition to being removed when cells are provided with fresh medium.

When looking at the expression of surface markers by irradiated B-LCLs, it was not clear how the results should be interpreted. As was seen from the resting B-LCLs, the expression of both MHC II and B7-2 seems to fluctuate quite a bit, so when expression levels changed after irradiation it was not obvious whether this was natural or due to the irradiation. It may have been beneficial to analyze more surface markers, and perhaps some that are more steadily expressed, to see if a general trend can be found. It may seem logical to think that breaks in the DNA caused by irradiation would lead to altered expression of surface molecules, but as we have seen it does not seem like that is the case. At least not in the cells that still appear to be alive. The fact that the expression of both MHC II and B7-2 increased in some cell lines after irradiation demonstrates the fact that many cells stay metabolically active even after irradiation. This fact was also demonstrated by the fact that expression of PD-L1 could be induced in cells after they had been irradiated.

4.5 Differential effects of anti-CD3 and PHA on proliferation

Compared to the uncertainty around the importance of costimulation, or signal 2, for expansion of T cells, there is definitely more certainty connected to the importance of the first signal; the activation of the TCR, which is absolutely necessary. The two options for providing this stimulation that were looked at during this study seemed to affect the T cells differently.

Colleagues in Amsterdam had reported great expansion success with using PHA to expand CD4 T cells. They had even managed to expand an HPA-1a specific T cell clone isolated at

the Immunology research group numerous times. This same clone had also grown well with the conventional anti-CD3 expansion methods at the Immunology research group, but recently started to show poor proliferation capacities like several other clones.

Based on this as well as on the fact that PHA is known to be a more potent stimulator than anti-CD3 (27), it was expected that the T cells expanded with PHA would proliferate more than those stimulated with anti-CD3. There are not a lot of reports available that directly compare the abilities of PHA and anti-CD3 to stimulate proliferation of T cells, but those that do generally report more proliferation with PHA (51-53). In one study, it was found that anti-CD3 was more effective than PHA for stimulating memory cells, whereas naïve T cells responded better to PHA (24). The CD4 T cells expanded in the present study should contain both cell types, whereas the HPA-1a specific T cell clones are neither naïve nor memory, which makes it difficult to say what this may indicate for our expansion cultures.

The results obtained from this study did not suggest that the PHA protocol for expansion is more effective than the already established anti-CD3 expansion protocol. For improved expansion in the future, there may be other changes that could be made. The use of anti-CD28 in addition to anti-CD3, coating plates with anti-CD3, or using beads coated with anti-CD3 and anti-CD28 may be better options than PHA. Knocking out PD-L1 or other inhibitory molecules in feeder cells may also be an option (discussed in detail later).

It is important keep in mind that the protocols used for expanding cells with either PHA or anti-CD3 in this study used different amounts of feeder cells. This could be the reason why the anti-CD3 expansion seemed more effective. For a direct comparison of the stimulatory effects of PHA and soluble anti-CD3, it would probably have been more accurate to use the same number of feeder cells and the same amount of IL-2. However, in this study, the objective was to compare the expansion methods routinely used by two different research groups rather than anti-CD3 and PHA specifically.

4.6 The effects of anti-CD3 and PHA on expression of immune checkpoint molecules

It was known that PHA is a more unspecific stimulator than anti-CD3 as it is able to bind to several cell membrane glycoproteins in addition to CD3 (27). The fact that PHA is such a strong activator is likely the reason why the levels of both CD28 and PD1 were noticeably higher in PHA stimulated cells compared to anti-CD3 stimulated cells. Whether or not this a

good thing is however not obvious. Higher CD28 could lead to better costimulation while higher PD1 could lead to inhibition.

The expression of PD1 was expected to be found, as T cells are known to express it upon stimulation trough the TCR (15). An article from 2005 also reported on the effects of anti-CD3 versus PHA stimulation of cells on the expression of PD1 (54). Similar to what was found here, they also saw that PHA led to a higher degree of PD1 expression than anti-CD3. In their results, the highest expression levels were found at 72 h for anti-CD3 stimulated cells and at 72-96 h for PHA stimulated cells. This also correlates with the results found here as levels decreased after day 4, but since this was the first day of measurement, we cannot say exactly when the expression was at its highest. Nevertheless, the fact that PD1 expression was found at all means that interaction with PD-L1 expressed by feeder cells is possible.

Having seen that PHA leads to higher expression of both CD28 and PD1 than anti-CD3 probably means that it induces high expression levels of other markers as well. Still, expression of CTLA4 was not detected in neither anti-CD3 nor PHA stimulated cells. This was somewhat unexpected as activated T cells are usually known to express CTLA4 (14). However, a study from 2004 that looked at CTLA4 expression in freshly isolated CD4 T cells stimulated with PMA and ionomycin found that the expression levels peaked after only four hours and then gradually decreased again (55). As our first measurements were performed on day 4 it may very well have been that the cells did express CTLA4 at some point before this. However, if that was the case, it did not seem like it had a significant impact on proliferation as the cells continued to proliferate for the entirety of the 11 days that the experiment lasted. It may still have been useful to perform a measurement of expression of all markers at an earlier time point. Both to see if CTLA4 could be detected and to see if higher expression levels of CD28 and PD1 could be revealed.

4.7 Induced expression of PD-L1 in B-LCLs

The initial results with resting B-LCLs indicated that they do not express PD-L1 at all. The fact that co-culturing them with CD4 T cells did not lead to PD-L1 expression either reinforced this finding. Therefore, it was a great surprise to find that the B-LCLs did in fact express PD-L1 after all, but only when used as feeder cells in expansion cultures with CD4 T cells and irradiated PBMCs. We know that expression of PD-L1 is induced in B cells when they are activated (15), but neither anti-CD3 nor PHA is known to activate B cells. The fact

that neither activated nor resting CD4 T cells cultured with the B-LCLs led to expression of PD-L1 indicates that it must have been one or more cell types present within the PBMCs that induced the expression. It would have been interesting to find out which cells had this effect and if they could induce it alone or if the expression was a result of being in a culture with many different cell types. One way to try to determine this would be to repeat the experiments performed in this study, but using PBMCs depleted for one cell type, for instance monocytes, natural killer cells, CD8 T cells or B cells. Unfortunately, due to time restraints, it was not possible to perform such an experiment.

As could be seen from the histograms displaying PD-L1 expression, there was a high degree of autofluorescence in the unstained samples, probably due to the large size of the B-LCLs as well as the flow cytometry channel used for the eFluor 450 conjugated antibody. This made it somewhat difficult to interpret the early results and it was not obvious whether these cells expressed some PD-L1 or none at all. However, the clear positive population that appeared when B-LCLs were cultured with PBMCs but not without them clearly indicated an increased expression in some cells. For future experiments it may however be beneficial to utilize an antibody conjugated to a fluorochrome that is not associated with such a high degree of autofluorescence. An isotype control antibody could also be used to compare with to make sure that observed expression is not due to unspecific binding.

4.8 Effects of PD-L1 present in expansion cultures

Knowing that expanding T cells upregulate their expression of PD1 and that B-LCLs express PD-L1 when in an expansion culture, there is reason to believe that interaction between these two molecules occur in expansion cultures. However, there are very few articles that report on the effects of PD-L1 on proliferation of T cells in vitro not in relation to cancer. Freeman et al., who were the first to identify the PD-L1 molecule, performed a selection of experiments on the effects of PD-L1 on proliferation of T cells (15). Among other things, they found that the proliferation of T cells stimulated with anti-CD3 was inhibited when PD-L1 was also present. These experiments used beads coated with anti-CD3 and PD-L1 instead of feeder cells, but they still represent conditions that are similar to the conditions that our T cells experience during expansion. Therefore there is reason to believe that PD-L1:PD1 interaction plays a role in our expansion cultures as well.

Interestingly, Freeman et al. also found that if cells received enough costimulation through CD28, then they could resist the inhibitory effects of PD-L1 (15). This again sparks an interest in measuring the expression of B7 in feeder cells when actually in the expansion cultures. It is possible that our T cells are not affected by the presence of PD-L1.

4.9 Knocking out PD-L1 in B-LCLs may improve feeder capacities

To study the effects of PD-L1 on B-LCLs used as feeder cells, the idea to use CRISPR/Cas genome editing to knock out the gene encoding PD-L1 in one or more strains of B-LCLs was created. PD-L1 knock out in antigen presenting cells used as feeder cells is something that has not previously been reported. It is therefore not certain what the effects of it would be. As already mentioned, PD-L1 may not play any role in expansion at all.

PD-L1 interactions are thoroughly studied in relation to cancer as many cancer cells express this ligand as a way of avoiding immune responses that may kill them (56). This has led to the development of new cancer immunotherapies based on the inhibition of the interaction between PD-L1 on the cancer cells with PD1 on tumor infiltrating lymphocytes. For obvious reasons, it is not possible to knock out PD-L1 in tumor cells inside the body by use of CRISPR/Cas technology. Instead, an option is to knock out the gene encoding PD1 in T cells in vitro and then reintroduce the cells to the body (19). Another option is to use antibody blockade, where anti-PD-L1 antibodies are administered to the cancer cells so that they can bind to PD-L1 and thereby inhibit it from binding to PD1 on the T cells. This may also be an option for in vitro culturing or to simply determine whether PD-L1 expression plays a role in expansion cultures. However, if it does play a role, then knocking the gene out permanently would seem more cost effective in the long run.

Both knock out of PD1 and use of PD-L1 blocking antibodies has shown promising results in terms of increased proliferation and survival of T cells in relation to cancer immunotherapy (19). With this in mind, there is reason to believe that will be the case for T cells in expansion cultures as well. However, another interesting point is that some studies have reported that PD-L1 can have positive effects on T cell proliferation under certain conditions (13, 57). One study found that when the TCR signal is low, PD-L1 and PD-L2 had costimulatory effects on T cells (13). Anti-CD3 and PD-L1 together has also been found to result in increased secretion of IL-10, IFN-γ and GM-CSF (13). These results indicate that the PD-L1 and PD-L2 ligands may take part of a similar mechanism as B7-1 and B7-2, where they are able to bind

two different receptors, one of which is stimulatory and the other is inhibitory (13). Taking this into consideration, it may be possible that knocking out PD-L1 in cells used to promote growth of T cells actually has a negative effect.

4.10 hTERT transfection of cells may be an option for better proliferation

Completely unrelated to interactions with feeder cells and expression of costimulatory molecules, there may exist another option for improved culturing of specific T cell clones. As explained in the introduction, a possible explanation for the decreased ability of a cell line to proliferate may be shortening of their telomeres; the short DNA repeats at the end of chromosomes that protect their integrity during replication. The enzyme telomerase functions to add telomeric repeats to chromosomes and is encoded by the hTERT gene (58). An option discussed during this study was to transfect the poorly proliferating T cell clones with this gene to see if that would increase their proliferative abilities. Lymphocytes are in fact among the few human cell types that naturally express this gene, and the telomerase activity has been found to be upregulated upon activation of lymphocytes (59). Therefore, hTERT transfection may seem unnecessary, but as we have seen, lymphocytes expanded in vitro do eventually reach a state of cellular senescence, which may or may not be caused by shortened telomeres. Furthermore, other groups who have transfected T cells with hTERT have achieved successful results in terms of improved proliferative abilities (60, 61).

However, due to lack of experience with this type of transfection as well as uncertainties about the possible effects of it, this was not prioritized. Ideally we do not want to change the isolated cells in any way as it may affect the cell’s characteristics in unexpected ways. Existing reports do however describe unchanged cytokine profiles and antigen specificities in transfected CD4 T cells (60). One study looked at CD28 expression in hTERT transfected CD8 T cells and found that they, like untransfected cells, eventually stopped expressing CD28, but were still able to proliferate extensively (61). This indicates that even though the problem with cellular senescence of T cells may be due to lack of CD28 expression, this problem could be surpassed by transfecting the cells with hTERT. Transfection of HPA-1a specific CD4 T cells at the Immunology research group thus remains an option.

4.11 Conclusion

The insights gained from this study have sparked an interest in understanding how CD4 T cells work, not only in relation to disease and immune responses, but also in everyday culturing and maintenance. We have experienced that clonal CD4 T cells eventually reach a point where their ability to proliferate is noticeably reduced, possibly due to loss of expression of CD28.

By studying some of the conditions of a standard CD4 T cell expansion culture, we have learned that the T cells increase their expression of both the stimulatory receptor CD28 and the inhibitory receptor PD1. The increase is greater when cells are stimulated with PHA than with anti-CD3. The B-LCLs that the T cells are cultured with express B7-1 and B7-2, which can bind to CD28, and the expression of the PD1 ligand PD-L1 is induced when B-LCLs are in an expansion culture.

In terms of stimulating proliferation of CD4 T cells, it did not seem like the levels of B7 expression played a significant role, which meant that different B-LCLs had similar stimulatory effects. When considering different options for the activation of the TCR, anti-CD3 seemed more effective at stimulating proliferation than PHA in the long run.

To conclude, some of the lessons learned from this study could hopefully be considered to establish better routines for culturing of CD4 T cells in relation to future work at the Immunology research group.

5 Future Perspectives

The work performed in association with this study has given some insights into the conditions of a CD4 T cell expansion culture. Still, there are many unanswered questions.

As already mentioned, studying the expression of B7-1 and B7-2 by feeder cells in expansion cultures to see if it is altered, like PD-L1, could be interesting.

One objective that will be pursued in the near future is to perform a CRISPR/Cas knockout of the PD-L1 gene in one or more of the B-LCLs. To make sure that the knock out is successful, the cells would then have to be stimulated in an expansion culture as before to determine whether the expression has disappeared. New parallel expansion cultures could also be set up, using the PD-L1 knock out cells in some wells and the original PD-L1 expressing cells in others. The amount of proliferation in these two systems could then be compared with each other to see if the knock-out had any effect. As mentioned it would also be interesting to try to determine exactly which cells or which interactions it is that cause the PD-L1 expression by B-LCLs.

As described in the introduction to this study, there exists several different checkpoint molecules and ligands that can be expressed by T cells and APCs. This study did not look into the expression of surface markers by PBMCs used as feeder cells, but that could also be interesting to study. Regarding inhibitory checkpoint molecules expressed by B-LCLs, this study focused on PD-L1, but there are other such molecules that could also be interesting to study closer, for instance GAL9. The expression of LAG3 and TIM3 on expanding T cells may also be interesting to look at.

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