Using NK Cell Mediated Cytolysis to Trigger Adaptive Immune Responses

Thesis for the Master's Degree in Molecular Biosciences

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Abstract

The vertebrate immune system is an enormously complex arrangement of a large number of interdependent cell types, responsible for fending off harmful invasive organisms as well as aberrant cells within the host itself. The system is divided into two broad arms; the innate and the adaptive immune compartments.

Whereas the cells of the innate arm recognizes threats based on certain characteristics common to most invaders, adaptive immune cells is able to recognize billions of unique antigens due to specialized receptors on their surface. This ability is dependent on an efficient means of exposing the cells to antigenic peptides, achieved through a mechanism known as antigen presentation. Several cell types, commonly referred to as professional antigen presenting cells (pAPCs), are able to carry out this function; most well known of these are the dendritic cells (DCs). However, difficulties in growing DCs *in vitro* has led to an increased interest in using alternative APCs to expand robust adaptive immune responses for clinical use. By using CD40 activated B cells as an alternative APC, we observed a significant difference in T cell expansions towards specific antigens.

Efficient antigen presentation requires the two distinct immune compartments to act in unison, where cell-mediated lysis carried out by the innate system generate antigenic peptides for activation of adaptive responses. Natural killer (NK) cells are innate immune cells, capable of eliminating virus infected or cancer-transformed cells without prior activation, an ability that coined the term “natural killer”. NK cells are regulated through signaling from a diverse array of activating and inhibiting receptors, with stress proteins acting as ligands for the activating receptors, and MHC molecules generally acting as ligands for inhibitory receptors. In this thesis we hypothesized that NK mediated cytolysis can bridge innate and adaptive immunity and prime systemic T cell reactivity. NK cells are able to respond quickly to cells that express stress markers, or lose expression of MHC molecules, characteristics that are generally associated with malignancy. It is possible that this innate recognition of transformed cells contributes to a more efficient priming of adaptive immune responses by generating peptide fragments readily available for presentation to T cells.

Using a step-wise *in vitro* model system of T cell priming we found evidence of improved T cell expansions as a result of being stimulated with peptide fragments obtained through NK cell mediated cytolysis of a melanoma cell line. This stimulation also led to an increase in

epitope specific cells against a known melanoma antigen, MART-1. Although further investigations are needed, exploiting NK cell cytolysis and natural fragmentation of tumor associated antigens may hold utility in the development of cancer vaccines by providing new insights into tumor specific epitopes and neo-antigens.

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1 Introduction

1.1 The immune system

The vertebrate immune system is composed of various structures and processes dedicated to defend the body against harmful foreign invaders or abnormal internal behavior. It encompasses a well-organized army of specialized cells, as well as structural barriers to prevent any harm to the host. A distinctive feature of the vertebrate immune system, as opposed to invertebrates, is the sub classification into the innate and the adaptive immune system[2]. Innate immunity is common to all species, both plants and animals as well as fungi, and is in terms of evolution a more primitive system that responds to infecting agents, known as pathogens, in a generic and highly consistent manner. Adaptive immunity is generally considered a feature of vertebrates, although there is evidence of a similar system present in some non-vertebrate species[3]. The focus of this project is on the cells and mechanisms of the vertebrate system however, which have the ability to respond to specific pathogens, mount an effective response, and then remember that pathogen to quickly respond to subsequent infections.

1.1.1 The innate immune system

The first obstacle a pathogen encounters as it tries to enter the body is the epithelial surfaces lining the parts of the body that are in direct contact with the environment. These form a physical barrier that efficiently blocks entry for most infectious agents[4]. Peristalsis and ciliary movement in the gastrointestinal and respiratory tract help removing invaders that become trapped on these surfaces. In addition, mucus, gut flora, tears, and saliva, all help in removing foreign invaders [4].

Some pathogens are still able to bypass these barriers however, causing infection. One of the first responses to infection is inflammation, leading to the classical signs of heat, pain, redness, swelling and loss of function. Inflammation is initiated by cells of the innate immune system, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells and

mastocytes. These cells release various chemicals that mediate the inflammation, and recruit other cells of the immune system to the infected area.

Innate immune cells express germline-encoded pattern recognition receptors (PRRs) that recognize various molecular signatures found only on pathogens, known as pathogen-associated molecular patterns (PAMPs). These moieties include lipopolysaccharides found on bacteria, hydrophobic lipids and proteins, as well as nucleic acids associated with viruses such as 5'triphosphate RNA[5]. There are several classes of PRRs, most extensively studied of these are perhaps the Toll-like receptors (TLRs), of which ten have been identified in humans[6].

Cells of the innate system are classified as white blood cells, known as leukocytes, that circulate in the blood stream searching for invaders. Most innate leukocytes are unable to divide or reproduce on their own, but are differentiated from hematopoietic stem cells in the bone marrow[7]. The innate leukocytes include mast cells, eosinophils, basophils, macrophages, neutrophils, dendritic cells and natural killer (NK) cells[2]. These cells are able to respond quickly to invasive pathogens, but the magnitude of the response is limited and does not vary between first and subsequent encounters. This is in contrast to the response profile of the adaptive immune system, which is slow in primary infection, but increase in scope and effectiveness during secondary infections due to generation of pathogen specific memory cells[8].

Some innate cells are classified as lymphocytes, the main cell type found in the lymphatic system. Recently, the family of innate lymphocytes has grown considerably, with the classification of innate lymphoid cells (ILCs); defined by a lack of rearranged antigen receptor genes, a lack of myeloid and dendritic cell phenotypical markers, and having a lymphoid morphology. The ILC family includes ILC1, ILC2, ILC3 and MAIT cells. Of special significance to this project are natural killer (NK) cells, which are a part of the ILC1 group, characterized by the production of interferon-γ (IFN- γ) and an inability to produce T_H2 and T_H17-cell associated cytokines[9]. The rather descriptive name of "natural killer" stems from the ability of these cells to quickly respond to and kill cells that have lost their "self" markers, MHC class I molecules, without activation[10]. NK cells will be described in more detail in section 1.3.

1.2 The adaptive immune system

Unique recognition of billions of different types of molecules is the defining ability of the adaptive immune system[2]. Being able identify not only dangerous foreign molecules as something that must be eliminated, but also the body's own materials as something that must be ignored, is both an accomplishment of evolution as well as crucial prerequisite for survival. This ability is obtained through a complex mechanism of random rearrangements in the genes coding for antigen receptors in the cells of the adaptive immune system, and the presentation of small peptide fragments called antigens, obtained through the destruction of invasive organisms[2, 11-14].

1.2.1 Antigen presentation

Antigens are presented to the immune system by two classes of molecules on the surface of the presenting cell, the MHC class I and MHC class II. These two molecules are similar, but have some important differences[15]. Firstly, they differ in the origin of the peptides presented. Class I molecules present antigens originating in the cytosol of the cell, while material picked up from the extracellular environment and broken down in the cell will be presented on MHC class II. A consequence of this difference is that MHCII is present on a much more limited selection of cell types than MHCI. Class I molecules are expressed on all cells in the human body with the exception of red blood cells. Peptide fragments from every protein produced by the cell are constantly presented on class I, and monitored by a type of adaptive lymphocytes called T cells[16]. When a cell is producing abnormal proteins as a result of an infection or mutation, it will be detected by the immune system through presentation on class I, and the cell will be eliminated. Class II however, is present mainly on cell types that are able to pick up invasive antigens from the environment and eliminate them, in other words mostly immune cells – dendritic cells, macrophages, B cells, T cells, and thymic cortical epithelial cells[2, 17]. The two classes of MHC molecules present their antigens to different co-receptors on T cells, CD4 and CD8. The presence of these co-receptors defines two major subtypes of T cells, cytotoxic (CD8) and helper (CD4) T cells. CD8 recognizes antigens presented on class I, and if identified as a foreign peptide, cytotoxic T cells will destroy the affected cell[18]. MHC II presents antigens to the CD4 found on helper T cells. Antigens displayed on class II molecules are derived from extracellular sources – usually as a result of a breakdown of invading pathogens, which are processed inside the

immune cell and transported to the MHCII to be presented on the cell surface[15]. Here they are recognized by CD4 positive helper T cells, which then in turn are activated and further drive the immune response to be more efficient versus this pathogen.

1.2.2 Cross-Presentation

Generally, MHC class I presents antigens derived from the intracellular environment, and MHCII present extracellular antigens. However, if this rule was strictly adhered to with no exceptions, the immune system would not be able to function. Naïve T cells need to be primed through interactions with professional antigen presenting cells (pAPCs), after which they will mediate infector function when they recognize the same antigen on an infected or transformed cell. In general, foreign antigens are presented on MHCI when the cell is infected, and an activated CD8 T cell recognizes the antigen and destroys the cell. However, if foreign antigen on MHCI is only presented by infected cells, and naïve T cells need to recognize a foreign antigen on an MHCI on an APC to become activated, then the APC would have to be infected to be able to present the antigen to activate the naïve T cell. Fortunately, this is not required, and this is where cross-presentation comes into play. Cross-presentation is an ability of professional APCs, in which antigens from all sources can be presented on both classes of MHC[19]. This means that APCs can ingest extracellular material, process it, and present it on MHCI, as well as on MHCII. Cross-presentation also occurs for endogenous antigens that normally would be loaded onto MHCI, meaning that they can also be presented on MHCII. This allows CD4 helper T cells to be activated towards pathogen derived intracellular antigens[20]. The mechanism behind cross-presentation is illustrated in **Figure 1**.

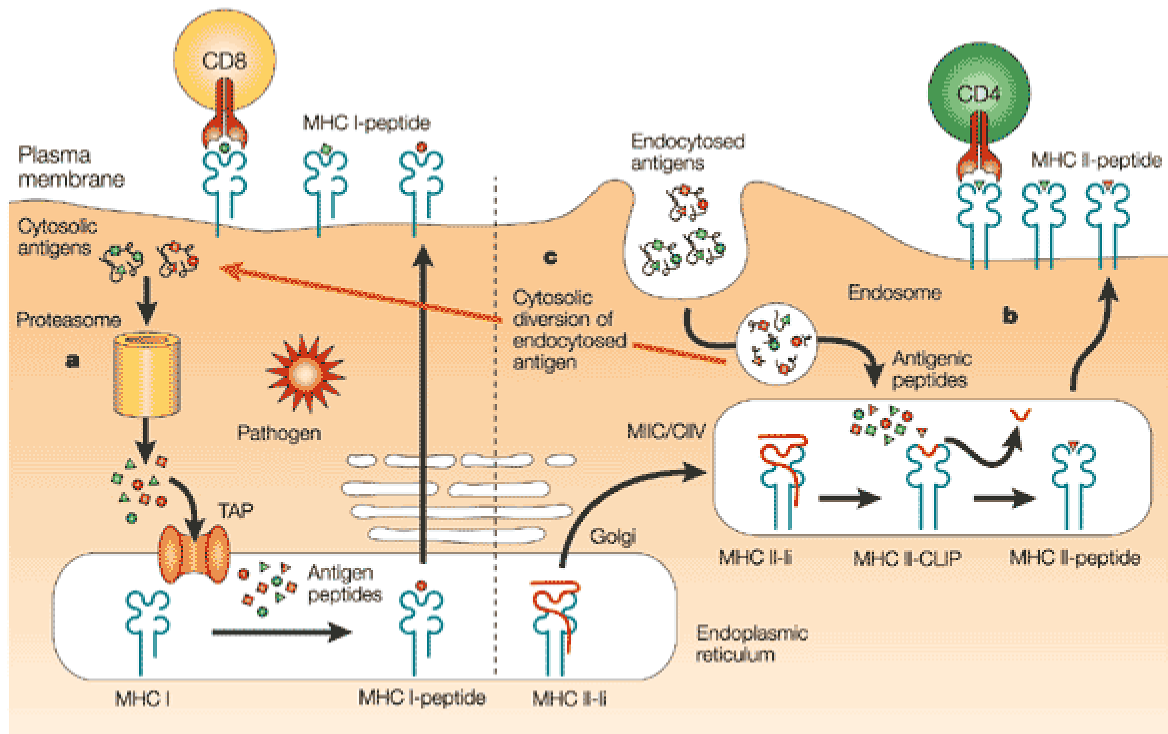


Figure 1 Schematic view of cross-presentation.

A) Endogenous proteins of either self or pathogen origin are degraded into peptides and transported into the ER by TAP molecules for loading on MHC I molecules. B) Extracellularly derived proteins are endocytocytosed into the cell and presented on MHC II. C) APCs are able to take endocytosed antigens and load them onto MHC class I molecules for presentation to CD8$^+$T cells, as well as load endogenous proteins onto MHC class II molecules for presentation to CD4$^+$T cells. Figure adopted from [21]

1.2.3 B cells

B cells are vitally important lymphocytes of the adaptive immune system. Their primary functions are to produce specific antibodies against invasive pathogens, and to present antigens to other parts of the immune system, functioning as an antigen presenting cell (APC). In this project, we are mostly concerned with their function as APCs.

Antigen is encountered by B cells, primarily in secondary lymphoid organs, and recognized by the B cell receptor (BCR) present on all B cells[22]. The BCRs affinity for a given antigen is directly proportional to the capacity of the B cell to present this antigen to T cells[23, 24]. When a pathogenic epitope is recognized by a BCR, the pathogen is internalized and processed inside the cell. Peptide fragments are then presented on MHC class II, or on MHC

class I through the cross-presentation mechanism described in 1.2.2. The processing of the epitopes bound to the receptor is blocked, causing these regions of the antigen to be presented[25, 26], thus shaping the immune response towards these epitopes.

However, as with most things in immunology, it is not as straight forward as it may seem at a first glance. A naïve B cell encountering a new antigen will not be allowed to instantly start initiating immune responses without confirmation that this antigen should be eradicated by the immune system. If the antigen is derived from a protein the B cell requires signals from CD4 T cells that recognize the same antigen to induce antibody responses. Without signals from the T cell, the B cell will not be able to induce a response. These antigens are known as thymus dependent, or TD antigens. The signal delivered by the CD4 T cell is sent when the T cell recognizes a peptide bound to MHCII on the B cell surface. CD40 ligand (CD40L or CD154) on the T cell interacts with CD40 on the B cell and contributes an essential part of the activating signal[27]. This interaction increases B cell proliferation, immunoglobulin class switching, somatic hypermutation, and an increase in expression of co-stimulatory molecules, most notably CD80 (B7-1) and CD86 (B7-2)[28]. Additional signals are delivered from the T cell in the form of secreted cytokines, IL-4 being one of the most important. Together with CD40 signaling, IL-4 is thought to drive the clonal expansion of B cells that precedes antibody production *in vivo*[29]. In this project, B cells are co-cultured with adherent feeder cells expressing CD40L, while at the same time being stimulated with IL-4. These conditions should maximize their antigen presenting potential.

1.2.4 T cells

T cells are the other major part of the adaptive immune response, the first being B cells. Similar to B cells, they express membrane bound receptors, known as T Cell Receptors (TCRs), capable of recognizing a staggering amount of different antigen epitopes[30, 31]. These highly variable receptors are the basis for adaptive immunity, as a cell expressing a receptor specific for an invading pathogen will be stimulated through various signals to duplicate and differentiate into various types of effector cells depending on the manner of antigen that is recognized. The two main families of T cells are known as CD4 T cells and CD8 T cells, classified by which class of MHC bound to a peptide they recognize. CD8 cells recognize peptides bound on MHC class I, and differentiate into cytotoxic effector cells that kill infected cells. CD4 cells recognize peptides bound on MHC class II, and can differentiate

into several distinct effector subsets, including multiple subsets of helper T cells and regulatory T cells[32]. Helper T cells are absolutely crucial for efficient activation of the adaptive immune system, including priming of B cells, while regulatory T cells inhibit undesirable immune activity such as self-reactive cells.

Similar to the way naïve B cells are dependent on T cells for activation, naïve T cells require signals from antigen presenting cells such as B cells or dendritic cells before they are allowed to carry out their effector functions. A naïve T cell requires three different types of signals to be activated[33]. The first of these is the signal that results from interaction between the MHC:peptide complex with the TCR. This signal is essential, but not sufficient for the T cell to become fully activated. Additional signaling from an APC is required. This signaling can be divided into co-stimulatory signals that promote the survival and expansion of T cells, and signals that contribute to the differentiation of the T cell into various effector subtypes[34]. Co-stimulatory signaling is provided by ligation of B7 molecules (primarily CD80 and CD86) on the APC to the CD28 receptor on the T cell, which is necessary for optimal clonal expansion[34]. For CD4 cells in particular, the type of signal provided by different cytokines released by the APC causes various pathways of differentiation that produces different subsets of CD4 T cells to carry out various effector functions that depends on the nature of the antigen, and the environment of the cells involved.

1.3 Natural Killer cells

As hinted by their name, natural killer (NK) cells have the ability to hunt down and eliminate foreign or abnormal cells in the body[35]. NK cells are innate immune cells, that are able to spontaneously respond to a target without prior sensitization, however their cytolytic activity does not differ in magnitude upon subsequent encounters with the same target. They are a crucial part of the innate immune system, where they function as cytolytic lymphocytes to directly induce lysis or apoptosis in virus infected cells or tumor cells. Despite their classification as a lymphocyte and their similarity to T and B cells, NK cells are not considered a part of the adaptive immune system, as they do not possess antigen specific surface receptors. Rather, they work in a complimentary manner to the adaptive system. Where the adaptive system responds to antigens presented on the MHC proteins on the surface of another cell, NK cells responds to a lack of the MHC class I proteins themselves [10], a state known as “missing self”[36-38]. Being able to respond to a lack of MHC I

expression is necessary, as certain viruses and cancers have developed ways to disrupt this expression to avoid detection by adaptive immune cells[35, 39]. However, the recognition of missing self molecules is not the only function NK cells serve in an immune response. NK cell-mediated killing of antigen expressing cells induces specific $CD8^+$ T cell responses to the antigen through cross-presentation[40, 41], thus acting as a link between the innate and adaptive immune system. They are major producers of cytokines such as interferon-γ (IFN-γ)[42], tumor necrosis factor α (TNF-α)[43] and interleukin(IL)-10[44]. The secretion of cytokines is a crucial step in activating further immune responses, having an impact on the production of dendritic cells (DC), macrophages, and neutrophils[45, 46]. Especially important is the impact on DCs, which are able to internalize the lysis fragments that result from NK cell mediated killing, and present the antigens on MHC I molecules to $CD8^+$T cells through cross-presentation[41], as well as to $CD4^+$T and B cells through presentation on MHC class II. DCs and NK cells have a symbiotic relationship, where they reciprocally activate each other, providing positive feedback mechanisms for both[47-49]. IFN-γ released by NK cells when they are activated, can cause maturation of DCs[50]. DC activation often results in production of type I IFN and IL-18, which serves to prime NK cells and increase cytolytic effector function [51]. As DCs are in turn important activators of both B and T cells, NK cell stimulation serves as a means of activating both the humoral and the cell-mediated arms of the adaptive immune system[52].

1.3.1 NK cell education

One of the most characteristic features of NK cells is an array of surface receptors that can either stimulate (activating receptors) or dampen (inhibitory receptors) their activity [53, 54]. To prevent aggression from NK cells towards the cells of the host, a variety of receptors that recognize self molecules inhibit NK cell activity. The predominant mechanism are inhibitory receptors that recognize self MHC I molecules. [55, 56]. However, NK cells lacking expression of inhibitory receptors will not react to all cells they encounter. This would lead to dramatic autoimmunity, and is prevented by a process known as NK cell education, where cells deficient for inhibitory receptors are rendered unresponsive[57, 58]. Several mechanisms behind this process have been proposed. Although each model has unique features, there are also many shared aspects between them, and their differences do not necessarily mean they are mutually exclusive and they could all be complementary biological processes involved in NK cell education.

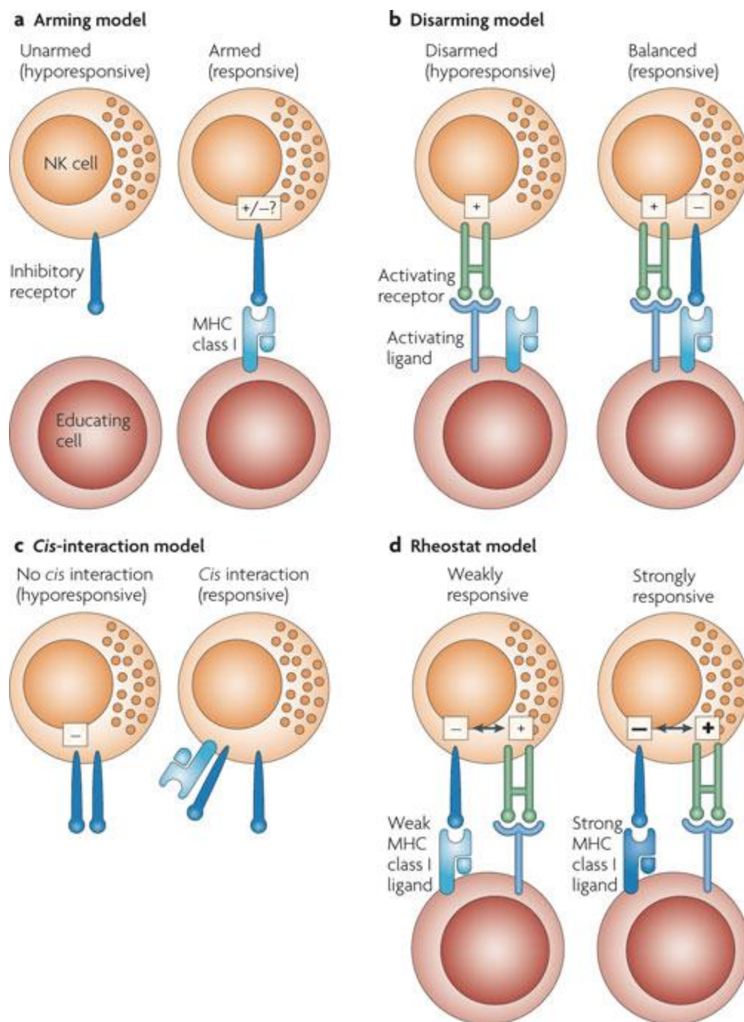


Figure 2 NK cell education models

A) In the arming model, NK cells only become responsive after receiving a signal from an inhibitory receptor. B) The disarming model suggests that NK cells become unresponsive in the absence of an inhibitory receptor. C) Shows the cis-interaction model, where inhibitory receptors can interact with MHC molecules in their own membrane. D) The rheostat model proposes a more dynamic view of education, where the balance between inhibitory and activating signals determines the strength of the response. Figure adopted from [1]

Figure 2 presents and overview of the various models for NK cell education. The “licensing”, or “arming”[58], model suggests that NK cells only become responsive after encountering MHC class I on an inhibitory receptor[58], meaning that signals from inhibitory receptors promote maturation. This view is supported by studies that show immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptor based signaling can lead to phosphorylation of downstream signaling substrates [59, 60].

The second model proposes a "disarming" hypothesis, where NK cells are rendered unresponsive by chronic stimulation by normal cells, unless opposed by inhibitory MHC I receptors[61]. This model suggests that NK cells are responsive in their initial state, and are rendered anergic by chronic stimulation, unless opposed by interactions with MHC I.

The cis-interaction model is based on observations of Ly49, a class murine NK cell inhibitory receptors that bind MHCI, similar to human KIR receptors. The Ly49 receptors are able to bind MHCI molecules in *cis,* that is, in the same cell membrane [62-64]. This model suggests that *cis* interactions between MHC I and Ly49 receptors sequesters the Ly49 receptors and prevents them from relocating to the immunological synapse at the target cell interphase. This sequestering of unengaged inhibitory receptors decreases inhibitory signaling and increases the responsiveness of the NK cell [1].

More recent studies have uncovered that NK cell effector function and responsivity are influenced by the number of inhibitory receptors they possess, with more receptors leading to higher activity levels[65]. The suggestion that NK cell education operates in a quantitative manner has been named the rheostat model [66]. Depending on the amount of inhibitory and activating signals it receives, the NK cell responsiveness can be tuned up and down [65, 67]. The rheostat model provides a framework, which is consistent with both the licensing and disarming models. It is also potentially mechanistically compatible with the cis-interaction model.

The licensing and disarming models of NK cell education are as mentioned not necessarily mutually exclusive mechanisms, and it is possible that they both occur in different contexts. Tumors might create an environment of constant inflammation, a context in which NK cells could become overwhelmed by a constant barrage of stress activating ligands and rendered hyporesponsive[68]. Uneducated NK cells that lack inhibitory receptors have also been shown to be more effective than educated cells in certain circumstances, after having been provided with a strong activating receptor. These circumstances could be antibody-dependent cellular cytotoxicity (ADCC) against MHC I positive cancer cells, where uneducated cells equipped with the Fc receptor CD16 outperformed educated cells in cancer clearance[69]. Nevertheless, cancer-transformed cells tend to alter the expression of inhibitory ligands such as MHC I to prevent recognition from cancer reactive T cells, or present stress-induced activating ligands, making them ideal targets for NK cells[70, 71]. The most potent effectors against these targets

are $CD56^{dim}$ NK cells[72], which are more mature and are terminally differentiated from $CD56^{bright}$ cells[73].

1.3.2 NK cell differentiation

So in what way does NK cell education manifest in the phenotype of the NK cell? The result is not as simple as a division into subsets of "educated" and "non-educated" NK cells. Traditionally, NK cells have been divided into two distinct subsets, defined by the amount of CD56 expressed on the cell surface, known as $CD56^{bright}$ and $CD56^{dim}$, bright expressing a high amount and dim expressing a low amount[74]. These subpopulations may be divided further into distinct subsets based on their expression of various other molecules and receptors such as NKG2A/CD94, KIR, NKG2C, CD62L, and CD57[75-77]. As the NK cell receives signaling, it differentiates and acquires or loses expression of these receptors. Mature NK cells starts differentiation from $CD56^{bright}$, who are not very cytotoxic but produce a lot of IFN-γ. As the cells differentiate, they lose some of their CD56 expression and move towards a $CD56^{dim}$ phenotype. These more mature $CD56^{dim}$ NK cells are more cytotoxic, but doesn´t generally produce as much IFN-γ. As the cells mature further, they acquire CD57. These $CD56^{dim}$ $CD57^{+}$ are the most differentiated and are able to produce more IFN-γ when triggered by activating surface receptors[78]. NK cell differentiation together with the stochastic expression of a wide range of inhibitory receptors diversifies the human NK cell repertoire. A recent study identified several thousand distinct subpopulations of NK cells in the peripheral blood of humans[79]. This reveals the extremely complex nature of the NK cell role in the immune response, and it seems that we as of yet have barely scratched the surface of understanding their function. **Figure 3** presents a flowchart of the maturation process for NK cells.

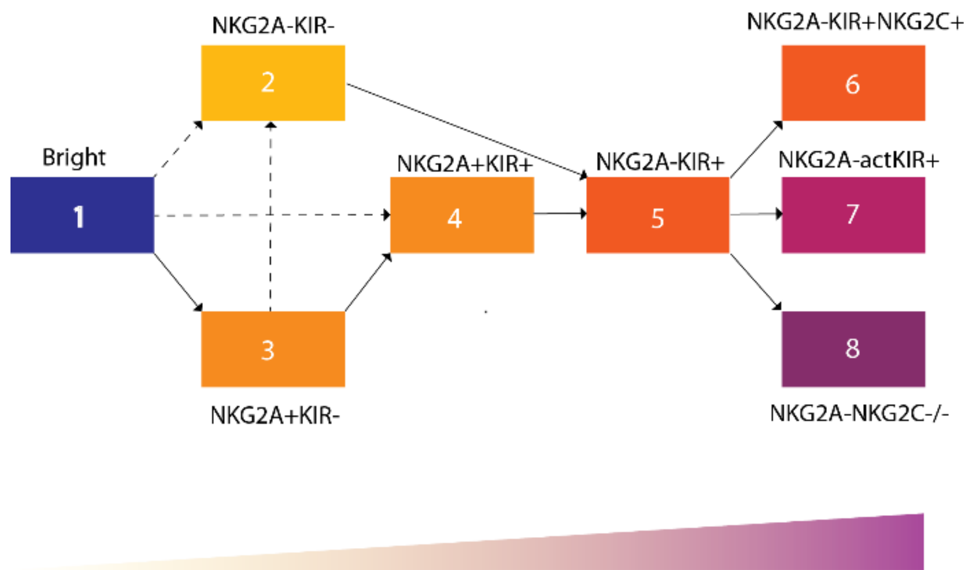


Figure 3 Differentiation of NK cells.

Shows the progression from CD56^{bright} (1) to CD56^{dim} CD57^{+} (8) with discrete stages in between. Note how NKG2A is both acquired and lost through the various stages. Figure by Jodie Goodridge

1.4 Summary in regards to the generation of immune functions

Natural killer cells are innate lymphocytes that are able to quickly respond to virus infected or cancer-transformed cells without activation, due to a vast array of inhibitory and activating receptors that recognize changes in expression levels of ligands for these receptors. Efficient NK cell mediated cytotoxicity generates peptide fragments from these threats, which can in turn be taken up, processed, and presented to the adaptive immune system by antigen presenting cells (APCs). In this manner, NK cell acts as a mediator between the innate and adaptive immune compartments by providing the antigenic peptides required for adaptive recognition.

B cells, though mostly regarded as producers of antibodies, are also highly efficient APCs, able to internalize the antigenic peptides generated through NK cytolysis of targets, and present target specific antigens to CD4 T cells on MHC II, and also to CD8 T cells through cross-presentation on MHC I. This allows for priming of antigen specific T cells, which will divide and proliferate resulting in an expansion of T cells able to recognize the target through various epitopes.

By using target cell lysate resulting from NK cell mediated toxicity, the hypothesis that motivated this project states that more epitopes should become available for presentation, resulting in efficient T cell expansions.

1.5 Description of project

The main focus of this project was to elucidate the molecular mechanisms that connect NK cell mediated lysis to generation of adaptive immune responses and memory, using a combination of the most current techniques in molecular biology, genetics, cell biology and flow cytometry. To approach this goal, a general 4-step process was used. First, target cell lines were destroyed through cytolysis. The lysates resulting from this were fractionated and then pulsed onto antigen presenting cells (APCs). These APCs were then used to generate adaptive T cell responses against the target cell line. **Figure 4** is an illustration representing the general process.

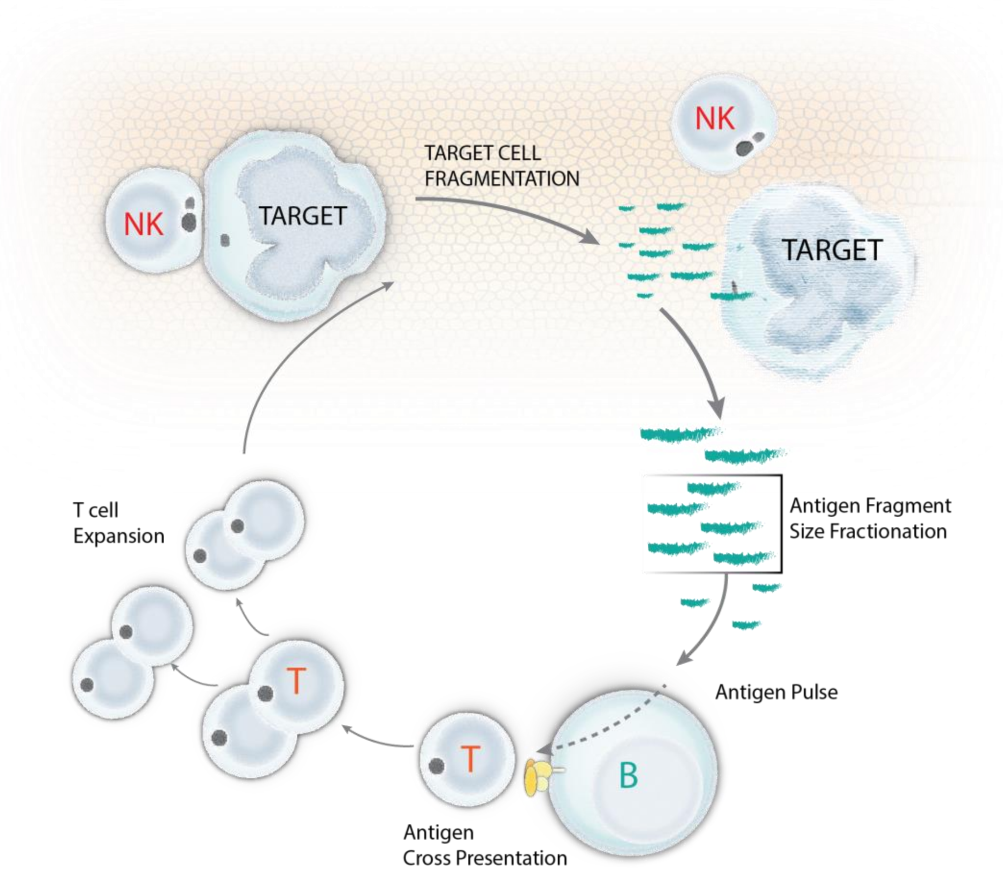


Figure 4 A Flowchart illustrating the main steps of the project.

A) NK cell destroying a target cell. B) The target cell is broken into fragments as a result of NK cytolysis. C) The cell lysate is filtered into size based fractions. D) the 3kD-10kD fraction is pulsed onto B cells, which will present them to T cells. E) Expansion of T cell specific for the target cell. Figure by Jodie Goodridge.

A-C. Killing of target cells

Using NK cells to kill target cells generates a lysate containing peptide fragments from the target cell line. This lysate can then be separated into discrete fractions containing peptides of a given size. Peptides of sizes between 3kD and 10kD can be used, as these are of theoretically ideal size for antigen presentation versus spontaneous formation of MHC complexes on the cell surface with smaller peptides of 1kD or less.

D. Presentation of antigens

Lysates from the previous step are pulsed onto antigen presenting cells (APCs), for cross-presentation on MHC class I to $CD8^+$ T cells. B cells co-cultured with adherent feeder cells expressing CD40L serve as APCs in these experiments. Co-culturing B cells with CD40L in combination with IL-4 leads to upregulation of expression of MHC class I and class II molecules, as well as the co-stimulatory molecules CD80 and CD86, which will enhance antigen presentation capabilities of the B cells. The expression levels of these molecules will be monitored over time.

E. T cell expansion

When a naïve T cell recognizes its cognate antigen, it needs stimulation from an APC to multiply. In this experimental layout, the CD40 activated B cells provide this stimulation. In the project, T cell cultures that have and have not been co-cultured with such B cells have been compared. The hypothesis is that the B cells provide a more efficient means of displaying antigen and therefore driving greater expansion of T cells versus PBMC alone. Cultures stimulated with peptide libraries of known antigenic peptides were compared to cells stimulated with the lysate fraction resulting from NK cell cytolytic activity. Pulsing with the lysate fraction should theoretically provide additional antigens toward a specific target that are not a part of the peptide libraries, which should result in improved T cell expansions.

E. Evaluating the nature of expanded T cells

A more detailed look at the biology of the expansions can be provided by looking at memory cell responses using fluorescently labelled antibodies toward CD45RA, CD45RO and CD67L. Tetrameric MHC molecules are used to assess their specificity towards the antigens presented to them.

1.6 Relevance of project

The ability to generate immune responses tailored against specific diseases has over the last few decades become increasingly relevant, with the advent of immunotherapy[80-85]. Harnessing the power of the body's natural defense mechanisms has the potential to help

combat some of the most widespread serious and deadly diseases, like cancers and autoimmune disorders, as well as infections of various pathogenic organisms. However, a solid understanding of the mechanisms involved in priming the immune system is required in order to maximize efficiency and minimize risks. This project aims to contribute to this understanding, by focusing on the early steps bridging innate immune responses to the priming of systemic immunity in the host.

An important part of the project is using antigen-presenting cells (APCs) to prime immune responses to various antigens. The APCs used will be B cells – most commonly known as antibody producers, but B cells are also highly efficient APCs[22, 86]. Activated APCs have been grown before, and used in clinical trials, but the focus has mainly been on using dendritic cells (DCs) [87-90]. While DCs are excellent APCs, they are difficult to expand *in vitro,* and are required to be in a specific differentiation state to exhibit antigen-presenting capabilities. The realization that B cells are capable of presenting specific antigens to generate immune responses, has led to increased interest in their clinical applications [22, 91]. The method described in the project presents a relatively simple way of expanding purified, activated B cells, and looks at the response of T cells co-cultured with them.

Activation of an immune response starts with the recognition of a foreign substance, known as an antigen (Ag). The origin of this antigen can be from a myriad of sources – viruses, bacteria, parasites are commonly known causes of immune responses – but can also originate from the body itself. Abnormal cells, like in cancer, that produce unusual proteins are for the most part swiftly taken care of and removed by the immune system[92, 93]. Unfortunately, sometimes this system fails, and allows the abnormal cells to live, and cancer can develop freely. So how can we strengthen the immune system to respond in these situations? Addressing this challenge is the focus of the field of immunotherapy. In this project, Natural Killer (NK) cells will be utilized to kill target cancer cell lines. As a result of this, the cell will be destroyed, leaving behind a fragmented profile of what the cell contained. These fragments will then be captured by activated B cells, and the B cells will then process and present these fragments as antigens to T cells. Hopefully, this will provide an efficient method for mass production of highly specific T cells toward the target cell.

2 Materials and Methods

2.1 Flow Cytometry

The principle behind flow cytometry is fluorescent labels, known as fluorophores, attached to antibodies specific for various cell markers. The antibodies bind their targets on the cells, a cell suspension is run through the flow cytometer, where lasers with different wavelengths excite the fluorophores, and the intensity of the resulting fluorescence is measured for each cell to determine the quantity of markers present. In addition to the information gathered from the fluorescence, the cytometer can also measure the physical properties of each cell, based on their interaction with the laser. From this interaction, information about size and internal complexity can be obtained. These physical properties are often characteristic of specific cell populations, and are very useful for sorting out unwanted material, such as dead cells or debris.

A flow cytometer has five main components[94]:

- -A flow cell, which ensures the cells pass in a single file through the light beam.
- -A measuring system, usually based on conductivity and optical properties.
- -A detector and a system that converts light into electrical output that can be processed by a computer.
- -An amplification system that strengthens the resulting signals.
- -A computer to process the data.

Flow cytometry can also be used to determine the amount of specific T cells, by staining with fluorescently labelled tetrameric MHC molecules. A tetramer is made up of four MHC monomeric molecules, bound together by a streptavidin. Tetrameric MHC molecules, as opposed to monomeric molecules, are used because monomeric MHC molecules have a high dissociation rate from T cell receptors. By using four MHC monomers bound together as a tetramer using streptavidin, each individual MHC molecule can bind a TCR, and in this way

increase the binding avidity. Tetramers are made up of four genetically engineered MHC molecules that have had a part of them replaced by a biotinylated domain, and have been stimulated with a peptide of interest. The MHC molecules are then mixed with streptavidin marked with fluorophore, and the biotinylated domains on the MHC molecules bind to the streptavidin, and form tetramers. These can then form complexes with TCR specific for the antigen presented, which can then be analyzed using flow cytometry. The T cells must, in addition to recognizing the presented antigen, be able to recognize the specific MHC haplotype of the tetramers. It is therefore necessary to determine the haplotype of the T cells before conducting this test[95].

2.2 Staining cells

Staining cells with fluorochrome-conjugated antibodies for flow cytometry was done in accordance to a general procedure described the BD Biosciences webpage[96]. 100-200µL cell suspension containing at least a few hundred thousand cells was spun down at 1700rpm for 5 minutes. The supernatant was flicked of, and the cells are resuspended in 50µL staining buffer containing an appropriate amount of fluorochrome-conjugated antibody. The cells were left in the dark at room temperature for 20 minutes. After incubation, 100µL (for microwell plates) or 1mL (for FACS tubes) staining buffer was added, and the cells are spun down. The cells were then washed twice by adding 100µL/1mL staining buffer, spun down, and the supernatant discarded. The cells were then fixed by re-suspending in 100µL paraformaldehyde. The staining panels used for B and T cell cultures are shown below.

B CELL FLUORESCENT MARKER PANEL

MARKER	Fluorophore	Manufacturer
CD45RO	Alexa Fluor 488	BioLegend
CD45RA	Brilliant Violet 421	BioLegend
CD80	APC-Cy7	BioLegend
CD86	Brilliant Violet 605	BioLegend
CD19	PE-Cy7	BioLegend
HLA-DR (CLASS II)	Alexa Fluor 700	BioLegend
HLA-ABC (CLASS I)	PE	Antibodiesonline.com

T CELL FLUORESCENT MARKER PANEL

MARKER	Fluorophore	Manufacturer
CD4	BV 421	BioLegend
CD8A	BV 785	BioLegend
CD3	PE	BioLegend
CD45RO	Alexa Fluor 488	BioLegend
CD62L	BV605	BioLegend
CD45RA	APC/Cy7	BioLegend

2.2.1 Tetramer staining

The protocol for staining cells with tetramers was performed in a similar way to staining with fluorochrome-conjugated antibodies. The only difference was a preparation step needed to make tetramers from MHC monomers, by assembling them on streptavidin. Class I MHC monomers refolded with specific 9mer peptides were obtained from the Jianhong Cao (Immune Monitoring Lab, Fred Hutchinson Cancer Research Center, Seattle Washington). 0,5µg/µL monomer/well on a 96 well plate was mixed with 0,75µg/µL streptavidin. PBS was added so the total volume amounted to enough for 5µL mixture per well to be stained. The mixture was then incubated for 30minutes at room temperature in the dark. 5µL of the tetramer solution was then added to each well to be stained. The rest followed the same procedure as for staining with antibodies.

2.3 Cell lines

2.3.1 NIH-3T3 tCD40L

Immortalized murine fibroblast cell line that is often used to provide growth factors to the cells they are coincubated with. The particular line used in this project provides CD40 ligand (CD40L), also known as CD154 to B cells[97]. CD40L binds to CD40 on B cells, which promotes their maturation, and regulates many important processes, including

immunoglobulin class switching, and formation of germinal centers[98]. Of particular importance, interaction with CD40L provides a resting B cell with a stimulatory signal that activates it and primes it for antigen presentation. This signal is normally acquired through interaction with T cells, but by using the feeder cell line as a co-culturant, the process is streamlined. IL-4 is provided to the culture, which causes up regulation of MHC class II expression and proliferation of the B cells[29], making them ideal for cross-presentation to CD8+ T cells.

2.3.2 NK cell lines

Several different NK cell lines exist, isolated from NK lymphomas that represent various stages of NK cell differentiation. These lines provide an excellent opportunity to test the cytotoxicity of NK cells at different education stages.

NK92

NK92 is an NK cell line characterized by a $CD56^{bright}$ phenotype, and a lack of inhibitory receptors such as KIR, as well as a lack of expression of the Fc receptor CD16[99]. This is a stage of differentiation relatively early in NK cell education, and cells with this phenotype is generally thought of as having low cytotoxicity, but capable of secreting a large amount of stimulatory cytokines, primarily interferon-γ (IFN-γ).

NKL

A cell line established from large granular lymphocyte leukemia[100], with a $CD56^{dim}$ $CD16^{+}$ phenotype, and expresses the inhibitory receptor CD94/NKG2[101]. This is charachteristic of a more mature state of differentiation.

2.4 Cytotoxicity assay

The adherent melanoma cell line FemX-V was killed using the NK cell lines NKL and NK92. A total of $9.5x10^7$ FemX cells were killed in two separate tubes, using $3.0x10^7$ NK92 in one tube, and $15.0x^7$ NKL in the other. This is an effector:target ratio of 0.3:1 for NK92:FemX, and 1.6:1 for NKL:FemX. The cells were suspended in 30mL CellGro GMP CD (*CellGenix*)

serum-free media to avoid serum-derived proteins and proteases in the supernatant. The tubes were then incubated at 37°C for 8 hours. After incubation, the tubes were spun down at 3000rpm for 12 minutes. The supernatant was kept, and protease inhibitors were added. The supernatant was then put at 4°C to keep for separation of antigens.

2.4.1 Time based Degranulation assay

To monitor the killing efficiency of the NK cells versus the target, a degranulation assay was set up to monitor expression of CD107a in the effector cells over time. 100µl of effector and target was pipetted into 10x2 wells on a 96 well plate. Anti-human CD107a antibody (*BD Pharmingen, Alexa Fluor 700*) was added to the effector cells at a concentration of 1:50. The effector cells were then transferred into the target well after 0, 1, 2, 3, 4, 5, 6, 7, 7,5, and 8 hours.

2.4.1 Fractionation of Lysates

The lysates generated from NK cell mediated killing of FemX were separated into fractions based on their molecular weight. This was done using Amicon Ultra-15 Centrifugal Filter (*Merck Millipore*) 3k and 10k devices. The lysates were first filtered through the 10k device, to separate into fractions of peptides above and below 10kDa. This was done by spinning the filter tubes at 4000rpm for 15 minutes. The flowthrough from this, containing the fraction below 10kDa, was then filtered through the 3k device. The filter tubes were spun at 4000rpm for 45 minutes. The flowthrough from this contained the fraction below 3kDa. The unfiltered volume left in the tube would then contain the fraction between 3kDa and 10kDa, at a concentration of about 50x. All fractions were then frozen down at -80°C.

2.5 Cell storage

2.5.1 Freezing cells

To freeze a cell culture down for storage, the culture was first spun down for 8 minutes at 1300rpm. The supernatant was discarded, and the cells resuspended in 10mL RPMI 1640 with 10% FCS and spun down again. This step was done twice, to wash the cells. The supernatant was then discarded, and the cells resuspended in 500µL RPMI1640 with 20%FCS. This

500µL cell suspension was then transferred into a cryotube. 500µL RPMI1640 with 15% DMSO was added dropwise into each cryotube. The vials were then put into -80°C overnight, before they were transferred to liquid nitrogen after 24 hours.

2.5.2 Thawing cells

To thaw cells for further use, they were taken from the freezer and put on dry ice. A 15mL tube containing 10 parts RPMI 1640 medium without serum and 1 part CTL wash was prepared. The sample was put at 37°C until it thawed almost fully, only containing a small ball of ice. 500µL of the washing media solution was then added drop wise into the sample. The sample was then pipetted drop wise from the sample vial into the media tube in lots of 500µL, until the entire sample was transferred. The cells were then spun down at 1500rpm for 5 minutes, resuspended, washed with 1 part CTL wash and 10 parts serum free RPMI 1640 medium, and spun down again at 1500rpm for 5 minutes. They were then resuspended in RPMI 1640 medium with 10% FCS, and either used or stored at 37°C and 5% CO_2.

2.6 Culturing CD40 activated B cells

CD-40 activated B cells to be used as APCs were generated as outlined in [102], with a few modifications. The B cells themselves were isolated from fresh blood or thawed PBMCs using AutoMACS magnetic separation.

2.6.1 AutoMACS separation

Isolation of B cells was done from thawed PBMCs, on the Miltenyi Biotech AutoMACS/AutoMACS Pro machines, following the Miltenyi provided protocol at their website[103]. The cells were counted, and spun down at 300xg for 10 minutes. The pellet was then resuspended in 80µL AutoMACS buffer per 10^7 total cells. On the AutoMACS Pro, the machine does the remaining steps. For the AutoMACS, it was done manually. 20µL CD19 Microbeads (Miltenyi Biotech) per 10^7 total cells was added, and mixed. The cells were then put at 4° for 15 minutes to incubate. After incubation, 1-2mL AutoMACS buffer per 10^7 cells were added, and spun down at 300xg for 10 minutes. The supernatant was discarded, and the cells were resuspended in 500µL AutoMACS buffer, now ready to be separated by positive selection on the AutoMACS.

2.7 B cell culture

B cells were expanded in 6 well plates, using NIH-3T3 cells expressing CD40L as feeder cells. First, feeder cells were irradiated, and a 2mL suspension was placed into the wells at a density of 0.1 x 10^6 /mL, for a total of 0.2 x 10^6 cells/well. The plates were then incubated for at least 4 hours, to allow the feeder cells to become adherent. The medium used was Dulbeccos Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 0,1% gentamicin.

Isolated B cells were suspended in RPMI1640 medium with 10% FBS and 0,1% gentamicin, supplemented with 4ng/mL IL-4 and 5µg/mL insulin. The medium from the preincubated feeder plates was removed, and replaced with 4ml B cell suspension containing 2 x 10^5 B cells/well. The plates were then incubated at 37°C and 5% CO_2 for 7 days. The adherent feeder cells were resuspended using 2mM EDTA in PBS. Trypsin was not used, as it had a damaging effect on the cells.

2.7.1 Pulsing with antigen

Antigen was pulsed onto cultures on day 7 of B cell culturing. Autologous PBMC was thawed from nitrogen storage, two fractions were made, one with 4 x 10^6 PBMC only, and one with 4 x 10^6 PBMC and 4 x 10^6 autologous B cells. The cells were resuspended in 400µL T cell medium (TCM, section 2.8.1). From these two fractions containing cells suspended in 400µL TCM, 100µL was dispensed into 4 wells for each tube for a total of 8 wells on a 96 well plate. The cells were then pulsed with antigen, by adding 2 µL antigen into each well, and set to incubate at 37°C for 60-90 minutes. The 100µL cell suspension in the well were then transferred into 10 wells, each now containing 10µL cell suspension. The volume in each well was then brought up to a total volume of 200µL with TCM. The plate was then incubated at 37°C and 5% CO_2.

2.7.2 Antigenic peptides used to pulse cell cultures

Epstein-Barr virus (EBV) peptides

BZLF-1

BZLF-1 is a transcriptional factor expressed by EBV that initiates disruption of its latent period. EBV is a ubiquitous virus that infects over 90% of the world's population, and is the cause of infectious mononucleosis, as well as having the potential to induce various lymphoid and epithelial-derived malignancies. BZLF-1 has been shown to be highly immunogenic[104], and has been suggested as a potential target for vaccine development[105].

EBNA-1

Another EBV protein, EBNA-1 involved in most of the functions of and latency phases of the virus. Although the protein is expressed in every phase of the EBV lifecycle[106], it is poorly recognized by the immune system due to a low expression level, and terminal Gly-Ala repeat sequences that prevents it from proteosomal breakdown and presentation on MHC molecules[107].

EBV consensus sequence

A library containing peptides from a consensus sequence of the EBV proteome.

Cytomegalovirus (CMV) peptides

PP65

PP65 is the major constituent of human cytomegelovirus (hCMV) virions, and has been found to be a highly recognized target for both humoral[108] and cell-mediated[109] immune responses. It has been shown to modulate antigen presentation[110], and to reduce the activation of NF-κB[111].

(Self) Tumor antigen

MART-1

Melanoma antigen recognized by T cells 1 (MART-1), also known as protein melan-A, is an antigenic protein found on the surface of melanocytes[112]. Its expression is a marker for melanoma, and is known to be recognized by $CD8^+$ T cells. MART-1 has successfully been used in clinical trials to pulse dendritic cells to induce expansion of melanoma specific T cells [113].

2.7.3 Pulsing with tumor cell Lysate fraction

Cultures were pulsed the lysate from NK cell killing, obtained from the procedures described in 2.4. The 10kDa to 3kDa fraction was used, as this fraction should contain peptide fragments of theoretically the right size for antigen presentation. The procedure was the same as the one described in 2.7.1, but the medium used for the duration of the pulsing was serum-free CellGro GMP CD (*CellGenix*), with lysate in a concentration of 1:5 lysate:medium. The cells were then incubated for 60-90 minutes at 37°C. After incubation, the cell suspension was pipetted over 10 wells, and added up to a total volume of 200µL with TCM.

2.8 T cell culture

T cells were grown from PBMCs and coincubated with B cells from the same donor. This was done by transferring 4 x 10^6 cells (PBMC only, and PBMC + B cells) into two 15mL tubes containing either PBMC only, or PBMC + B cells. The tubes were then spun down at 1500rm for 5 minutes. Cells were then pulsed with antigen according to the procedure described in 2.7.1. The next day, 50u IL2 was added into each well, and set back into incubation. The plate was incubated at 37°C and 5%CO_2 for 7 days.

On day 7, the 10 wells from each row were pooled together into 1 well on a 12 well plate, totaling a volume of 2mL in each well. This volume was brought up to 4mL with TCM. Around day 7 was when these T cell cultures started to proliferate heavily, and they were fed with new medium every 1-2 days past this point. Refeeding was done by carefully removing about half the volume in the well, and then adding the same volume of fresh TCM. Around

day 12 was when T cell proliferation reached its peak, so a sample of 200µL from each well was taken and prepared for analysis. The rest was frozen down for later use.

2.8.1 Media and supplements

Interleukin 2 (IL2) Drives the differentiation and proliferation of T cells

Interleukin 4 (IL4) Induces B cell proliferation, and up regulation of MHCII

Insulin Boosts metabolism of B cells, stimulating growth

Media

Suspension Cells were grown in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS)

Adherent cell lines were grown in Dulbeccos Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS)

T cell medium (TCM)

The T cell medium (TCM) used to grow T cells was made using RPMI 1640 with 10% human serum, supplemented with 1% non essential amino acids (NEAA), 1% sodium pyruvate, 0,1% Mercaptho-ethanol (ME), and 0,1% gentamicin.

3 Results

3.1 Establishing a B cell APC system for robust T cell responses

CD40 activated B cells were used as antigen presenting cells for this project. B cell cultures were established using magnetically purified primary B cells that were co-incubated with the feeder cell line NIH-3T3 tCD40L, a murine fibroblast line transfected with CD40L (CD154), normally expressed by activated $CD4^+$ T cells. Interaction with CD40L is mediated by CD40 expressed on the B cell, and the binding of this receptor to its ligand results in activation of the B cell[114]. This interaction also causes the $CD4^+$ T cell to secrete IL-4, a cytokine that is thought to synergize with CD40L in driving the clonal expansion of B cells[115]. IL-4 was therefore added as a supplement to the B cell culture to stimulate expansion in the absence of $CD4^+$ T cells. In addition, insulin was also given as a supplement to increase proliferation.

3.1.1 CD40 activated B cells exhibit a substantial increase in CD86 expression

To assess B cell activation in the primary B cell cultures, a timecourse for CD86 expression over seven days in culture with CD40L expressing feeder cells was set up. A sample of 200µl cell suspension was stained each day with CD86 antibody (Brilliant Violet 605, BioLegend) in a concentration 1:100. A mastermix of staining antibodies was made on Day 0 and used for every subsequent day to minimize variance. CD86 (B7-1) is a co-receptor expressed on activated B cells, as well as on monocytes. Its function is similar to CD80 (B7-2), namely to bind CD28 and CTLA-4 on a T cell, which triggers a co-stimulatory signal necessary for the activation and proliferation of naïve T cells[116]. Increased expression of CD86 will contribute to a more efficient means of activating T cells, and serves as a marker for B cell activation.

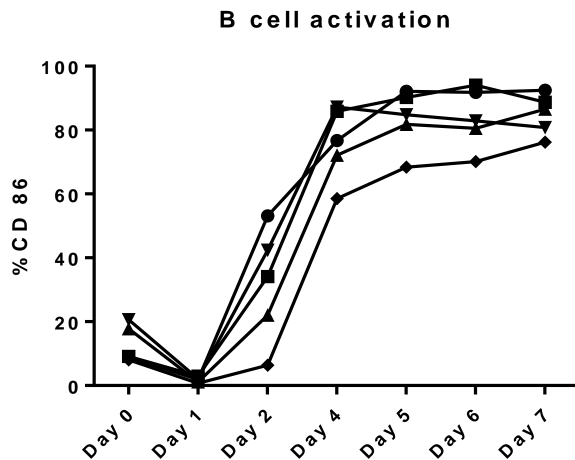


Figure 5 A representation of the expression of B cell activation marker CD86 over time.

The expression was measured on five donors, with each donor showing a similar curve. Every donor reached a peak expression at around day 4-5, after which point the level was maintained for the rest of the time period. The B cells were taken out of culture and pulsed with antigen at day 7.

Figure 5 shows the results. There is a substantial increase in expression of CD86 over the time period, a clear indication that the cells acquire an activated phenotype. The B cell protocol I followed to grow these cells[102] reports an increase in CD80 and HLA-DR (MHC class II) as well, but I was not able to reproduce these results, possibly due to problems with the staining. The same paper also reports a substantial increase in B cell numbers after about 10-12 days of culturing, but as I did not need a large amount of B cells, 7-8 days was sufficient for the purposes of the project.

3.1.2 Activated B cells were co-cultured with autologous PBMC and pulsed with antigen peptide libraries to induce expansion of T cells.

In order to determine the efficiency of the CD40 activated B cells as APCs, I pulsed the cells with antigenic peptide libraries, and co-cultured the cells with autologous PBMC to look for expansions of T cells. For comparison, PBMC from the same donors was stimulated with the same peptides in the same manner, but without B cells. This was done by adding $4x10^6$ PBMC into each of two tubes, and adding $4x10^6$ autologous B cells into one of these tubes. These cells were then resuspended in 400µL T cell medium (TCM, section 2.8.1), a medium with supplements meant to enhance T cell proliferation. The 400µL cell suspensions was then

dispensed into 4x2 wells on a 96 well plate, for a total of 8 wells with 100µL in each well. 2µL peptide was added to both the PBMC and the PBMC+B wells, with different peptides for each pair. Negative controls were wells without peptide added. Figure 6 illustrates how the plates were set up.

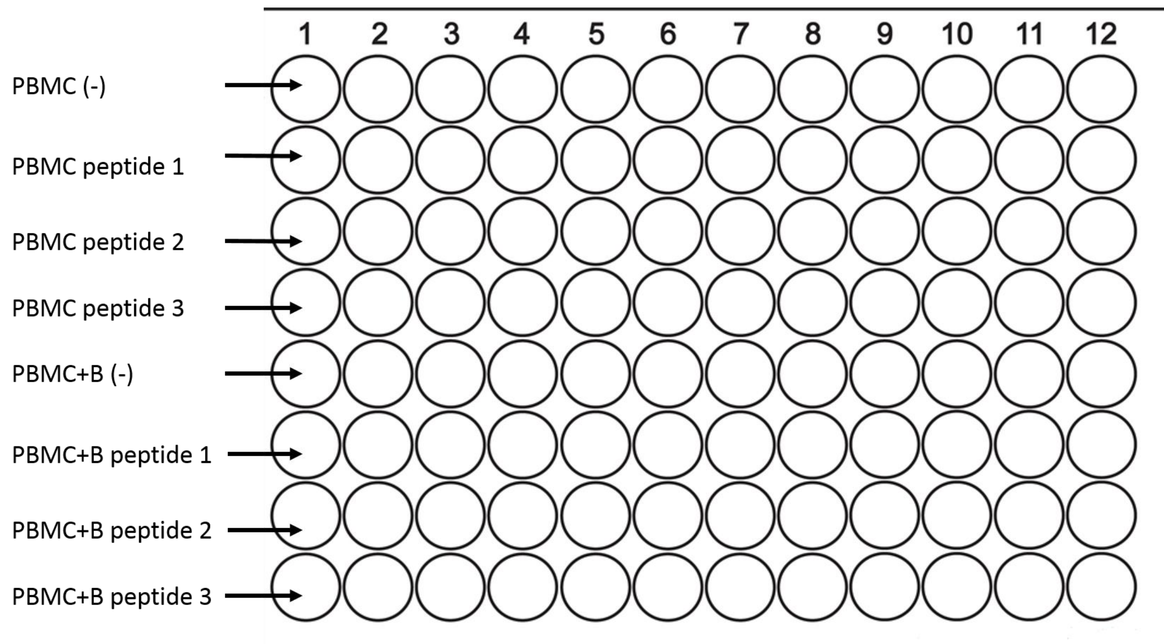


Figure 6 Plate set up for pulsing cells with antigen.

Antigen was added in such a way that wells with PBMC and PBMC+B was stimulated with the same peptides (peptide 1, 2, 3), as well as a negative control

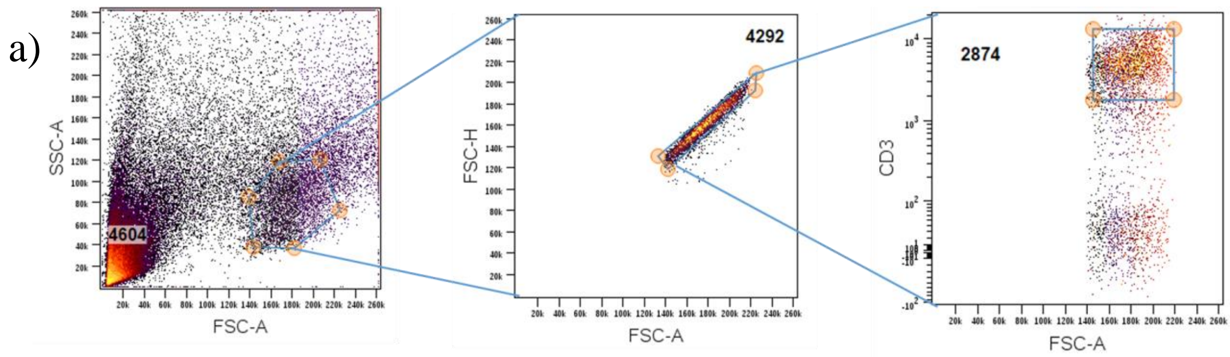
The cells were then set to incubate at 37°C for 60-90 minutes. After incubation, 10µL from each of the wells were dispensed into the next nine wells in the same rows, resulting in a plate with 8x10 wells with 10µL cell suspension in each. Each well was then filled to a total volume of 200µL with TCM, and set to incubation at 37°C and 5%CO_2 for at 7 days. 50u IL-2 was added to each well the next day. On day 7, the cells were pooled together and dispensed into 12 well plates, with one well for each culture. In the following days, the cultures were resuspended by pipetting to prevent too much clumping of cells.

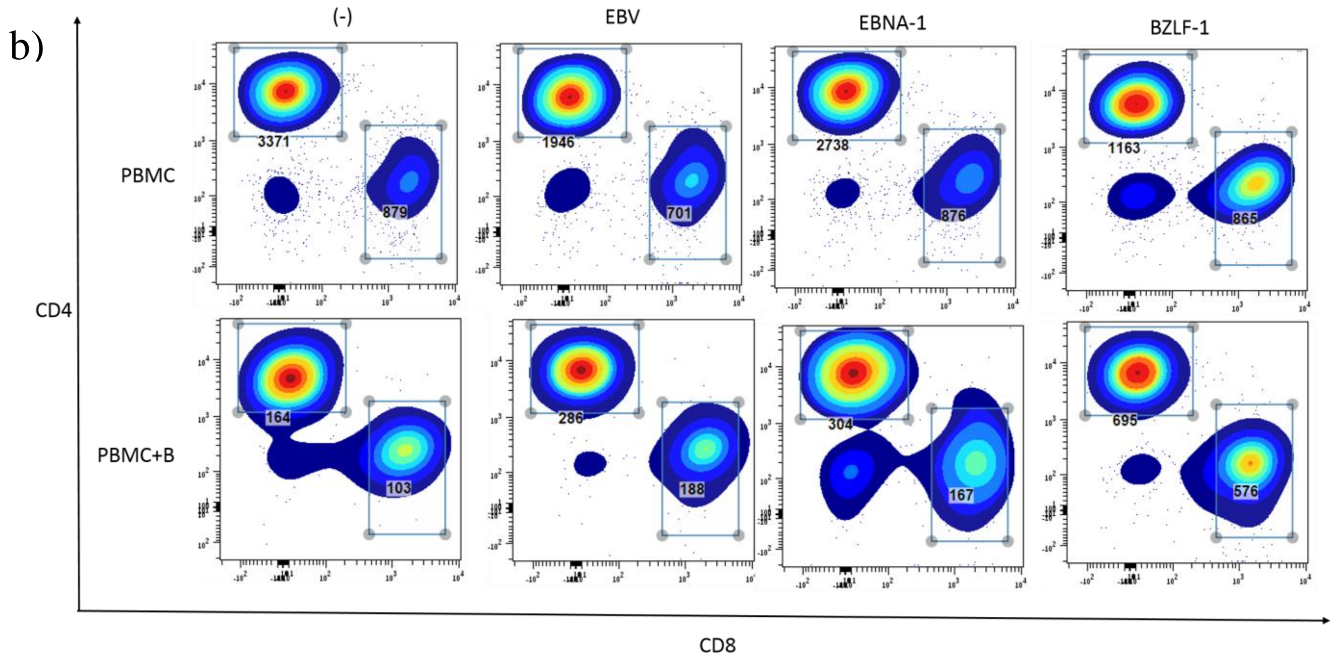
As this process was repeated several times, various peptides could be used for presentation. The peptides used were EBV consensus sequence, EBNA-1, BZLF-1, PP65 and MART-1. Out of these five, four of the peptides are of viral origin (EBV consensus, EBNA-1, BZLF-1 (from Epstein-Barr virus (EBV)) and PP65 (from cytomegalovirus(CMV)). MART-1 is a self

tumor antigen associated with melanoma. Through stimulation with these antigens, I wanted to determine to which extent T cells would expand in response, and whether or not co-culturing with CD40 activated B cells had an impact on magnitude of the expansions.

3.2 Expansion of T cells in response to antigens

The rationale behind co-culturing PBMCs with CD40 activated B cells, is that these B cells will provide a more efficient means of activating T cells, and thus result in a better expansion of effector T cells. To investigate this, a comparison between the cell cultures described in the previous section was done by flow cytometry analysis. The cells were stained with antibodies for CD3 (PE, BioLegend), CD4 (Brilliant Violet 421, BioLegend) and CD8a (Brilliant Violet 785, BioLegend). The results were gated in CytoBank by gating gating side scatter area (SSC-A) vs. forward scatter area (FSC-A) on the population with the size properties of PBMC, then gating on forward scatter area (FSC-A) vs. forward scatter height (FSC-H) to exclude non-single cells, and then on CD3 positive signal to obtain populations of $CD3^{+}$ PBMCs. Out of the CD3 positive cells, CD4 signal was plotted against CD8 signal to determine the size of these populations. In hindsight, a dead cell marker should also have been used, to exclude non-specific signals resulting from dead cells in the analysis. **Figure 7** shows an example of the gating strategy used to determine the $CD4^{+}$ and $CD8^{+}$ populations.





CD4	(-)	EBV	EBNA-1	BZLF-1
PBMC	3371	1946	2738	1163
PBMC+B	164	286	304	695
CD8	(-)	EBV	EBNA-1	BZLF-1
PBMC	879	701	876	865
PBMC+B	103	188	167	576

Figure 7 Example of the gating strategy used for analysis of T cell expansions.

a) Side scatter area (SSC-A) vs Forward scatter area (FSC-A) plot to select PBMCs from the total population of cells. Height (FSC-H) vs Area (FSC-A) to select single suspension cells. CD3 plot to select CD3+ cells (should be mostly T cells). Out of the CD3+ population, CD4 was plotted against CD8 to determine their counts. b) Contour plots of the cultures from one donor, with the cultures not stimulated with B cells (PBMC) in the upper row, and the cultures co-incubated with activated B cells (PBMC+B) in the lower row. The cultures have been stimulated with the same antigens (EBV consensus, EBNA-1 and BZLF-1). The negative control cultures (-) has not been stimulated with any peptide. The event counts of the plots are shown in the table below them.

To determine the extent of the T cell expansions, I first tried to look at the percentages of the resulting CD4$^+$ and CD8$^+$ cells. This turned out to be a non-optimal way of analysis, as the percentages did not really say much about whether or not an expansion had occurred, and it was confounded by the effect that percentage shift in one cell type affecting the other. It gave some indication about to what degree non-differentiated, double positive cells became differentiated as a result of being exposed to the antigen, but was not a very helpful way of determining if an expansion of effector cells were actually occurring.

A better way of looking for expansion was to look at the differences in the event count for the populations, as shown in **Figure 7**. Each well analyzed by flow cytometry was run in its entirety, giving comparable relative event counts per well between different conditions. The event count is simply the number of cells in a certain population, where one event is one cell. This should give a more representative view of the expansion of a cell population as a response of antigen stimulation. The hypothesis is that cultures stimulated with antigen should cause more cells to divide, resulting in more cells if the antigen is recognized, in other words, an expansion. Furthermore, if the culture contains CD40 activated B cells, the activation should be more efficient, thus resulting in a bigger expansion.

3.2.1 Co-culturing with CD40 activated B cells results in increased T cell expansions

In a perfect world, all the cultures would contain about the same amount of cells from the start, and give rise to about the same amount of events when analyzed, as they all started with what should be approximately the same amount of cells. However, the analysis of the different cultures showed at times very varied numbers of events from donor to donor, and from culture to culture. This variation could result from a lot of potential sources, from start to finish of the process, originating either from differences in biology from donor to donor, or, maybe more likely, the inexperience of the researcher. Even though every culture was treated the same way in theory, in practice there could be differences between certain conditions. These include parameters such as the freshness of reagents, health of thawed cell cultures, small differences in culture time, care and attention given to growing cultures, ongoing infections of the donor at the time of donation, or amount of dead cell debris in culture. Taking all this into consideration, the variance of the cell counts could be a result of external factors, however, one can only look at the numbers and analyze them as to the best of one’s

ability. Even though some donors showed some unexplained variation between cultures, each donor seemed to show a more or less consistent pattern after being stimulated with different antigens.

To standardize the magnitude of expansion between donors, fold change was used. Fold change is a simple way of showing the degree of change from one value to another, or the "x-fold" change in event count from one culture to another. The most common way of calculating fold change is to divide the final value B, with the initial value A, or $\frac{B}{A}$. This means that a positive change is above 1, and a negative change is between 0 and 1. A 4-fold change would mean a B value 4 times higher than the A value, while a 0.25-fold change would mean a B value 4 times lower that the A value. Another way of calculating fold change would be to take the negative of the inverse of a fold-value below 1, to end up with a negative number, in other words, a 0.25 fold change would be a fold change of -4. An obvious difference between these two ways of calculating fold change, is that when averaging out numbers, negative fold changes have a much higher impact when they are represented as their negative fold change, than when they are represented as a number between 0 and 1. This is clearly something one must take into consideration when trying to represent these numbers. Since I am looking for expansion of populations, I am really only interested in positive fold changes. Negative fold changes mean there is no expansion, while positive changes mean that there is. I will therefore use the standard way of measuring fold change, with negative fold changes represented as numbers between 1 and 0. The fold changes in T cell counts after being stimulated with antigen and incubated for 12 days are shown in **Figure 8**.

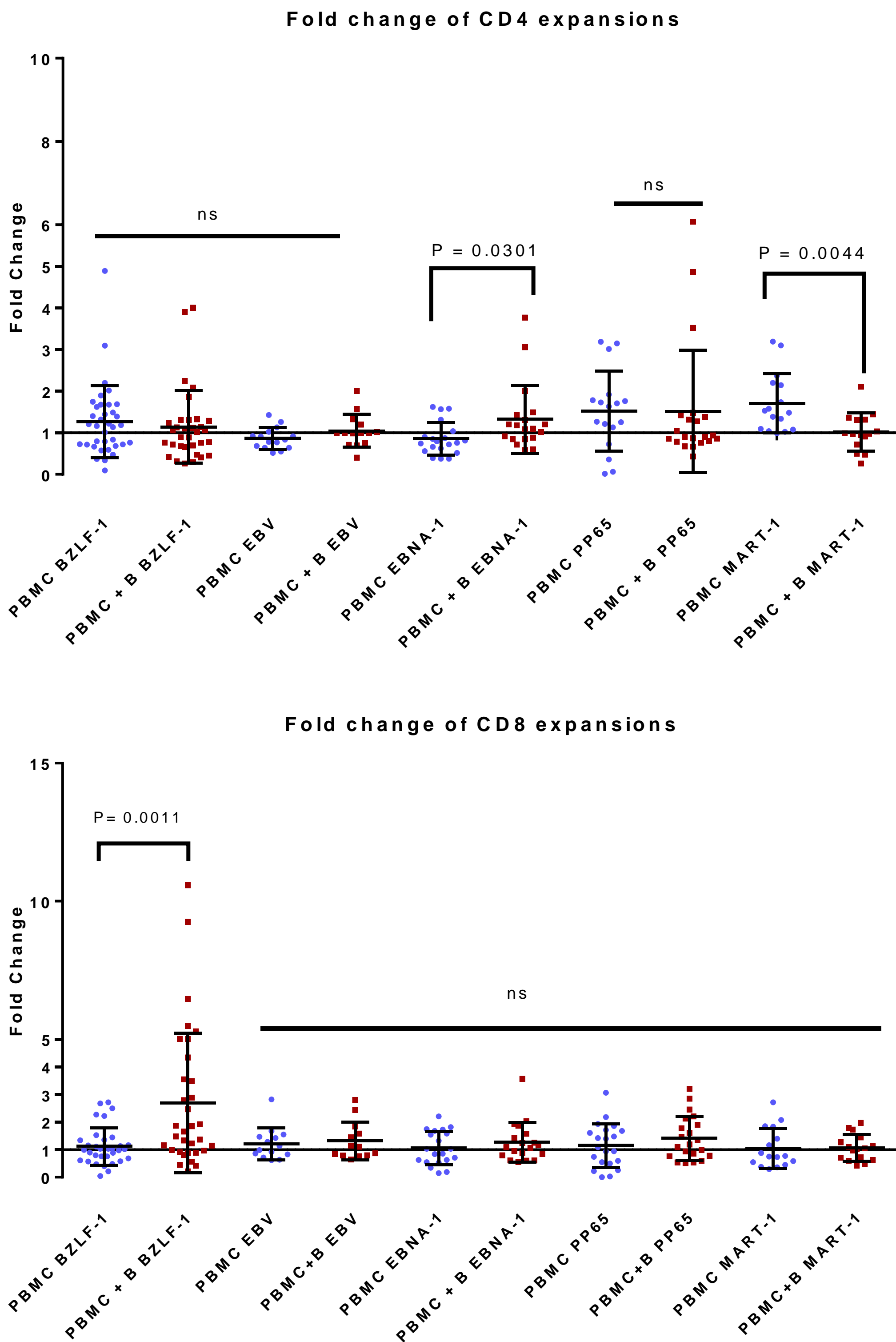


Figure 8 Graph of expansions in CD4+ and CD8+ T cells, represented by fold change.

Blue color represents cultures not co-cultured with CD40 activated B cells (PBMC), red color represents cultures that have been co-cultured with CD40 activated B cells (PBMC+B). The only significant differences in $CD4^+$ expansions are for cultures stimulated with EBNA-1 or MART-1 peptides. For the MART-1 expansions, there is significant increase for the cultures that have not been co-cultured with CD40 activated B cells. In the $CD8^+$ populations, there is a significant increase in expansion for cultures pulsed with BZLF-1 peptide. P values were calculated using a paired Students t-test.

Figure 8 shows the different T cell expansions represented by their fold change value. Cultures co-incubated with CD40 activated B cells (PBMC+B) are shown as red dots, cultures not co-incubated with CD40 activated B cells (PBMC) are shown in blue. The only significant increases in T cell expansions are displayed by EBNA-1 for CD4 and BZLF-1 for CD8. CD4 expansions for MART-1 actually show a significant decrease in numbers in the B cell stimulated cultures. The overall highest expansions are toward the BZLF-1 peptide, with $CD8^+$ cells showing the most dramatic response, suggesting that BZLF-1 is highly immunogenic. These findings are consistent with the reports of Hartlage et al., who found expansions of BZLF1-specific $CD8^+$ after co-culturing autologous PBMCs with dendritic cells pulsed with recombinant BZLF1[105]. It is strange that there are no significant expansions in the EBV consensus sequence stimulated cultures, as this peptide library contains sequences from all EBV proteins. A reason for this could be that the exact peptide sequences for the other peptides are not a part of the library, or at least contains a lower proportion of the recognized peptide. Overall, the results indicate that co-culturing with CD40 activated B cells results in better expansion, given that the cultures are maintained properly.

3.3 Generation of epitope specific T cell responses

Expansion of T cells come as a result of the clonal selection and proliferation of cells that have recognized a specific epitope and been given the signals required to divide and multiply. I wanted to investigate the T cell expansions to determine what percentage of the populations was epitope specific for the peptide they had been pulsed with. In order to assess this question, the populations were stained with tetramers. Tetramers are tetrameric molecules made up of MHC monomers complexed with antigen, bound together by streptavidin and marked with fluorescent labels. T cells specific for the peptide complexed with the MHC molecules are able to bind the fluorescent tetramers, and will show up as a positive signal in a flowcytometric analysis. In order for the cells to be able to bind the MHC molecules, they need to be of a matching haplotype, so donors need to be screened for the specific haplotype of the tetramers before staining.

3.3.1 $CD8^+$ T cell specific expansions towards PP65 antigen

A tetramer stain was used to test the specificity of the expanded T cells. The tetramers were of the HLA-A2 haplotype, so in order to assess the specificity of the cells, donors of this

haplotype were needed. A simple screen on eight donors, using anti-human HLA-A2 (*eBioScience, Pe-Cy7*) was set up to identify A2 positive samples. The results of this screen are shown in **Figure 9**.

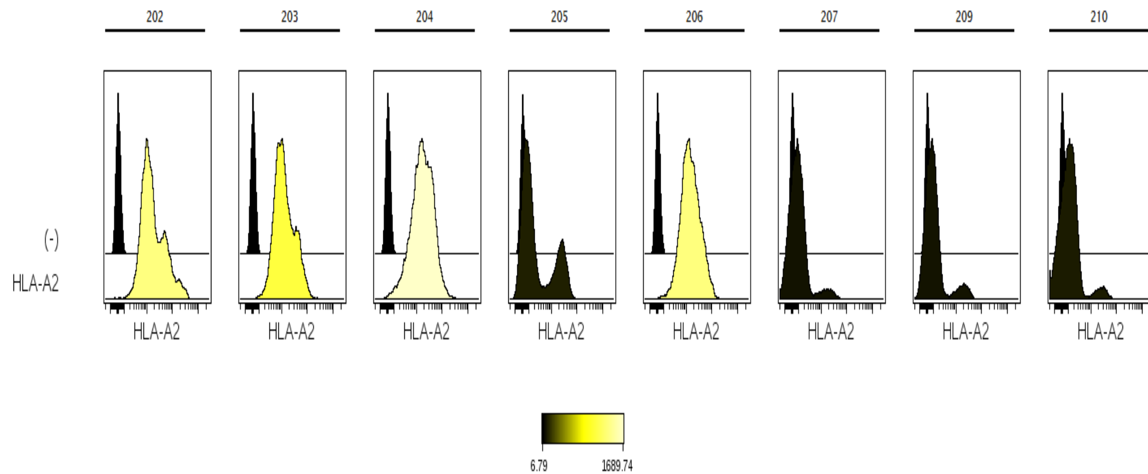


Figure 9 The result of the screen for HLA-A2 positive donors.

Donors 202, 203, 204, and 206 were identified as HLA-A2 positive.

Of these eight donors, four were identified as positive, and four were identified as negative, based on comparison to an unstained control. The cells were pulsed with peptides from PP65, MART-1 and BZLF-1 in the same manner as described in 3.1.2. The cells able to recognize the antigen presented on the tetramer, will bind the tetramer, and manifest as a positive signal on a flow cytometry dot plot. The percentage of cells specific for the tetramer were compared to the negative control, by gating for events trailing off from the main population. An example of this is shown in **Figure 10**

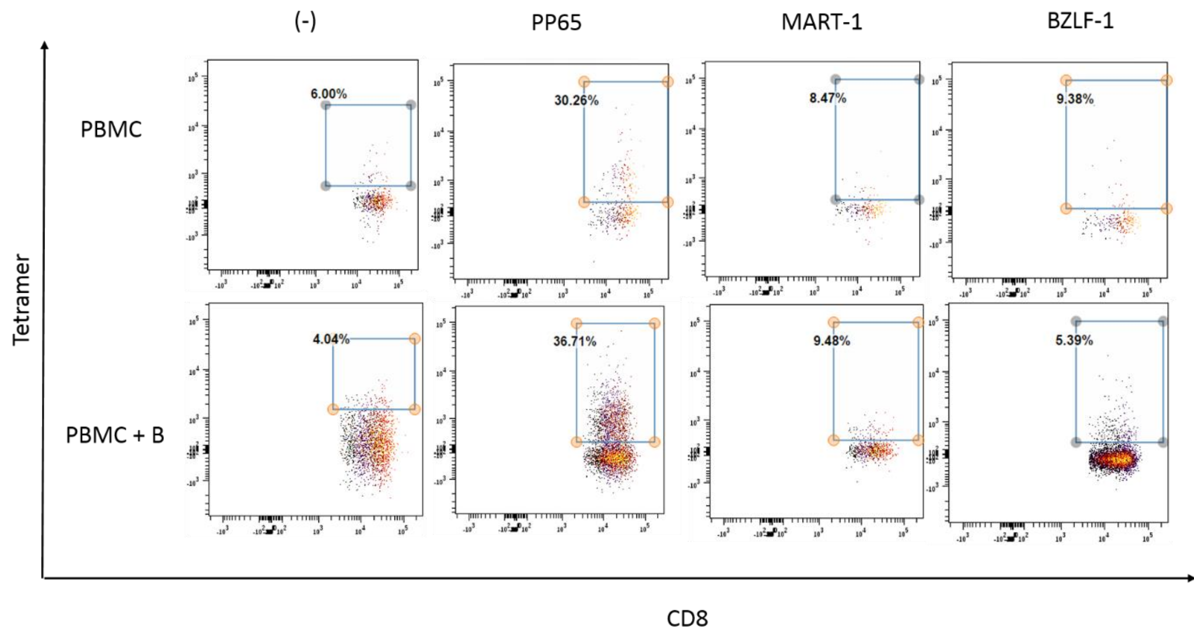


Figure 10 Tetramer stains of donor 206.

The dotplots show how the tetramer positive cells trail off from the main population.

	202⁺	203⁺	204⁺	205⁻	207⁻	206⁺	209⁻	210⁻
PBMC (-)	7,50 %	5,19 %	6,05 %	3,74 %	6,45 %	6,00 %	5,33 %	6,66 %
PBMC PP65	6,00 %	9,21 %	35,60 %	6,74 %	7,87 %	30,26 %	5,83 %	8,19 %
PBMC MART-1	7,18 %	7,14 %	5,89 %	7,09 %	9,81 %	8,47 %	3,34 %	5,07 %
PBMC BZLF-1	6,06 %	5,78 %	4,28 %	9,51 %	6,86 %	9,38 %	1,84 %	3,58 %
P+B (-)	4,47 %	4,87 %	4,69 %	6,54 %	5,02 %	4,04 %	2,91 %	4,99 %
P+B PP65	7,59 %	4,30 %	56,96 %	7,56 %	10,00 %	36,71 %	0,20 %	1,08 %
P+B MART-1	6,21 %	4,27 %	4,24 %	3,84 %	2,98 %	9,48 %	0,00 %	2,31 %
P+B BZLF-1	10,39 %	4,02 %	3,83 %	5,05 %	4,55 %	5,39 %	0,42 %	2,25 %

Table 1 Percentages of tetramer positive CD8 T cells.

Donor numbers are shown as HLA-A2 positive or negative (e.g. 204^+, 205^-). Donors 204 and 206 show a strong response to the PP65 tetramer, with minimal response towards the other tetramers. None of the HLA-A2 negative donors show any response.

Table 1 Percentages of tetramer positive CD8 T cells.sums up the results of the tetramer stain. Two donors, 204 and 206, show an obvious response to PP65, with no response to any other antigen. The HLA-A2 negative donors does not respond to any of the tetramers, as expected. None of the donors shows any strong signal from the BZLF-1 tetramer, even though BZLF-1 stimulated cultures had the highest $CD8^+$ expansions. This could either stem from a problem with the BZLF-1 tetramer, or it could be that the expanding cells are recognizing another epitope than the one presented on the tetramer.

3.4 Generation of NK derived cytolytic fragments for antigen presentation

Having established that B cells co-culture results in greater specific T cells expansion, I then proceeded with investigation into whether lysates derived from cytolysis of tumor cells could produce specific T cell expansions. The lysates were obtained by NK cell driven cytolysis of the tumor cell line FemX, with the NK cell lines NK92 and NKL as effector cells. A total of 14.25×10^7 FemX cells were used in the experiment, split equally into 3 tubes resulting in $4{,}75 \times 10^7$ FemX cells in each tube. 3.0×10^7 NK92 cells were added into one of the tubes, 15.0×10^7 NKL were added into the other tube, giving an effector:target ratio of 0.3:1 NK92:FemX and 1.6:1 NKL:FemX. The remaining tube containing FemX only, was kept to provide a negative control. The cells were suspended in 30ml CellGro GMP CD (*CellGenix*) serum-free media to avoid serum-derived proteins and proteases in the supernatant. These tubes were then set to incubate at 37C for 8 hours and a time based degranulation assay was set up to monitor NK cytotoxicity over the same time period. The next section will describe the results of the degranulation assay.

3.4.1 NKL exhibits a stronger degranulation than NK92 versus FemX

A degranulation assay was set up as described in 2.4.1 to assess the cytotoxicity of the NK92 and NKL NK cell lines versus the FemX target cell line. The FemX cell line has been tranfected with a plasmid that makes the cell express YFP when alive, and CFP when killed.

The experiment was conducted by adding the NK cells to wells at regular time points over 8 hours, to observe changes in CD107a expression over time. CD107a expression correlates with both cytokine secretion and NK cell mediated lysis of target cells, and is therefore used to measure NK cell cytolytic activity[117]. The results were analyzed in CytoBank, by first gating for YFP negative cells to eliminate target cells from the analysis, then forward scatter height vs forward scatter area (FSC-H vs FSC-A) to select for single suspension cells. Out of this population, the PBMC population was gated on a FSC-A vs side scatter (FSC-A vs SSC-A) plot. The %CD107a expression on this population was then assessed on a histogram plot. The gating strategy is shown in **Figure 11**.

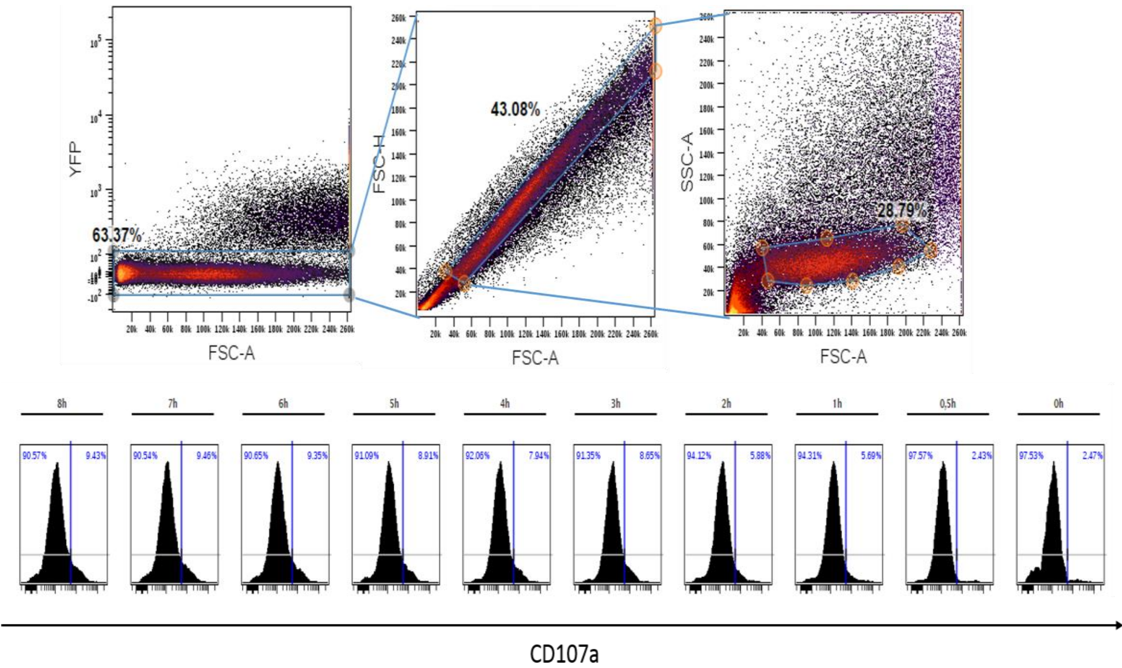


Figure 11 Gating strategy for the CD107a degranulation assays.

YFP positive cells are first gated away to eliminate target cells. Next, single suspension cells are gated on a FSC-A vs FSC-H plot. Out of the single cells, PBMCs are gated on a FSC-A vs SSC-A plot. Histogram plots are then used to assess the % expression of CD107a.

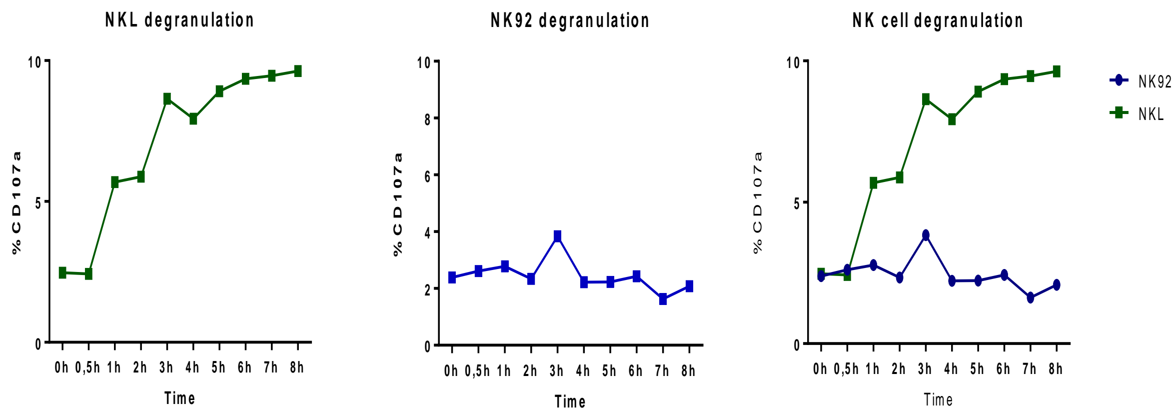


Figure 12 Results of the degranulation assays.

The graphs show the change in expression levels of CD107a over a time period of 8 hours. NKL exhibits a clear increase in degranulation, peaking out at about 10%, while NK92 shows no increase in expression of CD107a.

The results are shown in **Figure 12**. NKL exhibits a marked increase in degranulation over 8 hours peaking out at around 10%, with the highest increase between the 0,5h-4h marks. NKL has a more mature phenotype, $CD56^{dim}$ $CD16^{+}$ CD94/$NKG2^{+}$ [101, 118] associated with higher cytotoxicity, consistent with these results. $CD56^{dim}$ $CD16^{+}$ cells comprise the majority of NK cells found in human PBMC. CD16 being FCγ receptor III, these cells are able to bind the Fc portion of IgG and thus are capable of mediating antibody dependent cell-mediated cytotoxicity (ADCC)[119].

Also immediately obvious from these graphs, is that NK92 does not seem particularly effective at killing the target. This differs from the literature, which mostly seems to agree that NK92 is a highly efficient killer [99, 120-123], and the low cytotoxicity observed in this data could possibly be a result of the low effector:target ratio used in this experiment (0,3:1). However, NK92 has a $CD56^{bright}$ phenotype, a low expression of inhibitory receptors, and no expression of CD16. NK cells with these characteristics are generally considered to have a low cytotoxicity [119, 124]. The low degranulation exhibited by NK92 cells in my data could be a result of this, but it could also be an effect of keeping them in a state of continuous culture, which tends to have a negative effect on the cytolytic capacity of NK cell lines.

3.4.2 CD40 activated B cells has a significant effect on $CD8^+$ T cell expansions pulsed with lysate resulting from NK cytolysis

An important aspect of this project was to determine to what degree cytolysis by NK cells affect T cell responses. To assess this question, I used the lysate obtained from the NK cytotoxicity assay described in the previous sections (3.4) to pulse cultures of PBMC and PBMC+CD40 activated B cells. The lysates of NKL+FemX and NK92+FemX were used, as well as the lysate of the FemX only culture described in section 3.4. The lysates were sorted into fractions containing peptides of 3kDa-10kDa through the use of Amicon Ultra-15 Centrifugal Filter (*Merck Millipore*) 3k and 10k devices. The lysates were first filtered through the 10k device, to separate into fractions of peptides above and below 10kDa. This was done by spinning the filter tubes at 4000rpm for 15 minutes. The flowthrough from this, containing the fraction below 10kDa, was then filtered through the 3k device. The filter tubes were spun at 4000rpm for 45 minutes. The flowthrough from this contained the fraction below 3kDa. The unfiltered volume left in the tube would then contain the fraction between 3kDa and 10kDa, at a concentration of about 50x.

The reasoning behind using the fraction of peptides with molecular weights of 3kDa-10kDa is that these should contain peptides of the ideal size for antigen presentation. Pulsing of the cultures was done in much the same was as when pulsing with peptide libraries as described in section 3.1.2, but the medium used for the duration of the pulsing was serum-free CellGro GMP CD (*CellGenix*), with lysate in a concentration of 1:5 lysate:medium. The cells were then incubated for 60-90 minutes at 37C. After incubation, the cell suspension was pipetted over 10 wells, and added up to a total volume of 200µl with TCM. The fold changes of the T cell expansions that resulted from this are shown in **Figure 13**.

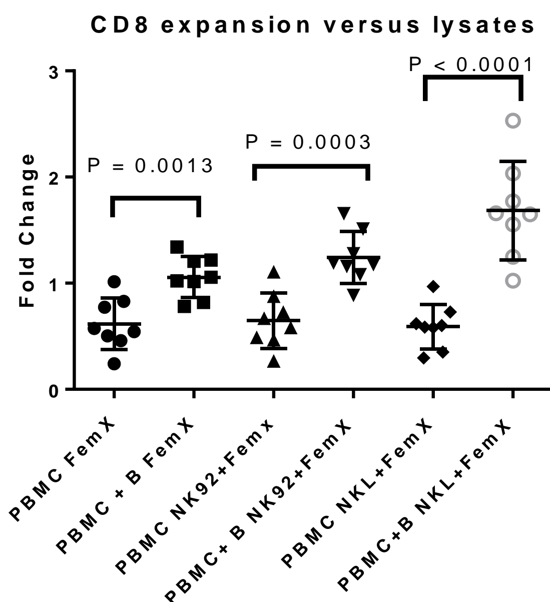


Figure 13 Shows expansions of CD8+ T cell pulsed with FemX lysate.

B cells have a significant effect on the expansions in all cultures, and non B cell cultures show a decrease in T cell counts.

While the $CD8^+$ expansions are not exceptionally high (they ranged from 1,0 to 2,5 fold change for the NKLvsFemX lysate), they do show a marked increase going from the FemX lysate to the NK92vsFemX, and the highest expansions for cells pulsed with NKLvsFemX lysate. This is in line with the results from the degranulation assay in 3.4.1, which showed a very low CD107a expression on NK92 cells towards FemX, and a higher CD107a expression for NKL. The higher cytotoxicity associated with the higher CD107a expression on NKL would result in more peptide fragments available for presentation, and thus lead to better T cell responses. If NK cell lysis fragments contribute to T cell responses, these results are what one would expect to see.

The non B cell cultures interestingly all show a similar median decreased fold change compared to the negative control (FemX: 0,6170 ± 0,08585, n=8, NK92+FemX: 0,6477 ± 0,09239, n=8, NKL+FemX: 0,5914 ± 0,07401, n=8). Why is this? A possible reason could be that there is some kind of immunosuppressive factor from the FemX cells present in the cell lysate. Melanoma cells have been shown to exploit several mechanisms to suppress immune

responses, such as down regulation of tumor associated antigens[125], various immune activating cell receptors like MHC class I[126], co-stimulatory molecules[127], and NK cell receptor ligands[128]. In addition, melanoma cells exhibit an intense secretion of immunosuppressive factors such as VEGF, TGF-β, IL-10, nitric oxide (NO), and prostaglandins [127, 129, 130]. These immunosuppressive factors could have been a part of the lysate, and thus caused a suppressive effect on the cells. However, the secreted agents mentioned have a molecular weight that should exclude them from the 3kDa-10kDa lysate fraction. Nonetheless, there does seem to be some kind of suppressive effect present. This suppressive effect is only apparent in the non B cell stimulated cultures, indicating that the CD40 B cells somehow counteract this suppression.

3.4.3 Pulsing with melanoma cell lysate induces expansion of MART-1 specific $CD8^+$ T cells

The same donors from section 3.3.1 was pulsed with the NK cell lysates of the melanoma line FemX as described in section 3.4.2. This caused expansions of $CD8^+$ T cells co-cultured with CD40 activated B cells (3.4.2), so I wanted to investigate if these expansions were specific towards the MART-1 antigen, a melanoma associated protein expressed by FemX. To answer this question, the cell cultures were stained with MART-1 specific pentamers (Proimmune, UK), and gated in the same manner as described in section 3.3.1.

	202+	203+	204+	205-	206+	207-	209-	210-
PBMC (-)	2,81 %	4,74 %	5,35 %	5,48 %	5,49 %	6,00 %	5,12 %	3,43 %
PBMC FemX	3,44 %	10,49 %	7,03 %	2,35 %	2,51 %	3,04 %	2,85 %	1,54 %
PBMC NK92+FemX	5,00 %	8,53 %	10,24 %	7,23 %	4,95 %	4,75 %	3,78 %	2,02 %
PBMC NKL+FemX	6,74 %	9,58 %	10,96 %	4,06 %	5,40 %	2,16 %	5,85 %	6,70 %
PBMC+B (-)	2,79 %	6,53 %	5,47 %	2,97 %	3,40 %	3,99 %	4,33 %	4,70 %
PBMC+B FemX	5,26 %	6,55 %	4,07 %	8,22 %	4,51 %	5,21 %	3,74 %	6,93 %
PBMC+B NK92+FemX	8,33 %	15,36 %	3,22 %	7,98 %	5,98 %	8,22 %	2,75 %	9,97 %
PBMC+B NKL+FemX	8,77 %	18,02 %	8,47 %	7,29 %	16,15 %	12,25 %	10,20 %	32,12 %

Table 2 Percentages of MART-1 tetramer specific $CD8^+$ T cells

Table 2 shows the results. The highest responses are from the cells co-cultured with B cells, and highest of those, are the cells pulsed with the NKL+FemX lysate. Because NKL had the highest degranulation versus FemX, these results support the hypothesis that NK cell mediated cytotoxic activity improves the responses of the adaptive immune system. A very striking observation here is that the strongest response is from donor 210. This donor was

identified as A2 negative, and therefore should not be able to bind to the tetramers. A possible explanation for this could be experimental error; however, it is possible that this donor is of a haplotype that is not A2, but a similar haplotype that is able to crosslink A2 specific tetramers. A DNA test of the donor would be required to confirm this.

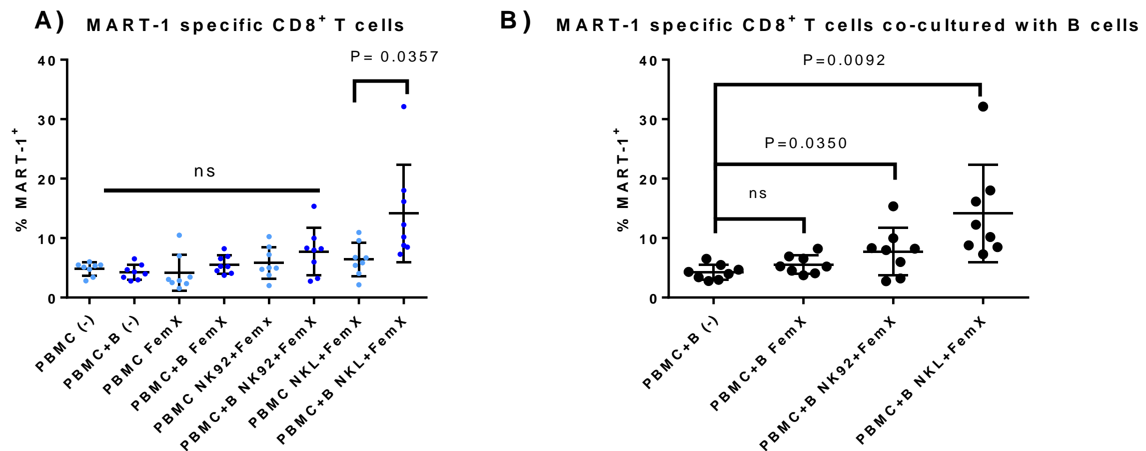


Figure 14 MART-1 tetramer specific CD8+ T cells.

A) Comparison between MART-1 specific CD8+ T cells with and without B cells in co-culture. B) A comparison of the MART-1 specific CD8+ T cells in cultures that was co-cultured with B cells. P-values was determined by a paired Students t-test.

The data is shown in **Figure 14**. I wanted to see if co-culturing with B cells would have a significant effect on the amount of MART-1 specific $CD8^+$ T cells induced. This can be seen in A). The figure shows that there is a significant difference (P=0,0357) between the stimulated and non-stimulated cultures pulsed with the NKL+FemX lysate, but not for the other lysates. NKL was the NK cell line with highest cytotoxicity towards FemX, and the observation that this lysate produced the highest amount of MART-1 specific T cells supports the hypothesis that NK cell mediated lysis produces antigenic fragments that improve adaptive immune responses. To further back up this hypothesis, I compared the amount of MART-1 tetramer specific $CD8^+$ T cells in the different cultures co-incubated with B cells. This is shown in **Figure 14** B). This figure has a similar pattern to the $CD8^+$ T cell expansions shown in **Figure 13** in 3.4.2, where FemX only lysate has a small effect, NK92+FemX has a higher effect, and NKL+FemX lysate has the highest effect.

3.4.4 MART-1$^+$ tetramer specific CD8$^+$ T cells are predominantly central memory cells

The adaptive immune compartment is a highly efficient system capable of remembering previously encountered threats, which will be eradicated faster and more efficiently than in the first exposure to the same threat. This is the mechanism behind vaccination, in which a harmless agent resembling a pathogen is injected into the body, to activate an immune response toward this pathogen. The agents responsible for this are memory cells, long-lived immune cells that have recognized the pathogen, become activated, and then kept after the infection has been fought off. These cells are then ready to immediately start fighting off the threat again if it should come back, without the need for going through the process of activation from APCs. The cell markers used in this project to identify memory populations are CD45RO, CD45RA, and CD62L. Naïve T cells express CD45RA, which they will lose after antigen experience, and start expressing CD45RO as they change to a memory cell phenotype. CD62L is a lymph node homing receptor, expressed by naïve T cells and central memory T cells that enables the cells to migrate to secondary lymphoid organs. Effector cells, both memory and non-memory, do not express CD62L which allows them to accumulate in peripheral tissues[131].

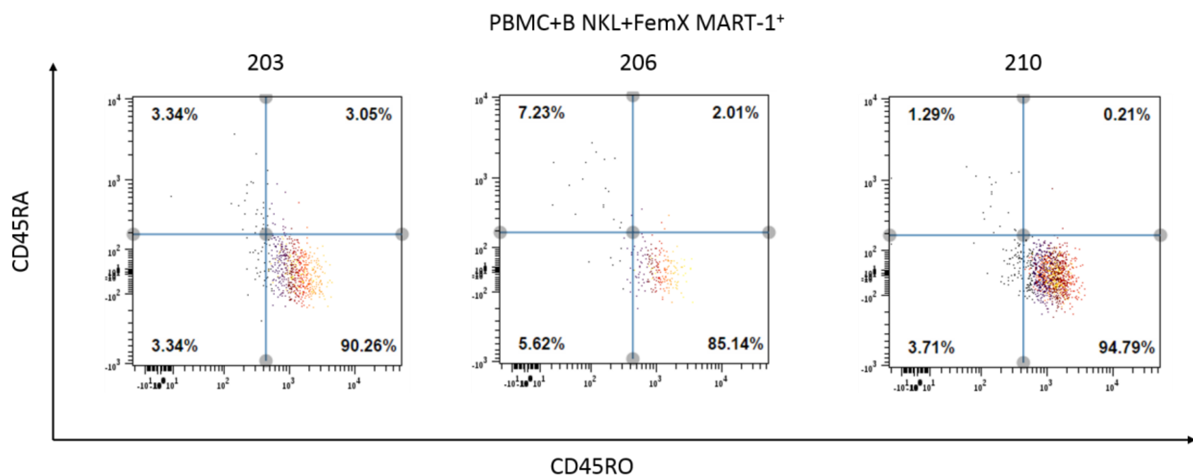


Figure 15 Determination of differentiation state of MART-1 tetramer$^+$ CD8$^+$ T cells.

The MART-1 specific cells are predominantly CD45RO$^+$ CD45RA$^-$, indicating a memory phenotype.

Figure 15 shows the flowcytometric analysis of the MART-1 positive $CD8^+$ T cells in the donors with highest responses. The cells are predominantly $CD45RO^+/CD45RA^-$, indicating a memory cell phenotype. To further investigate the phenotype of the MART-1^+ cells, the expression levels of CD62L were measured and compared to the MART-1^- population. Analysis dot plots are shown in Figure 16. The majority of the cells, both positive and negative for the MART-1 tetramer, are $CD62L^+$. A $CD45RO^+$ $CD62L^+$ phenotype is characteristic of central memory T cells (T_{CM}), experienced T cells that have little or no effector functions, but home to lymph nodes to proliferate and differentiate into effector cells in response to antigen encounter[131].

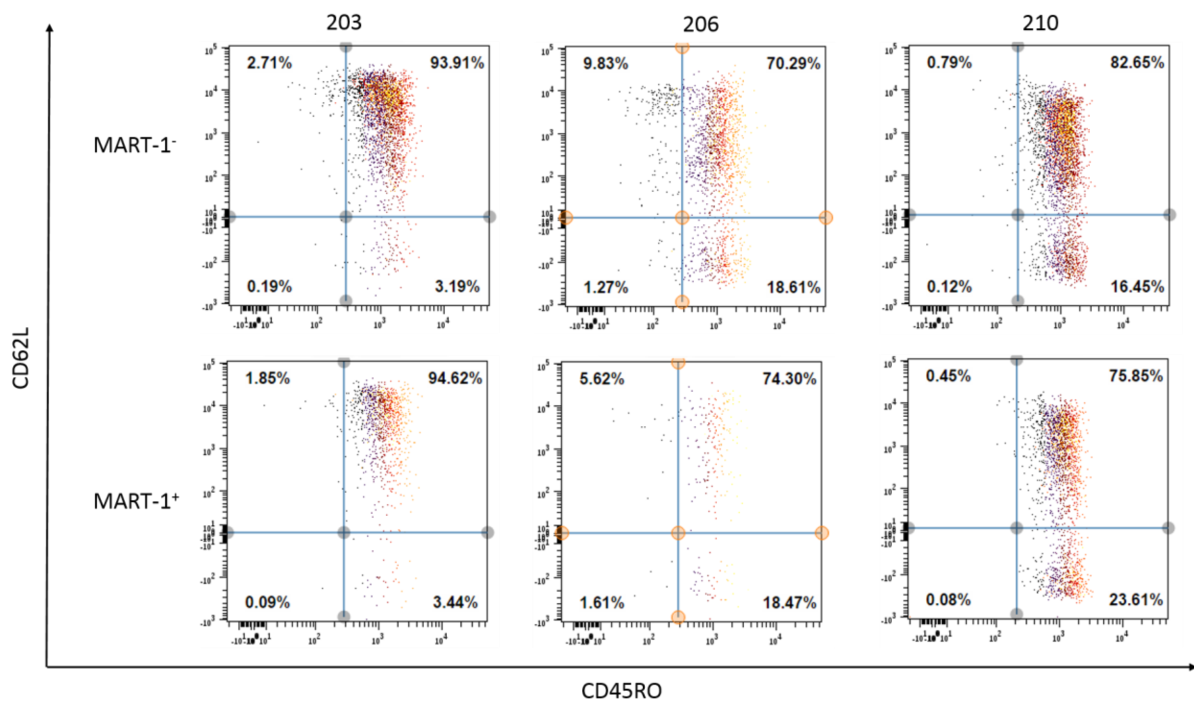


Figure 16 Levels of CD62L expression in the MART-1 positive and negative $CD8^+$ populations.

The majority of cells are $CD45RO^+$ $CD62L^+$, characteristic of central memory T cells (T_{CM}). A slight shift towards a lower expression of CD62L can be seen in the MART-1^+ population of donor 210.

The level of CD62L is equal between MART-1^+ and MART-1^- cells in donors 203 and 206, however in donor 210 there is a slight decrease in expression in the MART-1^+ population, indicating a higher percentage of effector memory T cells (T_{EM}). These are antigen experienced cells that are able to display immediate effector functions in response to antigen[131].

4 Discussion

The goal of this project was to investigate the connection between NK cell mediated cytolysis and adaptive immune responses, and as a part of this investigation I made use of CD40 activated B cells to improve the presentation of pathogen specific antigen. The B cells showed an increased expression of the activation signaling receptor CD86 after co-culturing with CD40L expressing feeder cells, indicating progression toward an activated phenotype. The B cells did not however, show an increased expression of CD80 and MHC class I and II . This contradicts the expected outcome, and the reports of the protocol[102]. This deviation from expectation can probably be attributed to experimental errors though, and repeated experiments under optimal conditions might show results more in line with the reports.

Nevertheless, CD40 activated B cells proved to be efficient for expanding antigen specific T cells, albeit only for a few of the antigens tested, and of these antigens, only BZLF-1 stimulated $CD8^+$ cells showed a very significant difference between cultures. BZLF-1 is known to be highly immunogenic [132], so this observation is in agreement with previous reports.

The observation that $CD8^+$ T cells are expanding in response to antigens in culture, and the strongest expansions are for cells co-cultured with CD40 activated B cells, is a demonstration of cross-presentation by these B cells. Naïve $CD8^+$ T cells require signaling from interaction with MHC class I to become activated and expand. MHC class I generally present endogenous antigens, and would thus normally require the cell to be infected to activate $CD8^+$ T cells. The ability of APCs to cross-present exogenous antigens picked up from outside the cell on MHC class I is responsible for the observed activation of $CD8^+$ T cells. Cross-presentation is usually associated with dendritic cells[133], and the feasibility of their use in expanding immune responses *in vivo* has been well documented[134-137]. However, difficulties in growing dendritic cells *in vitro,* as well as the requirement to use them at a specific stage of differentiation to generate protective T cell responses as opposed to regulatory or anergic responses[138-140], have led to the suggestion of using other APCs as an alternative to DCs. This project uses CD40 activated B cells as an alternative APC, and these results indicate the feasibility of this approach.

To test the hypothesis that NK cell mediated cytolysis generates antigenic epitope for efficient presentation to the adaptive immune system, the NK cell lines NK92 and NKL were used as

effector cell against the melanoma line FemX. A degranulation assay was set up to assess the cytotoxicity of the NK cell lines versus this target. NK92, a cell line characterized by a $CD56^{bright}$ $CD16^{-}$ and low expression of inhibitory receptors, proved to be an inefficient effector towards these cells. Although touted as a highly efficient killer [99, 120, 141], the receptor expression of NK92 indicates an immature phenotype that is not associated with high cytoxicity[57]. Conversely, the more mature $CD56^{dim}CD16^{+}$ $CD94/NKG2^{+}$ cell line NKL showed a higher cytotoxicity towards FemX, consistent with the view that cells with this phenotype are more efficient killers.

The lysate obtained from the NK cell cytolytic activity was then pulsed onto cell cultures. The B cells seemed to have a significant effect on T cell responses in this experiment. While FemX and the FemX+NK92 lysate did not result in high expansions, the non B cell stimulated cultures showed a significant decrease in T cell numbers, an indication that there might have been an immunosuppressive agent released from FemX present in the lysate. The CD40 activated B cells seemed to counteract this effect. As opposed to NK92, the more mature cell line NKL showed an increase in degranulation towards FemX. The lysate obtained from NKL+FemX proved to have a high impact on $CD8^{+}$ T cells, resulting in a significant expansion compared to the negative control. The higher degranulation exhibited by NKL versus FemX seemed to have increased the amount of antigenic peptides available for presentation, and thus improved the adaptive response.

Tetrameric stains of the expanded $CD8^{+}$ cells revealed a significant improvement in MART-1 specific T cells in the populations pulsed with FemX lysate. Again, the NKL+FemX lysate proved to be the most efficient for inducing antigen specific cells, and again co-culturing with B cells improved these responses. The donor with the highest response to the MART-1 tetramer was a donor typed as HLA-A2 negative. As the tetramer was A2 specific, this response is an indication that perhaps the donor is of a haplotype that is able to crosslink HLA-A2, however, the DNA test required to confirm this was not ready before the finalization of the project. The response could also have been an anomaly.

The MART-1 tetramer positive cells was predominantly of a memory cell phenotype, characterized by a $CD45RO^{+}$ $CD45RA^{-}$ expression pattern. Of these, the majority were also positive for the lymph node homing receptor CD62L, an indication of a central memory (T_{CM}) phenotype. One donor, 210, which had the strongest response to the MART-1 tetramer, showed a slight downregulation of CD62L in the MART-1^{+} T cell population, indicative of a

shift towards an effector memory cell phenotype(T_{EM}). Previous studies report that melanoma patients with T_{EM} cell positive for MART-1 (melan-A) positive cells had clear responses to *ex vivo* stimulation with MART-1[142, 143]. These findings indicate that the cells should be tumor reactive. While the majority of the MART-1 tetramer specific T cells where CD62L positive, this could come as a result of possible upregulation of CD62L during proliferation, and thus be an exaggeration of the amount of T_{CM}.

While outside the scopes of this project, a more detailed characterization of the adaptive responses to the FemX lysate would be an interesting project in itself. Analysis of recognized epitopes could perhaps reveal novel melanoma antigens, a promising prospect for future immunotherapies.

In conclusion, the research performed have produced results that seem to confirm the hypothesis that NK cell mediated cytolysis produces peptides able to induce efficient adaptive immune responses, acting as a mediator between the innate and adaptive compartments of the immune system.

4.1 Cell handling: A lesson in care

Something that became apparent during this project is the importance of how to handle growing cell cultures. Caring for cells is a subtle topic that is difficult to quantify in protocol, and is an acquired skill that can only be learned through experience. Yet, operator-dependent differences are huge, even if the described protocol has been followed. This section has therefore been added to describe the lessons learned over the course of the project.

One of the difficulties in growing cells, is to avoid accidently killing them. This seems obvious, but avoiding it is more difficult than it appears, simply because cells can not be seen with the naked eye. Rough handling, like pipetting, can cause fragile cells to die. Contaminations that are difficult to see can cause cells to die. Too low cell density can cause low growth, and too high density can cause clumping of cells, leading to lack of access to nutrients, which will cause the cells to die. To put it simply, there are many ways to kill a cell, and avoiding it is difficult and comes down to experience. Avoiding contaminations can for the most part be avoided by working in sterile conditions, and by being careful not to expose the cells to air outside the cell bench. Obviously, changing pipette tips for each well containing different cultures is a given. Keeping medium sterile and contamination free is of

huge importance. Still, following these rules, contaminations do sometimes happen, and it is important to be able to recognize a contaminated culture, so it can be thrown away to avoid contaminating other cultures.

Dense cultures containing a lot of cells, will clump together if left alone for too long. For this reason, it is important to resuspend the culture by mixing it with a pipette, to allow all, or at least most of the cells access to nutrients. My first attempts at growing T cells were unsuccessful, probably because I did not resuspend them enough. In addition, refeeding cells fresh media by throwing away half the volume of the culture and adding in new media is important to avoid a depletion of nutrients in the culture. Careful removal of media before resuspension of cells is necessary to avoid throwing away too much of the cells.

Another difficulty becomes apparent when transferring cell cultures between plates or test tubes. Cells will tend to stick to the plastic of a plate well or tube, making it important to thoroughly resuspend with a pipette to transfer as much of them as possible. Failure to do this will lead to transfer of a cull culture without the cells, something which is obviously not desirable. Proper pipetting technique is necessary to do this without too much complication.

In addition to these concerns, there are more small details in regard to technique and experience that add up over the course of culturing. My knowledge of maintaining cell cultures is far from perfect, but my experiences during the project has taught me very valuable lessons that lead to better and clearer results as time progressed.

4.2 Concluding remarks

The recent breakthrough in cancer immunotherapy has been paralleled by the insight that the immune system recognizes mutated tumor specific antigens, so called neo-antigens, which are largely patient specific. Using lysates resulting from NK cell mediated cytolysis mimic the naturally occurring bridging of innate and adaptive immunity and release protein fragments containing processed neo-antigens for presentation to the immune system.

The model system used in this project is relatively simple to set up, and can be stimulated with peptides from every type of origin, be it of cancer-transformed or infected cells. While this project only looked at responses to cytolysis fragments of melanoma cells and MART-1 specific responses, lysis fragments from other types of cancer or infected cells could be used.

A more thorough inspection of the epitopes recognized in expanded T cell populations has the potential to reveal a range of neo-antigens that can be used for efficient priming of immune responses in the individual patient paving the way for personalized immunotherapy. In order to identify new epitopes recognized as a consequence of lysate stimulation, one could isolate tetramer positive T cell clones and sequence their TCR.

Another approach to identifying immunogenic peptides could be further fractionation of the tumor cell lysate by using high-performance liquid chromatography (HPLC) to separate finer and finer fractions. HPLC is a way of separating the components of a complex sample in order to identify and quantify each component present. In this manner, peptides can be isolated in order to test the reactivity towards it, which could potentially lead to the discovery of novel antigens that can be synthesized for use in vaccines.

All in all, this projects describes an efficient and relatively simple system to study the priming of adaptive immune responses after stimulation with antigenic peptides. Antigens obtained through NK cell mediated cytolysis proved to be effective for expanding T cell responses, a strategy that could provide an efficient means in the development of new vaccines.

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