Exploring and exploiting cell cycle regulation of CD8+ T cells

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CHAPTER 1

General introduction

INNATE AND ADAPTIVE IMMUNITY

Our immune system is essential in the fight against many different diseases, including infections and cancer. A distinction can be made between the innate and the adaptive immune system. Whereas the innate immune system is rather non-specific, as it recognizes structural components, like damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), the adaptive immune system can recognize and kill specific pathogens or malignant cells. In addition, the adaptive immune system acquires memory in case of secondary encounters with the same pathogen, which can provide life-long protecting.

The innate immune system is the first line of defense and immediately recognizes the invasion of pathogens. After recognition of DAMPs and/or PAMPs, cells from the innate immune system become activated to directly combat pathogens but specialized innate immune cells also instruct cells from the adaptive immune system as a second line of defense. Especially, antigen presenting cells (APCs), such as dendritic cells (DCs), are the key players in bridging the innate and adaptive immune system. APCs take up and process antigens from pathogens and present these to cells from the adaptive immune system. A specific sequence of the presented antigen, an epitope, can be recognized by the antigen receptors on B and T cells, aptly named B cell receptor (BCR) and T cell receptor (TCR), respectively. After recognition of the antigen, B and T cells are primed and respectively start the production of antibodies to induce humoral immunity or induce cellular immunity.

T LYMPHOCYTES

Progenitor T cells are imported from the bone marrow into the thymus, where the entire T cell repertoire is generated throughout life[1,2]. In the thymus, T cell selection and development takes place. T cells start to express unique TCRs via DNA-recombination, which ensures enormous variation in T cells that can recognize many different pathogens. All generated TCRs are tested against reactivity to peptides presented in major histocompatibility complex (MHC) molecules: MHCI or MHCII[3]. Low reactivity of the TCR to self-peptide-MHC complex leads to positive selection while a high affinity of the TCR for self-peptides leads to negative selection. Interaction with MHCI-peptides results in $CD8^+$ T cell differentiation, while interaction with MHCII-peptides induces $CD4^+$ T cell differentation[2,4]. Next, $CD4^+$ and $CD8^+$ T cells can egress from the thymus into the circulation and migrate to secondary lymphoid tissues, such as the spleen and lymph nodes.

Once, naïve T cells have entered the circulation they migrate to secondary lymphoid organs where they can interact with APCs. During infection, APCs presenting non-self-antigens (such as derived from pathogens) stimulate the naïve T cells to proliferate and differentiate

to become effector and memory T cells, which are able to move towards the side of infection or malignancy to induce cell specific killing.

CD4+ and CD8+ T cells

CD4+ T cells can be further classified into two main subsets: conventional helper CD4+ T cells and regulatory CD4+ T cells. Conventional CD4+ helper T cells mediate the immune response by helping B cells and CD8+ T cells to become well-activated and induce a specific immune response[5-7]. Before CD4+ T cells can help CD8+ T cells, they interact with DCs via CD40/CD40L to induce cross-activation. Next, the CD4+ T cell is licensed to help installing a cytotoxic CD8+ T cell response[8]. B cell responses are supported by a specific subset of helper CD4+ T cells, called follicular T cells. Follicular T cells secrete cytokines such as IL-4 and IL-21, which induce B cell proliferation and differentiation of B cells into antibody secreting plasma cells[9].

In contrast to conventional CD4+ T cells, CD4+ regulatory T cells are fine tuning the magnitude of the immune response, by providing inhibitory signals to B cells and CD8+ T cells[7]. Without the presence of regulatory T cells, B cell and CD8+ T cell responses are not sufficiently controlled leading to severe immunopathology[10].

CD8+ T cells can specifically and directly kill cancer cells and infected target cells[11]. Accordingly, CD8+ T cells are called cytotoxic. Upon recognition of specific antigen, CD8+ T cells form a synaptic interaction with the target cell, and start secreting cytotoxic molecules, such as granzymes and perforin, into the synapse that directly and selectively kill the target cell to clear the infection or malignancy[12]. In addition, CD8+ T cells can secrete other cytotoxic cytokines such as interferon-gamma (IFN-g) and tumor necrosis factor (TNF) to further stimulate the local immune response[13].

CD8+ T CELL ACTIVATION AND PROLIFERATION

CD8+ T cells do not only require antigenic signals from the TCR (signal 1) to become activated, but other signals are necessary to induce a fully activated and functional CD8+ T cell; namely signals from costimulatory receptors (signal 2), and signals mediated by specific cytokines that bind to their respective receptors (signal 3) (**Figure 1**).

Signal 1 - TCR-triggering

The first signal that is required to start the activation cascade, is triggering of the TCR by a specific antigen presented in MHCI molecules on APCs[12]. After stimulation of the TCR, various downstream signaling cascades are activated that are involved in further activation of the CD8+ T cell[14,15].

Signal 2 - Costimulation

After TCR triggering, naïve CD8⁺ T cells require costimulation. In addition to MHCI-antigen complexes, activated APCs express costimulatory molecules, such as CD80/CD86 and CD70, which can be recognized by the costimulatory receptors, CD28 and CD27 respectively, on the cell surface of naïve CD8⁺ T cells[16]. In contrast to CD28 and CD27, expression of 4-1BB, another costimulatory molecule, is not present on naïve cells, but is induced after costimulation, further amplifying the response. After costimulation, several different signaling cascades are activated, which are involved in survival, proliferation and metabolism of CD8⁺ T cells. Interestingly, after receiving signal 1 and 2, CD8⁺ T cells increase their affinity for IL-2 by upregulation of the high-affinity IL-2 receptor (CD25). In addition, IL-2 is being produced leading to autocrine IL-2 signaling, which also induces signaling cascades involved in survival, proliferation and metabolism[17-19]. Besides costimulatory signals, there are also inhibitory interactions between the APC and CD8⁺ T cell, such as PD-L1-PD1 and CTLA4-CD80/CD86[16]. The balance between costimulation and inhibition determines the magnitude of the CD8⁺ T cell response.

Signal 3 - Cytokines

The last signal that is required to induce full expansion and functionality of CD8⁺ T cells, is signaling via cytokines. Especially, IL-12 and type I IFNs are important cytokines and are mainly produced by APCs. Both IL-12 and type I IFNs signaling contribute to increased expansion, cytotoxicity and differentiation of CD8⁺ T cells into effector and memory cells[20,21].

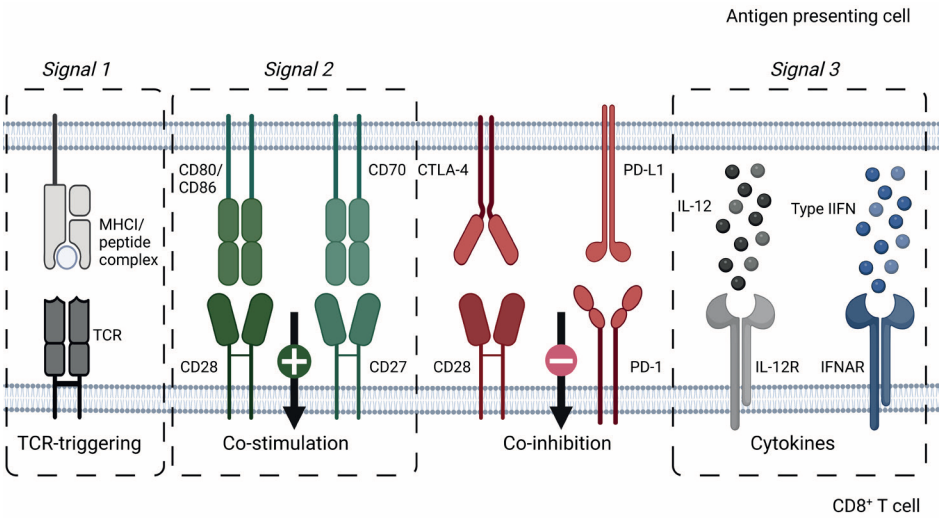

Figure 1. Costimulation and inhibition. Antigen presenting cells present a peptide in a MHCI complex to a CD8⁺ T cell. The CD8⁺ T cell recognizes its cognate antigen with its T cell receptor (TCR) and becomes activated (signal 1). Costimulatory (signal 2) and inhibitory interactions between the antigen presenting cells and the CD8⁺ T cell determine the magnitude of the T cell response. Cytokines IL-12 and type I IFN are providing signals to increase proliferation, cytotoxicity and shape differentiation.

Differentiation and proliferation

After activation, a CD8$^+$ T cell starts cell division, leading to generation of two daughter cells that share the same TCR[22]. After proliferation is initiated, CD8$^+$ T cells expand clonally, all originating from 1 precursor cell. During proliferation, CD8$^+$ T cells also start differentiation into effector and memory cells. While effector cells are crucial for initial clearance of pathogens or malignant cells, memory cells install lifelong protection in case of secondary encounter with the same pathogen. After combatting the pathogen or malignancy, CD8$^+$ effector T cells wane, but 5-10% of the CD8$^+$ T cells remain and become memory cells either in the circulation or in tissue. These memory cells constantly check whether antigen is cleared from the body. Upon re-encountering the antigen, memory cells are re-stimulated, which results in rapid proliferation and effector differentiation to again clear the host of the pathogen or malignancy[23,24].

Memory cells can be subclassified into 3 different types of circulating cells, which are central memory (TCM), effector memory (TEM) and terminally differentiated effector memory (TEMRA) T cells. Whereas TCM cells are restricted to blood and lymphoid organs, TEM cells can also traffic to other organs and are more cytotoxic then TCM cells[24,25]. Compared to TEM, TEMRA cells are even more cytotoxic, but are also more differentiated towards an exhausted phenotype[26]. Finally there is a subtype of non-circulating cells, the tissue-resident T (TRM) cells, which are residing in almost every organ to install tissue immunity and alarm the body to prevent or combat infection[27].

CD8$^+$ T CELL METABOLISM

Recently, the metabolism of immune cells, including CD8$^+$ T cells, has become a topic of major interest. In a simplified view, cells, including CD8$^+$ T cells, can make use of three different sources of nutrients: amino acids, lipids and glucose. These nutrients can be taken up from the environment or produced by CD8$^+$ T cells themselves[28,29].

There are 20 different amino acids, which can be further classified into non-essential and essential. Non-essential amino acids can be synthesized by the cell itself in different pathways, but the essential amino acids can only be acquired from exogenous nutrients[30].

There are multiple types of lipids, including phospholipids, triglycerides and cholesterol. Phospholipids and triglycerides can be synthesized via processing of glucose-derived glycerol in the cytosol or via β-oxidation of fatty acids in the mitochondria. Cholesterol can only be generated after β-oxidation of fatty acids[31].

Glucose that has been taken up from the environment can be processed into two main pathways: glycolysis and the pentose phosphate pathway. Glycolysis breaks down glucose to generate energy directly or provide intermediates for other metabolic pathways[32]. The pentose phosphate pathway is essential in providing precursors for nucleotides and amino acids[33]. An excess of glucose can be temporarily stored inside the cell in the form of glycogen, so that cells can convert glycogen back into glucose when needed[34]. Glucose can also be generated in a process called gluconeogenesis, in which lactate, glycerol or certain amino acids are the main substrates[35].

All these nutrients can be processed intracellularly to generate energy in the form of adenosine triphosphate (ATP) in two main metabolic pathways: oxidative phosphorylation (OXPHOS) and glycolysis (**Figure 2**). During OXPHOS, amino acids and fatty acids are being oxidized, resulting in a high ATP production of 30-32 molecules. During glycolysis, glucose is broken down into lactate, which results in a low ATP production of only 2 molecules[36]. Although the most efficient way for cells to produce energy is via OXPHOS, glycolysis is 100 times faster. Consequently, the situation and the associated needs, will determine if a cell will rely on OXPHOS or glycolysis to produce energy.

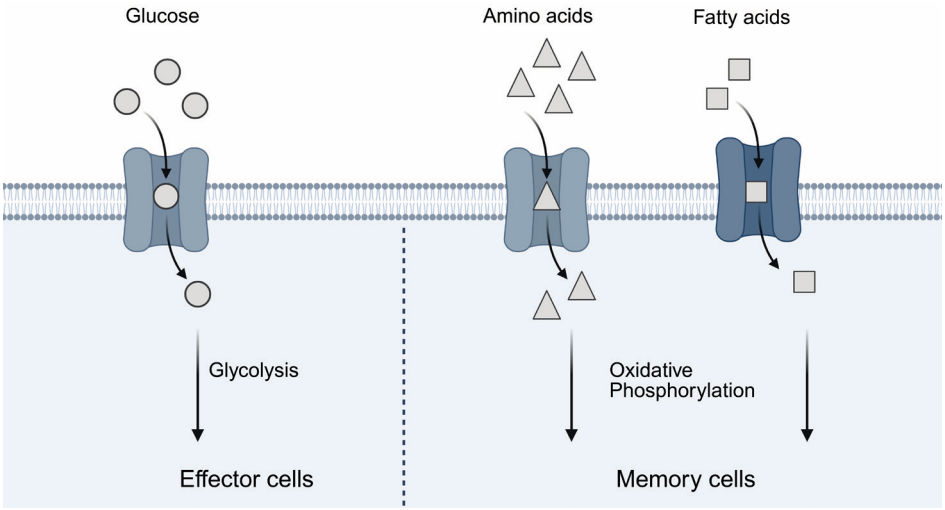

Figure 2. Glycolysis and oxidative phosphorylation. CD8⁺ T cells can take up different nutrients from the environment. Glucose is being processed in glycolysis and amino acids and fatty acids are being processed processes in oxidative phosphorylation (OXPHOS). Whereas effector cells mainly use glycolysis, memory cells are depending on OXPHOS.

Resting naïve CD8⁺ T cells are using OXPHOS to maintain homeostasis[37]. Shortly after activation, naïve CD8⁺ T cells switch their metabolism towards glycolysis to be able to rapidly

generate energy[38,39]. While, short lived effector cells remain mainly glycolytic, memory cells decrease glycolysis and switch their metabolism towards OXPHOS again[40,41]. However, upon re-stimulation, memory cells quickly switch to glycolysis to fuel fast proliferation and enable effector functions[42].

CANCER AND THE IMMUNE SYSTEM

A decade ago, the concept of the cancer immunity cycle was introduced by *Chen* et al. bridging the field of immunology and oncology[16]. It was shown that dying cancer cells release antigens, which results in priming of cytotoxic $CD8^+$ T cells. These activated $CD8^+$ T cells are then able to attack the cancer cells and induce killing. Accordingly, this is a cyclic self-propagating process. However, for continuation of this cycle there are two crucial points that must occur: 1. tumor cells must die to release tumor antigens that can then be recognized by the immune system and 2. $CD8^+$ T cells must be activated and become functional. Although the immune system is surveilling the body to check for and eliminate malignant cells, malignant cells can escape the immune system and form tumors[43].

Chemotherapy

To induce cancer cell killing, a large fraction of cancer patients is still being treated with chemotherapy. These drugs are designed to target rapidly proliferating cancer cells and inhibit their cell cycle. Since, cell cycle inhibitors are not cell specific, also other proliferating cell types, including activated $CD8^+$ T cells, can be inhibited in their proliferation. While the effects of cell cycle inhibitors on cancer cells are well established, the effects on the immune system are currently being investigated[44]. Chemotherapy can affect the immune system either directly or indirectly. Indirect effects are mostly observed in the tumor microenvironment, where the release of damage signals (such as heat shock proteins) after chemotherapeutic-induced tumor cell death can activate the immune system. The direct effects of chemotherapy on immune cells are more diverse and can have both activating or repressing effects[45]. Evidence is arising that T cells can tolerate these drugs and that it might even positively contribute to increased T cell immunity[46-49]. However, further research is needed to understand the direct effects of cell cycle inhibition on $CD8^+$ T cell functionality.

Immunotherapy for cancer

While conventional treatment with surgery, radio- and chemotherapy can cure cancer patients, unfortunately it often fails to cure all cancer patients[50]. Tumor cells can metastasize and/or adapt themselves to become resistant to the treatment. Research has been looking for new ways to treat cancer patients and immunotherapy appears to be an attractive and

successful approach. Immunotherapy includes antibody-based immune-checkpoint blocking, adoptive cell transfer and (therapeutic) vaccination. In the end, all these different methods aim to induce a new or enhance an existing anti-tumor immune response.

A major pilar in immunotherapy is immune checkpoint therapy (ICT). The goal is to prevent or inhibit the suppressive milieu that tumor cells create by attraction of immunosuppressive cells, secretion of immunosuppressive cytokines or expression of inhibitory molecules[51]. Better understanding of these mechanisms has led to the development of strategies to manipulate the immune system in such a way that malignant cells can be eliminated again. Immune checkpoint therapy is now implemented in the clinic as standard treatment of care for certain cancer types[52]. Impressive results are obtained in studies in which the interaction between the inhibitory molecule PD-L1 on tumor cells and myeloid cells and the receptor PD-1 on $CD8^+$ T cells is prevented by antibodies[53,54]. Treatment with these immune checkpoint blocking antibodies result in improved tumor clearance and survival in a subset of patients. Unfortunately, not all patients benefit from this type of treatment and therefore other methods to manipulate the immune system are explored. Pioneering studies are now looking for ways to not only prevent inhibitory signals but provide molecules that give stimulatory signals to $CD8^+$ T cells instead. Clinical trials in which agonistic antibodies target 4-1BB to provide costimulation, are showing promising results[55,56].

Besides ICT, also adoptive immune cell transfers (ACT) are efficacious. With this method, cells from patients are collected, after which these cells can be genetically modified or certain cell types are specifically selected. Next, these cells are expanded and reinfused into the patient[57]. The last decade, chimeric antigen receptor (CAR) T cell therapy has been established. These CAR T cells are genetically engineered in such a way that they can recognize tumor-specific epitopes[58]. CAR T cell therapy has emerged quickly and promising clinical results for defined tumors are obtained thus far[59,60].

The third pilar in immunotherapy, vaccination, has shown promising results in the treatment of cancer and even in the prevention of cancer. Whereas therapeutic vaccines aim to prime $CD8^+$ T cells to recognize neoantigens that are expressed by tumor cells to induce specific tumor cell killing[61]. Prophylactic vaccines, such as vaccines against HPV causing cervical cancer, are designed to induce memory responses, such that upon recognition of antigens malignant cells are immediately recognized and cleared from the body and tumors cannot be formed[62,63].

To further improve treatment options for cancer patients and increase the effectiveness of ICT, future research will optimize ways to manipulate and exploit the immune system. Since, $CD8^+$ T cells are the key players in specific tumor cell killing, we suggest that understanding the underlying molecular mechanisms of $CD8^+$ T cell proliferation and differentiation is of great importance to accomplish this.

SCOPE OF THESIS

Although there is extensive research showing the beneficial effects of chemotherapy on tumor cells, the effects of chemotherapy or other cell cycle inhibiting agents on the immune system, and in particular on $CD8^+$ T cells, is still incompletely understood. In this thesis we investigate how short-term cell cycle inhibition impact $CD8^+$ T cells in the context of cancer. We further discuss the possibilities how this knowledge can be used and exploited to improve treatment strategies for cancer patients.

In this thesis we dissected the direct and indirect effects of cell cycle inhibition on the phenotype and proliferation capacity of $CD8^+$ T cells. In **chapter 2**, we review the current knowledge and consensus of the regulation of cell cycle progression of $CD8^+$ T cells and how proliferation of $CD8^+$ T cells is affected by internal and external cues. We propose that understanding the processes involved in the regulation of proliferation can be used to tweak and exploit $CD8^+$ T cell expansion, which is essential to improve $CD8^+$ T cell-based therapies for cancer.

In **chapter 3 and 4**, we show, unexpectedly, that temporal cell cycle inhibition can positively affect $CD8^+$ T cell proliferation and effector cell differentiation, which might be used to improve $CD8^+$ T cell-based therapies.

In **chapter 3**, we investigate the direct effects of short-term cell cycle inhibition on $CD8^+$ T cells with a reductionist approach. We show the effects on proliferation and differentiation, with a specific focus on the metabolism. Furthermore, we show how our findings can be implemented in strategies to improve chemo-immunotherapy of cancer. In **chapter 4**, we elaborate further on the immunomodulatory effects of chemo-immunotherapy. We show synergy of chemotherapy with vaccination, resulting in a sustained anti-tumor immune response. Here, we show both the direct effects of chemotherapy on $CD8^+$ T cells, but also how indirect effects from the tumor microenvironment impact $CD8^+$ T cell proliferation and functionality.

Finally, we discuss all these studies in **chapter 5.** We will provide a short overview of the main findings in this thesis and focus on gaps in the current knowledge and understanding of T cell proliferation and metabolism. We will give suggestions how a better understanding of $CD8^+$ T cell proliferation can be used to improve immunotherapy for cancer patients.

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