# Exploring and exploiting cell cycle regulation of CD8+ T cells

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# CHAPTER 5

## Discussion

Chemotherapeutic agents are designed to inhibit and kill rapidly proliferating cancer cells. The downside of these drugs is that they are not specific in targeting only rapidly proliferating cancer cells, but that they can also affect other proliferating subsets in the body, including activated $CD8^+$ T cells. Given that $CD8^+$ T cells are actually essential in the fight against cancer, it is critical that the effects of chemotherapeutic drugs on $CD8^+$ T cells are better understood[1]. Although it was shown that cell cycle arresting agents such as doxorubicin or cis-platin are detrimental to B cells, $CD8^+$ T cells can survive this treatment[2]. However, how chemotherapy affects the phenotype of $CD8^+$ T cells is still poorly understood. Better understanding of the effects of chemotherapeutics and other cell cycle inhibiting chemotherapeutics on $CD8^+$ T cells, can directly result in improving immune-therapeutic treatments for cancer patients. In this thesis, we show, unexpectedly, that cell cycle inhibiting drugs can positively affect $CD8^+$ T cell proliferation and effector cell differentiation via modulation of their metabolism. This puts forward that cell cycle inhibition might actually be used to directly improve $CD8^+$ T cell-based therapies.

## UNCOUPLING DIFFERENTIATION AND PROLIFERATION

Under normal physiological settings, naïve $CD8^+$ T cells are activated upon encountering antigens in the context inflammatory conditions in which dendritic cells present antigens and simultaneously provide costimulation. After activation, naïve $CD8^+$ T cells start processes for differentiation and proliferation at the same time. However, in this thesis we show that it is possible to uncouple these two processes by providing a temporal cell cycle arrest following $CD8^+$ T cell priming (**chapter 3**). While these 'uncoupled $CD8^+$ T cells' are still activated and already start their differentiation, they are arrested in their cell cycle and therefore cannot proliferate. We show that uncoupling differentiation and proliferation by cell cycle inhibition during priming directly affects differentiation, resulting in improved effector T cell formation and increased proliferation potential once these cells are released from the cell cycle inhibitor. We propose that this set up is an attractive approach to exploit and implement as an immune-modulating therapy for more effective treatments. To accomplish this, it is essential to improve our understanding of the effects of TCR signaling, costimulation and cytokine signaling on the cell cycle of T cells both in the context of inhibition and release of short-term cell cycle inhibition (**Figure 1**).

## TCR signaling

It is known that, besides the antigen itself, also the duration and the strength of TCR stimulation determine the expansion potential and the magnitude of the CD8$^+$ T cell response (**chapter 2**)[3]. Therefore, we hypothesize that uncoupling differentiation and proliferation of CD8$^+$ T cells results in prolonged TCR triggering. Hyperactivation of TCRs results in massive proliferation and is often associated with autoimmunity[4]. In the context of cancer, it might actually be beneficial to induce massive CD8$^+$ T cell expansion in order to establish a large tumor specific CD8$^+$ T cell population that can fight the cancer cells. However, it is important to note that increasing TCR signal strength might not induce similar effects as prolonged TCR triggering, since high TCR signal strength can induce non-functional effector cells[5]. Further research should show whether CD8$^+$ T cells that are arrested in their cell cycle right after priming indeed have a prolonged interaction with the cognate antigen and whether this at least partly explains the phenotype that we observe.



## Costimulation and IL-2

Costimulation is essential in the formation of functional proliferating effector cells (**chapter 2**). In our set up in **chapter 3**, we focused on use of CD28-mediated costimulation. We showed that arrested CD8$^+$ T cells increased their autocrine IL-2 production after TCR triggering and CD28 costimulation. These increased IL-2 levels contributed to improved proliferation potential of cells that were released into a proliferation state after temporal cell cycle inhibition. Since CD28 is not the only costimulatory molecule on CD8$^+$ T cells, it is also interesting to know how other costimulatory molecules, such as CD27 and 4-1BB, can be used in this set up. While both CD27 and 4-1BB costimulation can induce autocrine IL-2 production, CD27 is expressed on naïve cells and 4-1BB expression is induced after TCR-triggering[6,7]. This makes CD27 a more attractive target for our set up, in which we provide a cell cycle arrest right after priming of CD8$^+$ T cells in combination with costimulation. In **chapter 4,** we show that blocking CD27 costimulation decreased the percentage of intratumoral CD8$^+$ T cells after treatment with chemotherapy with cisplatin and topotecan. In this set up we did not inhibit CD27 costimulation during priming, but 5 days after CD8$^+$ T cell activation, which might underestimate the effects and importance of CD27 costimulation on proliferation in combination with cell cycle arresting agents. Accordingly, we propose that it would be worthwhile to investigate how (a combination of) different costimulatory stimuli in combination with a temporal cell cycle arrest during priming will impact the proliferation potential of CD8$^+$ T cells (**Figure 1**).

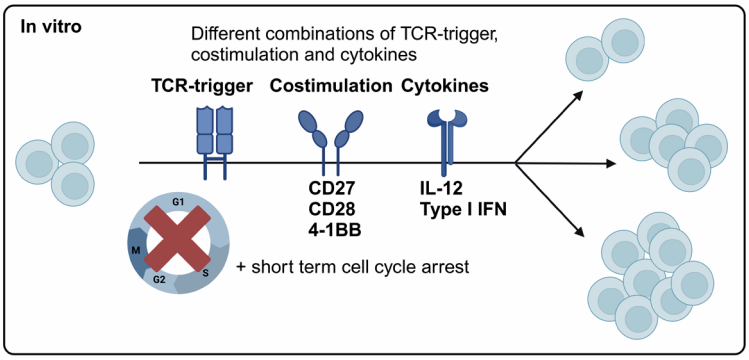


**Figure 1. Different activation stimuli in combination with a short-term cell cycle arrest can impact proliferation.** Different combinations of TCR-triggering, costimulation and cytokines could be combined with short cell cycle inhibition to optimize *in vitro* proliferation of $CD8^+$ T cells.

## IL-12 and type I IFN

In concert with TCR-triggering and costimulation, IL-12 and type I IFN cytokine signaling amplifies the proliferating $CD8^+$ T cell pool (**chapter 2**). These cytokines are produced by activated dendritic cells, and depending on the amount of inflammation are released during priming of the $CD8^+$ T cells. In **chapter 3**, we did not supplement our culture system with these cytokines, but it would be interesting to determine whether IL-12 and type I IFN can further enhance the vigorous proliferation observed after temporal cell cycle arrest (**Figure 1**). In addition, it is of great importance to understand the *in vivo* effects of chemotherapy and other cell cycle inhibiting agents on monocytes, dendritic cells and macrophages that produce IL-12 and type I IFN. It was shown that hydroxyurea, the same chemotherapeutic drug as we used in **chapter 3**, dampens the inflammatory responses of monocytes[8]. Additionally, small molecule inhibitors, which inhibit Cdk4/6, directly affected the number of circulating monocytes directly[9].

Extending our knowledge on the direct effects of TCR triggering, costimulation and IL-12 and type I IFN cytokine signaling on $CD8^+$ T cell proliferation in combination with cell cycle inhibition will help to optimize the immuno-modulating aspect of temporal cell cycle inhibition with the aim to implement this as immunotherapeutic intervention.

## NAÏVE VERSUS EFFECTOR AND MEMORY POPULATIONS

To study the effect of temporal cell cycle inhibition in newly primed CD8$^+$ T cells (**chapter 3**), it is essential to consider that we made use of naïve CD8$^+$ T cells. Naïve cells are resting in G0 and are not yet actively cycling. Although this set up provided valuable knowledge on the effects of cell cycle inhibition on newly primed CD8$^+$ T cells, it is not reflecting the complex situation in the body of cancer patients. When a malignancy is present there is already an ongoing immune response consisting of both effector and memory CD8$^+$ T cells. In particular, effector cells are rapidly proliferating and therefore cell cycle inhibition on this population might have other effects as compared to naïve or memory cells. Also, indirect effects on tissues and the tumor microenvironment of the various drugs are poorly understood. In **chapter 4** we show that short term treatment with cisplatin and topotecan can be tolerated by effector CD8$^+$ T cells. While these cells do not actively proliferate as long as the chemotherapeutic drugs are present, they regain their ability to do so after treatment has stopped. Similar to our observations, others have observed that effector CD8$^+$ T cells can recover proliferation after 1 cycle of chemotherapy and that short term chemotherapy treatment can even improve cytotoxicity[10]. Another interesting finding is that there seems to be a shift from terminally differentiated effector memory T cells (TEMRA) towards effector memory T cells (TEM) after chemotherapy[11]. In a recent study it was shown that long term treatment with the chemotherapeutic cisplatin in combination with PD-1 blockade impaired proliferation and cytotoxicity of effector cells, whereas non-proliferating naïve and stem-like cells were not affected[12]. Showing that timing and dosing of chemotherapy is essential in establishing and maintaining a functional effector response.

In our set up in **chapter 3,** we did not investigate how a short-term cell cycle arrest during priming of naïve CD8$^+$ T cells impacts memory differentiation. To induce a long-lasting anti-tumor response, it is essential that also proper memory T cell formation is installed. Therefore, we propose that future research should not only focus on the effect of temporal cell cycle inhibition on proliferation during activation of naïve and memory cells, but also on the long-term effect on differentiation. It is known that memory CD8$^+$ T cells can survive chemotherapy well by different mechanisms. Memory CD8$^+$ T cells acquire mechanisms to keep DNA integrity by improving their response to coop with DNA damage and therefore these cells are less sensitive to cytotoxic drugs[13]. In addition, memory CD8$^+$ T cells have developed a mechanism to enhance the capacity to efflux cytotoxic drugs, resulting in better survival[14]. In line with these findings, we show in **chapter 4** that after treatment with topotecan and cisplatin memory cells upregulate Bcl-2, an anti-apoptotic molecule that prevents cells from entering apoptosis. All these features make memory CD8$^+$ T cells

an attractive subset to use in our experimental set up, in which we improve proliferation by short term inhibition of the cell cycle. However, there are some features that must be considered when using memory CD8$^+$ T cells for this approach. In a recent study of *Heinzel* et al. it was shown that although TCR triggering induced similar proliferation of memory cells as compared to naïve cells, memory cells showed reduced proliferation after CD28 costimulation and IL-2 signaling compared to naïve cells[15]. Furthermore, it appears that memory cells cannot immediately start proliferation upon re-activation. It takes 2 to 3 days before memory cells start to divide and proliferation is initiated, which is similar to naïve cells[16]. In addition, *Whitmire* et al. suggest that the exponential increase in the number of antigen specific CD8$^+$ T cells is not because memory cells can divide more rapidly, but that there is a higher precursor frequency accommodating this. Although memory T cells might not show improved proliferation compared to naïve cells, it would still be interesting to investigate the effect on memory cells, because many cancer patients already have a memory response by the time the cancer is discovered.

To conclude, we suggest that future research should focus on how to establish both a functional effector and memory response after chemotherapy treatment. We hypothesize that temporal cell cycle inhibition can serve as a tool to manipulate cells and create a more attractive phenotype. This approach might be of great interest in the field of adoptive cell therapy (ACT) in particular, in which cells can be manipulated *ex vivo* and transfused back into the patients once the optimal phenotype has been established. This will be discussed below.

# METABOLISM

In this thesis, we also elaborate on how different metabolic pathways are generating energy to comply with the needs of CD8$^+$ T cells and that activation of CD8$^+$ T cells alters the metabolism of CD8$^+$ T cells (**chapter 1 & 2**). In **chapter 3** we show that short term cell cycle inhibition can positively impact CD8$^+$ T cell proliferation by largely affecting the metabolism in these cells. Recently, the metabolism of CD8$^+$ T cells has become a field of major interest and we and others suggest that understanding metabolic processes in CD8$^+$ T cells are essential to further improve immunotherapy for cancer patients[17].

## Stockpiling nutrients

In **chapter 3**, we postulate that despite a cell cycle arrest, newly activated CD8$^+$ T cells can already start preparing themselves at the metabolic level. We show that these cells start to upregulate receptors to take up nutrients, including glucose and amino acids, resulting in a

nutrient influx and storage of these nutrients. We demonstrate an interesting phenomenon that although these cells are stalled in the cell cycle and cannot continue to proliferation, they already start modifying their metabolism. Specifically, we further noticed that these arrested T cells behave actually like effector cells and start taking up glucose after activation, to generate energy quickly by glycolysis. However, these arrested cells do not need the glucose yet and therefore store the glucose in the form of glycogen. In that way, these T cells stockpile themselves with fuel and become energized. In addition, these T cells not only have a buildup of nutrients, but also have activated metabolic signaling cascades, including mTOR and c-Myc signaling. This stockpiling of nutrients in combination with an activated metabolism leads to kickstarting proliferation once their cell cycle arrest is released for proliferation. We hypothesize that manipulation of the metabolism of $CD8^+$ T cells is an attractive approach to influence their proliferation and should be explored further in the clinic such as in the context of adoptive cell transfers (**Figure 2**), in which large $CD8^+$ T cell numbers are required to obtain successful results.



## Altered glucose metabolism

In addition to stockpiling nutrients, we observed that cells that are released from a short-term cell cycle arrest are even more glycolytic than normal activated effector cells (**chapter 3**). While this results in quick energy production, contributing to vigorous proliferation, one might argue that this is also a concern for *in vivo* situations in the context of tumors and chemotherapy because $CD8^+$ T cells not only proliferate in lymphoid organs, but also intratumorally[18]. In the tumor microenvironment there can be substantial nutrient competition, and tumor cells are mainly using glucose as their source of energy, also known as the Warburg effect[19]. However, it appears that $CD8^+$ T cells are not deprived in their glucose uptake by the tumor cells[20]. If indeed $CD8^+$ T cells can use glucose from the tumor microenvironment it would be intriguing to examine this in a clinical situation where short-term chemotherapy is provided and extensive $CD8^+$ T cell proliferation occurs after the chemotherapy. Based on our findings in **chapter 4**, we do not anticipate that glucose competition in the tumor microenvironment will occur after chemotherapy as we observed beneficial effects of temporal chemotherapy. Basically, we show that $CD8^+$ T cells are still proliferating in the tumor microenvironment after treatment with chemotherapy. Although we did not check the glucose metabolism in great detail in vivo (**chapter 4**), we can assume here that effector cells are using glucose to fuel proliferation.

Another big concern arises on how this switch towards increased glucose metabolism after short term cell cycle inhibition will impact memory formation. It has been shown that high glycolytic activity negatively impacts differentiation of $CD8^+$ T cells into memory cells[21]. Since

memory cells are essential in long term protection, it is essential to understand how a short-term cell cycle arrest will impact memory differentiation. We do have indications (**chapter 3**) that, at least a subpopulation of, these cells still take up and process fatty acids, which is essential for memory cell differentiation [22]. This suggest that not all cells in this set up will become short lived effector T cells that rely on glycolysis, but that a fraction of cells switch toward oxidative phosphorylation and memory cell differentiation can still occur. However, future studies are needed to confirm this.

## mTOR: the master regulator of glucose metabolism

The mTOR pathway, consisting of mTORC1 and mTORC2, is important and critical for the activation of glucose metabolism (**chapter 2**)[23]. Research focusses mainly on mTORC1, and how mTORC1 regulates glucose metabolism. We showed that during a short-term cell cycle arrest, $CD8^+$ T cells activate mTORC1, however it seemed not to be crucial to install glycolysis in these cells. We showed that while $CD8^+$ T cells have improved glucose metabolism after a short-term cell cycle arrest, this is partly mTORC1 independent (**chapter 3**). There are multiple explanations of how this short term arrested $CD8^+$ T cells overcome the necessity of mTORC1 activity to induce glycolysis and proliferation. First, recent research has shown that besides mTORC1, mTORC2 can induce glycolysis[24]. mTORC2 can be activated by TCR-triggering and CD28 costimulation as well, which makes it possible in our set up that mTORC2 complements the role of mTORC1[25]. However, literature shows that mTORC2 deficient cells produce higher levels of IL-2, which is in sharp contrast with what we observed in our experiments in **chapter 3**[26]. We observed that short term arrested $CD8^+$ T cells produce high levels of IL-2, which makes regulation of glycolysis via mTORC2 a less plausible explanation. Interestingly, research has shown that IL-2 can install glycolysis independently of mTORC1 (**chapter 2**). In agreement with these data, we showed in **chapter 3** that IL-2 can increase expression of ALDOA in $CD8^+$ T cells, which is a glycolysis-related enzyme. We hypothesize that IL-2 induces transcription of c-MYC, which in turn can induce transcription of many genes including these encoding glycolysis-related enzymes[27,28]. Taken together, we propose that the metabolic programming of $CD8^+$ T cells during a temporal cell cycle arrest is unique, and that cells not only rely on mTORC1 to install glycolysis, but also on IL-2 and c-MYC. It is important that the underlying mechanism of altered metabolism after short term cell cycle arrest will be clarified, because it might help to target and steer the metabolism of $CD8^+$ T cells to alter proliferation. Recent research is already exploring methods to target the metabolism. It was shown that inhibiting glucose uptake by the small molecule inhibitor WZB117 directly impairs $CD8^+$ T cell proliferation[29]. While this might be beneficial for certain auto-immune disorders, this will not help cancer patients that need proliferating effector cells to kill the

tumor cells. Most studies design drugs that can inhibit glycolysis, since it is more difficult to find strategies to promote glycolysis. Therefor we propose that future research should focus on ways to target the metabolism specifically in order to generate a rapid proliferating $CD8^+$ T cell phenotype by promoting glucose uptake and processing.

# CLINICAL APPROACH

Our ultimate goal is that we can use and exploit our acquired fundamental knowledge regarding $CD8^+$ T cell cycle regulation and proliferation to improve immunotherapeutic modalities for cancer patients. We showed that $CD8^+$ T cells can survive short term treatment with cell cycle inhibiting agents and that it can even improve T cell-based therapies (**chapter 3**). In **chapter 4**, we examine direct effects of cell cycle inhibition on $CD8^+$ T cells, but we also assess how cell cycle inhibitors impact the tumor microenvironment, which is important since $CD8^+$ T cell functionality is impacted by other immune cells as well, including $CD4^+$ T cells and antigen presenting cells. Therefore, it is important to discriminate between the *in vivo* effects of cell cycle inhibiting agents during systemic treatment with chemotherapy and the *ex vivo* optimization of $CD8^+$ T cells that are used for adoptive cell transfers (ACT).



## Systemic effect

To eliminate rapidly proliferation cancer cells, 30% of cancer patients are treated with chemotherapy systemically. This not only induces tumor cell killing, which can in turn lead to activation of the immune system and tumor specific killing by immune cells. This immediately raises the question how the immune system is impacted by this systemic treatment with cell cycle inhibiting agents. In **chapter** 4, we show that systemic treatment with chemotherapeutic agents can positively impact the immune system by changing the tumor microenvironment. In other studies, it has been established that chemotherapy can have both positive and negative effects on the tumor microenvironment[30]. Chemotherapy can induce immunogenic cell death and thereby facilitates direct activation of the immune system. In addition, regulatory T cells can be depleted specifically by some chemotherapeutic drugs, which can result in an increased tumor killing responses by $CD8^+$ T cells[31]. However, macrophages and fibroblasts can produce cytokines and chemokines that provide pro-survival signals for tumor cells[32]. The complexity here is that the net anti-tumor immune effect is depending on several factors including the tumor type, the type of drugs that are used, and the patient's immune system itself. Understanding the effects on tumor microenvironment by chemotherapy will help to improve immunomodulatory therapies to induce a better anti-tumor response.

## Adoptive cell transfer

Besides systemic treatment with chemotherapy to modulate the immune system, new techniques have developed to modulate immune cells *ex vivo*. A well-known example that is widely being applied in the clinic now, is the use of CAR T cells[33]. In **chapter 3**, we showed that short term chemotherapy treatment can directly positively impact CD8$^+$ T cell effector cell formation and proliferation *ex vivo*. We hypothesize that this is an attractive approach to apply in the field of CAR T cells. It might be possible to establish large numbers of rapidly proliferating effector CD8$^+$ T cells. On the other hand, we suggest that it is important to investigate how other cell cycle modulating drugs and therapies can be used to tweak CD8$^+$ T cells in a different way. This is also a limitation of our studies, since we did not test how all different types of cell cycle inhibitors that are used in the clinic impact proliferation and differentiation. However, in the last decade, other research has focused on the effects of Cdk4/6 inhibition on CD8$^+$ T cell proliferation and differentiation (**chapter 2**). It appears that inhibition of Cdk4/6 in CD8$^+$ T cells promotes memory T cell formation, instead of effector T cell formation[34]. Therefore, we would like to suggest exploring and thereby utilize the possibilities to change the (metabolic) phenotype of CD8$^+$ T cells by using different types of cell cycle inhibitors and eventually combine different strategies to establish the desired immune phenotype for effective cancer therapy (**Figure 2**).

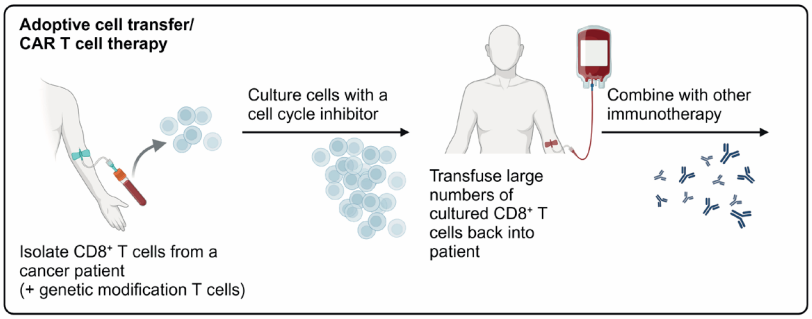


**Figure 2. Adoptive cell transfer can be combined with cell cycle inhibitors.** After collection of CD8$^+$ T cells from a cancer patient, the tumor specific CD8$^+$ T cells are cultured in the lab. To enhance proliferation, short term treatment with cell cycle inhibitors can be used. After culturing, these large number of tumor specific CD8$^+$ T cells can be transfused back into the patient. To optimize therapy further, adoptive cell therapy can be combined with other immunotherapeutic modalities, such as immune checkpoint blocking antibodies.

## CONCLUDING REMARKS

CD8$^+$ T cell proliferation is essential to establish a large enough anti-tumor immune response for efficient cancer therapy responses. In this thesis, we explored how the cell cycle of CD8$^+$ T cells is arranged and how external cues, such as TCR-triggering, costimulation and cytokines influence the continuation of the cell cycle. The sum of these signals determines the proliferation of CD8$^+$ T cells. We identified that a short-term cell cycle arrest during priming of newly activated CD8$^+$ T cells positively impacts the proliferation outcome, by changing the metabolism and cytokine production. We suggest that it is attractive to explore this intervention further to improve CD8$^+$ T cell numbers *ex vivo* for adoptive T cell transfers. In addition, understanding the *in vivo* effects of cell cycle inhibition on CD8$^+$ T cells and the tumor microenvironment will help to improve or combine chemotherapy with other types of immunotherapies, such as immune checkpoint blockade and vaccination. Understanding the cell cycle of CD8$^+$ T cells is essential to exploit the proliferation capacity of CD8$^+$ T cells to ultimately improve T cell-based therapies for cancer patients.

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