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Hyperthermia as a supporting strategy for cancer immunotherapy

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Zusammenfassung

Fieber ist ein natürliches Phänomen des menschlichen Körpers, welches das Immunsystem dazu anregt, Infektionen zu bekämpfen. Länger andauernde Fieberperioden wurden mit spontanen Rückbildungen von Tumoren in Verbindung gebracht. Diese Erkenntnis bildete die Grundlage dafür, künstliches Fieber oder äußere Hitzeeinwirkung in der Krebstherapie einzusetzen. Diese sogenannte Hyperthermie wird heutzutage als unterstützende Krebsbehandlung eingesetzt, da sie Tumorzellen für Chemo- und Strahlentherapie sensibilisiert. Die biologischen Auswirkungen von Hyperthermie umfassen unter anderem makroskopische Effekte wie erhöhte Durchblutung, zytotoxische Effekte auf Tumorzellen sowie Stimulierung des Immunsystems. Die grundlegenden zellulären und molekularen Vorgänge, welche diese Effekte verursachen, sind jedoch größtenteils unklar.

Das Ziel dieses Projektes war es, die Auswirkungen von Hyperthermie auf das Tötungspotential von zytotoxischen T-Lymphozyten (CTL) – einem Zelltyp des Immunsystems, der dazu in der Lage ist, Krebszellen zu erkennen und zu eliminieren – zu studieren. Im Fokus dieser Studie standen insbesondere die Untersuchung von verschiedenen Temperaturen und Behandlungszeitspannen, welche sich positiv auf die Tötungseffizienz der CTL auswirken, und von zellulären Vorgängen, welche diesen Effekt verursachen.

Mittels organotypischen 3D-kollagenbasierten Assays und live cell- Mikroskopie konnten wir zeigen, dass Fiebertemperaturen von 38.5 und 39.5 °C ausreichen, um die CTL-bedingte Abtötung von Melanomzellen in Mäusen signifikant zu erhöhen. Dabei waren klinisch relevante Behandlungsmethoden von jeweils einer Stunde pro Tag, an zwei aufeinanderfolgenden Tagen, dazu in der Lage, die CTL-Tötungseffizienz zu erhöhen. Weiters konnten wir einen unmittelbaren zytotoxischen und wachstumshemmenden Effekt auf Tumorzellen feststellen, was sich positiv auf die CTL-Tötungseffizienz auswirkte. Um die Ergebnisse an echten Tumoren zu validieren, wurde ein in-vivo-System etabliert, welches Ganzkörper-Hyperthermie Behandlung von Mäusen erlaubte. Durch anschließende intravital-Mikroskopie konnte der Therapieanspruch von Tumor- und Immunzellen auf zellulärer Ebene direkt untersucht

werden. Eine einstündige Ganzkörper-Hyperthermie Behandlung bei 39.5 °C führte eine unmittelbare Unterdrückung der Mitose herbei, was die direkten zytotoxischen Einwirkungen auf Tumorzellen, welche in vitro beobachtbar waren, bestätigten. Die Erkenntnisse dieses Projektes unterstützen daher eine Kombination von Hyperthermie und adoptivem T-Zell Transfer.

Abstract

Fever is a naturally occurring phenomenon of our body which stimulates the immune system to fight infections. High fever periods have been linked to spontaneous regression of tumors. This finding provided a rationale to induce artificial fever, or external heat application, in cancer therapy. Today, hyperthermia is clinically applied as supportive strategy to sensitize tumor cells to chemo- and radiotherapy. The biological effects of hyperthermia include macroscopic effects such as increased perfusion, cytotoxic effects on tumor cells and immunostimulation. However, the cellular and molecular mechanisms mediating the effects remain largely unclear.

The aim of this project was to study the effects of hyperthermia on the killing potential of cytotoxic T lymphocytes (CTL), a cell population of the immune system which is capable of specifically detecting and eliminating cancer cells. Particularly, the definition of the range of temperatures and treatment durations which are required to enhance CTL killing efficiency and the cellular mechanisms which mediate the effect were in the focus of this study.

Using 3D organotypic collagen-based cytotoxicity assays and live cell microscopy, we showed that fever-range temperatures of 38.5 and 39.5 C are sufficient to significantly enhance CTL-mediated killing of murine melanoma cells. Further, clinically relevant treatment schemes of 1 hour per day and repeated on 2 consecutive days were capable of increasing CTL killing efficiency. We further identified a direct cytotoxic and proliferation-inhibiting effect on the tumor cells which contributed to the increased CTL killing efficiency. To validate the results in live tumors, an *in vivo* system was set up to treat mice with whole-body hyperthermia followed by intravital imaging to evaluate the response of tumor and immune cells at cellular level. Application of 1 hour of whole-body hyperthermia at 39.5 °C induced an immediate inhibition of mitosis which confirmed the direct cytotoxic effect on tumor cells observed *in vitro*. Thus, the results obtained during this project support a combination of fever-range hyperthermia with adoptive CTL transfer.

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1. Introduction

1.1 Application of Hyperthermia in cancer therapy

The term hyperthermia refers to a form of thermotherapy where the effects of internally or externally applied heat are used to treat disease. Besides relieving symptoms of diseases, such as arthritis, hyperthermia has also been applied in cancer therapy. Commonly, hyperthermia is used as a supportive strategy in combination with established cancer therapies, but it also bears intrinsic tumor eliminating potential.[1]

In general, three major forms of application have found use in the past years, each following slightly different modes of action. Whole-body hyperthermia (WBH) is usually applied within a range of 38-41 °C mimicking commonly occurring fever-range temperatures in the body and is used for patients suffering from metastasis or tumors which are difficult to reach with surgery or laser-ablation.[1] This application method mostly aims at sensitizing tumor cells to radiotherapy or chemotherapeutical agents thereby functioning as a supportive treatment.[2,3] Recently, the potential of whole-body hyperthermia to enhance immune responses has gained increased attention due to the current success of immunotherapies.[4]

Regional hyperthermia uses similar temperature ranges as WBH, but rather focuses on heating parts of the body like limbs or organs. Thereby heating can be achieved via multiple approaches, ranging from thermal perfusion to external application with antennas emitting micro- or radiowaves.[3]

Local hyperthermia is usually applied above physiologically occurring temperatures reaching up to 100 °C and aims at directly destroying tumor tissue. This method results on the one hand in tumor apoptosis and coagulative necrosis and on the other hand promotes release of tumor-related antigens which are considered to trigger enhanced anti-tumor immune reactions. Besides ablation hyperthermia, local hyperthermia is also applied, although less frequently used, within fever-range temperatures to promote physiological effects of hyperthermia, like an increase of blood flow, without directly damaging tumor tissue.[5,6]

In general, the term hyperthermia comprises a brought variety of different application modes. Heating during whole-body hyperthermia treatments is usually achieved by infrared heated tent-like devices or simply by covering patients with heated blankets or vests.[7,8] Locally targeted therapies make use of externally applied radio frequency or use invasive strategies like direct perfusion of target regions. Moreover, delivery of heat towards target tissues is accomplished by injection of magnetic nanoparticles in combination with an externally applied electromagnetic field resulting in precise heating of the target tissue. [9]

1.2 History and evolution of hyperthermia

The first use of hyperthermia can be dated back to antiquity by use of the Egyptians. More recently the work of Dr. William B. Coley is being referred to as the origin of today's hyperthermia. He discovered a spontaneous regression of tumor tissue preceded by a high fever period. Based on these results he developed a vaccine consisting of two inactivated bacterial strains: *S. pyogenes* and *S. marcescens* later also referred to as Coley's Toxin, which triggered high fever periods upon inoculation. Patients suffering from sarcomas showed remarkable tumor regression after administration of Coley's Toxin, indicating a beneficial effect of fever in cancer therapy.[10]

Over the years, induction of fever was replaced by external application of heat mostly to avoid the dangerous side effects. Despite the early reports of the beneficial effects of fever on cancer regression, hyperthermia got its first impetus during the 1980s when radiation biologists performed first clinical trials testing its tumor cell sensitizing potential in combination with radiation therapy. [11]

At this time, application of heat was challenging due to underdeveloped equipment and insufficient heat delivery as well as burning, which caused a stagnation in hyperthermia research. Fortunately, development and establishment of new application techniques around 1990 lead again to an uplift, resulting in raised numbers of clinical phase III trials. [8]

Currently, hyperthermia is established as adjuvant combination with chemo- and radiation therapy, whereby according to ClinicalTrials.gov, hyperthermic intraperitoneal chemotherapy after cytoreductive surgery (HIPEC) had the largest quantity of clinical trials. Generally, the potency of hyperthermia in combination with established cancer therapies like radiation- and chemotherapy is emphasized by several phase III clinical trials with significant positive outcome in patient groups treated with hyperthermia. [12]

1.3 Effects of Hyperthermia

1.3.1 Effects on circulation and vasculature

The physiological effects of hyperthermia range from macroscopic effects, such as enhanced perfusion of tissues to molecular effects on individual tumor cells. One common characteristic of tumors is their capability to modify the physiological conditions of their microenvironment to such extents, that common cancer therapies fail to be effective. A major factor in tumor therapy resistance is the hypoxic environment originating from extensive cell proliferation and aberrant vascular structure within the tumor tissue.[13] These conditions narrow for example efficacy of radiation therapy due to low O_2 concentration and hence a failure to produce ROS via ionizing radiation.[14] Hyperthermia is thought to enhance radiation therapy by counteracting hypoxia through a raise of blood pressure and increased perfusion. Thereby tumor tissue O_2 concentrations raise which sensitize tumor cells towards radiotherapy.[15]

Generally, effects on tumor vasculature strongly depend on the duration and temperature range of applied hyperthermia. Temperatures within fever range are beneficial for blood flow and also enhance vessel permeability, whereas temperatures above the fever-range result in death of endothelial cells and overall vascular damage.[16]

1.3.2 Effects on tumor metabolism

Alterations in metabolic pathways have been shown to be essential for the generation and progression of malignant cells due to unusual physiological circumstances like a hypoxic environment or general nutrient deprivation. Those changes in metabolism are exploited to develop certain anti-cancer agents. [17]

First evidence that hyperthermia influences tumor cell metabolism almost reaches back 30 years. It has been found that tumors treated with hyperthermia (43 °C, 30 min) tend to accumulate lactate, whereas glucose and ATP levels remained unaffected. Main consequence of raised intracellular lactate concentrations is an acidification of the cell which in turn leads to enhanced vulnerability. [18]

Further, increased expression of HIF-1 after hyperthermia treatment was found, a factor usually activated through hypoxia, promoting vascularization and also playing a role in the shift from oxidative to glycolytic metabolism that leads to acidification of the tumor microenvironment. [19,20]

1.3.3 Effects on cell membrane and drug uptake

The cell membrane plays a crucial role in maintaining cellular homeostasis and enabling exchange of metabolites with its surrounding. Therefore, its integrity is ultimately decisive for cell survival. Hyperthermia has shown to be able to render cell membrane permeability and fluidity, which is often seen as the major rationale for enhanced chemotherapy efficiency.[21,22] Several reports state that uptake of chemotherapeutic agents like cisplatin is enhanced after hyperthermia treatment and results in raised intracellular concentrations.[23,24]

1.3.4 Effects on DNA repair mechanisms

There is a large body of evidence, that hyperthermia intervenes in various DNA repair mechanisms. Homologous recombination is a repair process used by tumor cells facing DNA double strand breaks. Essential for this repair mechanism is the presence of an intact DNA template strand and thus, this repair mechanism takes place during S- or G_2 phase of the cell cycle. Two proteins, RAD51, which binds to DNA and forms

nucleoprotein filaments, and BRCA2, which plays a role in RAD51 recruitment, are major components of the homologous repair system.[25] Hyperthermia in the range of 41 – 42.5 °C has been shown to effectively inhibit homologous repair by degradation of BRCA2 and hence causing a reduction in accumulated RAD51 at sites of DNA damage.[26] This finding especially supports the use of hyperthermia in combination with radiation therapy, where induction of DNA double strand breaks is the main mode of action, and provides a mechanistic explanation of the hyperthermia-induced tumor radio-sensitizing potential.

Moreover, it has been shown that BRCA1-deficient tumors fail to repair DNA double-strand breaks using homologous recombination. Tumors showing this genotype are especially susceptible to chemotherapeutic agents like PARP-1 inhibitors, which are capable of collapsing the replication fork and are known to be non-toxic to surrounding healthy tissue.[27] By locally inducing BRCA2 deficiency in tumors, hyperthermia could thus enable PARP-1 inhibitors to fully reveal their potential.[26,28]

Hyperthermia applied as temperatures above 42.2 °C further has an impact on base excision repair mediated by inhibition of DNA Pol β, a key component of this repair mechanism.[29,30] However, more studies are needed to directly link these findings to the hyperthermia-induced radiosensitization.[31]

Likewise, it is still unclear whether hyperthermia exerts effects on repair mechanisms like non-homologous end joining (NHEJ). [32]

1.4 Effects of hyperthermia on the immune system

The effects of hyperthermia on macroscopic processes like blood flow and oxygenation of tissues, but also on molecular processes like DNA repair, cellular metabolism and characteristics of cellular membranes have been studied in detail and are currently exploited in combination therapies of hyperthermia and chemo- and radiotherapy. Cancer, however, is a rapidly changing, complex disease which requires different combinations of treatment modalities to efficiently eradicate malignant cells. During the past years, immunotherapies have evolved as a promising new approach of targeting

cancer. Immunotherapies have the potential to target tumor cells with great specificity and to establish a memory to avoid tumor recurrence. While a subset of patients responds with great efficiency and partially reaches complete remission, immunotherapy fails in large fractions of patients. Thus, hyperthermia may be a possible supporting treatment to enhance immunotherapies.

1.4.1 Heat shock response and dendritic cells

Heat application is a potent strategy to induce cellular stress which results in misfolding of proteins and protein aggregation. Usually cells counteract these circumstances by upregulating a well conserved class of thermal stress induced proteins which are referred to as heat shock proteins (HSPs). This protein class helps maintaining cellular functions by removing those aggregates either by refolding proteins (chaperon function), or regulating proteolytic degradation of non-refoldable proteins.[33] Besides their role in heat stress, HSPs are also constitutively expressed, with an abundancy of 1-2 % of total cytosolic protein to guarantee correct protein conformation after synthesis and trafficking. [34]

It has been found that some tumors express increased levels of HSPs which is linked to poor prognosis. High levels of HSPs, for example HSP70, in some types of cancer correlate with inhibition of apoptosis and may also benefit processes like invasion, angiogenesis and immortalization.[35,36,37] This dependency on elevated HSP levels gains more and more interest as a potential target for anti-cancer therapy. [38]

However, besides these tumor promoting capacities, HSPs represent a potent inducer of immune reactions by acting as “danger signals”. It has long been assumed that HSPs were only active within the cell, until research demonstrated their release into the extracellular space. [39] Release mechanisms are still not fully understood, but there is evidence that release is mediated by exocytosis or necrosis.[40,41] Thereby HSPs transport bound tumor peptides or proteins out of the cell where they are subsequently taken up and presented by dendritic cells.

Presentation of unique tumor antigens, to cells of the adaptive immune system is a crucial step in autogenous tackling of cancer.[42] Especially HSP 70 has been shown to influence dendritic cell (DC) activity in two different ways. First, binding of HSP 70 to

surface receptors of DCs results in enhanced activation and changes their state from naive towards mature. Second, internalized tumor-associated antigens bound to HSP 70 can be presented by MHC-I on DCs through a mechanism called cross-presentation. Presentation of those antigens can lead to activation of tumor specific $CD8^+$ cytotoxic T-lymphocytes.[43,44]

Hyperthermia has been shown to significantly upregulate levels of HSPs not only in the range of ablation temperatures (42 - 45 °C) but also febrile temperatures as they occur naturally during fever periods (38 – 41 °C).[45,46] Thus, application of hyperthermia has the potential to enhance DC mediated immune responses. Current clinical approaches therefore target HSP based anti-cancer vaccines. Thereby anti-tumor responses are provoked by surgically removing parts of the tumor, isolating and subsequently reinjecting tumor-antigen loaded HSPs into the patient.[47]

In contrast, multiple consecutive hyperthermia treatments can also result in the unwanted negative side effect of thermotolerance. [48] HSPs like HSP 27 and HSP 70 have been found to exert essential functions in thermotolerance by restoring function of DNA Pol β which is usually disabled under hyperthermia. Thereby DNA repair is ensured contributing to enhanced cell survival under hyperthermia. [49]

1.4.2 Hyperthermia enhances trafficking of Lymphocytes

Trafficking of lymphocytes is a strictly-regulated process in immune responses and involves several adhesion steps of lymphocytes with endothelia to enable infiltration of tissues. In lymph nodes, whole-body hyperthermia within fever-range enhances this process by increasing expression of ICAM-1, a surface molecule of high endothelial venules (HEVs) of secondary lymphoid organs. This upregulation enhances entry of naïve cytotoxic T-cells into the lymph nodes where activation and maturation into effector cells takes place.[50] In this context, hyperthermia could lead to augmented activation of tumor reactive cytotoxic T-lymphocytes provided that tumor antigens are being presented by APCs in the lymph nodes. Moreover, hyperthermia also has shown to support lymphocyte trafficking into tumor tissue through a trans IL-6 signalling-dependent modification of tumor vasculature.[51]

Besides vasculature, adhesive properties of lymphocytes also seem to be affected by fever-range hyperthermia. L-selectin and $\alpha 4\beta 7$ integrin interaction with endothelial cells from HEVs have shown to be enhanced, promoting lymphocyte infiltration into lymph nodes. [52,53]

Both, adhesive properties on endothelial cells and on lymphocytes can contribute to enhanced immune surveillance and activation of naïve CTL through encounter of cognate tumor-specific antigens. Further research needs to be conducted to exploit the potential of hyperthermia to enhance homing of lymphocytes into tumor tissue in combination with current immunotherapies like adoptive cell transfer.

1.4.3 Effects of hyperthermia on cytotoxic T lymphocytes

As one of the main mediators of anti-tumor immune response CTL represent a cell type capable of specifically targeting and eliminating cancer cells. These properties are currently exploited by several therapeutic approaches like CAR (chimeric antigen receptor) T-cell or TIL (tumor infiltrating T-lymphocytes) therapy which have been established as potent anti-cancer treatments. [54] However, efficiency of those therapies still needs to be increased to guarantee full potential. In general, influence of hyperthermia on T-cells hasn't gained a lot of research interest yet. But since elevated temperatures during fever periods favor immune functions, it seems likely that hyperthermia could mediate enhanced T-cell function.

Fever-range hyperthermia has been shown to trigger differentiation from naïve T-cells towards effector T-cells, indicated by a switch from $CD62L^{high}$ $CD44^{low}$ to a $CD62L^{low}$ $CD44^{high}$ phenotype as a consequence of 39.5 °C hyperthermia treatment and antigen stimulation.[55] Further, signs of enhanced T-cell activity after stimulation with fever range hyperthermia have been revealed through clustering of $GM1^{+}$ CD-microdomains in the plasma membrane of T-cells. This mechanism is hypothesized to support T-cell activation by enhancing conjugate formation with APCs and clustering of proteins like TCRβ and the CD8 coreceptor. [55]

Other studies focused on local heating of tumors *in vivo* outside of physiological occurring fever-range temperatures (43 °C, 30 min) and discovered that mice, whose primary tumors were treated with hyperthermia were resistant to rechallenge with the

same tumor type for a period of up to 30 days. Concurrently, an activation of DC and tumor-specific CTL was found in the draining lymph node. Interestingly, heating of primary tumors to tissue-destructive temperatures (45 °C) allowed successful re-challenge with a subsequently-injected tumor, thereby revealing that the applied temperature range plays a crucial role in therapy success.[56]

Further evidence shows, that hyperthermia has a direct influence on the molecular killing mechanisms of CTL. An upregulation of Fas-L mRNA levels and Fas-L promotor activity in CTL could be detected after fever range hyperthermia treatment which correlated with increased apoptosis induction in tumor cells. [57]

Apart from these studies, the mechanisms of action and molecular effects of hyperthermia on effector functions of CTL remain to be elucidated.

1.5 Cytotoxic T cells

T-cells fulfill a crucial role of the adaptive immune system in fighting infections. Each T-cell expresses a unique TCR with binding capacities for a a specific antigen due to a series of genetic recombination events. CTL TCRs recognize peptides presented on MHC-I molecules, which present part of the cells own peptide repertoire or pathogen associated peptides in case of infection.[58] Upon successful binding of antigen by the TCR, CTL induce apoptosis of the infected cell by secretion of cytotoxic vesicles containing granzymes and perforin. [59]

Originally, CTL are derived from lymphoid progenitor cells of the bone marrow which travel via the blood stream to the thymus, where they mature to naïve $CD8^+$ T lymphocytes. During maturation in the thymus a crucial selection process takes place that tests premature lymphocytes for autoreactivity and eventually induces, if endogenous antigen-MHC complexes are either bound with too high or too low affinity, apoptosis in the target lymphocytes.[60] Only about 2 % of all entering T-lymphocytes survive the selection process, leave the thymus into the blood stream and are from then on being referred to as naïve T-lymphocytes. [61]

Naïve $CD8^+$ T cells continuously migrate through secondary lymphoid organs to scan

APCs for MHC-I-mediated presentation of their designated antigen.[62] Switching to an activated effector state requires their interaction with APCs such as DCs. Those cells take up antigen from peripheral tissues and travel to T-cell areas in lymph nodes to present their antigens to $CD8^+$ T cells via MHC-I.[63,64] For successful T-cell activation a sequence of signaling events are required. After recognition of antigens by the TCR, co-stimulation of the T-cell is achieved via binding of CD28, expressed on the T cell to B7, expressed on DCs. This triggers T-cell proliferation and thus the production of large numbers of antigen-specific effector T cells which is a phenomenon also known as clonal expansion. For CTL activation both co-stimulatory signaling from DCs and secreted IL-2 are necessary to become fully functional. Once antigen specific T-cells have been activated and their numbers have drastically increased, they leave the lymph node in order to travel to the tumor tissue to eliminate antigen-specific tumor cells.[65]

Guided by chemotactic signaling of inflamed tissues, CTL migrate out of the bloodstream into the tumor where they unveil their cytotoxic potential. CTL identify tumor cells by tumor-specific antigen bound to MHC-I on their cell surface. Upon successful recognition of those peptides through the TCR, CTLs are triggered to induce apoptosis in the target cell by release of cytotoxic vesicles or activation of death-receptors. [66] The most abundant pathway is the perforin/granzyme pathway which aims at permeating the cellular membrane using pore-forming perforines and simultaneous secretion of Granzyme B, a serine protease capable of inducing apoptosis via caspase activation . [67,68]

Besides this pathway CTL express FasL (CD95-L) , also known as the death ligand, on their surface which induces apoptosis upon binding to its counterpart Fas (CD95) on the target cell surface. [69] Lastly CTL also possess the ability to secrete cytokines like IFNγ, TNFα and β which promote tumor cell apoptosis and inhibit proliferation. [66]

1.6 Aim of the thesis

Through outstanding potential in recognizing and eliminating cancer cells, CTL are the key players in cancer immunotherapy. However, complete eradication of solid tumors is still challenging and an ongoing topic of research. Combined with the potential of

hyperthermia to enhance immune responses, this provided the rational to test whether hyperthermia enhances CTL-mediated killing of melanoma cells. Particularly, the identification of clinical relevant treatment schemes including the necessary duration of application and the required temperature range represented a main focus of this project.

2. Material and Methods

2.1 Chemicals

AlexaFluor750® 70 kDa Dextran	(Thermo Fisher)
BIC	(Gibco, 25080-060)
Bouin's solution	(Sigma-Aldrich, HT10132-1L)
BSA	(Aurion, 900.022)
Collagenase I	(Sigma-Aldrich, C0130)
FCS	(SIGMA, F7524)
HEPES	(Gibco, 15630)
IFNγ	(Biolegend)
IL-2	(AbD Serotec, PMP38)
$KHCO_3$	(Sigma-Aldrich, 298-14-6)
Lymphoprep	(Ficoll, 1114545)
MEM	(SIGMA, M0275)
Mouse Serum	(Abcam, ab7486)
Na_2EDTA	(Sigma-Aldrich, 6381-92-6)
NEAA	(Gibco, 11140)
NH_4Cl	(Sigma-Aldrich, 12125-02-9)
OVA-peptide	(Bachem, H4866)
PBS	(Department)
Penicillin and Streptomycin	(PAA, P11-010)
Propidium Iodide	(Sigma-Aldrich, P4170)
PureCol	(Advanced Biomatrix, 500-C)

RPMI 1640	(Gibco, 21857)
Sodium Pyruvate	(Gibco, 11360)
Triton X-100	(Sigma-Aldrich, 100-155)
Trypsin	(Gibco, 15090046)
β-mercaptoethanol	(Department)

2.2 Media and buffers

Complete culture medium

- RPMI 1640
- FCS, 10 %
- Penicillin and Streptomycin, 1 %
- Sodium Pyruvate, 1 %

T-cell medium (TCM)

- RPMI 1640
- FCS, 10 %
- Penicillin and Streptomycin, 1 %
- Sodium Pyruvate, 1 %
- NEAA, 0,1 mM
- β-mercaptoethanol, 500 µM
- HEPES, 10 mM

Red blood cell (RBC) lysis buffer

- NH_4Cl 4,15 g
- $KHCO_3$ 0,5 g
- Na_2EDTA 1,85 g
- Bidistilled H_2O 1 l

Trypsin

- 1x PBS
- 1x Trypsin
- EDTA (10 mM)

PureCol gel (135 µl)

- BIC, 5 µl
- MEM, 10 µl
- Cell suspension, 45 µl
- PureCol, 75 µl

2.3 Cell culture

2.3.1 Used cell lines

Table 1: Overview of used cell lines

Cell-Type	***Name***	***Properties***
Murine Melanoma Cell Lines	B16-F10/OVA	Cells are transfected with a gene encoding an ovalbumin peptide (SIINFEKL)
	B16-F10/OVA GFP	Expresses OVA peptide and GFP labelled histone-2B
	B16-F10/OVA mCh Ca^{2+}	Expresses OVA peptide and mCherry labelled H2B. Further, expresses GCaMP6s to detect fluctuations in intracellular Ca^{2+} concentrations.
	B16-F10/OVA NLS-GFP	Expresses OVA peptide and GFP tagged with a nuclear localisation signal.
Murine primary lymphocytes	OT1 dsRed $CD8^{+}$-T Lymphocytes	Primary cells obtained from spleens of transgenic mice expressing the OT1 T-cell receptor which specifically binds the OVA peptide SIINFEKL presented by MHC-I. Additionally express cytoplasmic dsRed.

2.3.2 Culture of murine melanoma cells

Murine melanoma cells were cultured using complete culture medium. For splitting the cells, the medium was removed and the cells were washed once using 1 x PBS. Cells were detached by incubation with Trypsin for approximately 5 min at 37 °C. The reaction was stopped by diluting the cells with fresh medium. Dependent on the splitting ratio a part of the cell suspension was discarded and the remaining part was filled up with fresh medium. Cells were cultured at 37, 38.5, 39.5 or 40.5 °C and 5 % CO_2.

2.3.3 Isolation, activation and harvesting of OT1-TCR $CD8^+$ T-lymphocytes

Spleens from transgenic OT1 C57/Bl6 J mice (Charles River), were removed and collected in a tube containing 1 x PBS. Cells were isolated by mashing the spleen through a 100 µm mesh size cell strainer (BD) and collected in T-cell medium. After a centrifugation step (1600 rpm, 4 °C, 5 min) the cell pellet was resuspended in RBC buffer and incubated for 5 min at RT. To stop the reaction T-cell medium was added and the cell suspension was again centrifuged (1600 rpm, 4 °C, 5 min). Cells were counted using a counting chamber (Marienfeld) and 0.5 x 10^6 cells were seeded per well in a 24-well plate. Additionally, cells were stimulated with 0.5 µg/ml OVA-peptide. Plates were sealed in plastic foil and cultured under a small angle at either 37, 38.5, 39.5 or 40.5 °C, at 5 % CO_2. Three days after isolation the medium was changed, cells were seeded at a density of 8 x 10^5 cells/well and stimulated with 20 ng/ml IL-2. Culture was performed again under a small angle.

Before each experiment $CD8^+$ T-lymphocytes were isolated by collecting the cells and using a Lymphoprep density grade centrifugation (2300 rpm, 10 min, 4 °C, no brake). The fraction containing OT1 $CD8^+$ T-lymphocytes was located on the interface between Lymphoprep and medium and was carefully pipetted off, spinned down (1600 rpm, 5 min, 4 °C) and counted.

Previous flow cytometry experiments confirmed the effectiveness of the isolation with a $CD8^+$ positive population of 95-98 %.

2.3.4 Isolation of B16-F10/OVA GFP melanoma cells from solid tumor tissue and preparation for FACS

2 x 10^5 B16-F10/OVA cells were injected intradermally into C57/Bl6 J mice (Charles River). 1 – 2 weeks after injection the tumor was cut out and incubated in 2000 U/ml Collagenase I at 37 °C. As soon as the tumor was fully digested the suspension was collected in 1 x PBS and centrifuged (1200 rpm, 5 min). The pellet was again washed using 1 x PBS, resuspended in 1 mM EDTA / 1 x PBS and incubated for 30 min at 37 °C. To stop the reaction 10 % FCS / 1 x PBS was added. After another washing step with 1 x PBS the pellet was incubated in RBC for 5 min at RT. The reaction was stopped by adding 10 % FCS / 1 x PBS followed by an additional washing step. Cells were counted and an amount of 2 x10^5 – 5 x10^5 cells was used for staining. Blocking was performed using 2 % mouse serum in 1 x PBS and incubation for 10 min on ice.

Staining was performed as described in section 2.4.2

2.4 Microscopy

2.4.1 Bright field killing movies

B16-F10/OVA cells were stimulated with 200 U/ml IFN-y 30 h before coculture with T cells. U-shaped chambers were formed with Wax (1:1 mixture Vaseline® and Wax) on microscopy glass slides (Marienfeld). Cells were harvested, restiumlated with IFN-y and seeded at a concentration of 0.15 x 10^6 cells/ml until the chamber was full. The top end was then sealed with wax and cells were incubated either on 37 or 39.5 °C overnight. OT1 $CD8^+$ T-lymphocytes were harvested as described in section 2.2.3 and embedded at a concentration of 0.6 x 10^6 c/ml in a PureCol gel .

Chambers were opened again on the top side using a sterile scalpel and the medium was removed. Chambers were filled up to half with the OT1 $CD8^+$ T-lymphocytes consisting gel. Polymerization of the gels was accomplished in an upright position in an incubator at 37 °C. As soon as gels were polymerized the rest of the chamber was filled up with TCM and sealed with wax.

Imaging was performed using live cell bright field microscopy (Leica DMIL) under 37 or 39.5 °C for up to 48 hours at a frame rate of 30 seconds. Heating of microscopic stages was accomplished via infrared lamps and covering of slides with transparent plastic boxes. Movies were analysed using Fiji/ImageJ.

2.4.2 Visualizing sublethal damage using molecular sensors (Ca^{2+}, NLS-GFP)

B16-F10/OVA mCh Ca^{2+} or B16-F10/OVA NLS-GFP cells were stimulated 1.5 days previous experiment with 200 U/ml IFN-y and cultured under 37 or 39.5 °C . Cells were harvested using 1 mM EDTA and seeded at a concentration of 0,15 $x10^6$ cells/ml in a WillCo® dish. To promote re-attachment to the glass surface cells were incubated for approximately 6 hours at 37 or 39.5 °C. OT1 $CD8^+$ T-lymphocytes were harvested as described in section 2.2.3., concentration was adjusted to 1.5 x 10^6 cells/ml and cells were embedded into a PureCol gel. Medium was removed from the dishes, CTL containing gel was filled up until one third and polymerized at 37 °C for 20 min. Imaging was performed overnight using fluorescence confocal microscopy (SP8, Leica) for up to 20 hours. 488 nm laser (excitation NLS-GFP and GCaMP6s) and 561 nm laser (excitation mCherry and dsRed) were used with 3 - 5 % and 3 % laser power respectively. Movies were analysed using Fiji/ImageJ.

2.4.3 Analysis of B16-F10/OVA GFP mitotic events before and after hyperthermia

7000 B16-F10/OVA GFP cells were seeded in 150 µl medium per well in a dark 96-well plate suited for fluorescence microscopy. One day after seeding nuclei of cells were imaged using a BD Pathway with an excitation wavelength of 488 nm. Cells were treated with hyperthermia (37, 38.5, 39.5 or 40.5 °C) for 1 hour and nuclei were imaged before and directly after treatment.

Fiji was used for analysing nuclear shape in order to determine mitotic vs non-mitotic cells. Healthy nuclei were segmented and counted by a self-written macro based on shape, size and intensity of the nucleus. Mitotic events were counted manually using the “cell counter” plug-in.

2.4.4 *In vivo* imaging of B16-F10 NLS-GFP OVA before and after hyperthermia treatment

8 to 14-week-old male C57/Bl6 J mice (Charles River) were transplanted with dorsal skin-fold chambers, as described.[70] Mounting of the chamber was accomplished by surgically attaching it to a skin-flap whereby one side of the skin was removed, as described.[71] One day post-surgery, approximately 2 x 10^5 pelleted B16-F10/OVA NLS-GFP cells were injected into the dermis at one or two sites within the dorsal skin-fold chamber imaging window. Four days post-surgery, *in vitro* activated 2 x 10^6 OT1-dsRed $CD8^+$ lymphocytes were injected intravenously in 100 ul NaCl.

On day 8 / 9 after tumor injection mice were treated with hyperthermia. Mice were anaesthetized with Isoflurane (1 - 3 % in oxygen) and placed in an air ventilated thermocage (Datesand). Once 39.5 °C core body temperature was reached, mice were heated stably for 1 hour under continuous monitoring of the core body temperature and the temperature next to the tumor.

During imaging mice were anaesthetized and immobilized by fixation of the chamber onto a temperature-controlled stage (37 °C). To visualize blood vessels, AlexaFluor® 750 labeled 70kDa-dextran (2 mg/mouse) was injected retro-orbitally. Imaging was performed before and directly after application of hyperthermia.

A customized multi-photon microscope (TriMScope-II, LaVision BioTec) was used for image acquisition. Excitation wavelengths were generated using three tunable Ti:Sa lasers (Coherent Ultra II Titanium:Sapphire) with a coupled Optical Parametric Oscillator (OPO PP, Coherent APE).[72]

All animal experiments were approved by the Ethical Committee on Animal Experiments and performed in the Central Animal Laboratory of the Radboud University, Nijmegen, in accordance with the Dutch Animal Experimentation Act and the European FELASA protocol (www.felasa.eu/guidelines.php). [72]

2.5 Flow Cytometry

2.5.1 Organotypic cytotoxicity assays combined with continuous or fractionated hyperthermia

B16-F10/OVA cells were stimulated with 200 U/ml IFN-y and cultured under 37, 38.5, 39.5 or 40.5 °C for 1.5 days prior to seeding. 7000 cells /well were seeded into a 96-well plate, stimulated with IFN-y and incubated under different temperatures overnight.

OT1 $CD8^+$ T-lymphocytes were harvested as described under section 2.2.3 and approximately 1875 cells were embedded in a PureCol gel. The medium of the wells containing B16-F10/OVA cells was removed and tumor cells were overlayed with 75 µl of the PureCol gel. Polymerization took place within 30 minutes at 37 °C. To prevent gels from drying out wells were topped with 75 µl of TCM. Samples were then incubated 48 hours at different temperatures (37, 38.5, 39.5 or 40.5 °C) or heat treated twice within 48 hours for a period of 1-3 hours per treatment at different temperatures (37, 38.5, 39.5 or 40.5 °C).

Cells were harvested by addition of 35 µl collagenase I and incubation for 30 min at 37 °C. Supernatants were collected and 30 µl Trypsin was added to the wells. After incubation for 10 min at 37 °C supernatants of the corresponding well were used to collect the detached cells.

10 µl of PI were added to each sample 1 minute prior to flow cytometry readout (BD FACS Calibur™). Samples were measured for 1 minute at high flow rate and the data was analysed using FlowJo X10 (LLC).

2.5.2 MHC-1 staining of B16-F10/OVA melanoma cells

B16-F10/OVA were treated under several different conditions (Temperature: 37, 38.5, 39.5 or 40.5 °C, culture without IFN-y/ with IFN-y) ranging from 48 hours up to 96 hours, harvested and 2 x 10^5 cells were transferred to a 96 U-bottom plate, spinned down (1200 rpm, 3 min, 4 °C), washed with 150 µl of 1 x PBS. Incubation with primary antibody (see table 1) was performed in 50 µl 1 x PBS for 45 min at 4 °C. Wells were topped with 150 µl 1 x PBS, spinned down and washed two more times. Secondary

antibodies (see table 1) were used the same way as primary ones. Stained cells were measured with a BD FACS Calibur™ whereby a gate on cell morphology was set in the FSC/SSC plot within which 5000 events were acquired.

2.5.3 Fixation with Bouin's solution and staining of OT1 CD8$^+$ T-lymphocytes

Cells were harvested and an according amount was washed with 1x PBS (1600 rpm, 5 min, 4°C). The pellet was resuspended in 1 ml of Bouin's solution and incubated for 20 minutes at room temperature. Cells were washed three times with 0.2 % BSA/1x PBS (550 rcf, 5 min, room temperature) and afterwards permeabilized with 1 ml of 0,1 % Triton X-100/1x PBS for 10 min at room temperature. Cells were washed again 3 times with 0.2 % BSA/1x PBS.

After fixation cells were counted using a counting chamber (Marienfeld). $3x10^5$ cells were used for each staining and incubation with primary antibody (see table 1) was performed in 50 µl 0.2% BSA/1x PBS for 30 min at 4°C. Cells were then washed 3 times (2300 rpm, 3 min, 4°C) using 0.2% BSA/1x PBS and incubated with secondary antibodies following the same procedure as primary antibodies.

Stained cells were measured with a BD FACS Calibur™ whereas upon measurement 5000 events were acquired.

Table 2: List of used primary and secondary antibodies

	Volume [µl]	Dilution	Species	Producer
MHC-1 IgG	2	1:25	Mouse	Biolegend
MHC-1	2	1:25	Mouse	Biolegend
CD 44 IgG (PI)	1	1:50	Rat	BD bioscience
CD 44 (PI)	1	1:50	Rat	BD bioscience
CD 137 IgG (Alexa 488)	0,1	1:500	Rat	AbDserotec
CD 137 (Alexa 488)	1	1:50	Rat	ebioscience
CD 25 IgG (Alexa 488)	0,5	1:100	Rat	ebioscience
CD 25 (Alexa 488)	0,5	1:100	Rat	ebioscience
CD 62-L IgG (FITC)	1	1:50	Rat	BD bioscience
CD 62-L (FITC)	1	1:50	Rat	BD bioscience
CD 8 IgG	1	1:50	Rat	BD bioscience
CD 8	1	1:50	Rat	BD bioscience
Granzyme B IgG (PE)	1	1:50	Rat	ebioscience
Granzyme B (PE)	1	1:50	Rat	ebioscience
anti-mouse 488	1	1:50	Goat	Thermo Fisher
anti-rabbit 488	1	1:50	Goat	Thermo Fisher
anti-mouse 647	1	1:50	Goat	Thermo Fisher
anti-rabbit 647	1	1:50	Goat	Thermo Fisher

2.6 ELISA - Measurement of CTL IFN-γ secretion under hyperthermia

B16-F10/OVA cells were seeded at a density of 7000 cells/well in a 96-well plate one day previous to the experiment. CTL were harvested and concentration was set to 1.25 x 10^4 cells/ml. Old medium was replaced with 150 µl of the cell suspension and plates were incubated at either 37, 38.5, 39.5 or 40.5 °C for 48 hours. After the incubation period supernatants were collected, spinned down (1600 rpm, 5 min) and stored at -20 °C until the measurement. IFN-γ secretion was detected using the Mouse IFN-γ Quantikine ELISA Kit purchased from R&D Systems (Abingdon, UK).

2.7 Analysis and Statistics

Microscopic images and movies were analysed using FIJI/ImageJ, FACS data were processed with FlowJo X10 and individual data was plotted using GraphPad Prism.

3. Results

3.1 Hyperthermia enhances CTL-mediated killing of B16-F10/OVA melanoma cells

3.1.1 Organotypic 3D cytotoxicity assay

To address the CTL killing efficiency under the influence of hyperthermia, 3D-organotypic cytotoxicity assays were performed. B16-F10/OVA cells were co-cultured with OT1- CTL in a collagen matrix to mimic the 3D *in vivo* tissue environment and to allow the CTL to migrate freely. Hyperthermia temperatures within the physiological fever range of 38.5 to 40.5 °C were continuously applied for the entire duration of the assay. After 48 hours of coculture, tumor cells and CTL were isolated from the collagen matrix and analysed by flow cytometry. Live/dead stainings using propidium iodide allowed the quantification of surviving tumor cells and viable CTL.

The combination of CTL and hyperthermia, additionally decreased the number of surviving tumor cells significantly (Figure 1C). To distinguish the direct cytotoxic effects of hyperthermia on the tumor cells from hyperthermia-enhanced CTL-mediated killing, the specific CTL killing efficiency was calculated by normalizing CTL-mediated killing to samples treated with hyperthermia alone (Figure 1D). This revealed a significant increase in CTL-mediated killing at 38.5 and 39.5 °C, while no increase in killing efficiency was detectable at 40.5 °C.

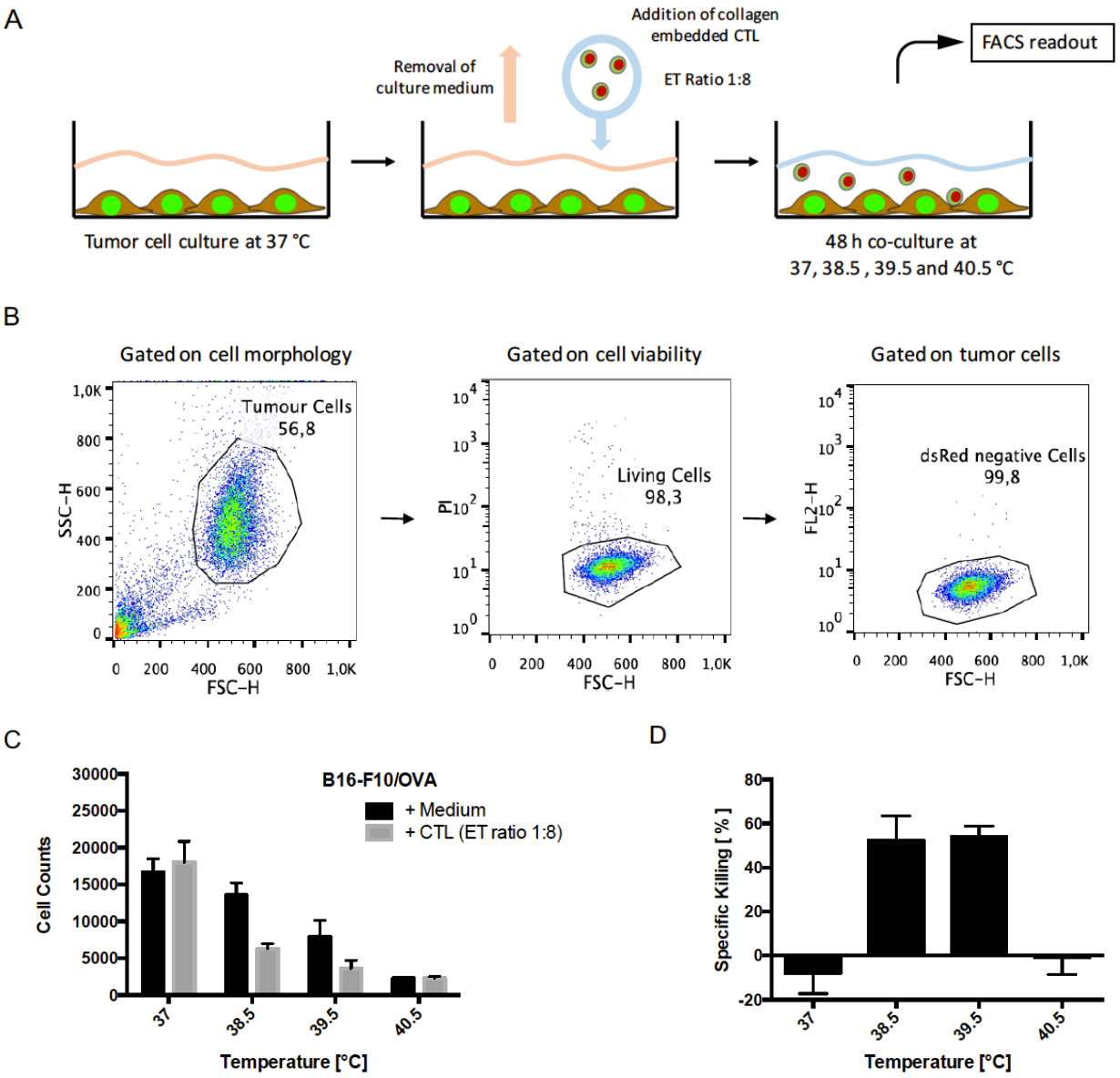


Figure 1: Continuous hyperthermia treatment increases CTL-mediated killing of B16F10-OVA. B16-F10/OVA melanoma cells were co-cultured with OT1- CTL in a 3D-collagen matrix gel for 48 hours under different temperatures at an effector-target ratio of 1:8. **(A)** Schematic course of an organotypic 3D cytotoxicity assay. **(B)** FACS readout gating procedure. Tumor cells were gated based on morphology. Samples were stained with PI to exclude dead cells. DsRed-positive CTL were gated out to exclusively obtain tumor cell counts. Counts were obtained by measuring each sample for 60 seconds at medium speed. **(C)** Comparison of tumor cell counts without CTL (+Medium) and samples containing both, tumor cells and CTL (+CTL ET ratio 1:8). **(D)** Specific-CTL killing efficiency obtained by normalizing samples to hyperthermia treatment alone, using the following formula: $(cell\ counts\ (Medium) - cell\ counts\ (CTL\ ET\ ratio\ 1{:}8))/(cell\ counts\ (Medium))$ Graphs display data from three independent experiments with SD.

3.1.2 Temperature-dependent upregulation of MHC-I levels

MHC-I complexes play a key role in the recognition process of target cells by CTL. Previous studies showed that the density of MHC-I/pOVA complexes determines CTL-mediated killing efficiency and that hyperthermia treatment induces an upregulation of MHC-I expression on the cell surface. Thus, we addressed if hyperthermia-induced upregulation of MHC-I expression in B16-F10/OVA cells was sufficient to explain the enhanced CTL killing efficiency.

B16-F10/OVA cells were cultured for 48 hours under 37, 38.5, 39.5 and 40.5 °C and subsequently analysed for MHC-I expression using flow cytometry. While MHC-I levels were below the detection limit at 37 °C, hyperthermia treatment of 38.5 and 39.5 °C significantly increased MHC-I levels on the cell surface (Figure 2, A+B). MHC-I levels after 48 hours incubation at 40.5 °C appeared to be unchanged, which may be explained by cytotoxic effects of the prolonged exposure to 40.5 °C.

To compare the range of MHC-1 upregulation by hyperthermia to physiological inducers of MHC-I expression, B16-F10/OVA cells were treated with IFN-γ.

IFNy treatment for 48 hours increased MHC-I levels on the cell surface to a maximum which exceeded the hyperthermia-induced upregulation by 10-fold. Additional treatment of IFNy and hyperthermia further increased MHC-I levels by 1.5-fold (38.5 °C) and 1.8-fold (39.5 °C), respectively (Figure 2, C+D).

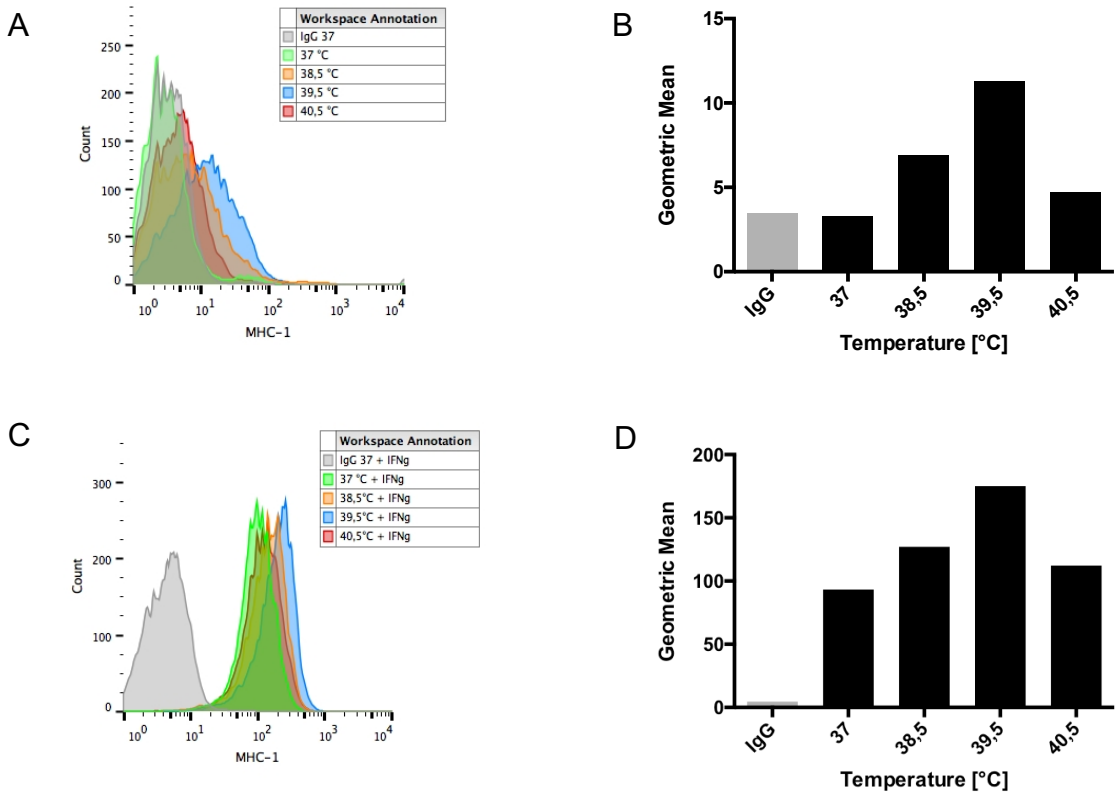


Figure 2: Increase of B16-F10/OVA MHC-1 expression after 48 hours hyperthermia treatment. Cells were cultured for 48 hours under different temperatures (37; 38.5; 39.5 ; 40.5 °C). 2x10^5 cells were stained using MHC-1 anti-mouse mAb (1:25 in PBS) and detected via secondary goat-anti-rabbit 647 (1:50 in PBS). Flow cytometry histogram and graphical depiction of geometric mean values of MHC-1 levels after 48 hours hyperthermia of untreated **(A+B)** and IFN-γ treated cells **(C+D)** are shown

3.1.3 Additional effects of CTL co-culture and hyperthermia treatment independent of MHC-I upregulation

In the tumor microenvironment, particularly during an anti-tumor immune response, IFN-y levels are considered to be upregulated, leading to increased MHC-I levels on tumor cells. Therefore, we repeated the cytotoxicity assay to determine if hyperthermia treatment was still beneficial despite a close-to maximum upregulation of MHC-I levels by IFN-y pre-treatment.

Overall, CTL-mediated killing was increased after IFN-γ pre-treatment with ca. 50 % reduction of surviving tumor cells at 37 °C (Figure 3) compared to no detectable killing without IFN-γ pre-treatment (Figure 1). The combination of hyperthermia and CTL co-culture resulted in additionally decreased numbers of surviving tumor cells compared

to hyperthermia treatment or CTL co-culture alone, suggesting additional mechanisms besides MHC-I upregulation mediating the effect. In line with previous results, hyperthermia treatment at 40.5 °C showed no increase in CTL-mediated killing.

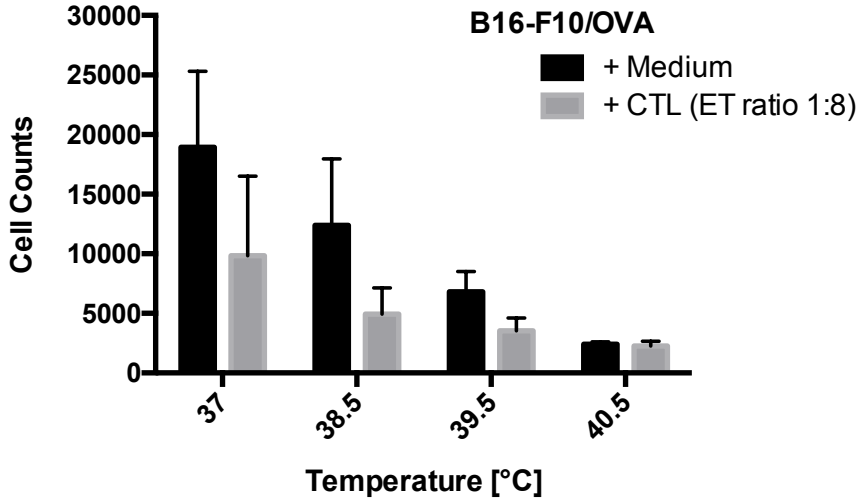


Figure 3: **Continuous hyperthermia treatment increases CTL-mediated killing of IFNy pre-treated B16-F10/OVA.** IFN-γ stimulated B16-F10/OVA melanoma cells were co-cultured with dsRed CTL in a 3D-collagen matrix gel for 48 hours under different temperatures (37, 38.5, 39.5, 40.5 °C) at an effector-target ratio of 1:8. A comparison of tumor cell counts (+Medium) without CTL and samples containing both, tumor cells and CTL (+CTL ET ratio 1:8) is shown. Graphs display data from three independent experiments with SD

To address if hyperthermia-treatment impacts CTL viability, the numbers of viable CTL were quantified by flow cytometry after 48 hours co-culture with tumor cells (Figure 4). 40.5 °C samples show, that CTL viability is significantly reduced when compared to tumor cells, which explains the lack of CTL killing efficiency at this temperature.

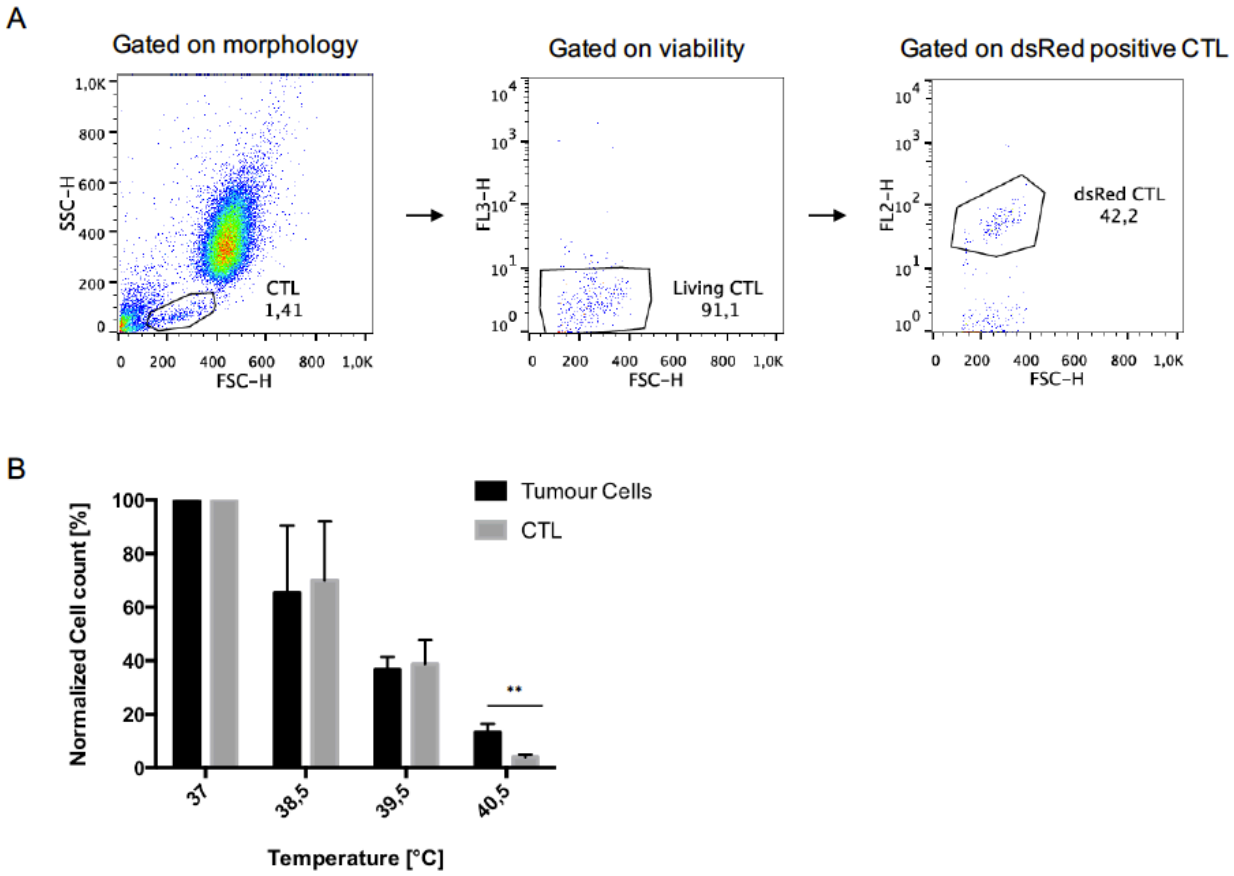


Figure 4: Hyperthermia decreases cell viability of B16-F10/OVA and dsRed CTL. (A) FACS readout gating procedure. CTL were gated based on morphology. Samples were stained with PI to exclude dead cells. Tumor cells were gated out to exclusively obtain CTL counts. Counts were obtained by measuring each sample for 60 seconds at medium speed. Tumor cells were gated according to figure 1. **(B)** Comparison of tumor cell and CTL viability after 48 hours hyperthermia treatment at 38.5, 39.5 and 40.5 °C. Cell counts were normalized to 37 °C samples. Graphs display data from three independent experiments with SD.

3.1.4 Hyperthermia prolongs CTL - tumor cell interactions and accelerates apoptosis induction

To address the cellular mechanism how hyperthermia enhanced CTL-mediated killing of tumor cells, time-lapse brightfield microscopy of CTL- tumor cell co-cultures was performed. B16-F10/OVA cells were pretreated with IFN-γ to monitor the effects of hyperthermia, independent of MHC-I upregulation. Live cell imaging was performed at 39.5 °C which showed the strongest effects in previous flow cytometry-based experiments. Similar to experiments conducted before, the culture of cells was performed in 3D-collagen based matrices to create an environment that mimics in vivo

tissue architecture. CTL- tumor cell interactions were monitored for up to 48 hours with a frame interval of 30 seconds and analysed manually for CTL – tumor cell interactions and apoptosis induction.

Analysis of contact durations between CTL and tumor cells showed 2 distinct populations of contacts: short-lived (< 3 hours) and long-lasting interactions (> 3 hours). The nature of contact duration was dependent on the tumor cell position in either clusters of tumor cells or isolated single tumor cells and both situations were separated in the analysis (Figure 5, A).

The contact duration of interactions < 3 hours was significantly increased in samples cultured at 39.5 °C compared to 37 °C (Figure 5, D). Long-lasting interactions of CTL with isolated tumor cells were not further prolonged by hyperthermia treatment.

Besides tumor cell – CTL contact durations, lag times to apoptosis of B16-F10/OVA cells were analysed. The time point of apoptosis was counted when first signs of cell membrane blebbing was observed. The lag time to apoptosis was defined as the time span between the first CTL - tumor cell contact and beginning of tumor cell apoptosis. Imaging revealed a trend of tumor cells undergoing apoptosis faster in hyperthermia treated samples than in 37 °C cultured samples (Figure 5, C).

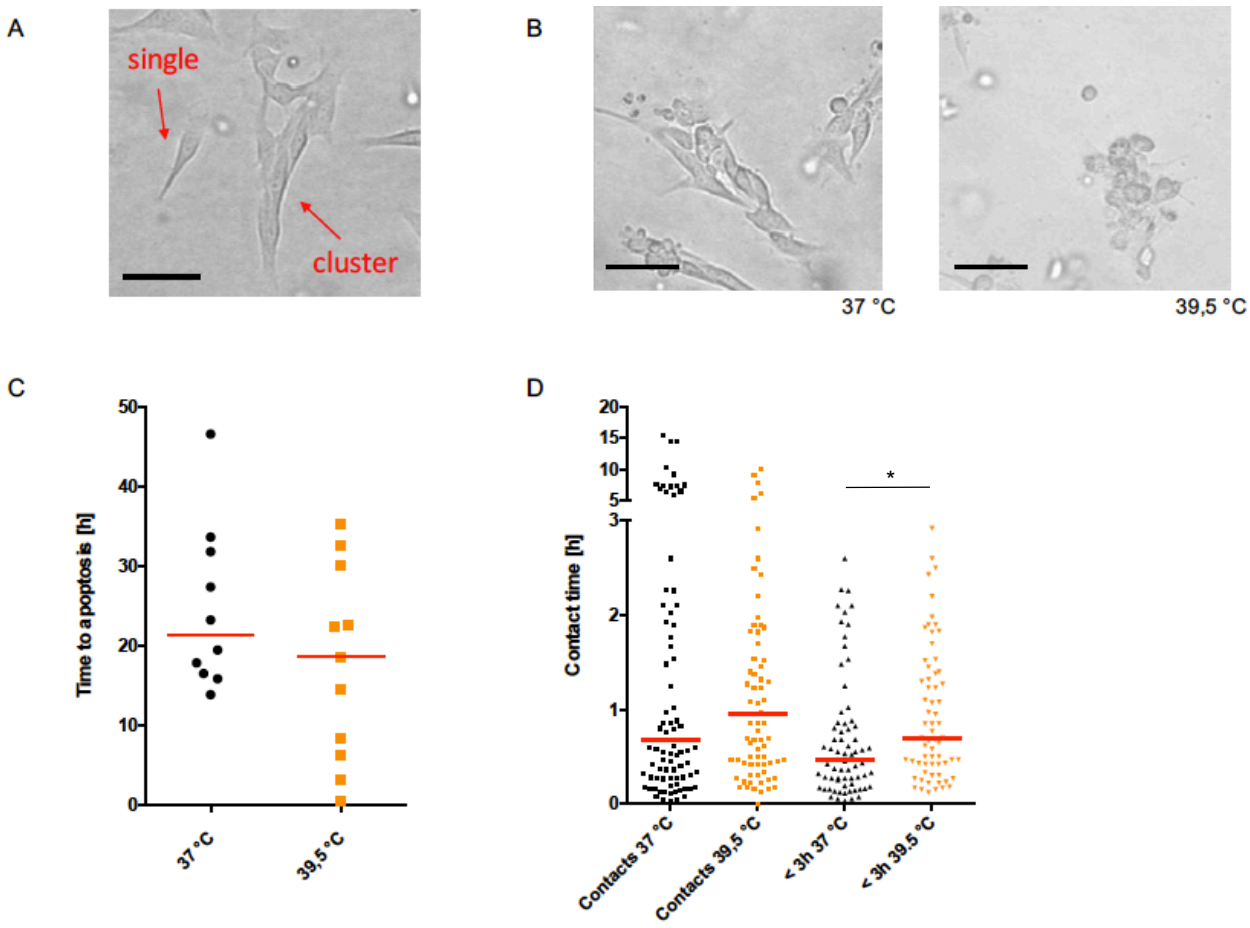


Figure 5: Hyperthermia-treatment prolongs CTL – tumor cell contact duration and shortens lag phase to apoptosis. B16-F10/OVA cells and CTL were co-cultured in 3D-collagen matrices at 37 and 39.5 °C and imaged for up to 48 hours. **(A)** Example image that depicts the difference between single vs clustered tumor cells. **(B)** Endpoint image of 37 and 39.5 °C cultured B16-F10/OVA with CTL after 48 hours. **(C)** B16-F10/OVA lag times to apoptosis after first contact with CTL. **(D)** Contact times of CTL with B16-F10/OVA divided up in all contacts and contacts shorter than 3 hours. Image depicts differences between single vs clustered tumor cells. Graphs show data from four independent experiments. Scalebar: 50 µm

3.1.5 Impaired recovery of CTL-mediated damage

Further investigation aimed at direct visualisation of CTL attacks to obtain further knowledge of CTL killing dynamics under hyperthermia. For this purpose, B16-F10/OVA cells expressing GFP tagged with a nuclear localization signal (NLS) were used in order to directly visualize Granzyme B mediated intracellular damage. Granzyme B is known to cleave multiple structural proteins within cells, including e.g. Lamin B and importins which maintain structural integrity of the nuclear envelope or the efficient transport of NLS-GFP into the nucleus, respectively. Granzyme B entry

into the cell upon CTL cytotoxic vesicle release will thus result in structural cell damage and the release of NLS-GFP into the cytoplasm. This allowed further analysis of possible altered response of tumor cells towards CTL attacks under hyperthermia.

Similar to experiments performed previously, B16-F10/OVA NLS-GFP cells were co-cultured with dsRed CTL in a 3D-collagen based matrix under 37 and 39.5 °C.

Figure 5 B shows the classic course of a CTL mediated nuclear envelope break where NLS-GFP leaks into the cytoplasm (Figure 6 B,b) and gets transported back into the nucleus upon successful repair of the envelope (Figure 6 B,c).

Analysis of movies revealed, that there was no significant difference in the occurrence of NLS-GFP leakage events in 37 and 39.5 °C. (Figure 7 A). However, cells cultured under 39.5 °C showed impaired recovery form NLS-GFP leakage events (Figure 7 B). Movies also allowed measurement of timespans needed for successful repair of the nuclear envelope after CTL mediated breaks. We assumed, that tumor cells facing hyperthermia weren't able to recover as quickly as cells cultured under normal conditions. Interestingly, comparison of recovery times didn't reveal any differences. (Figure 7 C) thereby indicating that susceptibility towards nuclear envelope breaks seems more relevant than the recovery of breaks in hyperthermia samples.

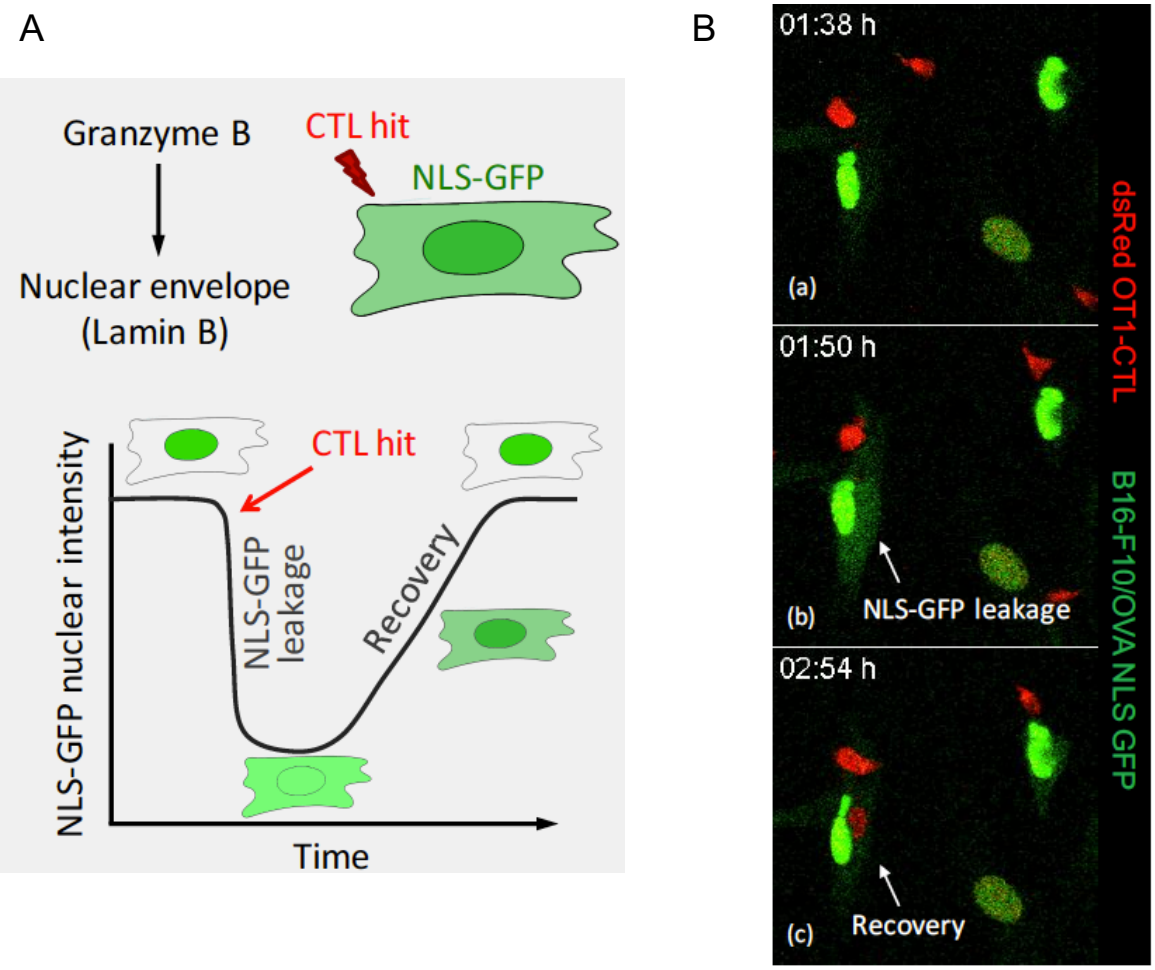


Figure 6: Working principle of the NLS-GFP sensor. (A) Strucutral damage of B16-F10/OVA nuclear envelope mediated by Granzyme B leads to a drop of NLS-GFP signal in the nucleus. Upon successful repair of nuclear envelope damage, NLS-GFP is slowly transported back to the nucleus, leading again to a nuclear signal increase. **(B)** Course of a NLS-GFP leakage event and following recovery within approximately 1 hour.

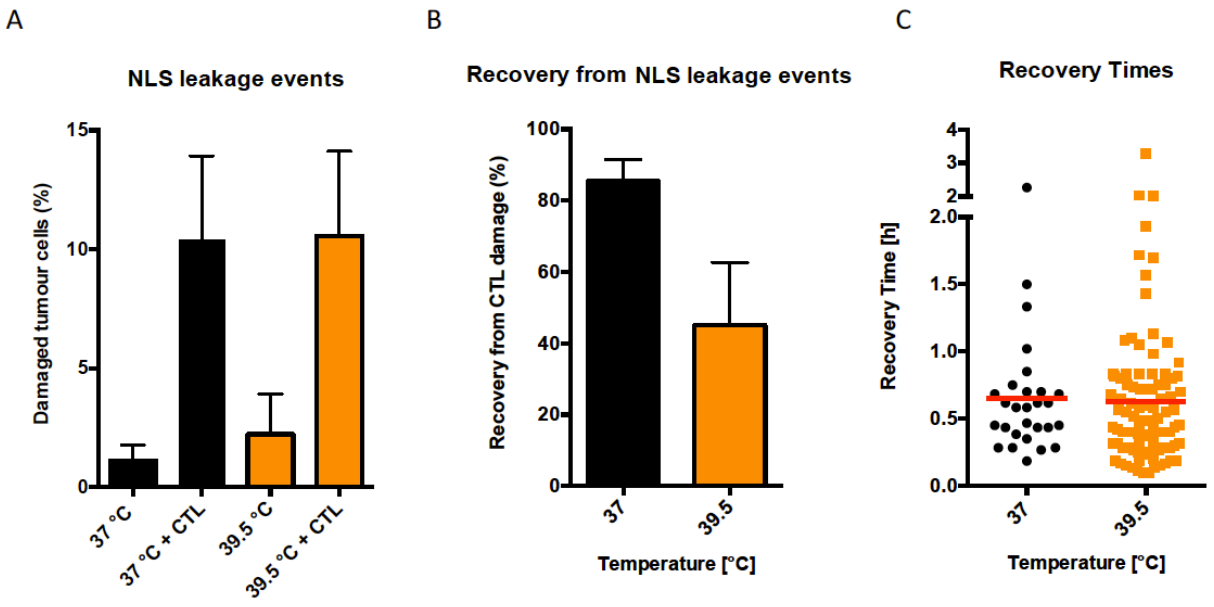


Figure 7: Hyperthermia impairs B16-F10/ OVA NLS-GFP recovery from nuclear leakage events. B16-F10/OVA NLS-GFP cells were co-cultured with dsRed CTL in a 3D-collagen based matrix and imaged overnight at either 37 or 39.5 °C. **(A)** Occurrence of NLS-GFP leakage events in B16-F10/OVA cells under 37 and 39.5 °C culture. **(B)** Amount of surviving tumor cells preceded by one or more NLS leakage events in percent of all tumor cells facing NLS leakage events. **(C)** Recovery time of nuclear signal of 37 and 39.5 °C cultured B16-F10/OVA NLS-GFP cells after NLS leakage event.

3.1.6 Hyperthermia decreases cytokine secretion of CTL

Besides the prevalent Perforin - Granzyme B pathway, CTL also bear cytotoxic functions mediated by secreted cytokines like IFN-γ or TNFα. We speculated, whether hyperthermia might lead to alterations in CTL cytokine secretion which could result in enhanced killing efficiency.

To address this hypothesis CTL were co-cultured with B16-F10/OVA cells under hyperthermia ranging from 37 to 40.5 °C for 48 hours. IFN-γ and TNFα secretion of CTL was measured from supernatants using ELISA (Figure 8).

Surprisingly preliminary data revealed that hyperthermia lead to severely decreased cytokine secretion with rising temperature, almost reaching detection limits of the assay in 40.5 °C treated samples.

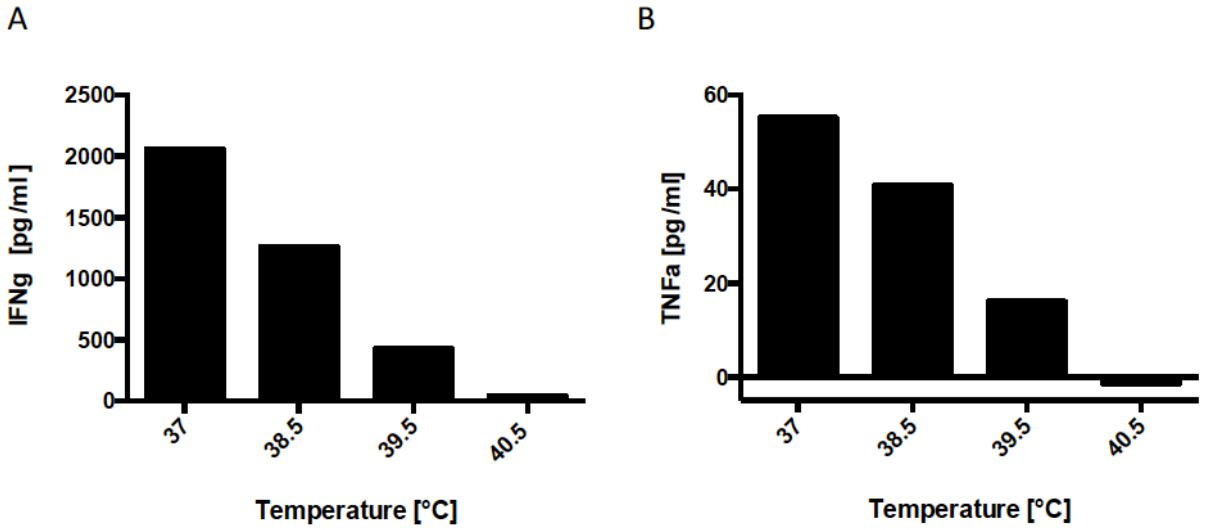


Figure 8: IFN-γ and TNFα secretion of CTL after 48 hour hyperthermia treatment. CTL were co-cultured with B16-F10/OVA cells for 48 hours under different temperatures (37; 38.5 ; 39.5 and 40.5 °C). Secreted IFN-γ (A) and TNFα (B) from supernatants were measured using ELISA. Graphs show data from one preliminary experiment.

3.2 Fractionated hyperthermia treatment schemes

3.2.1 Increased CTL-mediated killing in fractionated hyperthermia treatment schemes

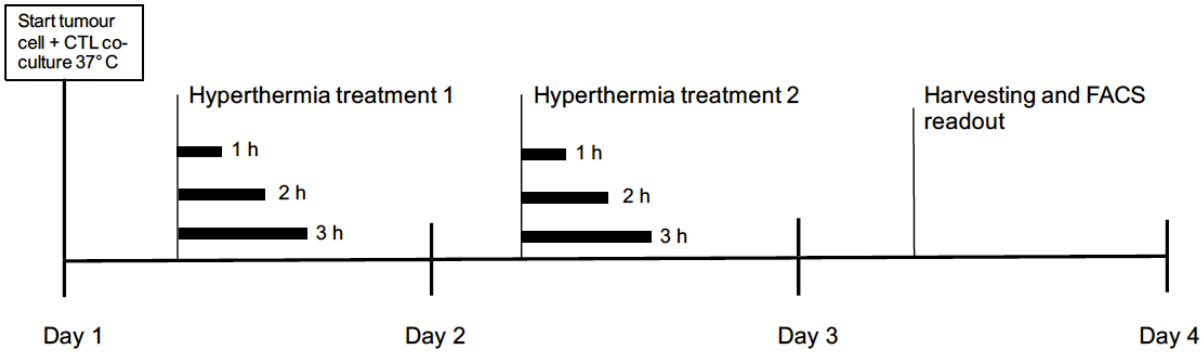
In clinical applications, whole-body hyperthermia can be applied for only a few hours per day to keep the treatment bearable for the patient. It is unclear how long each individual application must last and at which frequency applications must be repeated to observe clinical effects. Therefore, the following experiments addressed the application of repetitive short hyperthermia treatments to test clinical relevant settings.

3D-collagen based cytotoxicity assays were performed as described previously with application of hyperthermia (37, 38.5, 39.5 and 40.5 °C) ranging from 1 to 3 hours and applied on two consecutive days (Figure 9).

Fractionated treatment showed a trend of increased CTL killing efficiency already after two 1 hour treatments at 38.5 °C. Treatments at 38.5 and 39.5 °C showed similar increase in killing efficiency at 1 h, 2 h and 3 hours durations. Treatment with 40.5 °C for 1 h and 2 h showed similarly increased killing efficiency, however, 3 hours treatments lead to greatly reduced killing. Thus, fractionated treatment at 38.5 and 39.5

°C is sufficient to enhance CTL-mediated killing of B16-F10/OVA cells while 40.5 °C appears to impair CTL killing potential when incubation times of 2 h are exceeded.

A



B

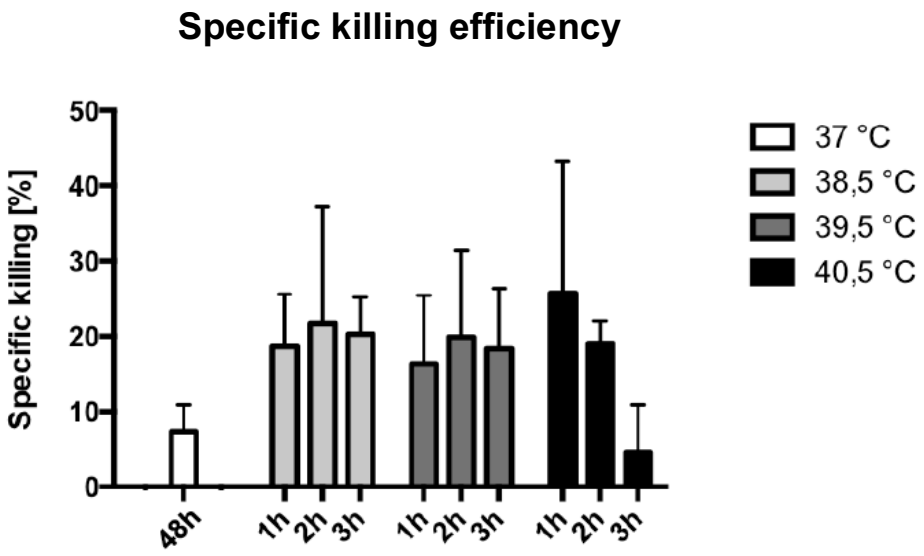


Figure 9: Fractionated hyperthermia treatment increases the killing potential of CTL. (A) B16-F10/OVA were co-cultured with CTL in a 3D-collagen matrix at an ET ratio of 1:8 for 48 hours. Within this period cells were treated twice with hyperthermia (38.5 ;39.5 and 40.5 °C) lasting 1-3 hours each. **(B)** Graph shows specific-CTL killing efficiency obtained by normalizing samples to hyperthermia treatment alone, using the following formula: $(cell\ counts\ (Medium) - cell\ counts\ (CTL\ ET\ ratio\ 1{:}8))/(cell\ counts\ (Medium))$ Graphs display data from three independent experiments with SD.

To address if direct cytotoxic effects on CTL are responsible for the lack of increased killing in the 40.5 °C samples, CTL viability was analysed at the endpoint of the assay. Tumor cell and CTL viability were compared side-by-side because a differential response to hyperthermia would alter the ET ratio and thereby affect CTL killing efficiency.

For this purpose, B16-F10/OVA and CTL counts at the assay endpoint were normalized to the according 37 °C counts and compared (Figure 10). 38.5 and 39.5 °C treatments ranging from 1 to 3 hours showed similar inhibiting effects on both cell types, whereas tumor cells seemed to be more resistant to 40.5 °C treatments of 1 to 2 hours. Thus, the enhanced killing efficiency in fractionated treatments of 38.5 and 39.5 °C are due to enhanced CTL function while the lack of killing efficiency of 3 hours treatments at 40.5 °C can be explained by direct cytotoxic effects on the CTL.

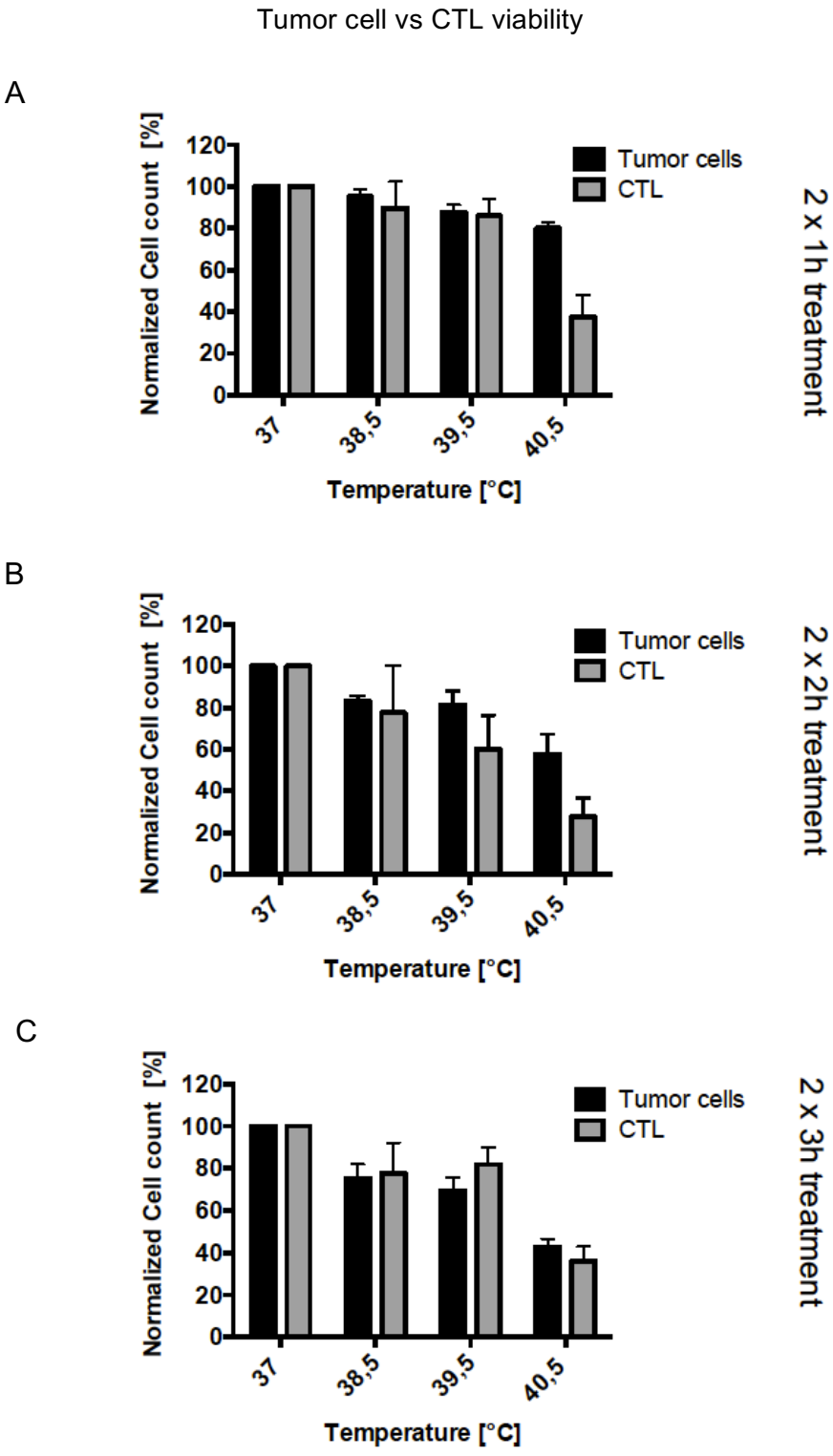


Figure 10: Comparison of tumor cell and CTL viability after fractionated hyperthermia treatment. B16-F10/OVA and CTL were cultured in a 3D-collagen matrix for 48 hours. Within this period cells were treated twice with hyperthermia (38.5 ;39.5 and 40.5 °C) lasting 1-3 hours each. Graphs show cell counts normalized to samples grown at 37 °C. Graphs show data from 3 individual experiments with SEM.

3.2.2 Minor MHC-I upregulation by fractionated hyperthermia treatment

Prior experiments showed that MHC-1 levels of B16-F10/OVA cells are temperature sensitive (Figure 2) and may contribute to enhanced killing potential of CTL especially under 38.5 and 39.5 °C. Follow up experiments were conducted in order to check whether fractionated hyperthermia treatment was capable of altering MHC-1 expression of tumor cells. For this purpose, B16-F10/OVA cells were cultured a total period of 96 hours either under continuous hyperthermia or were applied 4 times with heat treatments lasting 1 or 3 hours each. MHC-1 expression was then analysed using flow cytometry (Figure 11).

Treatment schemes of 1 h for 4 consecutive days had no effect on MHC-1 expression levels of tumor cells. Minor increase in expression was detected in samples treated for 3 hours for 4 consecutive days, but overall levels remained very low compared to continuous hyperthermia exposure (< 20-fold).

These results suggest that enhanced CTL killing efficiency during fractionated hyperthermia is supported by mechanisms other than MHC-1 upregulation.

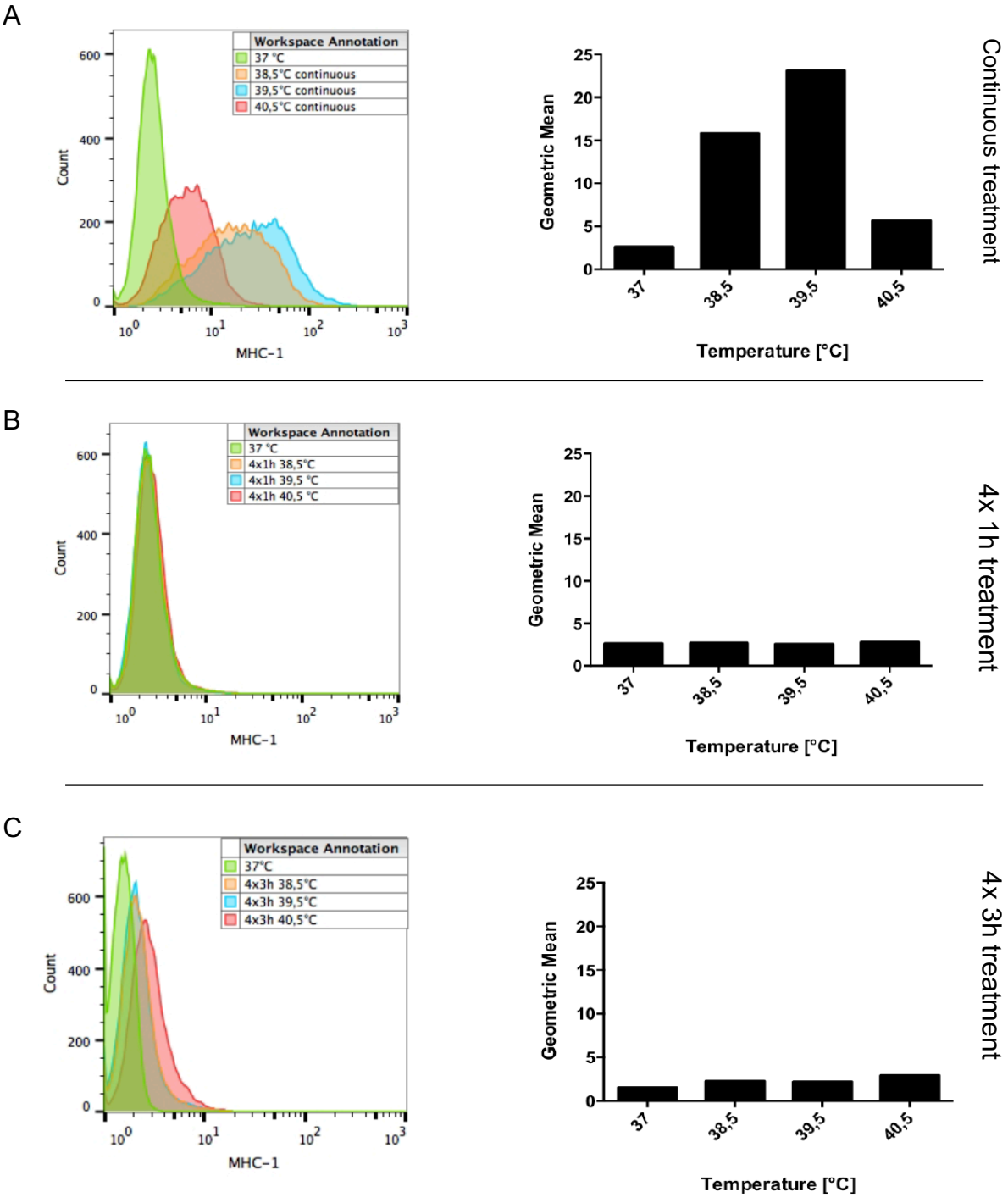


Figure 11: Changes in MHC-1 expression of B16-F10/OVA cells after application of different hyperthermia treatment schemes. B16-F10/OVA cells were cultured 96 hours in total either under continuous hyperthermia (A) or were applied with hyperthermia 4 times lasting either 1 hour (B) or 3 hours (C) each. Graphs show flow cytometry histograms and depiction of geometric mean values of MHC-1 expression.

3.2.3 Fractionated hyperthermia reduces proliferation of B16-F10/OVA

To address the recovery potential of tumor cells from fractionated heat-induced stress, B16-F10/OVA GFP cells were treated once with 1 hour 38.5, 39.5 or 40.5 °C and imaged shortly before and after application of hyperthermia (Figure 12 A)

With increasing temperature mitotic events dropped significantly directly after treatment, revealing a fast response towards elevated temperature. 40.5°C showed highest impact on the cells by almost completely abolishing mitotic events to a residual mitotic rate of 9 % (Figure 12 B).

Imaging of mitotic rates at 2 and 4 hours after the heat treatment showed quick recovery times with cells recovering to levels present before hyperthermia treatment.

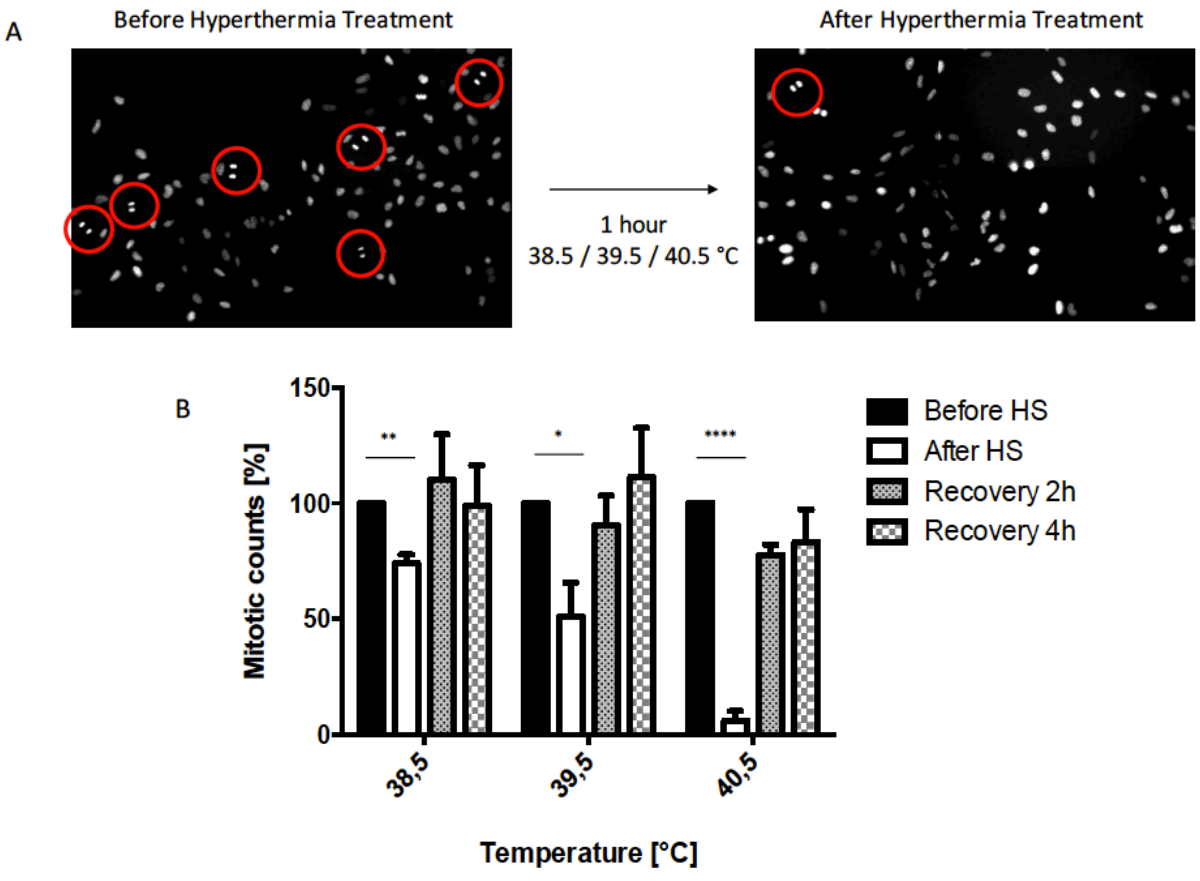


Figure 12: B16-F10/OVA GFP analysis of mitotic events before and after hyperthermia treatment. Cells were cultured under 37°C and were then treated with hyperthermia (38,5; 39,5 and 40,5 °C) for exactly 1 hour and imaged shortly before and after the treatment. **(A)** Example image of hyperthermia treatment effect on mitotic events. Red circles outline how mitotic events were counted. **(B)** Depiction of mitotic events normalized to events occurring at 37°C. Data show values from 3 technical replicates. Statistical test: Students t-test, two tailed.

To address if the temporary inhibition of mitosis has long-term effects or which treatment durations are required to impose long-lasting consequences, B16-F10/OVA were treated with different hyperthermia schemes ranging from fractionated heat treatments (1-3 hours) repeated on consecutive days up to continuous culture for 48 hours. Endpoint analysis of cell numbers by flow cytometry showed, that tumor cell proliferation was already affected by treatments of only 1 hour at 40.5 °C (Figure 13). 40.5 °C had the overall strongest effect on tumor cell proliferation and already showed a reduction of cell counts of about 20 % when cells were treated twice for 1 hour in comparison to cells grown at 37 °C. With prolonged treatment duration this effect got more predominant almost reducing cell counts to 40 % at treatment durations of 3 hours.

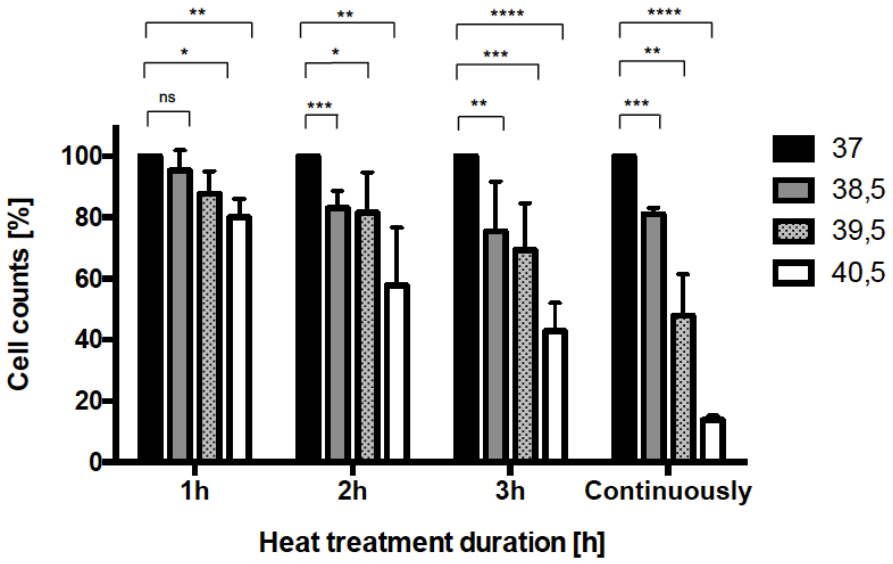


Figure 13: Decrease of proliferation of B16-F10/OVA cells after fractionated hyperthermia treatment. B16-F10/OVA cells were cultured 48 hours in total either under continuous hyperthermia or were treated twice with hyperthermia (38.5 ; 39.5 ;40.5 °C) lasting 1-3 hours each. Cell counts were observed via FACS and normalized to 37 °C samples. Data show values from three individual experiments with SD. Statistical test: Students t-test, two-tailed.

3.3 Upregulation of CTL activation markers by hyperthermia application during *in vitro* activation

During whole-body hyperthermia treatment, CTL are exposed to increased temperatures within the tumor as well as during their activation in the lymph nodes. To investigate if hyperthermia application during CTL activation results in CTL with better killing ability, CTL were analysed for the expression of activation markers (Figure 14).

CD25 is the IL-2 receptor alpha chain and considered as an activation marker of T-cells which is expressed upon antigen binding of the TCR. Binding and signalling of IL-2 has severe impact on CTL effector function and also enhances clonal expansion of CTL[73]. Results show, that CD25 was significantly upregulated in 39.5°C cultured samples.

Further, activated CTL show a CD62-L^{low}/CD44^{high} phenotype. Especially CD62-L, also known as L-Selectin plays a key role in lymphocyte homing into secondary lymphoid tissues and is predominantly upregulated in naïve CTL[74,75]. Stainings of CTL with anti-CD62-L revealed a significant downregulation in hyperthermia treated samples accompanied by an upregulation of CD44, indicating a strong activation phenotype.

Intracellular levels of Granzyme B could be detected, but preliminary data didn't reveal any differences between samples. However, since Granzyme B exerts its function upon secretion it remains unclear if possible enhanced secretion of Granzyme B could occur under the influence of hyperthermia.

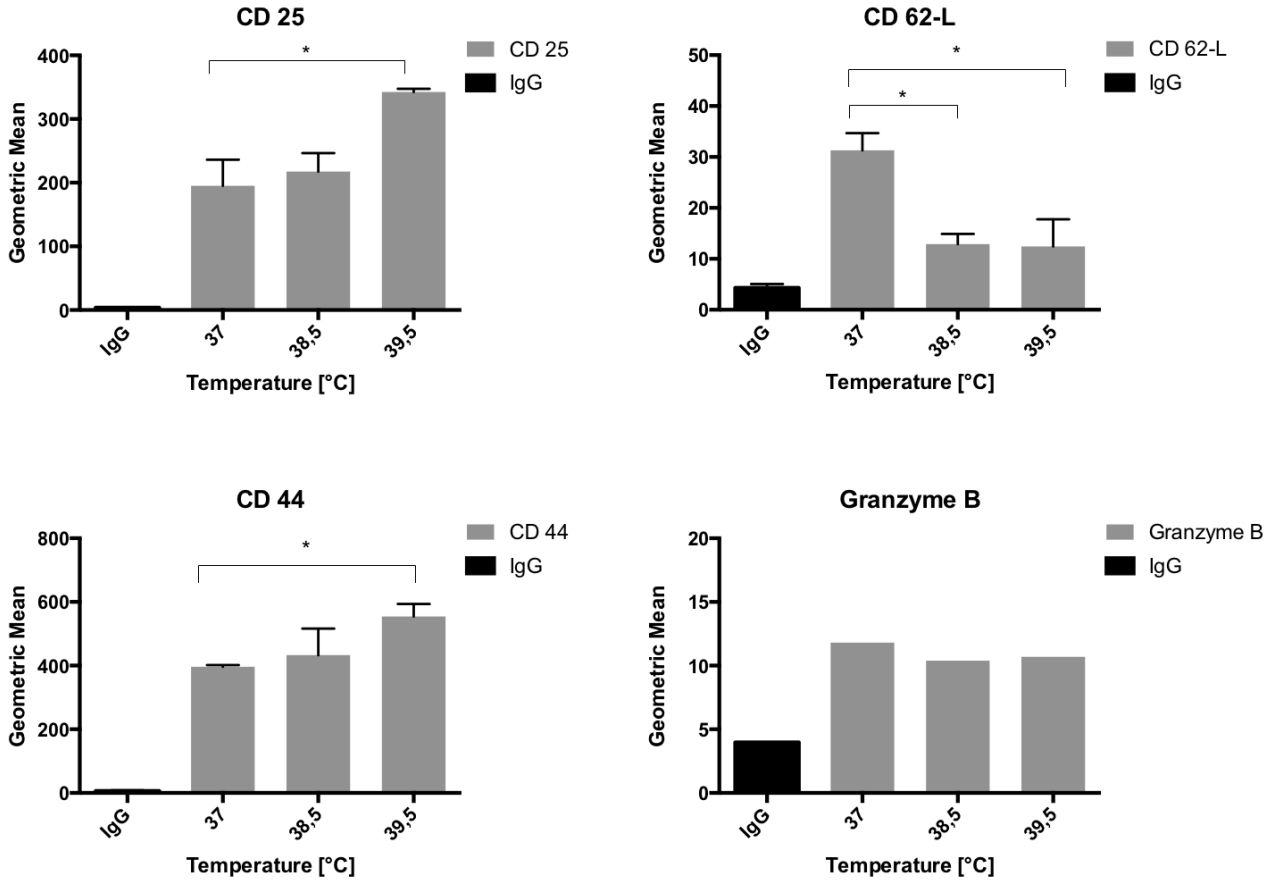


Figure 14: Activation marker expression of OT1 CTL after hyperthermia treatment during activation. OT1 splenocytes were isolated and cultured under hyperthermia (38.5, 39.5 °C) for 3 days in the presence of SIINFEKL followed by additional 2 days of culture with IL-2. CD25, CD62-L, CD44 and Granzyme-B expression levels were analysed by flow cytometry. Graphs show geometric mean values. (CD25, CD 62-L, CD44, n=2) , Statistical test: Students t-test, two tailed.

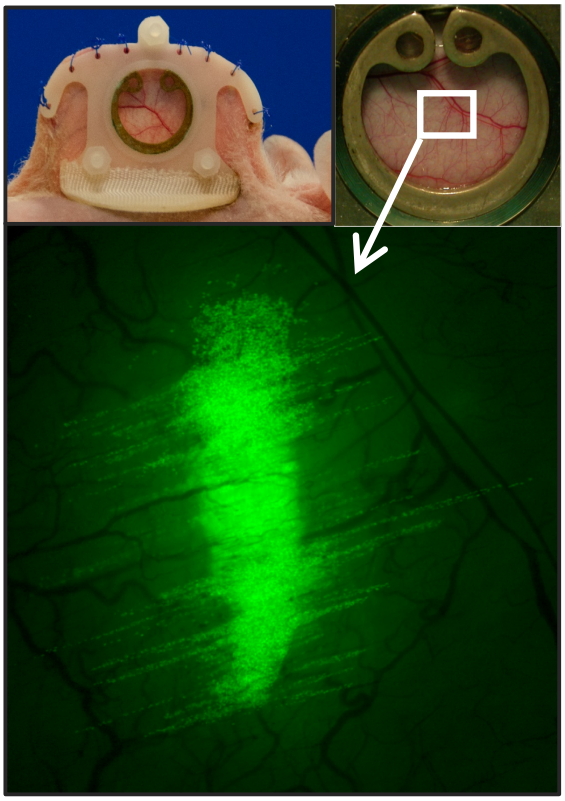
3.4 Intravital imaging of whole-body fever-range hyperthermia reveals reduction of proliferation of in vivo grown B16-F10/OVA NLS-GFP cells

Since previous experiments confirmed a severe effect of hyperthermia on tumor cells grown *in vitro* the next step was to validate whether tumor cell proliferation *in vivo* was affected in a similar way.

For this purpose mice were mounted with a dorsal skin-fold chamber for intravital imaging and were injected intradermally with B16-F10/OVA NLS-GFP cells. Following 6-8 days of tumor growth the mice were treated with whole-body hyperthermia (39.5 °C) for 1 hour and imaged directly before and after the treatment. For applying hyperthermia treatment a special setup was established (Figure 15) which enabled raising the core temperature of mice to 39.5 °C.

Validating the results obtained *in vitro*, hyperthermia showed a direct inhibition of mitotic events in B16-F10/OVA NLS-GFP tumors (Figure 16). Central and edge regions of the tumor were analysed, since distribution of dividing cells varies within living tissue. Drops of mitotic events directly after hyperthermia treatment could be observed in both regions of the tumor. Whether this reduction of proliferation can be sustained over a longer period of time remains to be investigated.

A



B

Figure 15: Intravital imaging of B16-F10/OVA NLS GFP tumors in mice and 39.5 °C whole body hyperthermia treatment set up. (A) Mice were surgically mounted with dorsal skinfold chambers. B16-F10/OVA NLS-GFP cells were injected underneath the imaging window and were grown approximately for 1.5 weeks. Intravital imaging of tumors was performed directly before and after whole body hyperthermia treatment to monitor direct effects on tumor cell proliferation. **(B)** Whole body hyperthermia application set up. Mice were anaesthetized with isofluroan and covered partly with aluminium foil to prevent heat loss and were placed in a pre-heated mini-thermacage (Datesand) to administer whole body hyperthermia. Mice were heated exactly 1h on 39.5°C under continuous monitoring of chamber and body temperature.

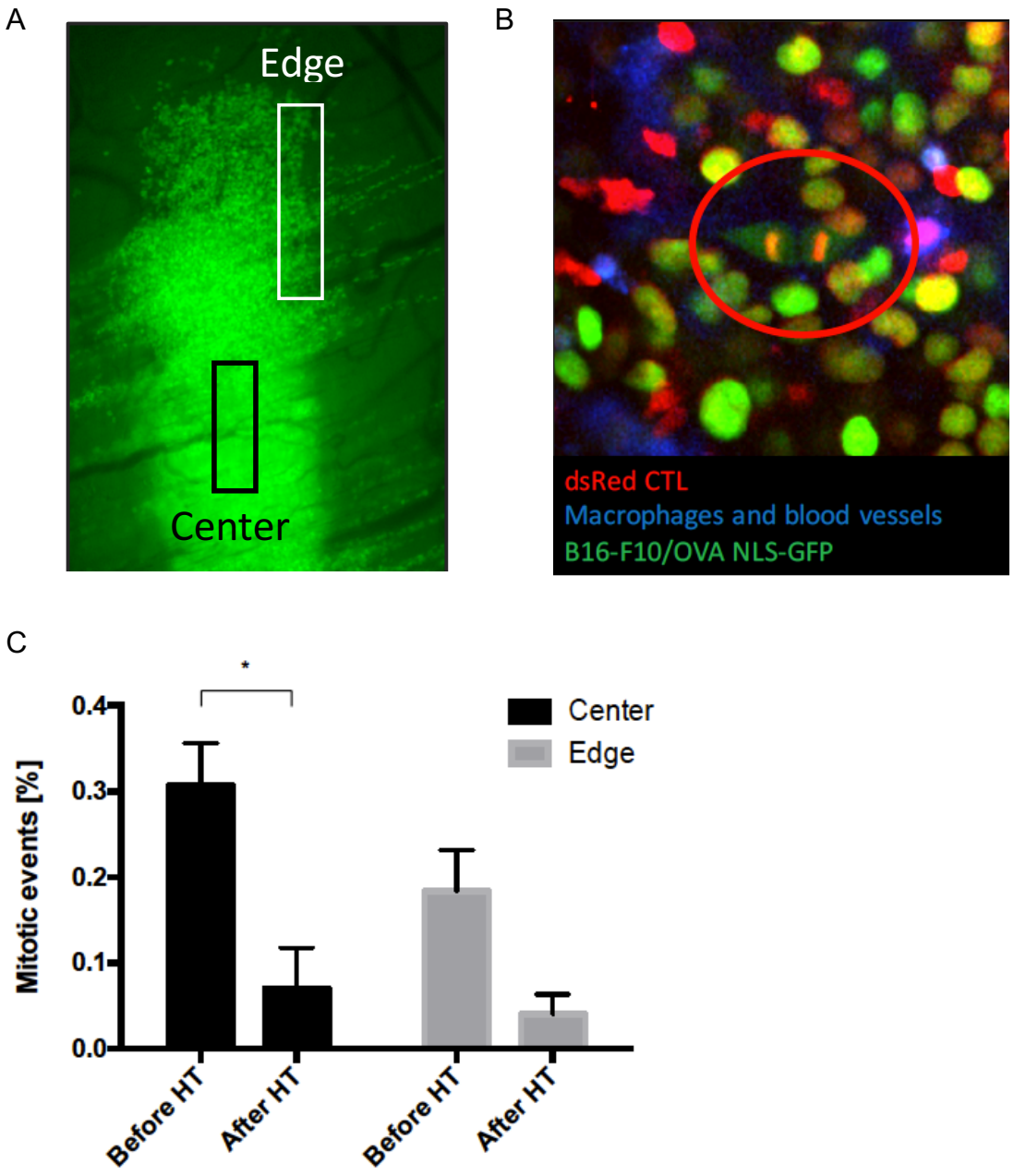


Figure 16: 39.5 °C whole body hyperthermia treatment reduces B16-F10/OVA NLS GFP tumor proliferation in mice. (A) Central and edge regions of the tumor were analysed for mitotic events. **(B)** Tumor cell shapes indicated by the red circle were counted as mitotic events. Colour scale: Green: NLS-GFP (Tumor cells); Blue: Alexa Fluor 750- Dextran (Blood vessels and macrophages), Red: dsRed (CTL). **(C)** Statistical depiction of mitotic events observed out of central and edge regions of the tumor. Mitotic events before and after hyperthermia treatment were analysed and are shown as percentage of total cell counts. Graphs represent data from 2 individual experiments with SD. Statistical test: Students t-test, two tailed.

4. Discussion

4.1 Fever-range hyperthermia in combination with CTL therapy decreases melanoma cell survival

The aim of this master thesis was to evaluate the potential of fever-range hyperthermia to enhance anti-cancer immune effector function and to get further insight into the underlying mechanisms. As melanoma model, we used the well-characterized murine B16-F10/OVA cell line combined with OVA specific CD8+ CTL isolated from transgenic OT1 mice. By using the OVA model, antigen expression was homogeneous in individual cells and variations in killing efficiency due to antigen specificity were absent, which allowed to determine the effects of hyperthermia treatment in individual CTL – tumor cell contacts. In the tissue, CTL are required to migrate and search for antigen-presenting tumor cells. This searching phase may be influenced by temperature and therefore we used a 3D collagen-based cytotoxicity assay which mimics the tissue architecture and requires CTL migration in order to interact and kill tumor cells.

First, we addressed which fever-range temperatures combined with CTL therapy result in reduced tumor cell survival and hence indicated a positive impact of hyperthermia. Melanoma cells and CTL were co-cultured for 48 hours under continuous hyperthermia temperatures and an increased killing efficiency was detected in samples incubated at 38.5 and 39.5 °C while 40.5 °C showed no effect. This lack of killing might be explained by cytotoxic effects on CTL by 40.5 °C treatment. In contrast, 38.5 °C and 39.5 °C exerted immune stimulatory functions by raising CTL killing efficiency. Since optimal temperature ranges for immunostimulation are widely unclear, 38.5 and 39.5 °C give first hints of treatment temperatures that bear therapeutic potential.

Fever-range hyperthermia has been shown to upregulate MHC-I expression levels on the cell surface. MHC-I density strongly correlates with CTL recognition and killing efficiency and thus, may explain the observed increased killing rates. We detected increased MHC-I levels in 38.5 and 39.5 °C samples at the assay endpoint which confirmed, that hyperthermia increases the immunogenicity of melanoma cells and might explain the increased killing rates.

To test if increased MHC-I levels are sufficient to explain the enhanced killing efficiency, tumor cells were stimulated with IFN-γ which increased MHC-I levels on the cell surface to a maximum in all samples, with and without hyperthermia treatment. As expected, IFNy pretreatment decreased the survival of tumor cells in 37 °C samples, however, despite equal MHC-I levels, the combination of CTL and fever-range temperature still further decreased the survival of tumor cells as compared to each treatment alone.

However, interpretation of endpoint measurements of tumor cell survival are difficult, due to the lack of dynamic data on cellular interactions and the kinetics of growth and apoptosis. Maintaining constant ET ratios during the duration of the assay is essential for comparing datasets, and hyperthermia treatment showed a severe impact on the proliferation of tumor cells and the viability of CTL. However we showed that hyperthermia affects both cell types to the same extent so that similar ET ratios were observed in samples treated with different temperatures. However, endpoint measurements provide no insight into cellular dynamics. CTL could be severely weakened by fever-range temperatures, but still achieve similar killing efficiencies compared to 37 °C if the susceptibility of tumor cells is enhanced. Thus, endpoint measurements do not allow to distinguish if the observed effects rely on weakening of tumor cells, enhanced effector function of CTL or a combination out of both.

4.2 Fever-range hyperthermia prolongs CTL–tumor cell interactions

To get further insight into the killing dynamics of CTL in combination with hyperthermia treatment, brightfield microscopy was used to monitor CTL mediated killing of B16-F10/OVA cells cultured in 3D collagen matrices at 39.5 °C. The time-lapse recordings revealed that CTL were still highly motile and showed prolonged contact durations with tumor cells. The tumor cells were pretreated with IFNy to achieve equal MHC-I levels in 37 and 39.5 °C samples. The prolonged contact duration is thus not due to increased MHC-I levels but could result from enhanced expression of adhesion molecules on either tumor cells (ICAM1, LFA3) or CTL (LFA-1, CD2, CD8) which stabilize the

immunological synapse.[77] The expression of these surface molecules could be tested in follow up experiments to explain the observed prolonged contact durations.

Prolonged interaction times between CTL and tumor cells hence could indicate more stable and more potent immunological synapses which deliver larger amounts of cytotoxic granules to the target cell. Alternatively, longer contacts may allow CTL to deliver repeated cytotoxic hits which, however, are of similar strength compared to hits delivered at 37 °C. The increased temperatures may further enhance the function of cytotoxic enzymes and thus increase the cytotoxic effects of individual hits in the target cell. Lastly, the target cells may be impaired in their capacity to repair CTL-induced damage and thus be more susceptible to CTL attacks.

Thus, to understand how prolonged interaction times increase the success of CTL-mediated apoptosis induction, we performed time-lapse confocal imaging using genetically encoded sensors for sublethal CTL-induced damage.

4.3 Hyperthermia impairs CTL-mediated sublethal damage repair

To visualize CTL-mediated sublethal damage in the target cell, we used B16-F10/OVA cells which expressed GFP tagged with a nuclear localization signal (NLS-GFP). NLS-GFP localizes to the nucleus in healthy cells and allows to visualize nuclear envelope breaks mediated by CTL secreted Granzyme B. NLS-GFP leakage events were specific for CTL-induced perforin-mediated damage as indicated by their absence in samples treated with hyperthermia alone or tumor cells co-cultured with perforin k.o. OT1 CTL (Data from previous projects). Sublethal damage to the nuclear membrane may be predominantly observed in resistant cells in which apoptosis is delayed long enough to observe sublethal damage. The fraction of resistant cells may be significantly reduced under hyperthermia, which is supported by an increase of apoptosis in these experiments from 10 % at 37 °C to 90 % at 39.5 °C. Within the population of cells that showed NLS-GFP leakage events, the average time needed by the cell to repair the damage appeared to be unaffected by the increased temperature. However, the average time needed for repair was very variable and larger data sets are needed to confirm the results.

Interestingly, the recovery rate of cells experiencing NLS-GFP leakage was significantly reduced in hyperthermia treated samples. While 75 % of all cells that showed NLS-GFP leakage, were able to recover from one or more NLS-GFP leakage events at 37 °C, only 40 % were able to recover at 39.5 °C. This further supports that hyperthermia reduces the resistance of the B16F10 melanoma towards CTL-mediated damage. In summary, the data indicate that the frequency of hits delivered by the individual CTL is not increased but the tumor cells capacity to recover from CTL-induced damage is impaired. However, the dataset needs to be independently validated in repeated studies to reach statistically meaningful results.

As this assay only visualizes GranzymeB-mediated nuclear envelope damage, it still remains unclear which other downstream targets of GranzymeB might result in enhanced tumor cell death due to hyperthermia treatment. Therefore, it is essential to further investigate other markers for sublethal CTL-induced damage. Usage of an intracellular sensor for visualizing DNA damage could give insight into the amount of damage acquired in tumor cells, accumulation of DNA damage over time and possible effects on DNA repair. Regarding that hyperthermia was already connected to inhibition of DNA-repair via blockage of DNA-Polβ it seems likely that a possible synergy of DNA-damaging proteins activated by GranzymeB and an induced DNA repair deficiency caused by hyperthermia result in premature or even increased tumor cell death.

It would further be interesting to visualize CTL-mediated cytotoxic hits directly to assess if CTL are more active or if the tumor cells are less resistant. This could be achieved by using an intracellular calcium sensor expressed in the tumor cells which enables visualization of perforin-mediated membrane pore formation. This would allow to measure frequency, intensity and recovery of CTL attacks based on the intensity of intracellular Ca^{2+} signals and to link CTL activity to temperature.

4.4 Hyperthermia decreases CTL cytokine secretion

Besides Perforin and GranzymeB, CTL also possess the ability to trigger target cell death via secretion of various cytokines.[66] Using ELISA we determined secretion of IFNγ and TNFα under fever range hyperthermia and simultaneous co-culture with target tumor cells. Surprisingly CTL secreted less cytokines with increasing temperature which for IFNγ was contradicting already published data. Mace et. al showed that effector CTL secreted higher amounts of IFNγ after they had been treated with fever-range hyperthermia for a period of 6 hours.[79] However, those experiments used shorter hyperthermia treatment periods and didn't include co-culture with target cells - both factors which are a source of variation. Data observed from our CTL killing assays revealed that hyperthermia decreased viability of CTL after a longer incubation period. This impact of hyperthermia on CTL could therefore be a reason for the decreased cytokine secretion.

4.5 Clinically-relevant treatment schemes

Considering a clinical perspective, treating cancer patients with hyperthermia for 48 hours is unrealistic due to side effects on circulation and dehydration during extensive heat exposure. [78] Therefore, we tested the effect of shorter, repeated hyperthermia treatments ranging from 1 - 3 hours per day for a total period of 48 hours.

Fractionated hyperthermia treatments strongly enhanced CTL killing efficiency, even though values observed were overall lower than in samples treated continuously for 48 hours. Nevertheless, considering the drastically reduced treatment duration it is remarkable that already hyperthermia treatments with 38.5 °C resulted in a 2-fold increase in killing efficiency compared to 37 °C samples. Further, 40.5 °C demonstrated the relatively narrow range of effective treatment conditions. While 1 and 2 hours treatment obviously were promoting CTL killing efficiency, 3 hours treatments resulted in a drastic decrease. FACS data confirmed that hyperthermia treatments didn't affect CTL viability.

Hence, a possible malfunction of CTL killing related enzymes, failure of cytotoxic vesicle release or destabilization of CTL- tumor cell contacts at 3 hours exposure to 40.5 °C could explain the decrease of CTL killing efficiency.

For these experiments, B16-F10/OVA cells were not pretreated with IFNy and therefore we quantified MHC-1 levels on the tumor cell surface at the assay endpoint. Hyperthermia treatments across all temperatures didn't change expression in 1 hour treated samples, whereas the 3 hour treatment resulted in a mild increase which however was insignificant when compared to cells incubated for 48 hours. These results confirm that MHC-1 upregulation might play a role in killing assays using 48 hour hyperthermia treatments, but is surely not the fundamental mechanism when applying multiple shorter hyperthermia treatments.

The data on fractionated hyperthermia treatment requires further validation in order to draw statistically relevant conclusions. Correct heating and temperature control for the required time periods is essential for conducting these experiments. Especially working with older incubators and simultaneous running of multiple experiments in one machine lead to significant fluctuations in temperature and reduced the number of successful experiments. Thus, the experiments should be repeated and validated to increase the independent experiments per dataset.

Especially in combination with immunotherapy effective temperature ranges and treatment durations in the clinic are hardly known. Our experiments confirmed that 38.5 °C and 39.5 °C promoted CTL killing efficiency and gave first evidence of functional temperature ranges. Since lower temperatures are better suited for cancer patient treatment due to fewer side effects, further experiments could target the effect of those temperatures combined with multiple consecutive hyperthermia treatments. Also hyperthermia treatment duration could be lowered in order to find the minimal duration requirements for enhancing CTL killing.

4.6 Reduction of proliferation in tumor cells

Reduction of proliferation is often an indicator for cellular stress. So we investigated whether hyperthermia was able to decrease tumor cell proliferation as a sign of stress

response. We found a temporary reduction of mitosis shortly after hyperthermia treatment as well as a long term inhibition of proliferation after 2 consecutive hyperthermia treatments. Interestingly reduction of proliferation after hyperthermia treatments was proportionally strong when compared to the decrease in proliferation during 48 hours culture under hyperthermia.

This finding of functional short hyperthermia treatment durations makes these results especially relevant regarding possible future treatment schemes for cancer patients. In general, lower temperatures and treatment durations are favorable in order to keep accompanying side effects of hyperthermia like dehydration or problems with circulation as low as possible.

To further validate the effect of hyperthermia on cell proliferation, usage of another cell line with different tissue origin could provide important data to compare to. Testing the effect of hyperthermia under physiologically relevant circumstances could be achieved by switching from 2D to 3D cell culture, where cells could respond differently to hyperthermia because of altered heat and nutrient delivery, cell morphology and cell-cell communication.

The decrease in proliferation caused by hyperthermia is a general sign of weakened tumor cells. Screening for altered protein expression under hyperthermia could be a first attempt to understand molecular dynamics of hyperthermia and to find possible deregulation of tumor cell proteins that are related to tumor cell response towards CTL attacks.

4.7 Hyperthermia changes CTL activation status

T-cell development, homing to tissues and exertion of effector functions is widely regulated by the expression of specific cell surface markers. Application of whole body hyperthermia therefore might intervene on all these three stages during the T-cell life cycle. We addressed if hyperthermia applied during *in vitro* activation was capable of changing the CTL activation status which might give further hints about CTL behavior under altered culture conditions.

We analyzed different markers for T cell activation and effector function using flow cytometry. Fever-range hyperthermia (39.5 °C) showed upregulation of CD25 which is involved in induction of clonal expansion of CTL upon receptor binding of IL-2.[73] As a matter of consequence CTL could respond more efficiently to IL-2 stimuli in vivo, which could lead to overall raised amounts of antigen specific CTL. As a matter of consequence, CTL could respond more efficiently to IL-2 stimuli in vivo, which could lead to overall raised amounts of antigen specific CTL.

Expression of the marker combination CD62-L and CD44 can be used to discriminate naïve (CD62-L^{high}/CD44^{low}) from activated CTL (CD62-L^{low}/CD44^{high}).[75] In our experiments hyperthermia lead to a decrease of CD62-L and an increase of CD44 levels with raised temperature. This gives reason to assume, that hyperthermia is involved in facilitating the transition from a naïve towards an effector CTL state, which might of course also include effects of hyperthermia on other immune cells playing a role in CTL activation.

CD25 and CD62L/CD44 are not directly connected to CTL killing and observed marker levels have to be reviewed critically, since results are observed out of *in vitro* experiments which look at an isolated part of the immune system. Nevertheless, these changes in marker expression provide evidence that hyperthermia exerts effects on CTL and definitively deserve validation in advanced *in vivo* hyperthermia settings.

As one of the main mediators of cytotoxicity GranzymeB expression was also analyzed. Intracellular levels remained equal when cells were treated with hyperthermia. However, intracellular stored GranzymeB isn't directly related to cytotoxicity but rather its secretion. So could further assays target GranzymeB release to get insight whether hyperthermia triggers its release and consequently promotes CTL killing efficiency.

CTL used for analysis of marker expression were cultured continuously under hyperthermia upon isolation and throughout activation. To find the optimal treatment scheme, validation experiments could investigate the use of fractionated hyperthermia treatment as applied during CTL killing assays. Thereby variation of hyperthermia duration as well as frequency of application could lead to increased CTL activation efficiency.

4.8 Fever-range hyperthermia rapidly blocks tumor cell proliferation in live melanoma tumors

Most of the results observed on tumor cell proliferation under hyperthermia culture have been obtained from *in vitro* experiments. Tissue structure, cell-cell and cell-matrix contacts or signaling molecules are only a couple of factors which are hard to simulate with normal cell culture but which are essential factors that influence tumor proliferation. Hence it was unavoidable to validate obtained *in vitro* results by performing in vivo experiments.

Because of this reason we established a set up which allowed controlled heating of mice to fever range temperatures. Usage of intravital multiphoton microscopy provided a measure to directly image effects of hyperthermia on an in vivo growing tumor. For these pilot studies, mice were treated once with whole-body hyperthermia and imaged directly before and after the treatment. Based on the *in vitro* results we decided to use 39.5 °C as target temperature. Intravital imaging allowed counting of mitotic events in tumor cells as an indicator for tumor proliferation. Observed data revealed, that the tumor immediately responded to an 1 hour whole body hyperthermia treatment with a drastic reduction of mitosis. This leads to the conclusion, that hyperthermia also induces severe stress to tumors grown in vivo.

Unfortunately, mice had to be sacrificed directly after the imaging procedure and hence long term effects on tumor proliferation couldn’t be studied, which would have provided essential data on possible recovery of the tumor.

Using our established hyperthermia setup in combination with intravital multiphoton microscopy opens up a brought range of research possibilities. First and foremost clinically relevant treatment schemes could be tested, where hyperthermia is combined with adoptive T-cell therapy. Thereby several factors like tumor shrinkage or CTL invasion and viability could be monitored over a certain time period to document possible supportive functions of hyperthermia in T-cell therapy. Further approaches could target different immune cell subsets and for example test invasion and antigen uptake by dendritic cells. Alterations of the immunosuppressive tumor microenvironment of the tumor that often limits effectivity of immunotherapies could be analyzed as well.

5. Conclusion

The goal of this master thesis was to evaluate whether fever range hyperthermia had an effect on the killing potential of CTL. Taken all data together, we can conclude that there is definitively an increase on CTL mediated killing of melanoma cells. Further, hyperthermia proved itself as a potent inducer of cellular stress resulting in drastic reduction of tumor cell proliferation *in vitro* as well as in vivo. Application of hyperthermia during CTL activation lead also to changes in marker expression which can be linked to an enhanced activation status of CTL.

One major goal for future investigations will be to determine which effect is predominating – a weakening of tumor cells, an enhancement of CTL effector functions or a combination out of both effects. For this purpose precise molecular analysis is needed to find major pathways in both cell types being responsible for raised tumor cell apoptosis. In conclusion, hyperthermia proves itself as a promising supporting strategy for current immunotherapy and will definitively get further into the focus of research in the upcoming years.

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