TITLE: **Immunological response in the mouse melanoma model after local hyperthermia**

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RUNNING TITLE**: Local hyperthermia in mouse melanoma model**

Summary:

Our study was aimed to characterize the phenotype and functional endpoints of local microwave hyperthermia (LHT, 42°C) on tumor infiltrating and spleen leukocytes. The effectiveness of LHT applied into the tumor of B16F10 melanoma-bearing C57/BL6 mice was compared with anaesthetized and non-treated animals. Subpopulations of leukocytes were analyzed using the flow cytometry, and the cytotoxic activity of splenocytes against syngeneic B16F10 melanoma and NK-sensitive YAC-1 tumor cell lines was evaluated in ^{51}Cr-release assay. Similarly, the *in vitro* modification of the heat treatment was performed using healthy and melanoma-bearing splenocytes.

We found a 40% increase of activated monocytes (CD11b+CD69+) infiltration into the tumor microenvironment. In the spleen of experimental animals, the numbers of cytotoxic T

lymphocytes (CTLs-CD3+CD8+) and NK cell (CD49b+NK1.1+) raised by 22% and 14%, respectively, while the NK1.1+ monocytes decreases by 37%. This was accompanied by an enhancement of cytotoxic effector function against B16F10 and YAC-1 targets in both *in vivo* and *in vitro* conditions.

These results demonstrate that LHT induces better killing of syngeneic melanoma targets.. Furthermore, LHT evokes the homing of activated monocytes into the tumor microenvironment and increases the counts of NK cells and CTL in the spleen.

Key words: local hyperthermia; melanoma; cytotoxic cells; monocytes; CD69

Introduction:

The use of hyperthermia (HT - heating of tissue to 41-44ºC) is one of the promising methods in cancer treatment (Van der Zee 2002, Baronzio *et al.* 2006). Although the molecular mechanisms of this process are not fully understood yet, possible effects are: 1) direct cytotoxicity (Milani and Noessner 2006), 2) inhibition of DNA-repair procedures (Kampinga *et al.* 2004), 3) changes in capillary blood flow and microvessels (Emami *et al.* 1980) and 4) immunomodulatory effects (Hildebrandt *et al.* 2002). Relative contributions of the above stated effects are still not fully clarified and are presumably tumor type dependent (Ostapenko *et al.* 2005).

Hyperthermia is being introduced either as localized (LHT) or as a whole body hyperthermia (WBH) in various temperature ranges. During WBH, the elevation of temperature near tumoricidal levels (T≥42ºC) leads to the inhibition of host immunocompetence (Huang *et al.* 1996, Shen *et al.* 1994), making such arrangement unsuitable for immunological studies. The fever-range temperatures (39-41ºC) on the other

hand show an immunoregulatory advantage by enhanced secretion of immunoglobulins (Ostapenko *et al.* 2005).

In case of LHT, even if the heating of tumor exceeds 42ºC, the surrounding healthy tissue usually remains at fever-range temperatures (39-41ºC). This selectivity is beneficial, because LHT combines the tumoricidal effect of the heating with the fever-induced immunostimulation.

Immunomodulatory effect of HT can consist of augmenting of antigenicity of tumor cells, followed by induction of the immune response. More efficient antigen presentation on tumor cells and stimulation of immunocompetent cells was demonstrated *in vitro* (Ito *et al.* 2001) and *in vivo* using LHT (Basu and Srivastava, 2003) or WBH (Atanackovic *et al.* 2002). The current knowledge of the LHT influence on immunological changes encompasses the activation of spleen cells (Vartak *et al.* 1996), natural killer (NK) cells (Szmigielski *et al.* 1991), CD8+ (Tc or CTL) cells (Ostapenko *et al.* 2005) and CD4+ (Th) cells (Stawarz *et al.* 1993) at temperatures within the fever-range (39-41ºC).

In our study, we investigated the effect of LHT on the *in vivo* and *in vitro* induction of NK and CTL-mediated cytotoxic activity against B16F10 melanoma targets. We evaluated changes in effector cell numbers, activation and killing activity in the spleen and the tumor site after *in vivo* LHT application. To distinguish effects on effector and target cells, we examined the LHT impact *in vitro*, with consequent functional studies. We demonstrated, that the response to LHT is not a single-step event, but a cascade of actions ranging from tumor infiltration, possible augmentation of antigen presentation and effector cells proliferation to the activation of terminal cytotoxic cell function.

Materials and methods:

Experimental animals

Eight-week-old inbred male C57/BL6 mice were purchased from Charles River (D). The mice were housed under natural day/night conditions (22 ^{o}C, 55% humidity) and fed on a commercial pelleted diet (Velaz - ST1) *ad libitum*. The *ex vivo* experiments started at least 2 weeks after the arrival of the animals to avoid immunological changes caused by transportation stress. The mouse melanoma cell line - B16F10 derived from C57/BL6 mice (kindly donated by Weizmann Institute, Rehovot, Israel) was used in the syngeneic tumor model. B16F10 cells (10^{6}/mouse in 0.1ml PBS) were inoculated subcutaneously (*s.c.*) into the lower back at day 0. Mice with tumors reaching 1-2 cm in greatest dimension were randomly divided into three groups: 1) anesthetized mice treated with hyperthermia (HT), 2) anesthetized mice (anest), and 3) non-treated controls (control). All procedures were conducted in accordance with the European Convention for the Care and Use of Laboratory Animals as approved by the Czech Animal Care and Use Committee.

Hyperthermia

Local hyperthermia was performed under anesthesia (ketamine: 1.9mg/mouse and xylazine: 0.25mg/mouse weighing 25-30g, *i.p.*). A Microwave generator with working frequency 2450MHz and power 10W was used for tumor heating, in connection with the applicator constructed at the Czech Technical University (Prague, CZ) based on conventional waveguide, with the effective aperture 2x2cm. The superficial, intra-tumor and rectal temperature of mice was monitored during the whole period of HT application by the fluoro-optic thermometer (LUXTRON, Luxtron Corporation CA). Target temperature (42^{o}C) in the tumor was reached during 1 minute and was maintained using pulses of microwaves for 7 minutes. Rectal temperature did not surpass 38^{o}C. We performed three consecutive heatings in the hyperthermia group at days 10, 14 and 17 after tumor cells inoculation. Animals in the anesthesia group were anesthetized in the same time intervals as mice in the HT group, but

without hyperthermia. Twelve hours after the third session of hyperthermia all mice were euthanized and tumors and spleens were removed for further separation.

Isolation of mouse spleen cells (SC) and tumor infiltrating leukocytes (TIL)

Spleens were dissociated through a nylon mesh, repeatedly washed and used immediately for flow cytometry (FACS) or *in vitro* experiments. Tumor infiltrating leukocytes (TILs) were prepared from individual melanomas minced with scissors, eluted by repeated pipetting, and mononuclear cells separated on Ficoll-Hypaque density gradient (1.086). Freshly separated mononuclear cells were used for further phenotype and functional assays.

Cell cultures and *in vitro* experiments

Established cell lines YAC-1 (mouse NK-sensitive T lymphoma) and B16F10 (mouse melanoma) were maintained in RPMI-1640 medium supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol, antibiotics (0.05mg/ml gentamycin, 25mg/ml amphotericin B) and 10% heat-inactivated fetal calf serum - FCS (Gibco, Grand Island, NY, USA).

The cytotoxic activity assays of mouse spleen cells were performed in RPMI 1640 medium supplemented with L-glutamine, gentamycin and 5% FCS. Incubation was carried out at 37°C in a humidified atmosphere containing 5% CO_2 in a CO_2 incubator (Jouan, France).

For *in vitro* modification of hyperthermia, B16F10 cell line (as target) and splenocytes (as effectors) were cultivated at 37°C or 42°C, in a humidified atmosphere, and 5% CO_2. The effector or target cells were transferred into pre-warmed cultivation medium for the heat treatment and the incubation at 42°C for 10 minutes followed, once or 3-times in the same schedule as in the *in vivo* model. The melanoma cells were then harvested and used as targets in cytotoxic assays.

Flow cytometry

Individual cell suspensions prepared from the spleen and tumor were resuspended in PBS with 0.1% gelatin (gelatin from cold water fish skin – Sigma) and 0.01% sodium azide. Phenotypes of cells were determined using specific surface markers for T lymphocytes – CD3-PECy5 (17A2), CD4-FITC (H129.19), CD8-PE (53-6.7), B-lymphocytes – CD45R/B220-Alexa405 (RA3-6B2), monocytes, APCs - CD11b-Pacific Blue (M1/70.15), CD11c-FITC (HL3), NK cells – CD49b-FITC (DX5), activation antigens NK1.1-PECy7 (PK136), CD69-PECy5 (H1.2F3) and analysis was performed by FACS LSR II (Becton-Dickinson, USA). Five- or six-color staining was performed according to the manufacturer's standard protocol. The CD45-PECy7 (30-F11) leukocyte common antigen (LCA) staining was applied to distinguish the leukocytes from residual melanoma cells in TIL fraction. Further, the PI negative (live) CD45 positive (leukocytes, Fig.1 A) cells with lymphocyte and monocyte morphology were gated (large lymphomonogate, SSC *vs* FSC, Fig.1 B) and analyzed for surface markers expression (Fig.1 C, D). Monoclonal antibodies were purchased either from Pharmingen (San Diego, CA, USA) or Caltag (San Francisco, CA). Evaluation of data was performed using FlowJo version 6.1.1 software (Tree Star Inc., Ashland, OR, USA).

Cytotoxicity assay

Cell-mediated cytotoxicity was estimated using standard ^{51}Cr-release assay with splenocytes of experimental animals as effectors. YAC-1, mouse NK-sensitive, and B16F10 melanoma cell lines were used as target cells and were labeled by 60-min. incubation with $Na_2{}^{51}CrO_4$ in round-bottomed 96-well microtiter plates (NUNC), at 37°C in a humidified atmosphere containing 5% CO_2. Evaluation of effector cell lytic activity against 10^4 target cells was performed after 4 hours of incubation as described previously (Fiserova *et al.* 1997). The cell free supernatants were harvested (0.025ml/sample), 0.1ml of scintilation cocktail (SuperMix, Wallac, Finland) was added, and radioactivity was measured using scintillation counter Microbeta Trilux (Wallac, Finland).

Statistical analysis

Results in groups were analyzed using one-way analysis of variance (ANOVA), P values ≤ 0.05 were considered significant (P≤0.05 - *, P≤0.01 - **).

Results:

Our results did not include the clinical parameters (tumor growth, survival), since the experiments were designed as short time schedules after three courses of LHT treatment dedicated to the changes of immune parameters. We performed four consecutive experiments with 22 animals in the heat treated group (HT), 17 in the anesthetized group (anest), and 16 animals in the untreated group (control) to evaluate LHT effects on the immune response of tumor-bearing animals and the distribution of leukocytes in different compartments (spleen, tumor). The subpopulation specific and activation markers were estimated using polychromatic FACS analysis for determination of the LHT effects on the immune cells proportion, and activation. Functional activities of cytotoxic cells (NK, CTL) were measured by standard ^{51}Cr-release assay in both *in vivo* and *in vitro* arrangements.

LHT evoked changes in tumor microenvironment

We have determined the distribution of tumor infiltrating leukocytes (TIL) in tumors using FACS. We did not find any significant changes between the control, anesthetized and the LHT groups in proportion of CTLs, NK or APC cells in tumor microenvironment.

Further we have investigated the changes of very early activation antigen CD69 on different TIL subpopulations – NK, CTL, APC, DC (Fig.1 E, F, G). Our results depicted in Fig.2 demonstrated enhanced expression of CD69 only in the monocyte population (CD8-/CD49b-/CD11b+/CD11c-). The counts of activated monocyte (CD11b+/CD69+) in both control and anesthetized mice were similar, 12±1,7%, while the LHT treated group showed a 40% increase (17±2,6%) in the TIL fraction.

LHT effect on immune cells distribution in the spleen

Our study revealed changes in the occurrence of cell subpopulations in secondary lymphoid organ, the spleen, after the application of LHT. The parameters of detected surface markers were similar as for the analysis of TILs represented in Fig.1 without CD45 staining. Cytotoxic T lymphocytes (CD3+/CD8+) increased their counts by 22%, natural killer cells (CD49b+/NK1.1+) increased by 14% and a subpopulation of monocytes, bearing NK cell markers (CD49b-/NK1.1+/CD11b+), decreased by 37% (Fig.3). However, except for the activated monocytes in the TIL fraction (Fig.2), similar results were observed in the anesthetized group (Fig.3), although these changes were not as strong as with the concurrent hyperthermic stress.

Cytotoxic effector function after LHT treatment *in vivo*

To determine the effect of LHT on cytotoxic effector functions, we performed the standard 4-hour ^{51}Cr-release assay using NK-sensitive (YAC-1) or syngeneic (B16F10) melanoma cell lines as target cells, and freshly isolated splenocytes of experimental animals as effectors. The results have shown a significant attenuation of cytotoxicity against B16F10 melanoma targets when anesthesia was applied. Nevertheless, the LHT countered this effect, returning the cytotoxicity to the control level (Fig.4 A). We did not observe this attenuation, when NK-sensitive YAC-1 target cell line was used (Fig.4 B), but NK cell effector function was significantly enhanced by LHT treatment as well.

Taken together, the hyperthermia stimulates the functional activity of effector cells, compared to the anesthetized controls, of both specific and natural cytotoxicity against B16F10 and YAC-1 targets, respectively.

Direct effect of heat treatment on melanoma and/or effector cells *in vitro*

To omit the effect of anesthesia, and to distinguish the role of hyperthermia on either tumor or lymphoid cell populations, we performed an *in vitro* modification of the experiments. In this procedure, we used splenocytes from healthy or B16F10 melanoma-

bearing mice as effectors and B16F10 melanomas from *in vitro* cultures as targets. Hyperthermia was applied to B16F10 cells in 1 or 3 courses of 10-minute incubation in 42°C, 5% CO_2 atmosphere in the same time schedule as was done during *in vivo* treatment (3xHT on days 0, 4, and 7). The non-treated cells were kept under the same conditions in 37°C. Freshly isolated spleen cells of either tumor-bearing or healthy animals were heated for 10 minutes at 42°C (Fig.5 C, D) immediately before the experiment, while non-treated effector cells were kept in 37°C (Fig.5 A, B) in a round-bottomed 96 well plate. ^{51}Cr-labeled B16F10 target cells after HT treatment (none, 1xHT and 3xHT as indicated in Fig.5) were washed and seeded onto splenocytes in E:T ratio 32:1. The cytotoxicity was measured in a standard 4-hour ^{51}Cr-release assay.

Heat treatment of effector cells exhibited a strong inhibition of cytotoxicity against untreated B16F10 targets that was more pronounced in healthy animals (down to 26%, Fig.5 A vs C) then in tumor-bearing ones (to 41%, Fig.5 B vs D). Further significant suppression was noted in cytotoxic activity of healthy splenocytes, when heat treated melanoma cells were used as targets (Fig.5 A). No marked difference was seen between the 1 or 3 courses of HT. On the other hand, splenocytes cytotoxicity of tumor-bearing animals responded to 1xHT B16F10 melanoma target cells by significant increase of cytotoxic effector function, not present in 3-course HT (Fig.5 B).

The presented results demonstrate an inhibitory influence of HT on effector cells, which is well known in the case of tumoricidal temperature ranges (T≥42ºC). However, the response of effectors to heat-treated melanoma cells evoke an opposite cytotoxicity effect in healthy (remain suppressed – Fig.5 A, C) and tumor-bearing animals (stimulated – Fig.5 B, D). Moreover, the results in Fig.5 B, D showed preferable use of one course of hyperthermia.

Discussion:

One of the causes of cancer progression is the failure of tumor cells recognition by the immune system.

In this study we revealed an increased count of activated monocytes in B16F10 melanoma microenvironment, which is poorly recognized by the immune system. These monocytes are potent antigen presenting cells (APCs), facilitating more effective identification of tumors by other immunocompetent cells. Here we showed an increase of cytotoxicity against the melanoma cells (Fig.4), which we also observed *in vitro* in the case of splenocytes, derived from melanoma-bearing animals (Fig.5 B). The lack of antigen presentation accounts for the decreased cytotoxicity from our *in vitro* studies, where splenocytes from healthy animals were not primed for the killing of target cells by the shedded antigens (Davies and Lindmo 1990) (Fig.5 A, C). The prior contact with melanoma cells and subsequent priming of the immune system seems to be crucial for effector function of cytotoxic cells.

Ex vivo functional assays using B16F10 cell line as target demonstrated an attenuation of cytotoxicity by anesthetics, which returned to the control level after LHT application (Fig.4 A). On the other hand, the natural cytotoxicity against YAC-1 targets was not impaired by the use of anesthetics (Fig.4 B). Assuming that the total cytotoxicity against B16F10 targets involves both the natural and specific components, we have found the specific immunity (CTLs) to be predominant in B16F10 melanoma killing. Thus, the anesthetics affect either the specific immunity itself, or the antigen presentation, which is required for its proper function.

According to the literature, HT-induced changes involve an increase of tumor immunogenicity via induction of hsp70 expression (Clark and Menoret 2001), MHC II- (Michalek *et al*. 1992) and MHC I-mediated antigen presentation (Ito *et al*. 2001). Our results showing the tumor infiltrating monocyte activation, increase in CTL and NK cell numbers in

the spleen and stimulation of specific and natural cytotoxic activity under local hyperthermia are a contribution to the data on the involved cell subpopulations.

In conclusion our study demonstrates significant changes in the distribution, phenotype, activation and effector function of immune cell subpopulations in tumors and spleens of experimental animals after LHT. We suggest that the hyperthermia enhances tumor cell antigenicity, thus leading to improved recognition of these cells by the infiltrating monocytes. Activated monocytes then presumably present antigens to further cell subpopulations (Th, CTL), priming them for the effector function, as reflected in the increased counts of NK cells and CTLs in the spleen and their cytotoxic activity. As presented in the *in vitro* studies, the prior contact with tumor cells seems to be crucial for mounting this response. There are probably several pathways involved in the immunostimulatory effects of hyperthermia. The exact mechanisms and involved subpopulations require further investigation.

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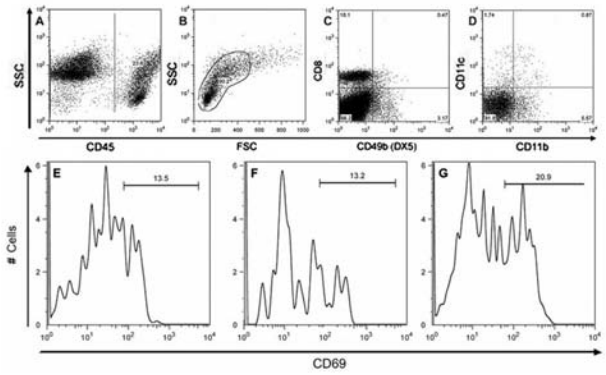

Fig.1: Cell surface markers FACS analysis. PI negative (not shown) and CD45 positive cells (A), with lymphocyte/monocyte morphology (B), were analysed for expression of surface markers of NK/CTL cells (C) and monocyte/DC subpopulation (D). These subsets were further analysed for CD69 expression (E, F, G), which changed significantly only in case of CD45+/CD49b-CD8-/CD11b+CD11c- cells (monocytes) comparing the hyperthermed animals (G) to controls (E) and anesthesised groups (F). Histograms (E, F, G) represent the percentage of CD69 positive cells out of the gated parental population (CD45+, CD49b-/CD8-/CD11b+/CD11c-). Figure shows an illustrative example of four consecutive experiments with similar results.

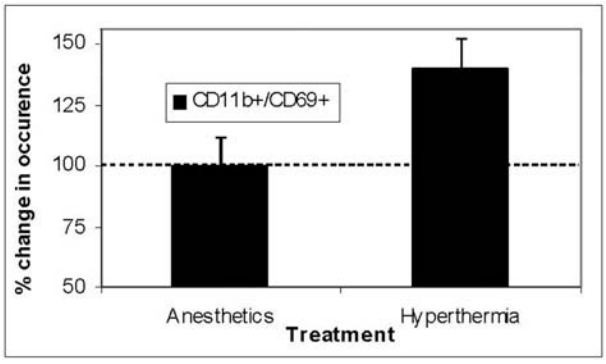

Fig.2: Proportion of activated monocytes (CD11b+/CD69+) occurrence in TIL fraction. Both control and anesthetized groups showed 12±1,7% of CD11b+/CD69+, while HT treated group had 17±2,6% of CD11b+/CD69+, showing a 40% increase. The percentage was counted from CD45 positive cells in the TIL fraction and is shown as percentage change relative to control stated as 100% (dashed line).

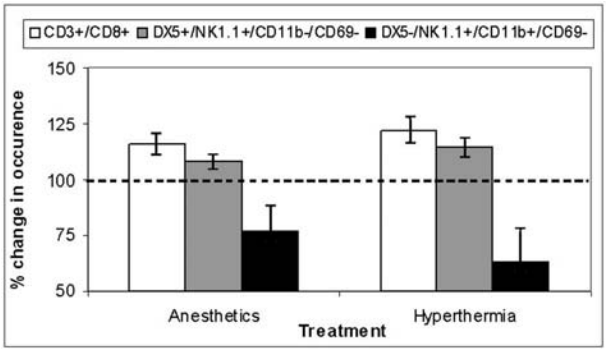

Fig.3: Distribution of immune cell subpopulations in the spleen after local hyperthermia. The values are presented as percentage changes in occurrence relative to individual controls. The control values (stated as 100%, dashed line) were for CTLs – 55% (CD8+ out of CD3+); for NK cells – 36% (NK1.1+ out of CD49b+); for NK1.1 positive monocytes – 17,5% (NK1.1+ out of CD11b+). The data represent Average ± StDev. of three performed experiments (5-7 animals per group).

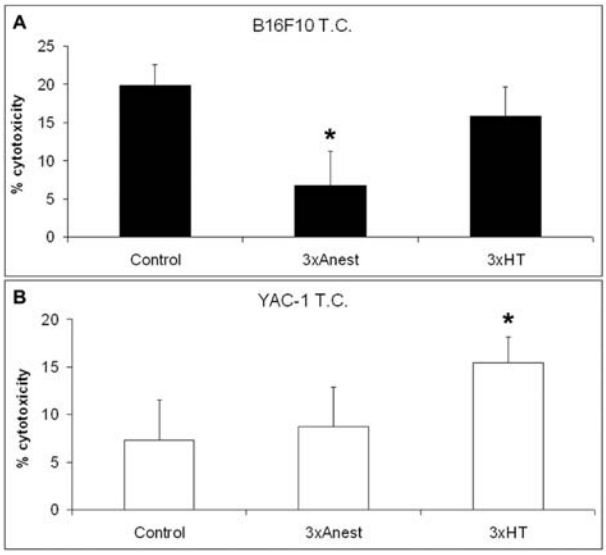

Fig.4: The specific (A) and natural (B) cytotoxic activity of splenocytes against syngeneic B16F10 melanoma or NK-sensitive YAC-1 target cells in E:T ratio of 32:1 after *in vivo* applied local hyperthermia. Specific cytotoxicity against B16F10 melanomas was impaired by the use of anesthetics – this effect was countered by a concurrent use of hyperthermia (A). No attenuating effect of anesthetics was observed in NK-mediated cytotoxicity (B). Significant changes relative to non-treated mice are marked by asterisk (* $p \leq 0.05$; ** $p \leq 0.01$).

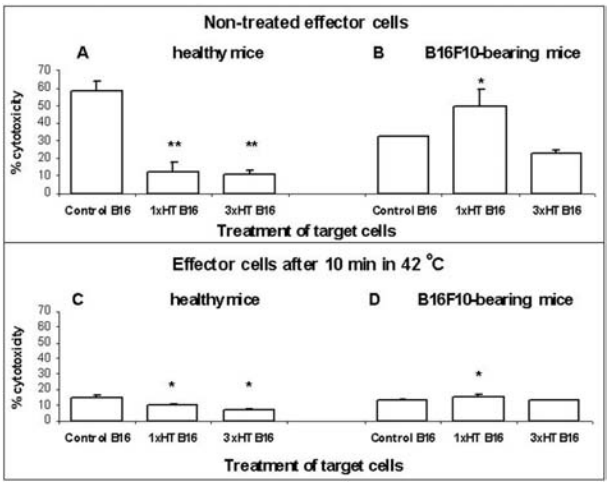

Fig.5: Splenocyte cytotoxicity against B16F10 melanoma targets after *in vitro* applied HT to effectors and/or target cells. Cytotoxicity of splenocytes isolated from healthy (A, C) or B16F10 melanoma-bearing (B, D) mice against pretreated syngeneic B16F10 cell line: Control - untreated, 1x HT B16 or 3x HT B16 - hyperthermed B16F10 cells one or three-times, respectively (10min. at 42ºC). Effector cells were incubated for 10 min. in either 37°C (A, B) or 42°C (C, D). Cytotoxic assay was performed in E:T ratio 32:1, data represent average±StDev of three experiments done in quintuplicates for each sample. Significant changes relative to non-treated mice are marked by asterisk (* $p \leq 0.05$; ** $p \leq 0.01$).