The dual role of NK cells in antitumor reactions triggered by ionizing radiation in combination with hyperthermia

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ABSTRACT

Classical tumor therapy consists of surgery, radio(RT)- and/or chemotherapy. Additive immunotherapy has gained in impact and antitumor in situ immunization strategies are promising to strengthen innate and adaptive immune responses. Immunological effects of RT and especially in combination with immune stimulation are mostly described for melanoma. Since hyperthermia (HT) in multimodal settings is capable of rendering tumor cells immunogenic, we analyzed the in vivo immunogenic potential of RT plus HT-treated B16 melanoma cells with an immunization and therapeutic assay. We focused on the role of natural killer (NK) cells in the triggered antitumor reactions. In vitro experiments showed that RT plus HT-treated B16 melanoma cells died via apoptosis and necrosis and released especially the danger signal HMGB1. The in vivo analyses revealed that melanoma cells are rendered immunogenic by RT plus HT. Especially, the repetitive immunization with treated melanoma cells led to an increase in NK cell number in draining lymph nodes, particularly of the immune regulatory CD27+CD11b+ NK cell subpopulation. While permanent NK cell depletion after immunization led to a significant acceleration of tumor outgrowth, a single NK cell depletion two days before immunization resulted in significant tumor growth retardation. The therapeutic model, a local in situ immunization closely resembling the clinical situation when solid tumors are exposed locally to RT plus HT, confirmed these effects. We conclude that a dual and time-dependent impact of NK cells on the efficacy of antitumor immune reactions induced by immunogenic tumor cells generated with RT plus HT exists.

Abbreviations: AnxV, AnnexinV; APCs, antigen presenting cells; ATP, adenosine triphosphate; CD, cluster of differentiation; CT, chemotherapy; DAMPs, damage associated molecular patterns; DCs, dendritic cells; depl., depletion; DNA, deoxyribonucleic acid; GM-CSF, granulocyte macrophage colony-stimulating factor; HMGB1, high mobility group box 1; HSP, heat shock proteins; HT, hyperthermia; ICD, immunogenic cell death; IFN, Interferon; IL, Interleukin; NK, natural killer; ns, not significant; RCT, radiochemotherapy; rep., repetitive; RT, radiotherapy

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Introduction

A promising approach to treat cancer is the use of immunization strategies in combination with radiochemotherapy (RCT) to further improve the antitumor immunity. For modifying the immune response to tumor cells, the immune suppressive microenvironment has to be shifted to an active one. One central event is the induction of an immunogenic cell death (ICD) in vivo.2 Depend on morphological, biochemical and immunological features apoptosis, necrosis, oncosis,3 pyroptosis,4 autophagy,5 necroptosis,6 and NETosis7 can be distinguished. Current studies identified particularly a mixture of apoptotic and necrotic cells with a high potential of immune activation.8 On the one hand, apoptotic cells stimulate and mature dendritic cells (DCs) and initialize anticancer T cell response by exposing calreticulin (CRT) and heat shock proteins (HSP) on their surface.9,10 On the other hand, primary and secondary necrotic cells that have lost their membrane integrity release inflammatory cytosolic immune activating damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), adenosine triphosphate (ATP) or HSP70. These DAMPs are particularly increased in secondary necrotic cells and lead to DC maturation, NK cell activation and priming of Th1 effector cells.11,12 Basal levels of HSP70 and its inducible form are generally higher in tumor cells than in normal cells. Additionally, HSP70 is exclusively expressed on the surface of human tumor cells.13 RT further fosters this surface expression. Surface HSP70 on the one hand is a marker for more aggressive
tumors, but on the other hand a specific target structure for the cytolytic attack mediated by activated NK cells.\textsuperscript{14,15}

RT belongs to the classical cancer therapies and is an integral part in over 50\% of all protocols to treat solid tumors.\textsuperscript{16} The main function of RT is the local control of the tumor. The well-known mechanism of damaging DNA by RT with ionizing radiation (X-ray) triggers a network of events finally leading to cell cycle arrest and cell death.\textsuperscript{17} Several recent studies verified not only local cytotoxicity, but also immune mediated, systemic, and so-called abscopal effects of RT.\textsuperscript{18} Preclinical models demonstrated that irradiation results in tumor shrinkage outside the irradiation field suggesting an activation of systemic immune response by locally applied RT.\textsuperscript{19} In line with that, clinical studies also give hints of abscopal and immune mediated responses initiated by RT.\textsuperscript{20,21} Summarized, RT applied in distinct fractionation schemes has been proven to be capable of rendering tumor cells more immunogenic.\textsuperscript{22-24} While RT or RCT alone in most cases do not result in abscopal antitumor responses, combination with further immune stimulation seems to be most beneficial to induce innate and adaptive anti-tumor immune responses.\textsuperscript{11,25}

Mild HT is an additive tumor therapy. The tumor tissue is locally heated to temperatures of 40–44°C for a time period of maximum one hour. HT enhances the effect of many chemotherapeutic agents and sensitizes the tumor cells for RT.\textsuperscript{26} By HT, the blood circulation can be modified and oxygen is delivered into the tumor. Furthermore, HT increases the metabolism leading to reduced ATP levels and increased anaerobic metabolites, fosters protein aggregation and thereby aggravates DNA repair.\textsuperscript{27} In addition, local HT might act as in situ tumor vaccine by the induction of a systemic antitumor response.\textsuperscript{28,29} This is in part due to activation of DCs and NK cells by thermal stress over 40°C.\textsuperscript{30} An exposure to HT improves DC functions during immune activation inter alia by upregulation of CD80, CD83, and CD86 on DCs.\textsuperscript{31} HT further enhances the NK cell cytotoxicity by induction of the NKG2D receptor.\textsuperscript{30} RT especially fosters surface exposure of HSP70\textsuperscript{14} and in combination with HT its release.\textsuperscript{32} Another important advantage of HT is its low systemic toxicity.\textsuperscript{33} Hints exist that immune stimulation by HT is capable of augmenting the efficacy of CT and RT treatments in melanoma\textsuperscript{4} that is known for its susceptibility to immune therapeutic approaches.\textsuperscript{35,36} Preclinical models revealed that CD8\textsuperscript{+} T cell responses are initiated when combining RT with further immune modulation for the treatment of melanoma.\textsuperscript{34,37} An increased NK cell infiltration into the tumor was also reported. However, the role of NK cells in this scenario is still scarcely understood.

NK cells, firstly described by Kiessling et al.,\textsuperscript{38} are an important component of innate immunity. Regulated by an impressive diversity of activating and inhibiting receptors NK cells acquire self-tolerance and get licensed to recognize foreign or altered cells.\textsuperscript{39,40} By release of cytoplasmic perforin and granzyme, NK cells contribute to a rapid immune response against foreign, infected, malignant, and stressed cells.\textsuperscript{41}

Human NK cells can be divided into at least two phenotypically and functional distinct subsets based on their surface expression of CD56 and CD16, the immune regulatory CD56\textsuperscript{bright}CD16\textsuperscript{dim} and the cytotoxic CD56\textsuperscript{dim}CD16\textsuperscript{bright} NK cells. Mouse NK cells do not express CD56, but can be subdivided by the expression of CD27 and CD11b into CD27\textsuperscript{high}CD11b\textsuperscript{low} NK cells with immune regulatory and CD27\textsuperscript{low}CD11b\textsuperscript{high} with cytotoxic properties.\textsuperscript{42,43} CD11b\textsuperscript{+} NK cells are fully mature and show the highest cytotoxic potential.\textsuperscript{44,45} Influenced by splenocytes, NK cells mature from CD27\textsuperscript{high}CD11b\textsuperscript{low} to CD27\textsuperscript{high}CD11b\textsuperscript{high} and differentiate terminaly to stable CD27\textsuperscript{low}CD11b\textsuperscript{high} NK cells.\textsuperscript{43,45,46} Moreover, NK cell induced production of IFN\textgamma, TNF-\alpha, lymphotixin, granzyne, perforin, IL-10, IL-13, and GM-CSF seems to be crucial for activation and migration of components of the adaptive immune system.\textsuperscript{47,48}

Whereas the importance of NK cells in advanced tumor stages has been circumstantially investigated, their role during immunization remains still unclear. On the one hand, it has been reported that successful DC-vaccination increased NK cell activation by upregulation of Nkp46 and NKG2D.\textsuperscript{59} On the other hand, in a B16OVA C57BL/6 vaccination model, activated NK cells have been shown to lyse CD8\textsuperscript{+} T cells in a perforin- and NKG2D-dependent manner that might impair the adaptive immune response.\textsuperscript{50} These examples of controversial studies prompted us to re-examine the role of NK cells during the immune activation period, and here especially that induced by RT in combination with HT.

**Results**

*RT alone and in combination with HT induces apoptosis and necrosis in B16 melanoma cells*

While the sole treatment with HT (41.5°C for 1 h) did not result in significant cell death induction of B16 melanoma cells, irradiation with 15 Gy or the combination of 15 Gy plus HT resulted in a mixture of about 20\% apoptotic and 30\% necrotic B16 cells, as early as 48 h after the in vitro treatment (Fig. 1). Furthermore, the danger signals HMGB1 and HSP70 were increasingly released in the B16 tumor cell supernatant after irradiation with 15 Gy combined with HT treatment. (Fig. S3)

**Immunization with RT or RT plus HT-treated B16 melanoma cells results in tumor growth retardation**

Next, we tested the in vivo immunogenicity of the therapy-modified B16 melanoma cells. For that purpose, C57BL/6 mice were immunized with subcutaneously injected RT or RT plus HT pre-treated B16 cells. Seven days afterwards, mice were challenged with viable B16 cells subcutaneously injected in the contralateral flank (Fig. 2A). This assay is strongly suggested for the evaluation of ICD in vivo.\textsuperscript{51} By measuring local tumor growth at the tumor injection side, we checked the basal immunogenic potential of RT or RT plus HT-treated melanoma cells (Fig. 2B–C). Since the repetitive treatment of RT plus HT is successfully used for treating solid and heatable tumors,\textsuperscript{33,52,53} we also tested repetitive immunizations, which were significantly more effective than a single one (Fig. 2D). We therefore
Figure 1. Cell death kinetics and forms of B16 melanoma cells after irradiation and/or hyperthermia. The cell death forms of B16 mouse melanoma cells were analyzed with two color flow cytometry after staining with AnnexinV-FITC and DAPI 24, 48 or 72 h after the respective treatment with ionizing radiation with 15 Gy and/or hyperthermia (HT, 41.5°C for 1 h). Viable cells are defined as AnxV−/DAPI−, apoptotic cells as AnxV+/DAPI− and necrotic ones as AnxV+/DAPI+. Representative data of four independent experiments, each performed in triplicates, are presented as mean ± SD.

Figure 2. In vivo immunogenicity of treated B16 melanoma cells. B16 mouse melanoma cells were either irradiated with 15 Gy or additionally exposed to hyperthermia (HT, 41.5°C for 1 h) and injected 30 h later s.c. into the flank of syngenic C57BL/6 mice. After 7 d, viable B16 cells were injected in the contralateral flank (A). Tumor growth was followed after immunization with B16 cells pre-treated with RT (B), RT in combination with HT (C) and RT plus HT repetitively (rep.) twice the week (D) until day 25 at the side of injected viable B16 cells. Representative data of two independent experiments, each with five mice per group, are presented as mean ± SD ***p < 0.001 determined by two-way ANOVA, Bonferroni post-test; control: PBS immunized mice.
focused on this immunization protocol in the following experiments.

Repetitive immunization with RT plus HT-treated B16 melanoma cells especially augments NK cells in draining lymph nodes

We then analyzed the draining inguinal lymph nodes of immunized mice for the presence and amount of cells from the innate and adaptive immune system. Quantifying lymphocytes of immunized versus PBS-injected mock mice (control) indicated a significant general growth in cell numbers after immunization which however was varying (Fig. 3A). Flow cytometry analysis further revealed a significant increase of CD3⁺NK1.1⁺ NK cells, CD3⁺CD19⁺ B cells, CD3⁺CD8⁺CD4⁺, and CD3⁺CD8⁻CD4⁻ T cells (Fig. 3B). Of note is that the NK cell population showed the highest relative increase in cell numbers. Specific characterization of NK cell subpopulations revealed that the immune regulatory CD27⁺CD11b⁻ population increased most extensively (Fig. 3C).

Figure 3. Immune cells in tumor draining lymph nodes of mice immunized repetitively with 15 Gy plus HT-treated B16 cells. Total cell count in tumor draining lymph nodes (sentinel) of C57BL/6 mice after repetitive (rep.) immunization with 15 Gy plus hyperthermia (HT, 41.5°C for 1 h) treated B16 cells is displayed in (A). The amount of infiltrated B cells (CD3⁺CD19⁺), NK cells (CD3⁺NK1.1⁺), T cell (CD3⁺) subpopulations (NK1.1⁺, CD8⁺CD4⁺, CD8⁻CD4⁻, CD8⁻CD4⁺) is shown in (B) and that of NK cell subpopulations (CD27⁺CD11b⁻, CD27⁺CD11b⁺, CD27⁻CD11b⁻) in (C). The analyses were performed by flow cytometry and are presented as mean ± SD. **p < 0.01; ***p < 0.001 calculated by unpaired student’s t-test.

NK cells impact on the immunization-induced retardation of B16 tumor growth in a time-dependent manner

We then asked whether and how NK cell depletion either before or after immunization might impact on tumor growth. Therefore, we eliminated NK cells systemically by NK1.1-depleting antibodies that were administered at different time points (Fig. 4A). Our experiments revealed that a lasting, weekly NK depletion during the entire experiment led to a significant acceleration of tumor outgrowth, in both immunized and control animals (Fig. 4B). Whereas the weekly NK depletion after immunization led to an accelerated tumor progress compared to immunocompetent mice, a single NK depletion 2 days before immunization contrariwise resulted in significant tumor growth retardation (Fig. 4C) as also indicated by prolonged time to tumor growth >150 mm² (Fig. 4D).

NK cell depletion before immunization with RT plus HT-treated tumor cells increases the amount of CD8⁺ and CD4⁺ T cells in lymph nodes

To further explore the influence of NK cells during the immune activation period, we examined again the draining lymph nodes and performed immune monitoring analysis in order to compare lymphocyte populations of PBS-mock (control) and immunized mice. Interestingly, we observed that a single NK depletion before the immunization with RT plus HT-treated tumor cells induced especially a significant increase of CD8⁺ T lymphocytes in the lymph nodes (Fig. 5).

Local treatment of established tumors with RT plus HT also revealed a time-dependent role of NK cell presence in tumor growth retardation

Finally, we established a therapeutic model, namely a local in situ immunization model closely resembling the clinical situation when solid tumors are exposed to local RT plus HT treatment (Fig. 6A). For this, viable B16 melanoma cells were subcutaneously injected in the right flank of the mice. Ten days later, the resulting solid tumor was locally exposed to 15 Gy of irradiation and HT with 41.5°C. Already one single local RT plus HT application sufficed to significantly retard tumor growth (Fig. 6B). Again, a long-lasting depletion of NK cells 2 days after local treatment led to an accelerated tumor growth compared to immunocompetent mice. In contrast, one single NK depletion before RT plus HT significantly decelerated tumor growth (Fig. 6B–C) in coherence to the before reported results obtained with the immunization assay (Fig. 4).

Discussion

Malignant melanoma is a very aggressive, fast growing, and early metastasizing tumor with high mortality. Beside surgery, CT and RT, a promising additional treatment option is immunotherapy including the use of cytokines, checkpoint-inhibitors, cellular immunotherapeutics as well as vaccines.54-57 Further, hints exist that HT is capable of augmenting the efficacy of RT and CT treatments in melanoma.33
In particular, the induction of an immunogenic tumor cell death has been shown to foster T-cell-mediated antitumor immune responses.9-11 Pre-treatment of B16 melanoma cells with either 15 Gy of RT or a combination with HT, resulted in both apoptotic and necrotic tumor cells (Fig. 1) and an increased release of especially HMGB1 in the tumor cell supernatant (Fig. S3). Apoptotic cells secrete lipid attraction signals for immune cells,58 and necrotic cells release DAMPs such as HMGB1.59 The latter is a highly conserved chromatin-associated nuclear protein with pleiotropic character. Extracellular HMGB1 acts as danger signal and binds with high affinity to the receptor for advanced glycation end products (RAGE) and toll-like receptors (TLR)-2, -4, and -9. It thereby activates DCs and mediates processing and cross-presentation of antigen derived from dying and dead tumor cells. Immunogenic CT and irradiation induce the release of HMGB1 and consecutive immune activation.60 Besides its role in maturing DCs and polarizing Th1 cells,61 it also enhances INFγ release of macrophage-stimulated NK cells.62 We found that the danger signals HMGB1 and HSP7063 are increasingly released in the B16 tumor cell supernatant after irradiation with 15 Gy combined with HT treatment (Fig. S3). This might contribute to enhanced immune responses against the B16 melanoma cells. Thereby, HSP70 might especially foster the activation of NK cells.64

Besides the role of HMGB1 for DC and NK cell activation, it also contributes to leukocyte attraction.65 The latter has just recently been shown to be also fostered by tumor cell autophagy.66 Since we injected the B16 tumor cells 30 h after treatment, in vivo all, apoptosis, necrosis, and autophagy might contribute to ICD of the melanoma cells. Furthermore, injected NK cells into growing tumors could provide a source of dying cells for cross-presentation and deliver stimuli for DC maturation.67 This suggests that cells of the innate and adaptive immune system act in concerted action to fight the tumor.

To analyse this in vivo immunogenicity of the melanoma cells, we followed the consensus guidelines for the detection of ICD.51 The B16 cancer cells were exposed in vitro to RT
determined in dependence of NK-depletion once before immunization. The analyses were performed by flow cytometry and are presented as mean ± SD ("p < 0.01; ""p < 0.001 calculated by unpaired student’s t-test.

Figure 5. Impact of NK cells on immune cells in tumor draining lymph nodes of mice immunized with 15 Gy plus HT-treated B16 cells. Total cell count of immune cells (B cells (CD3^−CD19^−), NK cells (CD3^+NK1.1^+), and T cell (CD3^+CD4^+CD8^+)) in tumor-draining lymph nodes (sentinel) was determined in dependence of NK-depletion once before immunization. The analyses were performed by flow cytometry and are presented as mean ± SD ("p < 0.01; ""p < 0.001).

(15 Gy) or RT plus HT, both treatments as putative inducers of ICD. These cells were then s.c. injected into one flank (immunization site) of immunocompetent syngeneic mice. 7 days later, the mice were challenged with living cancer cells of the same type, which were inoculated s.c. into the contralateral flank (challenge site). We observed that the tumor growth at the challenge site was significantly reduced when the mice were especially repetitively immunized before with RT plus HT-treated B16 cells (Fig. 2). This indicates that the immunogenicity of B16 melanoma cells can be induced by these combined treatments and is further substantiated by the fact that a significant increase of CD4^+ and CD8^+ T cells in the sentinel lymph node in immunized compared to not immunized mice was detected (Fig. 3B). Furthermore, especially immune regulatory CD27^−CD11b^− NK cells augmented in lymph nodes of before immunized mice (Fig. 3C).

As NK cells impressively increased in immunized compared to PBS-mock injected mice (Fig. 3B), we further quantified the increase of the different NK cell subpopulations CD27^+CD11b^−, CD27^+CD11b^+ and CD27^−CD11b^+ known for their distinct functional characteristics. Interestingly, a major increase in CD27^−CD11b^− NK cells known for their immune regulatory ability was observed (Fig. 3C). These results are in line with previous reports in the B16 melanoma model indicating that besides mature NK cells with cytotoxic function, a high number of CD27-expressing IFNγ-producing NK cells efficiently protects from metastatic spread and supports antitumor functions. Furthermore, our data underlines the highly immunogenic potential of local tumor treatment with RT and further immune stimulation. In immunized animals, a higher variation of the total cell count in tumor draining lymph nodes was observed (Fig. 3A). However, no correlation between the number of total cells and intensity of tumor growth retardation was observed.

By experiments in immune-deficient NK-cell-depleted mice, we showed that the high immunogenic capacity of repetitive injected irradiated and heated tumor cells is not only mediated through the adaptive, but also the innate arm of immunity (Figs. 4–6). To especially examine whether NK cells are only important as antitumor effector cells or also during the immune activation period, we depleted NK cells for the entire period or before a sole immunization with the treated melanoma cells (Fig. 4A). Permanent depletion of NK cells during the entire experiment accelerated the tumor growth in both PBS-mock treated and immunized mice, thereby confirming their important role for antitumor protection. We here got first hints that a memory response is inducible by immunization with RT plus HT-treated B16 cells, since more tumor cells were specifically stained by antibodies present in serum of immunized mice. This is dependent on NK cells, since the percentage of stained tumor cells was decreased when NK cells were depleted in the immunized mice (Fig. S4). It is important to note, that a lasting NK cell-depletion weekly after immunization ended up in accelerated tumor growth, whereas one singular NK-depletion before the immunization significantly retarded tumor growth (Fig. 4C–D). These results suggest that NK cells might prevent a competent immune activation during the immunization phase. The detailed mechanistic basis for this should be subject of intensive future research.

We speculate that besides the important cross-talk between NK cells and DCs, NK cells lyse not only malignant tumor cells but also T cells in the tumor milieu induced by their degranulation after activation. One already described mechanism is that NK cells shape the CD8^+ T cell fate by killing recently activated CD8^+ T cells in NKG2D- and perforin-dependent manner. In line with that we observed a significant increase of CD8^+ T cells in the absence of NK cells (Fig. 5). This suggests an elimination of cytotoxic local T cells by activated NK cells in the draining lymph node.
We found that tumor growth can be significantly delayed. Secondly, as also suggested by the ICD recommendations, we performed the therapeutic assay, namely a local irradiation of an established B16 tumor with 15 Gy and additional local HT treatment (Fig. 6A), as described just recently by our group. We found that tumor growth can be significantly delayed.

In both scenarios, namely in the immunization and the therapeutic assay, our main aim was to examine for the first time which role NK cells do play in the immunization phase and later on in the tumor response phase. To achieve complete tumor rejection, future studies are needed with fractionated irradiation of the tumor with e.g. 10 × 2 Gy and repeated local HT treatment. For this, large animal cohorts will be necessary to define the best fractionation scheme and chronological sequence of the applications. According the findings of the immunization model with pre-treated B16 cells (Fig. 4C–D), again a weekly NK cell depletion after local treatment accelerated the tumor growth, while a single NK cell depletion before treatment retarded the tumor growth significantly (Fig. 6B–C).

To summarize, combined treatment of melanoma cells with X-ray and HT results in immunogenic tumor cells and consecutive involvement of T cells and NK cells in antitumor responses. Hereby, we identified a dual role of NK cells. On the one hand, NK cells are important for tumor cell killing after initiation of antitumor immunity, but on the other hand NK cells might be disadvantageous during the immune activation period. This knowledge is important for the design of future radio-immunotherapies, since not only the right combination but also the chronological sequence combining RT with further immune therapies matters. Despite their direct antitumor effects, NK cells might have a negative influence on the generation of the favored T-cell-mediated immunity induced during the immunization period.

Materials and methods

Animals

C57BL/6 mice were obtained from Elevage Janvier (C57BL/6NRj) and used at 7–9 weeks of age. All mice were kept and bred under sterile atmosphere at the animal facility of the Universitätsklinikum Erlangen. The animal procedures have been approved by the "Regierung of Mittelfranken" and were conducted in accordance with the guidelines of Federation of European Laboratory Animal Science Associations (FELASA). Mice were euthanized when reaching a tumor volume of 1700 mm³. This volume was calculated three times the week by a well-established formula: \( V_{\text{Tumor}} = \frac{1}{2} \cdot (L \cdot B^2). \)

Culture and treatment of B16 cells

The mouse melanoma cell line B16-F10 (ATCC, # CRL-6475) derived from C57BL/6 mice was cultured in RPMI 1640 medium with stable glutamine (Sigma-Aldrich, # R8758), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom, # S0615), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, # 15140–122). The cells were tested negatively for mycoplasma contamination and maintained in 5% CO₂ atmosphere at 37°C and 95% relative humidity. The cells were used when they reached 90% confluence. 4 × 10⁶ B16 cells were cultured in culture bottles (surface of growth 75 cm²; Greiner BioOne, # 658175) with 15 mL of the described culture medium.

The tumor cells were irradiated with an X-ray generator (120 kV, 22.7 mA, 2 min; GE Inspection Technologies) with a single dose of 15 Gy. For HT, the melanoma cells were exposed to heat in a homemade device placed in a cell incubator as described previously. The variations of the temperature during the treatment were less than 0.2°C. The cells remained at stable 41.5°C for 1 h. For combined applications, the tumor cells were stored at 37°C for 4 h between RT and HT treatment.

\[V_{\text{Tumor}} = \frac{1}{2} \cdot (L \cdot B^2).\]
**Analysis of tumor cell death**

To detect viable, apoptotic, and necrotic melanoma cells, we used the AnnexinV-DAPI-Assay. The treated melanoma cells were stained at different time points with AnnexinV-FITC (Immunotools, # 31490013) and DAPI (Applichem, # A1001). After 30 min of incubation at 4°C in the dark, the samples were analyzed by flow cytometry. Double negative cells were defined as viable, AnxV⁺/DAPI⁻ as apoptotic, and AnxV⁺/DAPI⁺ as necrotic ones. The gating strategy as well as the dot blots are depicted in Fig. S1.

**Analyses of HMGB1 and HSP70**

The expression of intracellular HMGB1 and HSP70 was semi-quantitatively determined in cell lysates by Western blot technique using standard protocols. To detect the concentration of the DAMP HMGB1 in the supernatant of treated B16 cells, we also used the Western Blot technique. 30 h after the respective treatments, the supernatants were concentrated by centrifugation (Millipore-strainers (NMWI = 3000) 40 min, 4°C, 4000 rpm). 25 μg of protein was loaded on SDS-pagess and separation was performed by electrophoresis. After blotting, the protein on nitrocellulose membranes, the proteins of interest were stained with the following antibodies: mouse mAb anti-HMGB1 (abcam, # ab12029) and mouse mAb anti-HSP70 (BD Transduction, # 610608). Anti-mouse HRP conjugated Ab (upstate, Merck Millipore, # 12–349) was used as second step reagent and chemiluminescence was detected with Amersham ECL hyperfilms (GE Healthcare, # 2896836). For detection and quantification of total HSP70 in supernatants of tumor cells, the ELISA DuoSet IC Kit (R&D Systems, # DYC1663) was used according to the manufacturer’s instructions.

**Determination of the in vivo immunogenicity of treated melanoma cells**

0.5 × 10⁶ pre-treated B16 melanoma cells were subcutaneously injected at day -7 in the left hind flank (near thigh) of the mice. Thirty hours before injection, these cells were either irradiated with 15 Gy (RT), exposed to 60 min of HT or a combination of RT plus HT. Seven days later (d0) the mice were challenged by injecting subcutaneously 0.2 × 10⁶ viable melanoma cells in the contralateral right hind flank (near thigh, Fig. 2A). Hereafter, the tumor growth was measured at the side of tumor injection at least three times/week. This volume was calculated by the well-established formula: \( V_{\text{Tumor}} = \frac{1}{2} \cdot (L \cdot B^2) \). We compared single and repeated immunization. The repetitive immunization protocol consisted of immunization with 0.5 × 10⁶ pre-treated B16 melanoma cells twice/week. In several experiments, NK cells were depleted a single time at day -9 (single NK-depl. before immunization) or continuously starting at day -9 (weekly NK-depl.) or starting at day -2 (weekly NK-depl. after immunization) (Fig. 4A).

**Local in situ immunization model**

The therapeutic in situ immunization model (Fig. 6A) closely resembles the clinical local treatment of solid tumors by RT and HT. For this, 0.2 × 10⁶ untreated B16 melanoma cells were subcutaneously injected in the right hind flank (near thigh) of the mice. 10 d afterwards, the melanomas were solid visible in the skin and represented good targets for local treatments. To irradiate the tumor bearing mice, a Plexiglas® box was manufactured which allows the locally irradiation of three mice at once. Before placing them into the box, the mice were anesthetized with Isoflurane. Then, the tumors were locally irradiated with a dose of 15 Gy using a linear accelerator (6MV, PRIMART, Siemens). The planning of the irradiation was conducted using a computer tomography image of the irradiation box and tumor bearing mice with Philips pinnacle software to obtain an optimal target volume. To further protect normal tissue, the gantry of the 6MV linear accelerator was drifted to 340°. HT was performed 4 h after irradiation. For this, the mice were anesthetized and the tumors were heated locally under temperature control to 41.5°C for 30 min using the BSD50 HT system (Dr Sennewald Medizintechnik), as described earlier.34

**Analysis of immune cells in draining lymph nodes**

To analyze immune cell populations in the draining lymph nodes, the cells were isolated by slitting the organs and pressing them through 100 μm mesh cups (Corning, # 52360) to generate single-cell suspensions. 2 × 10⁶ cells were stained with different combination of the following antibodies: CD11c-FITC (# 553801), CD4-PE (# 553730), CD27-PE (# 558754), NK1.1-PE-Cy7 (# 552878), 7AAD (# 559925) CD19-PE-Cy5, (# 560375), CD11b-PE-Cy5 (# 560455) all from BD Biosciences; CD3e-APC (# 17–0031) from eBioscience; AnnexinV-FITC from Immunotools (# 31490013) and CD8a-FITC from Miltenyi (# 130–102–806). A minimum of 5 × 10⁶ events were detected per measurement. Flow cytometry was performed on a FACS Canto II (BD Biosciences, Heidelberg, Germany) and analyzed using FlowJo Software v7.6.5 (Treestar). The gating strategy is representatively depicted in Fig. S2.

**Detection of tumor cell-specific antibodies in serum of mice**

The tumor cell-specific antibodies (against B16-F10 cells) in the sera of mice were analyzed by the indirect immune fluorescence method as described previously.78 At the end of the observation period the blood of the mice was drawn into BD microtainer SST™ tubes (BD, # 365951) and serum was prepared by centrifugation of the tubes according to the manufacturer’s instructions. Serum was freshly used or stored at −80°C. For detection of the indirect immunofluorescence, viable target cells were incubated with the serum of the respective mouse for 60 min at 4°C in the dark. Afterwards, the cells were washed with PBS (SigmaAldrich, # D8537) including 10% inactivated FBS to block unspecific binding and to remove unbound antibodies, respectively. The tumor cell specific antibodies were detected by flow cytometry using a fluorescein-tagged F(ab′)2 fragment of goat anti-mouse IgG (Invitrogen, Molecular Probes, # F11021).
**NK cell depletion in vivo**

NK cell depletion was performed by using NK1.1-depleting antibodies (PK136). Antibodies were obtained from ascites of nu/nu mice following intraperitoneal application of PK136 hybridoma cells that was kindly provided by Prof. Zitvogel (Institute Gustave Roussy, Villejuif, France). The purification of the monoclonal NK1.1-depleting antibody was performed by affinity chromatography using HiTrap column, prepacked with Protein G Sepharose High Performance (GE Healthcare Life Sciences). Former experiments showed that NK cells proliferate at the time point when tumor growth exceeded a surface of 150 mm². We repeated the depletion weekly.

**Statistical analysis**

Statistical analysis was performed as indicated in the figure legends by use of Graphpad Prism Software v5.0. One-way ANOVA Bonferroni was used to compare the correlation between tumor growths in different treatment groups. One-way ANOVA Bonferroni post-test was performed to analyze the legends by use of Graphpad Prism Software v5.0. 2way ANOVA Bonferroni post-test was performed to analyze the correlation between tumor growths in different treatment groups.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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