Dissecting the role of hyperthermia in natural killer cell mediated anti-tumor responses

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Online Publication Date: 01 January 2008
To cite this Article: Dayanc, Baris E., Beachy, Sarah H., Ostberg, Julie R. and Repasky, Elizabeth A. (2008) 'Dissecting the role of hyperthermia in natural killer cell mediated anti-tumor responses', International Journal of Hyperthermia, 24:1, 41 - 56
To link to this article: DOI: 10.1080/02656730701858297
URL: http://dx.doi.org/10.1080/02656730701858297
Dissecting the role of hyperthermia in natural killer cell mediated anti-tumor responses

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(Received 17 October 2007; revised 21 November 2007; accepted 11 December 2007)

Abstract
The effects of hyperthermia on natural killer (NK) cell cytotoxicity against tumor cell targets are not yet fully understood. A more complete understanding of these effects could be important for maximizing the clinical benefits obtained by using hyperthermia for cancer therapy. Here, we summarize results in the literature regarding the effects of elevated temperatures on NK cells and our own recent data on the effects of fever-range temperatures. At treatment temperatures above 40 °C, (which is above the physiological body temperatures normally achieved during fever or exercise), both enhancing and inhibitory effects on cytotoxic activity of NK cells against tumor cells have been reported. Our own results have shown that fever-range thermal stress (using a temperature of 39.5 °C) enhances human NK cell cytotoxicity against tumor target cells. This effect requires function of the NKG2D receptor of NK cells, and is maximal when both NK and tumor cell targets are heated. Reported heat sensitive cellular targets affected by hyperthermia on tumor cells include heat shock proteins, MICA and MHC Class I. In NK cells, plasma membrane reorganization may occur after mild heat stress. We conclude this review by listing several unresolved questions that should be addressed for a more complete understanding of the molecular mechanisms which underlie the effects of thermal stress on the function of NK cells. Altogether, the available data indicate a strong potential for heat-induced enhancement of NK cell activity in mediating, at least in part, the improved clinical responses seen when hyperthermia is used in combination with other therapies.

Keywords: Fever-range hyperthermia, tumor, heat shock, NK cell cytotoxicity, NK cell receptors and ligands

Introduction
Reports that link the occurrence of fevers with improved recovery from cancer have been cited in the literature for well over a century [1], and various protocols to artificially induce hyperthermia have also been investigated as tools for treating cancer patients in combination with other therapies [2]. Recent clinical trials utilizing the addition of heat to the tumor site during treatment with radiation continue to provide very encouraging data regarding overall clinical benefit (i.e. complete response rate, overall and relapse free survival) of this combination [3–7, 8]). These studies have renewed enthusiasm for combining hyperthermia with radiation, and recent studies also confirm the strong clinical benefit of combining hyperthermia with chemotherapy [9]. However, the molecular or cellular events contributing to overall survival benefits seen by adding hyperthermia to radiation or chemotherapy are not entirely clear. While the historical origin of the use of hyperthermia has been linked to the potential anti-cancer benefits observed when patients developed high fevers, modern studies have largely focused on two non-exclusive explanations of why hyperthermia enhances efficacy of radiation or chemotherapy: (1) the direct cytotoxic effects of high temperatures
observed in tumor cells and intra-tumoral vascular structures, which could be particularly feasible during local hyperthermia treatments [4, 10]; or (2) the indirect effects of elevated temperatures on blood flow, or on other aspects of the tumor microenvironment. Most literature on the indirect effects of hyperthermia center on tumor re-oxygenation which could result in enhanced efficacy of radiation treatment [11–14] and reviewed in [4, 10]. However, an emerging set of literature [15] identifies the potential of hyperthermia to serve as an adjuvant to the immune system, helping to provoke stronger anti-tumor responses. Hyperthermia, particularly in the mild, physiologically relevant temperature range, may trigger long-conserved, heat sensitive molecules in cells of the immune system that have evolved to respond to the thermal element of fever, which occurs as a host inflammatory response to viral or bacterial challenges to the immune system. Recent evaluations [16–20] strongly support thermal enhancement of anti-tumor immunity as at least part of the basis for improved long-term survival of patients given hyperthermia since intra-tumoral temperatures high enough to cause direct thermal killing of tumor cells is not as commonly achieved in the clinical setting as was previously believed [4]. However, even if non-physiologically high temperatures are used in the clinic, delivered locally at the site of the tumor, causing rapid tumor or vascular endothelial cell death, other regions of the body will eventually be warmed by dissipated heat; thus, even under tumor ablative thermal conditions, there is still considerable potential for enhancement of the immune system by milder hyperthermia in the surrounding tissue.

In terms of identifying which immune effector cells could play critical roles in mediating the improved survival seen when hyperthermia is combined with cancer treatment, NK cells have emerged as one likely possibility. NK cells have already been recognized as being able to recognize and kill tumor cells treated at heat shock temperatures [21] through recognition of heat shock proteins on the surface of the tumor cell. Major questions which need to be addressed include whether there is an optimal temperature range or duration of heating for maximal enhancement of NK cell cytotoxicity, or whether various forms of heating (e.g. ultrasound, microwave or infrared) differentially affect NK cell function. In order to gain some understanding of these questions, it is important to review previous literature on cellular and molecular effects of increased temperature on NK cells, with a particular focus on studies that address anti-tumor activity. We summarize studies on the effects of elevated temperatures on NK cell distribution and number, tumor infiltration and cytotoxic function, and on temperature dependent sensitivity of tumor cells to NK cell killing. We attempt to separate enhancing and inhibitory effects, and to correlate these effects with the temperatures used for the study (e.g. heat shock conditions versus mild, physiologically relevant elevations in temperature) (see Table I). We conclude this review with a set of questions which should be addressed in order to fully understand the role of NK cells in mediating the positive benefits of clinical hyperthermia.

Key role of NK cells in anti-tumor immunity

NK cells are a subset of the innate immune system and play a major role in the control of tumor cells via direct cytotoxicity against target cells exhibiting altered MHC Class I structure or levels on their cell surface [22, 23]. Unlike adaptive immune responses, NK cells do not require prior sensitization with an antigen, but when sensitized to haptens, recent studies have shown that NK cells have potential for generating memory responses [24]. An NK cell response precedes the adaptive anti-tumor immune response and is critical for downstream tumor-specific T cell memory responses mediated by CD27–CD70 interaction [25].

The earliest phase of NK cell activation, termed the ‘non-specific phase’, precedes the triggering of any receptors on the surface of NK cells. Pro-inflammatory cytokines in the microenvironment have been shown to regulate the non-specific phase of NK cell activation [26]. TNF-α is known to recruit NK cells to the inflammatory sites [27, 28], where pro-inflammatory cytokines like IL-2, IL-7, IL-12, IL-15 and Type I IFNs [26, 29–32] are known to rapidly activate them. Importantly, some of these cytokines that activate NK cells (e.g. TNF-α, IFN-α/β) are also ‘pyrogenic’ in that they stimulate an increase in body temperature (fever), which occurs during infection and inflammation [33, 34]. Although there is a temporal link between NK cell activation and the pyrogenicity of these cytokines, whether there is a specific role for increased temperature in regulation of NK cell function is not yet fully understood.

Following the cytokine-associated ‘non-specific’ activation phase, induction of cytotoxicity for NK cells is further regulated by a complex mechanism of balance between activating, e.g. NKG2D [35–37], natural cytotoxicity receptors (NCR) (i.e. NKp46 [38], NKp30 [39] and NKp44 [40, 41], and several inhibitory receptors (i.e. KIR and CD94-NKG2 heterodimers [42, 43]). The magnitude of NK cell activation is a function of altering the balance in the signaling strength between activating and inhibitory receptors. If the balance shifts towards...
activating receptors, NK cells respond with secretion of effector molecules leading to the lysis of their targets. This fine tune of activation causes NK cells to play a major role in immunosurveillance of tumors [44]. During the formation of an immunological synapse between an NK cell and its target cell, receptors on NK cells reorganize into clusters that are associated with the lipid raft fraction of the NK cell plasma membrane [45]. Intriguing experimental evidence (summarized below) suggests that such receptor-associated activity of NK cells may indeed be thermally sensitive. However, further characterization of the mechanisms by which physiologically relevant temperatures affect NK cell-mediated cytotoxicity is essential.

NK cells in clinical hyperthermia studies

Identification of NK cells as critical effector cells in immune responses mediated by clinical use of local and systemic hyperthermia resulted in further interest for the study of this cell type in detail. Clinical studies reviewed below have investigated effects of hyperthermia on several parameters of NK cell function in patients, including NK cell cytotoxicity and changes in peripheral NK cell numbers (summarized in Figure 1 and Table I).

Effects on NK cell cytotoxic activity

The role of hyperthermia on NK cell function was first investigated over 25 years ago, in a study by Zanker et al., which reported an enhancement of NK cell cytotoxicity with whole body hyperthermia at 41.8°C in a patient with Ewing’s sarcoma [46]. In support of this observation, isolated limb perfusion in patients with malignant melanomas (40.5–41.8°C for 1 hour) resulted in enhanced NK cytotoxicity of peripheral blood cells during heating [47]. Furthermore, treatment of prostate carcinoma patients with local transrectal microwave hyperthermia (45°C, 30 min, six treatments) resulted in a significant increase of their NK cytotoxic activity against K562 target cells in vitro [48]. Another study found that cancer patients who received whole body hyperthermia (40.5°C, 75 min) and IFN-γ therapy also demonstrated increased NK cell cytotoxicity, but this increase was not statistically significant when compared to the group who received IFN-γ alone [49]. Application of local hyperthermia to healthy human subjects had no effect on the cytotoxic activity of peripheral blood-derived NK cells in this study. When mild whole body hyperthermia at 38°C core temperature was applied to healthy volunteers, an enhanced NK cell activity was again observed [50]. In summary, although the extent of the effect varies, clinical studies appear to generally reveal an enhancement of NK mediated cytotoxic activity upon hyperthermia treatment of either cancer patients or healthy subjects.

Effects on peripheral NK cell numbers or distribution

Increased NK cell numbers in peripheral blood can concur with enhanced cytotoxic activity measurements, and thus support the clinical outcome.

Figure 1. Summary of preclinical and clinical studies that have investigated NK cell responses to hyperthermia. NK cells can be activated through a series of activating and inhibitory receptors, and the balance of these signals decides the fate of NK cell cytotoxic activity. The reported effects of hyperthermia on NK cell cytotoxicity, distribution in the periphery and cell number and infiltration into tumor beds are summarized in this schematic.
Table I. Chronological summary of studies investigating the effects of hyperthermia on NK cell function.

<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
<th>Model</th>
<th>Temperature &amp; duration</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>[60]</td>
<td>Human: peripheral blood lymphocytes of healthy donors against K562 cells, <em>in vitro</em> hyperthermia</td>
<td>38.5–40°C, 2,4,12 hr</td>
<td>NK cell activity decreased with hyperthermia</td>
</tr>
<tr>
<td>1982</td>
<td>[46]</td>
<td>Human: whole body hyperthermia of patients with Ewing’s sarcoma</td>
<td>41.8°C</td>
<td>Increased NK activity</td>
</tr>
<tr>
<td>1982</td>
<td>[61]</td>
<td>Human: peripheral blood lymphocytes versus K562 cells, <em>in vitro</em> hyperthermia</td>
<td>38–40°C during cytotoxicity assays</td>
<td>Decreased NK cell activity at 38°C and above</td>
</tr>
<tr>
<td>1983</td>
<td>[110]</td>
<td>Human: lymphocytes from healthy volunteers. NK activity measured against K562, <em>in vitro</em> hyperthermia</td>
<td>37–42°C, 3 hr</td>
<td>At 42°C, 90% of cytotoxic activity was lost, slight decrease in lymphocyte viability with thermal stress</td>
</tr>
<tr>
<td>1983</td>
<td>[111]</td>
<td>Human: peripheral blood NK cells from volunteers, <em>in vitro</em> hyperthermia</td>
<td>42°C, 1 hr</td>
<td>Viable cells, abolished NK activity</td>
</tr>
<tr>
<td>1986</td>
<td>[112]</td>
<td>Hamster: <em>in vivo</em> ultrasound hyperthermia on spleen</td>
<td>38–43°C, 8 min (500 sec)</td>
<td>Significant depression of NK cytotoxic activity in 4 hr, back to normal in 24 hr. Lymphopenia at 4, 8, 16 hr, recovers within 48 hr</td>
</tr>
<tr>
<td>1987</td>
<td>[113]</td>
<td>Human: PBMC, <em>in vivo</em> hyperthermia</td>
<td>39°C</td>
<td>NK activity was increased with elevation of core body temperature</td>
</tr>
<tr>
<td>1987</td>
<td>[114]</td>
<td>Mouse: spectrin distribution in lymphocytes, whole body hyperthermia</td>
<td>40.5°C, 1.5 hr</td>
<td>Increase in polar spectrin aggregates in splenic lymphocytes, but not in thymic lymphocytes</td>
</tr>
<tr>
<td>1988</td>
<td>[115]</td>
<td>Mouse: whole body hyperthermia, in combination with cyclophosphamide</td>
<td>39.5–40°C, 30 min</td>
<td>Higher NK activity, with hyperthermia or in combination. Prolonged lifespan is probably NK mediated</td>
</tr>
<tr>
<td>1989</td>
<td>[116]</td>
<td>Human: <em>in vitro</em> hyperthermia of PBMC</td>
<td>44°C</td>
<td>NK activity reduced to 4.3% after treatment</td>
</tr>
<tr>
<td>1990</td>
<td>[117]</td>
<td>Rat: <em>in vitro</em> hyperthermia from rat lung, peripheral blood and spleen</td>
<td>42.5°C, 30 min</td>
<td>95% inhibition of NK activity</td>
</tr>
<tr>
<td>1990</td>
<td>[118]</td>
<td>Mouse: NK activity from C3H mice, local hyperthermia</td>
<td>43°C, 45 min</td>
<td>NK activity suppressed, lowest at 2 days then significantly enhanced on day 7, counteracted with liposome encapsulated recombinant human superoxide dismutase</td>
</tr>
<tr>
<td>1991</td>
<td>[119]</td>
<td>Human: PBMC from healthy volunteers, water immersion whole body hyperthermia</td>
<td>41°C, 39.5°C, 2 h</td>
<td>Number and IL2 enhanced NK cell activity was increased during hyperthermia. Elevated stress hormone levels</td>
</tr>
<tr>
<td>1991</td>
<td>[120]</td>
<td>Mouse: virally infected mice, treated with M-CSF and hyperthermia, splenic NK activity measured. Whole body microwave hyperthermia</td>
<td>38.8°C–40.2°C</td>
<td>M-CSF or hyperthermia treatment alone has no effect; combination therapy restores NK activity to normal</td>
</tr>
<tr>
<td></td>
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<td>43°C, 30 min</td>
<td>80–90% decrease</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>44–45°C, 30 min</td>
<td>100% decrease, (NK cells still show recognition and binding, competitive inhibition of unheated NK cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Same treatments for target cells</td>
<td>No effect</td>
</tr>
<tr>
<td>1991</td>
<td>[122]</td>
<td>Mouse: OK-432 (Picibanil) effects on C3Hf1/Sld mice with fibrosarcoma (FSa-II), <em>in vivo</em> and <em>in vitro</em> hyperthermia</td>
<td>44°C, 30 min</td>
<td>Thermal enhancement is eliminated with asialo-GM1. NK activity is stimulated when OK-432 is locally administered with hyperthermia</td>
</tr>
<tr>
<td>1991</td>
<td>[48]</td>
<td>Human: transrectal hyperthermia of prostate</td>
<td>45°C, 30 min</td>
<td>Significant increase in NK cytotoxic activity</td>
</tr>
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<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
<th>Model</th>
<th>Temperature &amp; duration</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>[123]</td>
<td>Rat: splenic NK cells, <em>in vitro</em> hyperthermia using a water bath</td>
<td>41°–42.5°C, 30 min</td>
<td>Binding of NK to targets is not affected by hyperthermia. MTOC reorientation reduced by 40%, 62–77% inhibition of NK lytic activity, lytic activity peaked at 6 hr</td>
</tr>
<tr>
<td>1993</td>
<td>[69]</td>
<td>Mouse: local microwave hyperthermia of mice with B16. F10 melanoma in combination with IL2</td>
<td>42°C, 30 min 43°C, 15 min</td>
<td>Infiltration of macrophages and NK cells observed</td>
</tr>
<tr>
<td>1993</td>
<td>[67]</td>
<td>Mouse: local microwave hyperthermia of B16. F10 melanoma in combination with IFN-β</td>
<td>43°C, 15 min</td>
<td>Tumor infiltration of NK cells with hyperthermia. Infiltration is lost with combined therapy</td>
</tr>
<tr>
<td>1993</td>
<td>[124]</td>
<td>Mouse: M-CSF and local hyperthermia treatment of B16 melanoma</td>
<td>43°C, 30 min twice a week for 2 weeks</td>
<td>Combined therapy prolonged survival, restoration of NK activity and TNF-α levels</td>
</tr>
<tr>
<td>1994</td>
<td>[125]</td>
<td>Human: effects of exercise to NK cell function</td>
<td>Natural body temperature increase after exercise</td>
<td>Acute exercise increases NK cell function, possible relation to hyperthermia. Severe exercise suppresses NK cell function through prostaglandins</td>
</tr>
<tr>
<td>1994</td>
<td>[76]</td>
<td>Mouse: Splenic lymphocytes from C3H/HeN mice treated with poly I:C and IFN-α, measured against YAC-1 targets, <em>in vitro</em> hyperthermia</td>
<td>41°–43°C 40°C, 1 hr</td>
<td>Thermotolerance Poly I: C or IFN-α treated cells rendered resistant to hyperthermia at 42°C. Viral infection, fever and IFN-α can render NK cells resistant to stress</td>
</tr>
<tr>
<td>1997</td>
<td>[78]</td>
<td>Human: Ocular Melanoma, <em>in vitro</em> hyperthermia using a water bath</td>
<td>39°–45°C 45°C, 30 min</td>
<td>No significant difference in NK cell susceptibility of heated versus unheated cells Reduced HLA-I and β-microglobin levels, elevated Hsp70, but unchanged Hsp60 levels</td>
</tr>
<tr>
<td>1997</td>
<td>[79]</td>
<td>Human: Ewing’s sarcoma, K562 leukemia and EBV transformed B cells from peripheral blood of healthy volunteers, <em>in vitro</em> hyperthermia</td>
<td>41.8°C followed by 37°C recovery</td>
<td><em>De novo</em> increase in HSP72 levels after hyperthermia is related to increased sensitivity of lysis by NK cells</td>
</tr>
<tr>
<td>1997</td>
<td>[66]</td>
<td>Mouse: B16 melanomas of different sizes treated with ethanol injection, IL-2 and local microwave hyperthermia</td>
<td>43°C, 15 min</td>
<td>Increased infiltration of NK cells</td>
</tr>
<tr>
<td>1997</td>
<td>[47]</td>
<td>Human: hyperthermic isolated limb perfusion (HILP) of melanoma patients, local hyperthermia</td>
<td>40.5–41.8°C, 60 min</td>
<td>NK cell activity increased with HILP</td>
</tr>
<tr>
<td>1998</td>
<td>[58]</td>
<td>Mouse: SCID mice with human breast xenografts, whole body hyperthermia</td>
<td>39.8°C, 6–8 hr</td>
<td>Increased NK cell infiltration, depletion of NK cells inhibits tumor cell apoptosis</td>
</tr>
<tr>
<td>1998</td>
<td>[126]</td>
<td>Human: healthy volunteers with catecholamine, growth hormone and β-endorphin blockade, immersion in water bath up to the neck</td>
<td>39.5°C, 2 hr</td>
<td>Hyperthermia induced recruitment of NK cells to peripheral blood, unaffected with hormone blockade. NK activity (both naïve and IFN-α/ IL-2 stimulated) did not change</td>
</tr>
<tr>
<td>1998</td>
<td>[82]</td>
<td>Human: renal cell carcinoma ACHN <em>in vitro</em> hyperthermia</td>
<td>41.8°C, 3 hr</td>
<td>Hyperthermia enhanced NK cytotoxicity of ACHN cells, blocked with anti-Hsp72 antibodies</td>
</tr>
<tr>
<td>1999</td>
<td>[64]</td>
<td>Rat: hyperthermia with immersion into waterbath, induced peritonitis 8 hours after hyperthermia</td>
<td>42°C, 15 min</td>
<td>Hyperthermia protective in sepsis reducing severity of peritonitis. Possible enhancement of immune effectors including NK cells</td>
</tr>
<tr>
<td>2000</td>
<td>[127]</td>
<td>Human: effect of <em>in vitro</em> hyperthermia on activity and generation of IL-2 activated NK cells</td>
<td>42°C, 1 hr</td>
<td>Hyperthermia transiently reduced lytic activity of NK cells. Heating target cells alone did not substantially modify their susceptibility to lysis induced by either NK or IL-2 activated NK cells</td>
</tr>
<tr>
<td>Year</td>
<td>Reference</td>
<td>Model</td>
<td>Temperature &amp; duration</td>
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<tr>
<td>2000</td>
<td>[128]</td>
<td>Human and mouse: fibroblasts and NK cells, <em>in vitro</em> hyperthermia</td>
<td>39°C, 4-24 hr</td>
<td>Suppression of IFN mediated enhancement of NK cell activity in human and murine cells. Significant increase of both antiviral and antiproliferative activities of all three human interferons</td>
</tr>
<tr>
<td>2000</td>
<td>[50]</td>
<td>Human: healthy volunteers with whole body hyperthermia in 39°C water bath</td>
<td>38°C core temperatures</td>
<td>Increased NK cell count and activity</td>
</tr>
<tr>
<td>2000</td>
<td>[75]</td>
<td>Human: peripheral blood non-adherent cells, cytotoxicity measured against K562 and Raji cells, <em>in vitro</em> hyperthermia</td>
<td>42-42°C 1 hr, then cultured 7 days with IL-2</td>
<td>Cytotoxicity decreased at 42°C, but did not change at 40°C</td>
</tr>
<tr>
<td>2000</td>
<td>[129]</td>
<td>Mouse: IL-2 activated NK cells against T cell blasts, Cr51 assay, <em>in vitro</em> hyperthermia</td>
<td>40–41°C, 1–4 hr</td>
<td>Enhanced susceptibility to killing by syngenic IL-2 activated NK cells</td>
</tr>
<tr>
<td>2001</td>
<td>[130]</td>
<td>Rat: jaundice model, translocation of bacteria from gut and immune response, <em>in vivo</em> hyperthermia with water bath immersion</td>
<td>42°C, 15 min</td>
<td>NK cell number is elevated in hyperthermia group with respect to control</td>
</tr>
<tr>
<td>2001</td>
<td>[80]</td>
<td>Rat: T9 glioma cells, <em>in vitro</em> hyperthermia in a water bath</td>
<td>43°C 1 hr</td>
<td>Elevated MHC I and HSP70 levels (stable MHC II and ICAM levels). Splenic lymphocytes of rats injected with heated T9 cells displayed enhanced specific cytotoxicity</td>
</tr>
<tr>
<td>2002</td>
<td>[51]</td>
<td>Human: patients with advanced solid tumors, whole body hyperthermia</td>
<td>39–39.5°C, 3–6 hr 39.5–40°C, 6 hr</td>
<td>Transient decrease in number of circulating lymphocytes</td>
</tr>
<tr>
<td>2002</td>
<td>[81]</td>
<td>Human: osteo and chondrosarcoma cell targets, NK cells from peripheral blood of healthy donors, <em>in vitro</em> hyperthermia</td>
<td>42.5°C, 90 min</td>
<td>Lysis by NK cells was increased by heat treatment of the target cells. Hyperthermia increases HSP72 expression in sarcoma cells and their susceptibility to NK cell mediated lysis</td>
</tr>
<tr>
<td>2002</td>
<td>[65]</td>
<td>Rat: peritonitis model, <em>in vivo</em> hyperthermia</td>
<td>40°C and 42°C, 4 hr</td>
<td>NK cell amounts are found to be increased in the control peritonitis group. Hyperthermic preconditioning prior to peritonitis induction returned parameters to control levels</td>
</tr>
<tr>
<td>2002</td>
<td>[131]</td>
<td>Human: patients with various malignant diseases, whole body hyperthermia</td>
<td>41.8°C, 1 hr</td>
<td>Immediately after hyperthermia, drastic increase in peripheral NK cells, lost within 5 hr. CD56+ cells reach maximum number 48 hr after hyperthermia</td>
</tr>
<tr>
<td>2003</td>
<td>[132]</td>
<td>Human: colorectal, germ line and ovarian cancer patients, multiple hyperthermia treatments, whole body hyperthermia</td>
<td>41.8°C, 1 hr</td>
<td>Significant elevation of NK cell numbers during hyperthermia. Levels dropped significantly 20 hr after with apoptosis</td>
</tr>
<tr>
<td>2005</td>
<td>[90]</td>
<td>Mouse: B16 melanoma in combination therapy with hyperthermia and DC, hyperthermia with magnetic cationic liposomes</td>
<td>43°C, 30 min</td>
<td>Increased NK cell activity of splenocytes from combination-therapy cured mice</td>
</tr>
<tr>
<td>2005</td>
<td>[52]</td>
<td>Human: patients with advanced malignancies, whole body hyperthermia</td>
<td>41.8°C–42.2°C</td>
<td>NK cell numbers increased, plasma IL-12 levels and IFN-γ/IL-10 ratio were decreased transiently</td>
</tr>
<tr>
<td>2005</td>
<td>[86]</td>
<td>Mouse: Tumors in C57BL/6 mice, whole body hyperthermia</td>
<td>40°C, 2 hr daily</td>
<td>No change in splenic NK cell population</td>
</tr>
<tr>
<td>2005</td>
<td>[133]</td>
<td>Human: patients with hepatocellular carcinoma, local hyperthermia with RF heating device powered to 614-1363W no thermometry</td>
<td>1 hr, 1–2 times a week</td>
<td>Early (20 min) enhanced activation of NK cells</td>
</tr>
<tr>
<td>2005</td>
<td>[134]</td>
<td>Mice: C57BL/6 and rag2 −/− mice whole body hyperthermia</td>
<td>42°C 1 hr</td>
<td>Hyperthermia decreased perforin and granzyme message in spleen</td>
</tr>
<tr>
<td>2006</td>
<td>[53]</td>
<td>Human: cancer patients with solid tumors and healthy volunteers, whole body hyperthermia</td>
<td>41–41.8°C, 1 hr</td>
<td>Increased NK cells shortly after WBH</td>
</tr>
</tbody>
</table>

(continued)
of hyperthermia against tumors, thus here we summarize the studies reporting NK cell counts in patients after clinical hyperthermia. Our group observed that NK cell numbers in peripheral blood of cancer patients were increased immediately after a 6-hour whole body hyperthermia treatment of 39.5–40°C [51]. Moreover, when cancer patients with various metastatic tumors were treated with whole body hyperthermia of 41.8–42.2°C for 1 hour, absolute numbers and percentage of NK cells in the peripheral blood increased significantly starting from 2 hours after hyperthermia [52]. Most recently, similar observations regarding increased numbers of NK cells circulating in peripheral blood of patients with various tumors were made by two groups using whole body hyperthermia protocols of 41°C–41.8°C for 1 hour [53], or 39.4°C for 30 min [54]. In healthy human subjects, mild whole body hyperthermia of 38°C also increased peripheral blood NK cell counts, while local hyperthermia had no effect [50].

In conclusion, these clinical studies propose a role for hyperthermia to affect the distribution of NK cells and their cytolytic function, which supports possible use of hyperthermia as an adjuvant for cancer therapies.

**NK cells in pre-clinical hyperthermia studies**

Experimental application of elevated temperatures in vitro, using either human or animal cells, or in vivo, using animal models, have been performed by some investigators to better define the effects of hyperthermia on NK cell function, as well as on the tumor target cells. Here we summarize many of these studies regarding the application of hyperthermia in preclinical studies, where analyses of NK cytotoxicity, peripheral NK cell numbers, and NK cell infiltration into tumor beds were performed.

**Table I. Continued.**

<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
<th>Model</th>
<th>Temperature &amp; duration</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>[56]</td>
<td>Human: melanoma, colon carcinoma, non-small cell lung cancer and cervical carcinoma cell lines used as targets for IL-2 activated NK cells from healthy donors, in vitro hyperthermia in a water bath</td>
<td>42°C 1 hr</td>
<td>Increased NK cell cytotoxicity in vitro against heat shocked cell lines</td>
</tr>
<tr>
<td>2007</td>
<td>[59]</td>
<td>Mouse: B16F10 melanoma, in vivo local microwave or in vitro hyperthermia using an incubator</td>
<td>42°C, 7–10 min, 1–3 times</td>
<td>Splenocytes from hyperthermia treated mice show increased cytotoxicity against YAC-1 tumor cells. In vitro heating of NK and/or tumor targets enhances specific cytotoxicity</td>
</tr>
<tr>
<td>2007</td>
<td>[57]</td>
<td>Human: peripheral blood from healthy volunteers, in vitro hyperthermia</td>
<td>39.5–42°C, 6 hr</td>
<td>Thermal stress enhanced NK cytotoxicity against Colo205 cells. In vitro heating of NK and/or tumor targets enhanced specific cytotoxicity</td>
</tr>
<tr>
<td>2007</td>
<td>[54]</td>
<td>Human: peripheral blood from healthy volunteers, whole body hyperthermia with water bath immersion</td>
<td>39.4°C, 30 min</td>
<td>Increased numbers of NK cells circulating in peripheral blood</td>
</tr>
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</table>

**Effects on NK cell cytotoxicity**

In the first study to describe the effect of hyperthermia on tumor cells, malignant thyroid cells from patients were found to be more sensitive to killing by IL-2 activated NK cells when they were heated ex vivo to heat shock temperatures (44°C for 20 min) and used for cytotoxicity assays [55]. Interestingly, this observation was thought to be dependent on de novo protein synthesis by tumor cells. In recent studies, heat shock temperatures (42°C, 1 hr) on tumor cells significantly enhanced NK cell killing of KM12 colon carcinoma cells [56], but not Colo205 cells [57]. In our studies, cytotoxicity of purified human peripheral blood NK cells against Colo205 human colon adenocarcinoma cells was found to be significantly enhanced at fever range temperatures (39.5°C, 6 hours) [57]. More specifically, the cytotoxic activity of human peripheral blood NK cells, but not NK cell lines or IL-2 activated NK cells, was enhanced when both NK effectors and tumor target cells were exposed to fever-range temperatures. Thermal enhancement of cytotoxicity was not observed when autologous PBMC were used as targets for NK cells, indicating that thermal induction of NK cytolytic activity was specific.
for the Colo205 cells [57]. In an earlier study from our group, similar fever-range temperatures applied via whole body hyperthermia enhanced NK cell killing in a manner that was responsible for the growth delay of human breast tumor xenografts in SCID mice in vivo [58]. Splenocytes taken from B16.F10 melanoma bearing C57BL/6, mice treated with local hyperthermia (42°C, 10 min) also displayed increased natural cytotoxicity against YAC-1 cells in vitro [59].

In contradiction to these studies describing thermal enhancement of NK cell cytotoxicity, there have also been studies which indicate that hyperthermia can inhibit NK cell cytotoxicity. Azocar [60] and Nurmi et al. [61] observed a decrease in human NK cell cytotoxic activity at 40°C in vitro, which was also supported by observations of Dinarello et al., using 39°C for 18 hours [62]. In a mouse model of hyperthermia, long-term (up to 16 days), continuous mild whole body hyperthermia of 38.5°C in C3H/HeNCrj mice suppressed the natural cytotoxic activity of splenocytes against YAC-1 cells [63].

In conclusion, there is conflicting information regarding the effects of hyperthermia on human and mouse models for NK cell cytotoxicity, which in part, may relate to duration of heating at various temperatures. A consensus on thermal regulation of NK cell anti-tumor responses may require consideration of additional parameters such as NK cell numbers in the periphery or infiltration into tumor beds and the tumor model used, and also duration of heating at various temperatures.

Effects on peripheral NK cell numbers or tissue distribution

Similar to the cytotoxicity studies discussed above, there are also conflicting reports on the effects of hyperthermia on the numbers of NK cells in tissues and peripheral blood. These differences could be due in part to temperatures used, as well as duration of heating. Local hyperthermia for 42°C for 10 min on B16.F10 melanoma-bearing C57BL/6, mice was found to increase [59], whereas long-term, continuous mild hyperthermia (38.5°C) was found to decrease the number of NK cells in the spleen [63]. In a rat model of peritonitis, hyperthermia (42°C, 15 min) either did not change [64] or decreased (with hyperthermic preconditioning at 40°C for 4 hours) [65] the percentage of NK cells in peripheral blood.

It is important to recognize that differences in the distribution of NK cells in the periphery after hyperthermia may reflect their altered trafficking to tumors or other sites of inflammation in response to temperature changes. Thus, since NK cells must make physical contact with tumor cells in order to recognize their targets, we next examined the literature regarding the effects of hyperthermia on the presence of NK cells within the tumor microenvironment.

Effects on tumor infiltration of NK cells

In mouse models, an increased number of NK cells and other leukocytes were found to infiltrate the tumor bed of B16 melanomas in C57BL/6, mice given local hyperthermia at 43°C for 15 min [66–68] or 42°C for 30 min [69]. However, a recently published study found no significant change in NK cell infiltration between control and local hyperthermia (42°C for 7 min) groups using the same melanoma model [59]. An increased number of NK cells were found to infiltrate the tumor bed of breast xenografts in SCID mice treated with whole body hyperthermia (39.8°C, 6–8 hours) [58]. Thus, in mouse models that investigated the changes of NK cell infiltration, there is some support for the possibility of thermal enhancement of NK cell infiltration at the tumor site, however, much more work should be done on this question.

Potential mechanisms for thermal regulation of NK cell function

In the clinical setting, it would be expected that NK cells in the heated field and their tumor targets would each experience hyperthermia conditions. To understand the effects of hyperthermia on NK cells and their tumor cell targets observed in clinical and preclinical studies, several molecular mechanisms have been proposed and investigated. Here we summarize many of these mechanisms, focusing first on thermally induced changes that occur in NK cells and second on thermally induced molecular changes in tumor cells (which may make them more sensitive to NK cell killing).

Effects on NK cells

The nature of heat induced changes in NK cells, resulting in altered cytotoxic function is still undefined. However, it is at least thought to be independent of MHC Class I/peptide complexes [70] as reviewed by Milani et al. [71]. Early on, Fleischmann et al. showed that elevated temperatures (39.4°C) greatly enhanced anti-proliferative function of IFN-γ on tumor cells, which is one of the major effector molecules secreted by NK cells [72]. On NK cells, clustering of activating receptor NKG2D occurs at the site of target cell contact during the formation of conjugates between NK cells and target cells during IL-2 enhanced NK cell cytotoxicity [45]. Thus, we investigated the effect of mild thermal stress on NKG2D localization on the NK
Effects on tumor cells and other immune cells

Thermal stress triggers a variety of physiological changes in tumor cells. Studies investigating effects of hyperthermia on tumor cells concentrate on three molecules with potential to affect NK cell functions.

MHC Class I molecules

MHC Class I molecules have fundamental roles in recognition of target cells by NK cells. Down-regulation of these molecules on the cell surface was hypothesized to result in their recognition by NK cells, as described in the 'missing-self' hypothesis [22, 77]. Hyperthermia at 45°C for 30 minutes has been shown to down-regulate MHC class I molecules on melanoma cells [78], which would directly enhance NK cell target recognition and cytotoxic activity. In contrast, MHC Class I expression was found to be unchanged on K562 cells cultured at 41.8°C [79], or on Colo205, HCT116, HT29 cells cultured at fever-range temperatures (39.5°C for 6 hours) [57]. However, in rat glioma cells, cell surface presentation of class I antigens were increased with hyperthermia (43°C 1 hr), leading to suppressed tumor growth in vivo [80]. Thus, although MHC Class I might be altered with hyperthermia in some circumstances, it appears that possible down-regulation may not be a predominant mediator of enhanced NK cytotoxicity seen at temperatures normally achieved during clinical hyperthermia treatment.

Heat shock proteins

An important change induced by heat shock on tumor cells is the synthesis of heat-inducible proteins, collectively known as heat shock proteins (HSPs). These proteins appear to play key roles in the activation of NK cells, and might mediate the NK cell response to thermal stress. NK cell recognition of heat shock inducible immunologic determinants on tumors was first described by Multhoff et al. [21]. In this study the induction of HSP72, a member of the HSP70 family, was observed on the surface of Ewing's sarcoma and osteosarcoma cells with heat shock. Heat shock also up-regulated HSP70 expression on osteosarcoma and chondrosarcoma cells (42.5°C, 90 minutes) [81], and on human ocular melanoma cells (45°C, 30 minutes) [78], resulting in increased susceptibility to NK cells. In addition, a more moderate hyperthermia (41.8°C, 3 hours) was found to enhance cytotoxicity of IL-2 activated NK cells through induction of HSP70 expression on the surface of human renal cell carcinoma ACHN cells [82]. HSP70 has also been found to be induced by mild thermal stress (below 40°C) on CT26 tumor cells [83] and several tissues of...
BALB/c mice [51], and with heat shock on human melanoma cell lines [78]. An HSP70-derived peptide, as well as HSP70 positive tumor exosomes, has been shown to induce chemotaxis and cytotoxic activity of purified NK cells [84, 85]. Furthermore, an increase in HSP70 levels of B16 tumors after whole body hyperthermia treatment (40°C for 2 hours daily for 18 days) correlated with a significant decrease in tumor weight [86]. Similarly, melanoma cell lines transfected to express HSP25 were shown to be more susceptible to killing from IL-2 activated NK cells, despite unaltered surface MHC class I levels [87]. Also, HSP70 transfected human colon adenocarcinoma cells show an enhanced sensitivity to human NK cells that can be blocked with anti-HSP70 antibodies [88]. While CD94 has been proposed to be a receptor for HSP70 on NK cells [89] and potentially mediate the ability of HSP70 to stimulate both the proliferation and activity of NK cells, it has also been proposed that it is the binding of HSP70 to granzyme B that renders tumor cells more sensitive to the cytolytic attack of NK cells [88].

Interestingly, when B16 melanoma cells were heated at 43°C for 30 minutes in vitro, the high levels of HSP70 observed in the viable cells correlated with enhanced maturation of dendritic cells that were pulsed with these tumor cells [90]. In vivo, B16 bearing mice treated with hyperthermia (43°C, 30 min) and injected intratumorally with immature dendritic cells displayed enhanced survival [90]. When in vitro NK cell activity was measured in these surviving mice, NK cell activity was found higher in this group than in the naïve mice. The role of HSP70 activation in antigen presentation and the resulting immune stimulation (including NK cell stimulation) is reviewed by Milani and Multhoff [91, 92].

The effects of hyperthermia were also investigated on HSP90, another member of the heat shock protein family, whose role as a molecular chaperone under heat shock is well established. Indirect effects of HSP up-regulation on immune cells were found to regulate NK cell activation. For example, HSP90 was shown to be up-regulated in bone marrow derived dendritic cells via treatment with fever-range thermal stress. Fever range thermal stress was found to activate these cells to secrete IFN-γ, a pro-inflammatory cytokine that sustains NK cell activation as well as causing dendritic cells to mature [83].

Another HSP capable of triggering NK cell responses is the endoplasmic reticulum resident gp96/grp94 (HSP96) [93]. Although heat stress does not up-regulate gp96 levels significantly [94], this member of the HSP90 heat shock family was indicated for its role in inducing NK cell responses. An enhanced NK activity in peripheral blood mononuclear cells of colorectal cancer patients was observed (18 out of 26 patients) after their vaccination with autologous tumor-derived gp96. This activity is accompanied by an expansion in the CD3-CD56+ NK cell population, up-regulation of activating receptors NKG2D and NKp46, enhanced IFN-γ secretion and cytotoxicity against K562 targets [95]. Dendritic cells pulsed with tumor-derived gp96 show tumor growth delay in C57BL/6 mice carrying an OVA-transfected lung tumor cell line LLC, and this delay is lost with the depletion of NK cells [96].

Overall, it is obvious through these studies that HSP induction on tumor cells and other immune cells by hyperthermia has significant potential to affect NK cell functions.

**MHC Class I like molecule A (MICA)**

Another candidate molecule on tumor cells that might play a mechanistic role in the thermal enhancement of NK cell activity is the stress inducible, non-classical MHC Class I ligand A (MICA) which is recognized by the NKG2D activating receptor on NK cells [97]. The gene for MICA contains heat shock response element-like sequences in its promoter region and its expression has been shown to be enhanced with heat shock (42°C) in HeLa cells [98, 99] and HCT116 cells [100]. Our group has found that a milder fever-range thermal stress (39.5°C, 6 hr) is capable of inducing up-regulation of MICA expression in human colon adenocarcinoma cell lines [57]. MICA is encoded within the MHC cluster, and transcription factors Hsf1 and Sp1 have been suggested to regulate its transcription during heat shock [100]. Rodriguez-Rodero et al. also found that the MICA promoter is not polymorphic and mutation in the Hsf1 binding site, together with inhibitors of Sp1, can cause significant loss of activity in the MICA promoter in HeLa and Caco-2 cells [101].

Altogether, MHC Class I molecules, HSPs and MICA are three current candidates involved in hyperthermia-induced changes on tumor cells that might result in enhanced NK cell recognition and killing of tumor targets (see Figure 2).

Together, these findings suggest that the overall effect of hyperthermia on NK cell function depends on alterations in both the effector and target cells, as we have previously proposed [57].

**Unresolved questions concerning the effects of hyperthermia on NK cells**

The studies summarized above point out changes in NK cell function and distribution in various models in response to hyperthermia. While these results have significantly contributed to the understanding of the potential for thermal enhancement of NK cells.
during clinical hyperthermia, the available information is still insufficient (and often conflicting).

Several studies reviewed here support an increase in NK cell infiltration into tumors during hyperthermia. On the other hand, results in pre-clinical in vivo and in vitro models of hyperthermia measuring NK cell cytotoxicity and distribution are conflicting. It is important to note, though, that these studies use the same model to investigate the NK cell infiltration in several published studies and fail to distinguish NK cells from NKT cells in their models. Therefore, a more complete characterization of infiltrating cells into the tumor bed constitutes an important future goal. Moreover, it is still not clear how heat triggers a change in NK cell trafficking patterns or tumor infiltration that enhances their numbers within the tumor microenvironment.

Apart from the recruitment of NK cells to the tumors, a critical parameter for NK cell mediated control of tumor is the recognition and formation of an immunological synapse between NK cells and their targets (NKIS) [102]. Although several aspects of NKIS have been previously examined with regards to its role in NK signal transduction for cytotoxicity [103] and the intercellular exchange of molecules between NK cells and targets [104, 105], the role of hyperthermia in NKIS formation has not been investigated. As NK mediated tumor killing could potentially be affected by the cells on both sides of the NKIS (i.e. NK cells or tumor targets), hyperthermia could also be affecting cell surface molecules on both sides. Furthermore, it has been previously proposed that an activating NK immune synapse, resulting in NK cell activation, is different from an inhibitory synapse with regard to the recruitment of distinct proteins to the synapse site [106]. Thus, one possible mechanism for enhanced NK cell function with hyperthermia is the increase of stimulatory receptor levels recruited to the activating immunological synapse site on NK cells. We have previously shown that total surface NKG2D levels on peripheral blood NK cells do not change with hyperthermia [57]. However, it is yet to be determined whether other stimulatory receptors such as CD16, CD56, NKp30, NKp44, NKp46, CD94/NKG2C, DNAM-1, KIR2DS, 2B4, NKp80, NKR-P1C are affected during hyperthermia treatments of NK cells. Conversely, the corresponding ligands to these activating receptors, such as HSPs, MICB, ULBP family members, pp65, HLA-C, CD48, AICL, and Nectin-2, as well as MICA (which has already been shown to localize into the NK cell synapse site on tumor cells [73]), need to be investigated with regard to their expression levels and/or localization on the tumor/target cell membrane with hyperthermia. Furthermore, as the cytotoxic function of NK cells is a balance between their activating and inhibitory receptors, a decrease on the expression of inhibitory receptors or their corresponding ligands on tumor cells could result in enhanced NK cell function with hyperthermia. We investigated the surface expression of the major inhibitory receptor for NK cells (HLA-A,B,C) and found that their expression does not change with fever-range thermal stress [57].

Figure 2. Hypothetical model for effects of hyperthermia enhanced cytotoxicity. (1) Hyperthermia acts on both NK cells and tumor targets independently. (2) Thermal enhancement of the membrane clustering of the NKG2D stimulatory receptors on the NK cell surface membrane has been reported on NK cells. (3) Increased expression of stimulatory ligands on tumor cells (e.g. MICA, Hsp70) has been found with hyperthermia, and this increase might be accompanied by (4) a decrease of MHC class I expression on tumor cell surface, which could further activate NK cells. (5) Tumor derived Hsp70 has also been suggested to be presented to NK cells indirectly by antigen presenting cells. (6) These changes result in enhanced killing of tumor targets by NK cells.
However, numerous NK cell inhibitory receptor members such as KIR2DL and the KIR3DL family, the CD158/KIR2DL5 family, p70/AIRM (Siglec 7), Siglec 9, LAIR 1, NKG2A, and the Ly49 family as well as their ligands have not been investigated at all following hyperthermia.

Changes in effector molecule function of NK cells such as IFNγ (as mentioned above), TNF-α, GM-CSF, perforin, and granzymes might be another way of affecting NK cell functions by hyperthermia. As NK cells depend on these effector molecules for target cell cytotoxicity, as well as recruiting other immune cells to the tumor site, investigation of these effector molecules could answer fundamental questions about hyperthermia effects on NK cell responses.

Activation of stimulatory signaling pathways of NK cells by various kinases or inhibition of these signaling pathways by phosphatases could play a regulatory role in transmitting the effects of hyperthermia into a functional outcome for NK cells, which needs further studies to address. Recent publications suggest a transfer of activating receptor ligands such as MICA [104] and MICB [105] between interacting tumor and NK cells with functional consequences in cytotoxicity. The rate of this exchange with thermal stress has never been investigated.

Finally, MICA as a soluble ligand for the activating receptor NKG2D was found to be secreted through metalloproteinase activity in patients with tumors [107–109]. Yet it is unknown whether other stimulatory or inhibitory ligands could be solubilized and how hyperthermia might affect this mechanism in cancer patients. However, our group has already found that soluble MICA levels were not affected with hyperthermia in Colo205 cells [57].

**Conclusions**

In this review we have summarized the literature regarding the effects of hyperthermia on NK cytotoxic function, their number and distribution in the peripheral blood and spleen, as well as tumor infiltration. We have discussed possible molecular targets of heat on the anti-tumor activity of NK cells.

Potential mechanisms by which hyperthermia affects NK cell function include the involvement of MHC class I, HSPs (especially HSP70) and MICA on tumor cells, and changes in surface NKG2D and lipid raft clustering on NK cells. These mechanisms are summarized in Figure 2. Several other mechanisms proposed in this review such as formation of NK cell synapse, changes in the levels of activating receptors and their corresponding ligands, effector molecules and downstream activation signaling that might play a role on the effects of hyperthermia on NK function are currently under investigation.

Clinical studies agree on enhanced cytotoxicity as well as increased NK cell numbers in the tissues of cancer patients and healthy subjects with hyperthermia. Several clinical and preclinical results of hyperthermia are summarized in Figure 1 and Table I. Collectively, we believe that the data generally support the idea that hyperthermia can serve as an adjuvant, enhancing NK cell mediated tumor killing. Furthermore, we have recently demonstrated that the ability of hyperthermia to act as a adjuvant is not limited to cancer immunotherapy, but is also effective in regulating immune cell function in the setting of autoimmune disease. Recent studies have demonstrated that administration of weekly fever-range whole body hyperthermia in a non-obese diabetic mouse model can significantly prevent disease progression, and this effect is associated with an increase in NK cell cytotoxicity which may be important for suppressing autoimmune T cells [135]. Further dissection of the role of temperature on NK cell mediated cytotoxic activity will greatly assist the efforts of clinical and laboratory scientists to identify optimal hyperthermia protocols for more successful therapies in patients that can harness long term disease control by the immune systems.

**Acknowledgements**

This work was supported by grants NIH P01 CA94045, R01 CA71599, R21 CA098852, and the Roswell Park Cancer Institute Core grant CA16056. The authors would like to thank Melissa Grimm, Chen-ting Lee and Thomas Mace for their helpful comments on the manuscript.

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