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RESEARCH ARTICLE

Effector CD8$^+$ T cell IFN-γ production and cytotoxicity are enhanced by mild hyperthermia

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Abstract

Purpose: Clinical trials combining hyperthermia with radiation and/or chemotherapy for cancer treatment have resulted in improved overall survival and control of local recurrences. The contribution of thermally enhanced anti-immune function in these effects is of considerable interest, but not understood; studies on the fundamental effects of elevated temperature on immune effector cells are needed. The goal of this study is to investigate the potential of mild hyperthermia to impact tumour antigen-specific (Ag) effector CD8$^+$ T cell functions.

Method: Pmel-1 Ag-specific CD8$^+$ T cells were exposed to mild hyperthermia and tested for changes in IFN-γ production and cytotoxicity. Additionally, overall plasma membrane organisation and the phosphorylation of signalling proteins were also investigated following heat treatment.

Results: Exposing effector Pmel-1-specific CD8$^+$ T cells to mild hyperthermia (39.5°C) resulted in significantly enhanced Ag-specific IFN-γ production and tumour target cell killing compared to that seen using lower temperatures (33° and 37°C). Further, inhibition of protein synthesis during hyperthermia did not reduce subsequent Ag-induced IFN-γ production by CD8$^+$ T cells. Correlated with these effects, we observed a distinct clustering of GM1$^+$ lipid microdomains at the plasma membrane and enhanced phosphorylation of LAT and PKCθ which may be related to an observed enhancement of Ag-specific effector CD8$^+$ T cell IFN-γ gene transcription following mild hyperthermia. However, mitogen-mediated production of IFN-γ, which bypasses T cell receptor activation with antigen, was not enhanced.

Conclusions: Antigen-dependent effector T cell activity is enhanced following mild hyperthermia. These effects could potentially occur in patients being treated with thermal therapies. These data also provide support for the use of thermal therapy as an adjuvant for immunotherapies to improve CD8$^+$ effector cell function.

Keywords: *cytotoxicity, fever, hyperthermia, interferon-γ, T lymphocytes*

Introduction

Hyperthermia, used to elevate the temperature of tumours to between 40° and 45°C, is proving to be an important adjuvant in cancer therapy when combined with chemo- and/or radiation therapy. More specifically, hyperthermia has been shown to improve clinical responses to radiation in several clinical trials in patients with breast [1, 2], head and neck [3, 4], melanoma [5], cervical [6, 7], and brain [8, 9] cancers and improves response to chemo-radiation therapy in patients with soft tissue sarcoma [10]. Despite the growing documentation of the positive impact of hyperthermia as an adjuvant in cancer treatment, its use is still quite rare in comparison to other therapies. In part, this is due to a relative lack of new clinical trials that could document its effectiveness in various applications. However, the increased use of hyperthermia in cancer treatment may also be limited by a still-incomplete understanding of the precise mechanisms or cellular events by which temperature manipulation affects the tumour microenvironment or the efficacy of radiation or chemotherapy. Without more documentation of the complete range of cellular and/or

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molecular effects of temperature elevation, it is difficult to design the most effective clinical protocols.

Recently, there has been a growing appreciation for that fact that, due to physiological heat dissipating processes, the hyperthermic temperatures achieved in large regions of the tumour and surrounding area may be as mild as 39–40°C. Because this temperature range is not considered cytotoxic (at least not in treatment periods used in the clinic) the possibility exists that thermally sensitive activation of various cellular processes could be contributing to the beneficial effects seen with the addition of hyperthermia. One particularly attractive hypothesis is that the function of cells important for the anti-tumour immune system may be sensitive to thermal signals, possibly because of a natural, long-conserved sensitivity to elevated temperatures during fever. Based on a growing literature, it is apparent that the effect of hyperthermia on anti-tumour immunity is quite complex impacting various arms of the innate and adaptive immune response [11, 12]. Excitingly, very recent studies in a clinically relevant animal model have demonstrated that adding hyperthermia to chemotherapy results in a curative therapy that is immunologically mediated [13]. It has been widely reported that tumour infiltrating lymphocytes are essential for the inhibition of tumour growth and improved prognosis in various cancer types [14–19]. Moreover, the specific immune cells located within tumours have been shown to be a favourable predictor of survival among colorectal cancer patients, even more accurate than histology [20]. In particular, many of these observations indicate that the infiltration of $CD8^+$ T cells into the tumour microenvironment is an immune mechanism benefiting patient survival. The effect of hyperthermia on effector $CD8^+$ T cells is unknown in terms of antigen-specific and T cell-dependent events. However, our lab has shown that hyperthermia enhances the rate of antigen-dependent contact hypersensitivity through a T cell-mediated response [21]. This increased rate of hypersensitivity leads to an increased infiltration of lymphocytes accompanied by swelling at the site of antigen contact and blood vessel dilation. Further, our recent studies have indicated that increasing temperature enhances the early activation and differentiation of naïve $CD8^+$ T cells into effector cells [22]. Moreover, work by others [23] has shown that the differentiation of $CD4^+$ T cells into T_H1 cells is accompanied by increased interferon gamma (IFN-γ) production and subsequent promotion of $CD8^+$ T cell-mediated adaptive immune response. Hyperthermia trials have shown an effect on serum cytokine levels [24], but whether thermal treatment can directly influence IFN-γ production is also not known. However, since effector, but not naïve $CD8^+$ T cells are able to directly kill tumour cells, it would be very important to determine whether hyperthermia could enhance the functions of this differentiated subset.

Here, we address the question of whether mild heat treatment can enhance tumour antigen-specific, effector $CD8^+$ T cell functions, and in particular, their cytotoxicity and IFN-γ production. We also examined the effects of mild heating on the signalling pathway known to be engaged when $CD8^+$ T cells are activated. Finally, we show that temperature-induced effects on $CD8^+$ T cell function are acting through events and signals mediated by the TCR in the plasma membrane. Collectively our data suggest that fever-range temperature can enhance important $CD8^+$ T cell functions and thereby may help to promote tumour control and improve long-term survival in patients receiving hyperthermia as part of their therapy.

Methods

Animals and cell lines

C57BL/6 mice were obtained from National Cancer Institute (Bethesda, MA). B6.Cg-*Thy1*^{a}/Cy Tg (TcraTcrb)8Rest/J (Pmel-1) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at Roswell Park Cancer Institute (Buffalo, NY). B16.F10 melanoma cells and EL-4 lymphoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in 10% FBS, l0 mM L-glutamine, and 100 μg/mL penicillin/streptomycin in RPMI 1640 (Gibco, Grand Island, NY).

Antibodies, peptide, and reagents

PE-conjugated anti-CD8 mAb (53–6.7), FITC-conjugated Thy1.1 mAb (OX-7), FITC-conjugated CD8 mAb (Ly-2), and PE-Cy5-conjugated CD8 (53–6.7) were purchased from BD Pharmingen (San Diego, CA). p-LAT was purchased from Millipore and p-PKCθ, and β-actin antibodies were purchased from Cell Signaling Technology (Boston, MA) for western blot analysis. $gp100_{22–33}$ peptide was synthesised and purchased from JPT peptide (Berlin, Germany). FITC-cholera toxin B (CTxB) was purchased from Sigma (St Louis, MO). Recombinant human IL-2 was purchased from R&D Systems (Minneapolis, MN).

In vitro generation of Pmel-1 effector CD8$^+$ T cells

Pmel-1 splenocytes were cultured with 0.1 μg/mL of $gp100_{25–33}$ peptide and 30 IU of rhIL-2 (R&D Systems) in 10% FBS, 2mM l0mM L-glutamine, and 100 μg/mL penicillin/streptomycin in RPMI (Gibco) for 5 days at 37°C. Cells were harvested and run over a Ficoll gradient. Effector CD8$^+$ T cell phenotype was confirmed by FACS analysis.

^{51}Cr release assay for Ag-specific target cell killing

Target cells were labelled with 100–150 μCi of $Na^{51}CrO_4$ (^{51}Cr) (PerkinElmer, Waltham, MA) at 37°C for 1 h in FBS. After washing targets three times with 5% FBS in RPMI 1640 they were plated at 10^4 ^{51}Cr-labelled cells/well and mixed with effector Pmel-1 CD8$^+$ T cells (incubated at indicated temperatures) in round bottom microtiter plates at indicated E:T ratios in triplicate. After 4 h incubation at 37°C, plates were centrifuged for 5 min at 500 × *g*, and 50 μL of supernatant was collected and counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer). Percent-specific lysis was determined as (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100.

ELISA

Supernatants were collected 18 h after stimulating CD8$^+$ T cells with C57BL/6 Ag-pulsed splenocytes. IFN-γ levels were measured in a sandwich ELISA using anti-IFN-γ capture mAb and biotin conjugated anti-IFN-γ mAb (XMG1.2) purchased from BD Pharmingen.

Cell staining

To examine GM1 clustering, cells were adhered onto Alcian blue-coated cover-slips, washed with PBS, and fixed with 4% paraformaldehyde. Cells were stained with FITC-CTxB, visualised, and quantified by fluorescent microscopy.

Western blot analysis

Cells were collected by centrifugation and lysed in lysis buffer (20 mM Tris (ph 7.5), 120 mM NaCl, 100 mM NaF, 0.5% Nonionic P40) containing protease inhibitors (10 mM sodium pyrophosphate (NaPPi), 4 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/mL Lleupeptin, 2 mM benzamidine, and 10 μg/mL aprotinin) and phosphatase inhibitors (200 μM sodium vanadate and 50 mM b-glycerophosphate) for 30 min on ice. Lysates were centrifuged at 12,000 × *g* for 10 min at 4°C and the supernatants were collected. Protein concentration in the supernatants was determined using the Pierce BCA protein assay kit. Lysates were boiled for 5 min in the presence of 1 × SDS-PAGE loading buffer and electrophoresced on a 10% SDS-polyacrylamide gel for 1.5 h at 100 V. The proteins were transferred to nitrocellulose and probed with indicated antibodies. The membrane was washed three times for 5 min with 0.1% Tween 20 and 1 × TBS. Detection was performed with the appropriate secondary Abs conjugated with HRP followed by ECL detection reagent (Pierce).

Quantitative RT-PCR analysis

Total RNA was prepared from CD8$^+$ T cells stimulated with Ag-pulsed splenocytes using RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesised using oligo-dT primers and SuperScript® polymerase (Invitrogen, Grand Island, NY). Quantitative real-time PCR was performed in an Applied Biosystems (Carlsbad, CA) 7900HT Fast Real-Time PCR system using SYBR Green PCR kit from Applied Biosystems and specific primers to amplify 200 bp fragments from different genes analysed. A threshold was set in the linear part of the amplification curve and the number of cycles needed to reach it was calculated for each gene. Normalisation was performed using primers to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Results are expressed as mean ± standard deviation. Student's two-tailed t test was used for comparing experimental groups with p value < 0.05 considered significant.

Results

Ag-specific effector CD8$^+$ T cell IFN-α production is temperature sensitive

Previous work has shown that non-antigen-specific T cell function has been enhanced with hyperthermia [25, 26]. Moreover, our earlier work has demonstrated that elevated temperatures can enhance the differentiation of naïve CD8$^+$ T cells into effector cells [22]. However, whether increased temperature affects Ag-specific, effector CD8$^+$ T cell function is still unclear. Thus, we tested whether different physiological temperatures could regulate Ag-specific effector CD8$^+$ T cell function, using antigen to achieve activation, rather than non-specific mitogens or crosslinking antibodies against surface receptors. Effector CD8$^+$ T cells were generated from Pmel-1 splenocytes by stimulating cells for 5 days with 0.1 μg/mL $gp100_{25–33}$ peptide and 30 IU rIL-2. Effector CD8$^+$ T cells were then incubated at 33°, 37°, or 39.5°C for 6 h and

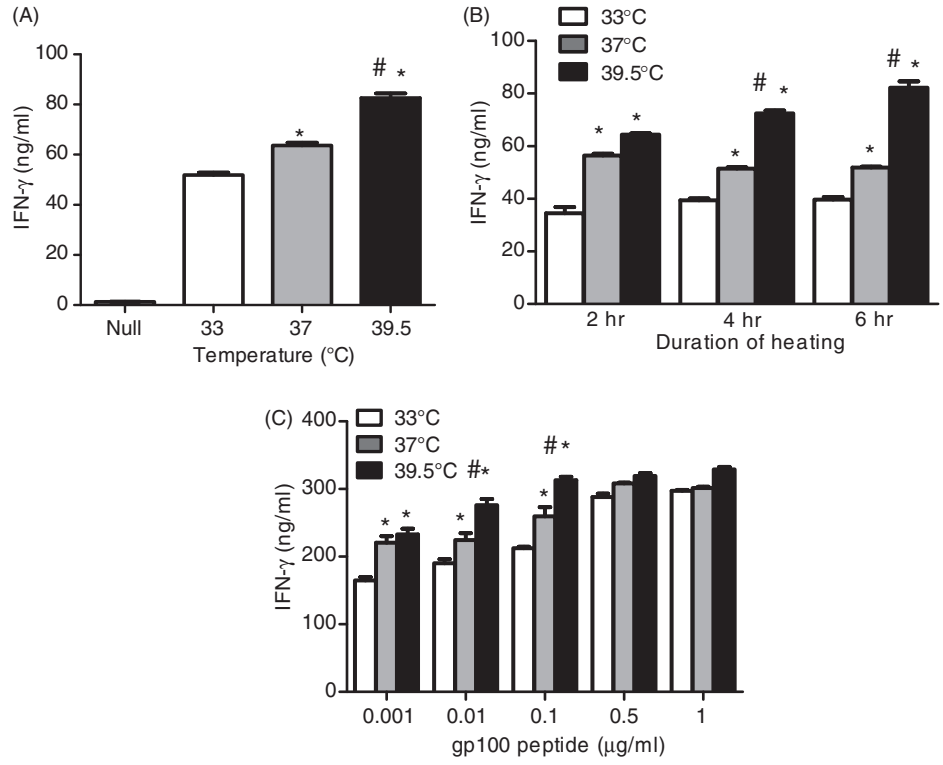

Figure 1. Ag-specific effector CD8$^+$ T cell IFN-γ production is temperature sensitive. Effector Pmel-1 CD8$^+$ T cells were generated by pulsing Pmel-1 splenocytes with 0.1 μg/mL gp100$_{25\text{-}33}$ peptide (at 37°C) and 30 IU of rIL-2 for 5 days. (A) Effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, and 39.5°C for 6 h and re-stimulated with C57BL/6 splenocytes pulsed with 0.1 μg/mL gp100$_{25\text{-}33}$ peptide. Supernatants were removed after 18 h of co-incubation at 37°C and analysed for IFN-γ by ELISA. (B) Effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, or 39.5°C for 2 to 6 h and activated with pulsed C57BL/6 splenocytes. Supernatants were analysed for IFN-γ by ELISA 18 h after pulsing. (C) Effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, or 39.5°C for 6 h and stimulated with varying concentrations of gp100$_{25\text{-}33}$ peptide pulsed C57BL/6 splenocytes. Results are reported as the mean ± SD. These results are representative of two independent experiments ($p < 0.05$, #vs 37°C and ⋆vs 33°C).

re-stimulated with gp100$_{23\text{-}33}$ peptide-pulsed C57BL/6 splenocytes for 18 h. First we examined production of IFN-γ because of the importance of this molecule in up-regulating MHC class I expression on tumour cells, allowing them to be recognised by the immune system. We found that Ag-specific production of IFN-γ by CD8$^+$ T cells is temperature sensitive with the highest production of IFN-γ observed with incubation at 39.5°C (Figure 1A). Cells pre-incubated at 37°C had higher IFN-γ production than those incubated at 33°C. Re-stimulation of the CD8$^+$ T cells with cognate peptide is still required, as temperature alone does not result in production of IFN-γ. The temperature sensitive effect on IFN-γ production by CD8$^+$ T cells is enhanced with a greater duration of heating (Figure 1B). Pre-incubation at 39.5°C for 6 h resulted in the highest level of IFN-γ production by effector CD8$^+$ T cells. Consistent with previously published results [27, 28], maximum effect on other immune cell subsets occurs by heating at 39.5°C for 6 h. We next determined the range of Ag concentrations at which thermal enhancement of IFN-γ production could be observed. We incubated effector CD8$^+$ T cells at 33°, 37°, and 39.5°C for 6 h and then stimulated them with splenocytes pulsed with varying concentrations of gp100$_{25\text{–}33}$ peptide. The effect of higher temperatures on enhancing IFN-γ production is maximal at a peptide concentration of 0.1 to 0.01 μg/mL. This enhancement is lost at higher peptide concentrations where IFN-γ production by cells incubated at 33° or 37°C is comparable to production by cells incubated at 39.5°C (Figure 1C). Thus, if Ag stimulation is optimal, exposure to elevated temperature does not further enhance IFN-γ. When CD8$^+$ T cells are stimulated at sub-optimal Ag concentrations, higher temperatures can enhance CD8$^+$ T cell IFN-γ production.

Ag-specific effector CD8$^+$ T cell cytotoxicity is temperature sensitive

Another important anti-tumour effector function of CD8$^+$ T cells is cytotoxicity. A previous study has shown that Fas ligand-specific killing is enhanced

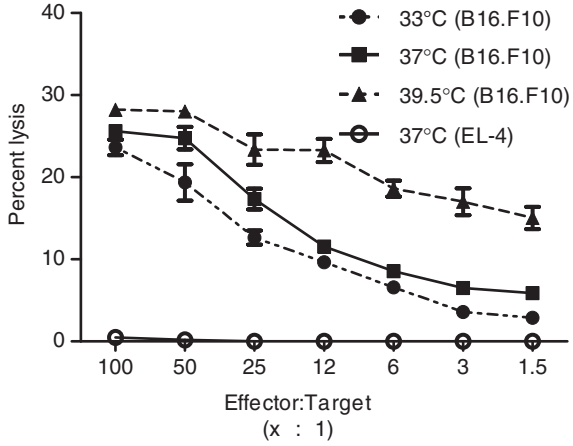

Figure 2. Mild hyperthermia enhances Ag-specific CD8$^+$ cytotoxicity. Effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, or 39.5°C for 6 h and then co-incubated with Cr^{51} labelled EL-4 (gp100 negative) or B16.F10 (gp100 positive) target cells for 4 h at 37°C. Supernatant was collected and percentage lysis was determined by chromium release by lysed target cells. Results are reported as the mean ± SD. These results are representative of two independent experiments.

with hyperthermia [29]; however, little is known about how Ag-specific T cell cytotoxicity is impacted by hyperthermia. To test Ag-specific CD8$^+$ T cell cytotoxicity, effector Pmel-1 CD8$^+$ T cells were generated in vitro by activating with C57BL/6 splenocytes pulsed with peptide for 5 days, and then effector cells were incubated at 33°, 37°, and 39.5°C for 6 h and then co-incubated with B16.F10 (gp100 expressing) or EL-4 (gp100 negative) ^{51}Cr labelled tumour cells. Similar to the effect of temperature on IFN-γ production, effector Pmel-1 CD8$^+$ T cells incubated at higher temperatures displayed an enhanced cytotoxicity against B16.F10 cells (Figure 2). Cytotoxicity was antigen-specific as no lysis was observed when effector CD8$^+$ T cells were co-incubated with chromium labelled EL-4 cells (Figure 2). These results demonstrate that antigen-specific CD8$^+$ T cell effector responses are highly dependent on temperature. These observations suggest an important role for mildly elevated temperature (i.e. during a fever or hyperthermia) in enhancing a cellular mediated T cell response (IFN-γ production and cytotoxicity) to antigen.

Temperature-dependent IFN-γ production does not depend on synthesis of new protein but does induce GM-1 clustering

To investigate the mechanisms by which increased temperature could affect IFN-γ production in effector CD8$^+$ T cells, we first asked whether additional protein synthesis occurs during heating which could be mediating the thermal regulation of IFN-γ production when the CD8$^+$ T cells are activated with Ag-pulsed splenocytes. CD8$^+$ T cells were incubated with 10 μm cycloheximide (to block protein synthesis) during incubation at 33°, 37°, and 39.5°C and then washed three times with pre-warmed media and subsequently stimulated with Ag-pulsed splenocytes from C57BL/6 mice. After incubation for 18 h at 37°C supernatants were collected and analysed by ELISA for IFN-γ. Overall, there is a slight decrease in IFN-γ production compared to DMSO controls as some cycloheximide may have remained in culture after subsequent washes. However, cells incubated at 39.5°C, even when co-cultured with cycloheximide during the heating process, still resulted in more IFN-γ secretion compared to cells incubated at 33° and 37°C (Figure 3A). Thus, additional protein synthesis is not required for the temperature-dependent effect on Ag-specific IFN-γ production by CD8$^+$ T cells.

Recent work has shown that incubating naïve CD8$^+$ T cells at 39.5°C for 6 h induces the clustering of GM1$^+$ cholesterol-dependent (CD)-microdomains in the plasma membrane [22]. We asked whether effector CD8$^+$ T cells were similarly impacted, thus changing the signalling required for IFN-γ production. Effector CD8$^+$ T cells were incubated at 33°, 37°, and 39.5°C for 6 h and GM1 was stained using CTxB to look at possible alterations in GM1 organisation in the plasma membrane. GM1 proteins appeared either diffuse or clustered depending on thermal treatment (Figure 3B). Staining patterns were quantified by fluorescent microscopy, and we observed a higher percentage of effector CD8$^+$ T cells with GM1 clustering at 39.5°C compared to cells incubated at 33° or 37°C (Figure 3C). This data suggests that temperature-induced changes at the plasma membrane may be associated with the enhanced Ag-specific CD8$^+$ effector function.

Increased temperature does not affect non-antigen-specific CD8$^+$ signalling that is independent of the TCR

Since elevated temperatures impact CD8$^+$ T cell functions through altering the distribution of CD-microdomains, we hypothesised that CD8$^+$ T cell activation through a stimulation signal bypassing the cellular membrane would not be influenced by temperature changes. Treatment with mitogen and ionophore (PMA and ionomycin) bypasses the need for TCR engagement on the membrane. We did not observe differences in IFN-γ production between any of the groups when the effector CD8$^+$ T cells were incubated at 33°, 37°, 39.5°C for 6 h and then stimulated with PMA and ionomycin (Figure 4).

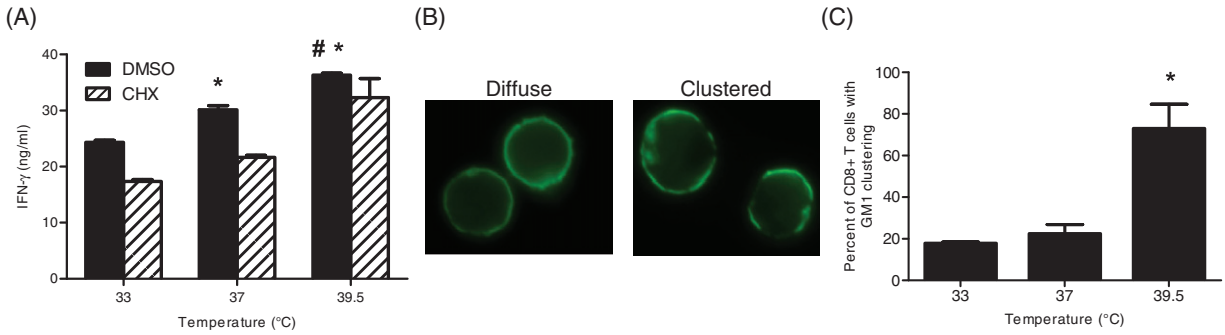

Figure 3. Mild hyperthermia does not affect new protein synthesis but induces GM1 clustering in the plasma membrane. (A) Effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, or 39.5°C for 6 h in the presence of 10 μm cycloheximide then stimulated with gp100$_{25–33}$ peptide pulsed C57BL/6 splenocytes for 18 h. Supernatants were analysed for IFN-γ production by ELISA. (B) Effector cells were incubated at 33°, 37°, 39.5°C for 6 h and adhered onto alcian blue coated coverslips, fixed with paraformaldehyde. Cells were stained with FITC-CTxB, visualised for clustering by fluorescent microscopy, and (C) quantified. Results are reported as the mean ± SD. These results are representative of two independent experiments ($p < 0.05$, #vs 37°C and *vs 33°C).

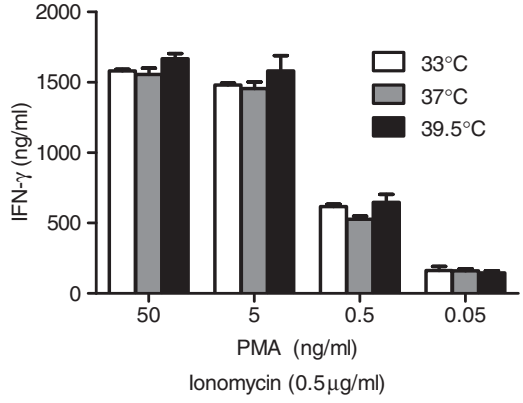

Figure 4. Mild hyperthermia does not affect effector CD8$^+$ T cell activation when TCR signalling is bypassed. Effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, 39.5°C for 6 h and activated with 0.5 μg/mL of ionomycin and varying concentrations of PMA for 18 h at 37°C. Supernatants were collected and analysed for IFN-γ by ELISA. Results are reported as the mean ± SD. These results are representative of two independent experiments.

This indicates that the temperature-dependent effect on CD8$^+$ T cell function is acting through events and signals involving the plasma membrane.

Elevated physiological temperatures enhance CD8$^+$ T cell TCR signalling

We next investigated how temperature could affect early signalling events following ligation of TCR during an Ag-specific CD8$^+$ T cell response. Phosphorylation of PKCθ and LAT plays an important role in mediating TCR signalling [30–32] and previous work from our lab has shown that following hyperthermia, PKC reorganisation and activity is enhanced in T cells [33]. Therefore we asked how the phosphorylation of PKCθ and LAT might differ in antigen-stimulated Pmel-1 CD8$^+$ T cells following pre-treatment with different temperatures. The effector Pmel-1 CD8$^+$ T cells were incubated at 33°, 37°, and 39.5°C for 6 h and then activated for 0–30 min at 37°C (Figure 5D). Lysates were analysed by western blot for PKCθ and LAT and densitometry was used to quantify the relative phosphorylated protein levels compared to control β-actin expression. The phosphorylation of LAT and PKCθ were enhanced after 5 min of Ag-stimulation when pre-incubated at 39.5°C compared to a delayed phosphorylation when cells were pre-incubated at 33° or 37°C prior to Ag-stimulation (Figure 5B and 5D). These results suggest that TCR signalling is enhanced in a temperature-dependent fashion and that higher temperatures increase the rate of activation. At later time points, the phosphorylation of LAT and PKC in the 37° and 39.5°C groups begins to decrease but remains higher in the 33°C pre-incubated cells. This suggests that signalling may not be occurring efficiently at lower temperatures (33°C).

Ag-specific effector CD8$^+$ T cell IFN-γ gene transcription is regulated by temperature

To test whether these signalling effects resulted in enhanced IFN-γ gene transcription, CD8$^+$ T cells were similarly incubated at 33°, 37°, and 39.5°C for 6 h and re-stimulated with Ag-pulsed C57BL/6 splenocytes for 0 to 2 h after which RNA was isolated. Ag-specific IFN-γ mRNA expression is regulated by temperature with higher-fold message levels in cells incubated at 39.5°C (Figure 6). Cells pre-incubated at 33°C showed a slower rate of IFN-γ transcription than cells incubated at 37° and 39.5°C temperatures. This demonstrates that mild hyperthermia can result in changes in genes downstream of

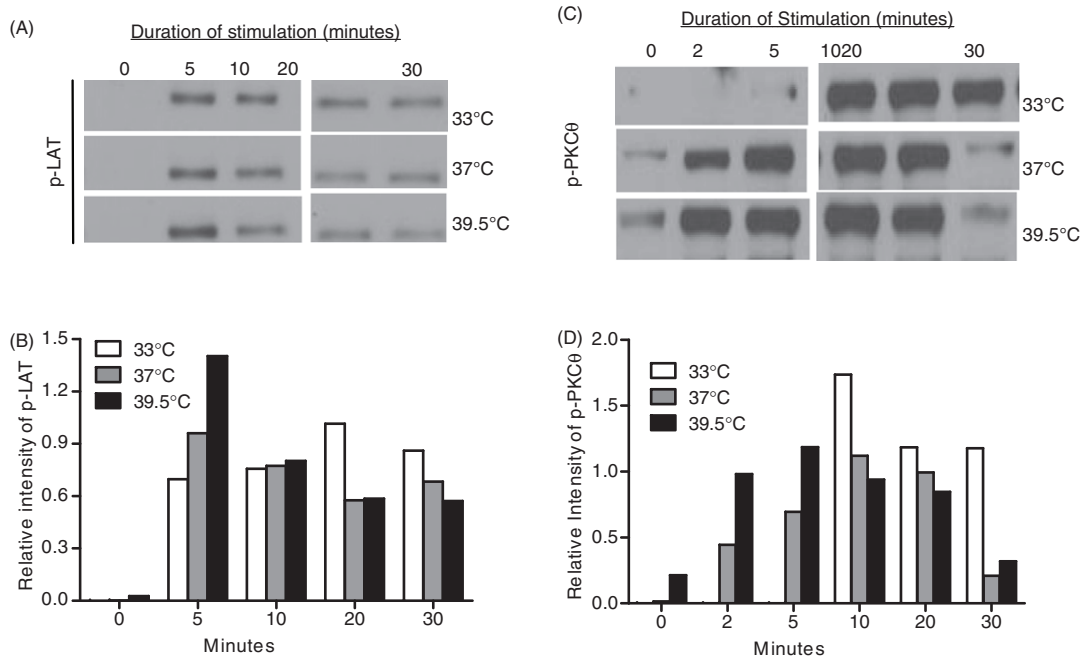

Figure 5. Effector CD8$^+$ T cell Ag-specific signalling is enhanced by mild hyperthermia. Cells were incubated at 33°, 37°, or 39.5°C for 6 h and stimulated with 0.1 μg/mL gp100$_{25–33}$ peptide pulsed C57BL/6 splenocytes at 37°C. Stimulation was varied between 0–30 min. (A–D) Expression of phosphorylated LAT and PKCθ were determined by western blot. (A, C) Western blots. (B, D) Densitometry was performed using total β-actin levels as background controls. These results are representative of two independent experiments.

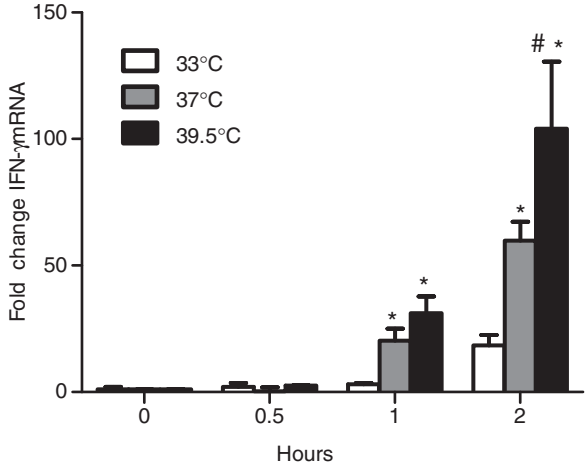

Figure 6. Effector CD8$^+$ T cell IFN-γ transcription is regulated by temperature. Effector CD8$^+$ T cells were incubated at 33°, 37°, 39.5°C for 6 h and stimulated with 0.1 μg/mL gp100$_{25–33}$ peptide pulsed C57BL/6 splenocytes for 0–2 h at 37°C. RNA was isolated and cDNA was synthesised. mRNA levels were assessed by real-time PCR. All message levels are relative to GAPDH controls and experimental gene expression is relative to cells activated with null peptide. Results are reported as the mean ± SD. These results are representative of two independent experiments ($p < 0.05$, #vs 37°C and *vs 33°C).

TCR signalling, thus resulting in the enhanced IFN-γ production observed at higher temperatures (Figure 1).

Discussion

New strategies to enhance the activity of CD8$^+$ T cells are critical for achieving long-term protection from cancer recurrence. T cell infiltration of tumours has been documented to be very important in overall survival, but substantial evidence exists that a suppressive microenvironment within the tumour limits the activation and function of CD8$^+$ T cells. Indeed, many different factors and cell types within the tumour exert suppressive forces against a CD8$^+$ T cell anti-tumour response. For example, factors such as IL-6, VEGF, MMPs, GM-CSF, IL-10 and many other cytokines and chemokines can be found within the tumour microenvironment and are responsible for the suppression of an anti-tumour immune response [34–36].

These factors and others promote the accumulation of cells including myeloid-derived suppressor cells (MDSCs), which suppress CD8$^+$ T cell activity by secreting ROS, depleting arginine, and decreasing TCR-associated signalling proteins [37–39]. MDSCs also secrete anti-inflammatory cytokines including IL-10 and TGF-β which promote

differentiation of T regulatory (T_{reg}) cells that also inhibit $CD8^+$ T cell anti-tumour activity. A recent study suggested that infiltrating DCs can suppress $CD8^+$ T cell function by inhibiting T cell L-arginine metabolism in a spontaneous mammary tumour model leading to decreased T cell expansion and IFN-γ production, ultimately resulting in anergy [40].

Since multiple factors and cell types within the tumour microenvironment can suppress T cell activity, new therapies or adjuvants that could help to increase the activation and function of these cells could overcome this suppression would be important in generating an improved anti-tumour response. In this report we document the ability of mild temperature elevation to significantly enhance activities (IFN-γ production and cytotoxicity) that would be important in tumour control, and we have associated these effects with changes in the organisation of the plasma membrane and downstream signalling pathways, alterations which may be involved in the underlying mechanisms by which elevated temperature affects cellular function. Earlier studies in our laboratory have reported that heating mice in vivo enhances PKC activity and results in the aggregation of PKC and the cytoskeletal proteins spectrin and vimentin [27, 41]. We have shown in this study that elevated temperature results in enhanced downstream signalling, with greater levels of phosphorylated PKCθ and LAT. Subsequent enhancement in IFN-γ gene transcription was observed when effector $CD8^+$ T cells were incubated at higher temperatures and that these changes are also associated temporally with a thermally induced reorganisation of membrane domains.

It will now be important to determine whether these thermally enhanced $CD8^+$ T cell functions could play a role in controlling tumour growth in vivo. Previous studies have demonstrated general enhancement of immune activity against tumours following mild hyperthermia, but these studies have not studied a tumour antigen-dependent response. For example, in mice, fever-range hyperthermia has been shown to enhance anti-tumour immunity through both the innate and adaptive immune systems [42]. Hyperthermia has also been shown to significantly enhance efficiency of heat shock protein vaccines [43]. When combined with intratumoural DC injection, hyperthermia induces DC migration to the tumour draining lymph nodes and enhances the priming of CTLs in animal melanoma models [44]. Clinically, Guo et al. have shown that patients with advanced melanoma treated three times a week with local hyperthermia followed by intratumoural injections of immature DCs experienced significantly longer time to tumour progression ($p < 0.05$) [45]. Furthermore, this study demonstrated that DC vaccination in combination with hyperthermia resulted in an increased infiltration of activated $CD8^+$ cells into the tumour site accompanied by decreased infiltration of immune suppressive T_{reg} cells, possibly creating an environment for improved tumour control [45]. Recently, long-term anti-tumour immunity was achieved in murine models; treatment with oxaliplatin chemotherapy followed 24 h later by 6 h whole body hyperthermia was able to cure all primary and metastatic tumours in 50% of MTLn3 tumour-bearing rats [13].

We have shown that mild hyperthermia increases the clustering of GM-1^+ regions within the plasma membrane (Figure 3). These GM-1 regions contain important signalling molecules, such as TCR, Lck, LAT and many other proteins important in recognising antigen presented by an APC or target cell [46]. Formation of the immunological synapse between an APC and target cells requires the aggregation and localisation of these signalling enriched GM-1 membrane domains to occur at this synapse [47]. The hyperthermia-induced increase in GM-1 clustering could potentially pre-condition effector T cells and allow them to react faster to antigen presentation and initiate killing mechanisms than at lower temperatures.

In summary, this study presents novel information describing the role of hyperthermia in improving tumour antigen-specific, effector $CD8^+$ T cell function. Our studies were preformed in Pmel-1 transgenic mice which carry a rearranged TCR specific for gp100 [48]. While most prior work has been on non-specific antigen-dependent immune responses, the gp100 protein is expressed by most melanoma cells, making this an important model for future in vivo work on the antigen-specific immune response. Furthermore, the fundamental observations made here may expedite the design and implementation of new clinical protocols utilising hyperthermia as an adjuvant immunotherapy.

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