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Emerging evidence indicates that physiologically relevant thermal stress regulates dendritic cell function

Julie R. Ostberg and **Elizabeth A. Repasky**
Department of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263

Abstract

Elevations in temperature that are associated with inflammation or fever have been linked to improved survival from infections, enhanced immunological functions, and increased control of tumor growth. Over the past few years, several groups have begun to explore the possible linkage among these observations and have tested the hypothesis that various cells of immune response are especially sensitive to thermal stimulation. However, relatively little is known regarding the effects of thermal stimulation on antigen presenting cells. Very recently, several groups have begun to examine the ability of thermal stimuli to regulate the function of these cells which are known to play a pivotal role in the efficacy of vaccines and other immunotherapies. In this review, we summarize what has been discovered about the role of mild thermal stress in regulating various DC activities. Excitingly, it appears that mild elevations of temperature have the potential to enhance antigen uptake, activation associated migration, maturation, cytokine expression and T cell stimulatory activity of DCs. While these studies reveal that the timing, temperature and duration of heating is important, they also set the stage for essential questions that now need to be investigated regarding the molecular mechanisms by which elevated temperatures regulate DC function. With this information, we may soon be able to maximize the strategic use of heat as an adjuvant, i.e., combining its use with cancer immunotherapies such as vaccines, which depend upon the function of DCs. Several possible strategies and timepoints involving clinical application of hyperthermia in combination with immunotherapy are presented.

Keywords

dendritic cells; antigen presentation; thermal stress; hyperthermia; danger signal

Introduction

The presence of fever has been linked to improved survival upon infection in multiple vertebrate species [4,5,10,15,16,26,31], strongly suggesting a role for physiological thermal stress in the enhancement of host defense mechanisms. In the past decade, an increasing number of studies have begun to pinpoint various immunological functions which appear to be temperature sensitive (reviewed in [14,24]), including enhancement of anti-tumor immune activity [6]. These studies recall some of the earliest literature in the hyperthermia field in which high fevers that were induced by deliberate or naturally occurring bacterial infections were associated with increased clinical benefit to cancer patients (reviewed in [19]). Thus, studies such as these have led to the hypothesis that strategic applications of heat could be useful in cancer therapy, particularly in combination with immunotherapy.

Corresponding Author: J.R. Ostberg, Ph.D., Department of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, email: julie.ostberg@roswellpark.org; phone: 716-845-3350; FAX: 716-845-8906..

Dendritic cells (DCs) have been recognized as playing a central role in the generation of effective anti-tumor immunity. Not only do they have the potential to recruit, select and expand T cells that are specific for tumor cell antigens within lymphoid organs, but tumor-associated or vaccine-delivered DCs may also help recruit T cells to the tumor site and help maintain their survival once they arrive [17]. Thus, *if the activity of DCs were found to be thermally sensitive, it would provide a strong rationale for using hyperthermia in combination with immunotherapy strategies that are known to be dependent upon these cells, such as vaccination.* Of all immune effector cells, however, antigen presenting cells (APCs) or DCs are the least well studied with regards to their potential regulation by thermal stress. In fact, until recently, there had been no study on the effects of the thermal microenvironment on DC activity [14]. This is despite the crucial role that these cells play in the transition between innate and adaptive immune responses [25], as well as the potential temporal association of their activity with elevated temperatures either locally, in the case of inflammation, or systemically in the case of fever responses. However, in just the past few years, there have been increasing steps taken to help clarify the role of mild thermal stress in controlling the function of these cells. The goal of this review is to summarize these new findings on the ability of mild thermal stress to control APC/DC activities such as antigen uptake, migration, maturation and T cell stimulatory activity, and to identify the critical questions that must still be addressed so that we can maximize the use of thermal therapy in the cancer clinic.

Thermal regulation of antigen uptake

It is currently understood that a primary function of immature DCs is antigen capture at the time of initial exposure to antigen (e.g., directly following vaccine injection in the skin), so that upon the activation associated maturation of these cells, this antigen is then processed and presented to T cells to help drive antigen specific immune responses [1]. While the effects of the thermal microenvironment on antigen uptake, either by phagocytosis or pinocytosis, has not been directly investigated in DCs at this time, other studies have examined the thermal regulation of the phagocytic capacity of another type of professional APC, the macrophage. Short term culture of murine resident peritoneal macrophages at temperatures up to 40 °C have been reported to enhance phagocytosis by approximately 40% compared to cultures kept at 37 °C [30,34]. This finding suggests that elevated temperatures have the ability to enhance antigen uptake by macrophages, and perhaps APCs in general. Obtaining this information is important for determining whether application of heat could be helpful at the time of initial contact of DCs with antigen, such as that associated with vaccines, since phagocytosis is an important aspect of this initial stage of the treatment (see Discussion below).

Thermal regulation of activation associated migration

When antigen is encountered by a peripheral DC, the DC internalizes it, as mentioned above, and then the DC initiates a process of activation associated migration to the draining lymph node where it can then encounter T cells [13]. The effects of mild thermal stress on this epidermal DC migration have been examined both *in vivo* and *in vitro*. After administering mild, fever-like whole body hyperthermia (WBH) (in which the internal body temperature of mice is raised to approximately 39.5 °C for several hours), it was found that the epidermal DCs, also known as Langerhans cells (LCs), had a more punctate or rounded morphology and fewer processes compared to control LCs –suggestive of the cells retracting their dendritic processes to facilitate emigration [23]. Furthermore, decreased LC numbers in the ear epidermis of BALB/c mice were seen between 2 to 5 days after WBH, with numbers returning to normal within 7 days [23]. This lower epidermal DC density (of approximately 10%) was observed despite the fact that new cells apparently continue to migrate back into the skin in this *in vivo* system. This report was thus the first to suggest that mild thermal stress in the form of WBH might have the ability to enhance LC activation and migration out of the epidermis [23]. In another study, FITC was applied to the skin of the mouse abdomen to track the

migration of fluorescent epidermal cells to the draining inguinal lymph nodes (LNs) [20]. Here it was found that FITC application prior to WBH administration resulted in an approximately two-fold increase in numbers of FITC+ CD11b+ APCs in the LNs when compared to that of non-heated mice [20]. However, if WBH was administered before FITC application to the abdomen, decreased numbers of FITC+ APCs in the draining LN were observed compared to controls. It was not clear what happened to the WBH pretreated DCs, as total numbers of CD11c + or CD11b+ cells were either decreased or unchanged in the draining LNs of WBH treated mice, and they were also not increased in other lymphoid organs such as spleen. *In vitro* studies, as better described below, did not indicate that the epidermal DCs were being destroyed. Thus, instead it was hypothesized that WBH pretreatment results in fewer LCs in the skin that are efficient at picking up the FITC antigen for transport to the draining lymph nodes. These data highlight the importance of studies which examine the *timing* of thermal application in a clinical setting since it is clear that applying thermal treatment *prior* to the injection of antigen might actually inhibit the subsequent response.

Ear skin organ cultures, where epidermal DC naturally migrate out of the skin into the media of these cultures, further characterized the ability of mild thermal stress to affect epidermal DC or LC migration *in vitro*. First, increased numbers of viable cells (via trypan blue) with dendritic morphology were seen in the media of one- or two-day ear skin cultures that were incubated at 40°C for the first 8hrs [23]. Further studies revealed that epidermal DCs in the hyperthermia treated explants displayed more ‘inflamed’ morphology (i.e., enlarged cells with erratic processes) at one day of culture, and more of the DCs in the epidermis were rounded with fewer dendritic processes at two days of culture when compared to non-heated control explants [21]. In addition, not only did the numbers or density of MHC class II+ DCs in the skin decrease faster in the heated explants, but two- to four-fold increases in numbers of emigrated MHC II + and CD86+ cells were also observed in the media of 2 and 3 day cultures after *in vitro* hyperthermia treatment [21]. However, although mRNA levels of CCR7, the chemokine receptor involved in LC migration from the periphery to lymph nodes, were upregulated in skin explants as expected, differences between heated and unheated cultures were undetectable [21]. Nonetheless, both the *in vivo* hyperthermia studies described above, and the *in vitro* ear skin explant studies indicate that mild thermal stress enhances the activation associated migration kinetics of DC out of the skin. Thus, application of heat immediately after peripheral DCs have come in contact with the antigens of a vaccine may have the potential to enhance their activation associated migration to lymph nodes where they can then initiate immune response cascades (**see Discussion below**).

Thermal regulation of DC maturation

The maturation of DCs is accompanied by alterations in surface molecule expression, such as enhanced MHC class II, and the costimulatory molecules CD80, CD86 and CD40 [1]. In the skin organ culture systems described above, mild thermal stress *in vitro* was found to enhance the expression levels of both MHC class II and CD86 on the emigrated cells [21]. In contrast, when murine bone marrow derived DCs (BMDC) that had been cultured for 8 days were incubated for 3h at 39°C or 40°C followed by 15h incubation at 37°C, the expression levels of CD11c, CD40, CD80, CD86, or I-A^d were not altered compared to those cells that were continuously cultured at 37°C, nor were the maturation-associated surface marker changes induced by LPS or *S. typhimurium* stimulation affected by mild thermal stress [29]. These results are in agreement with our own investigations on the effects of mild thermal stress on human monocyte derived DCs directly after incubation at 40°C for 7h with or without LPS, where no difference in MHC class I, MHC class II, CD40L, CD54, CD80, CD83 or CD86 levels were observed compared to those incubated at 37°C (data not shown). However, other groups have shown that 5 day or 6 day BMDC cultures incubated at 39.5°C or 40°C for 24h followed by a recovery at 37°C for 12h displayed enhanced MHC class I, MHC class II, CD40,

CD80 and CD86 expression [3,35]. An *in vivo* WBH where mouse core temperatures were elevated to 41°C and then the mice were allowed to recover for 18h at room temperature resulted in similar upregulation of CD86 and MHC class II expression on the surface of CD11c + splenic DC, to levels comparable to that induced by LPS injection [35]. Overall, it appears that the source of DCs, along with the duration of thermal stimulation followed by a period of recovery is important in detecting any effects of mild thermal stress on DC phenotypic maturation. This indicates that it may be necessary to develop and test multiple strategies for the clinical application of heat which may differ in terms of the source of DCs and/or the duration of heating.

Thermal regulation of DC cytokine release

Besides changes in phenotypic marker expression, the synthesis and release of high levels of important cytokines is another feature of DC maturation. For example, IL-12 production is considered largely responsible for the DC mediated stimulation of both innate (e.g., NK cells) and adaptive immune cells (i.e., T and B cells) [2]. Two studies to date have specifically reported on the ability of fever like thermal conditions to stimulate BMDC production of IL-12. In the study performed by Tournier *et al*, *in vitro* hyperthermia was found to enhance the BMDC production of IL-12p70 that was induced by LPS or live bacteria by 10% or more [29]. This hyperthermic stimulation was also reported to reduce the production of IL-10 and TNF-α. Intracellular staining suggested that the thermal regulation of mature DC cytokine production occurs at the single cell level, as it did not alter the percent of cytokine positive cells [29]. Non-activated BMDC did not produce detectable levels of these cytokines and elevated temperatures did not significantly alter their level of cytokines. This is consistent with the other study, performed by Zheng *et al*, which also reported that BMDC treated with mild thermal stress alone did not produce significant levels of IL-12, TNF-α, or IL-1β [35].

Thermal regulation of DC mediated T cell stimulation

Mature DCs are the most potent APCs for stimulating quiescent, naïve T cells [2]. Thus, potentially the most important test of the physiological relevance of thermal activation or maturation of DCs is the examination of their ability to stimulate T cells. When emigrated cells from heat treated skin explants were tested in an allo-MLR, they displayed a two- to three-fold enhanced stimulatory function [21]. Heat treated BMDCs were also found to have a two-fold enhancement or more in their ability to stimulate allogeneic T cells [3,29,35]. In addition, BMDC loaded with OVA and then treated with hyperthermia (41°C, 6h) were better at stimulating IFNγ production by the Moja T cell line, specific for the SIINFEKL epitope of OVA [3]. Use of heat treated SIINFEKL pulsed DC to immunize mice that had been adoptively transferred with CFSE labeled CD8+ OT-1 cells revealed an increased proliferation of these antigen-specific T cells when CFSE profiles of splenocytes were analyzed 3 days after the immunization [35]. Based on FACS analysis of MHC class I-SIINFEKL complexes on the DC surface, the increased *in vivo* T cell proliferation induced by the heated DCs was not due to higher peptide loading efficiency [35].

In vivo studies examining the effects of mild thermal stress on the ability of DCs to stimulate T cells are more difficult to interpret. When fever-like WBH was given *before* application of FITC as a model antigen to the abdominal skin of mice, proliferation of the inguinal LN T cells collected 5 days later was decreased, although polyconal stimulation of the inguinal LN T cell proliferation was not affected [20]. This might be attributed to the idea mentioned above that pre-stimulating the epidermal DCs makes them less efficient at picking up the FITC antigen for transport to the draining lymph nodes. However, WBH treatment directly after FITC application to the skin did not affect FITC specific proliferation of inguinal LN T cells collected 5 days later [20]. This lack of an enhanced response when WBH was administered after antigen application was suggested to reflect the inability of WBH to enhance the already strong

sensitizing capacity of the hapten acetone:dibutyl phthalate stimulus [20]. Another *in vivo* study, where WBH was administered directly after intradermal OVA immunization, and CD11c+ DC were harvested from the draining LNs one day later, the DCs from heated mice were four times better at stimulating B3Z T cells, which specifically recognize the immunodominant OVA peptide SIINFEKL, than DCs from non-heated mice [3]. This suggests that the time at which DCs are collected from the heated mice may be crucial in identifying the effects of *in vivo* mild thermal stress on DC mediated T cell stimulation.

Potential mechanisms underlying thermal regulation of DCs

Because mild thermal stress has been reported to induce heat shock protein (HSP) expression in various systems [9,18,22], it has been suggested that thermal regulation of various immune functions involves pathways or molecules similar to that associated with classic, higher temperature, heat shock protocols. This idea seems to be further supported by the fact that extracellular HSPs are now known to have significant stimulatory effects on APCs (reviewed in [27]). With regard to studies performed specifically with DCs, in the ear skin culture systems, while HSP70 expression in the ear skin explants was upregulated by hyperthermia, differences in extracellular HSP70 expression levels or HSP70 expression within the emigrated cells were not observed [21]. Fever-like temperatures have been found to induce HSP90 but not HSP70 expression in BMDC, and the use of geldanamycin to sequester the HSP90 prevented the heat induced BMDC maturation [3]. However, the BMDC maturation induced by mild thermal stress appears to be independent of the heat shock associated transcription factor Hsf-1 [35]. Furthermore, heat treated BMDC from Hsf-1 null mice could stimulate allogeneic CD4+ T cell proliferation and IFN-γ production more efficiently than untreated DC [35]. In addition, studies of heated and non-heated BMDC co-cultures, where only the heated DCs were found to enhance their CD86 expression, indicated that the thermally-induced DC maturation is not due to the release of endogenous DC activators including extracellular HSPs [35]. Thus, the mechanisms by which the maturation of BMDCs is induced by mild thermal stress does not appear to be dependent on either hsf-1 mediated transcriptional events or extracellar HSPs.

One study has revealed that the maturation of BMDC in response to fever-like temperature appears to involve the degradation of IkB in the cytosol and translocation of NFkB into the nucleus [3]. These authors then suggested that hyperthermia mediates DC maturation following the same pathways as utilized by LPS in activating macrophages [3]. However, because LPS mediated NFkB translocation drives TNF-α expression in macrophages, this report is contradictory to that of Tournier *et al*, described above, where mild thermal stress reduced the expression of TNF-α by DCs [29].

Mild thermal stress has been reported to induce the formation of DC aggresome-like induced structures (DALIS), which are accumulations of ubiquitinated proteins, specifically in BMDCs, as they were not observed in heated T cells or fibrosarcoma cell lines[11]. The correlation of DALIS formation with the thermally enhanced ability of BMDCs to cross-present exogenous antigen to T cells led to the suggestion that these structures might facilitate the loading of peptides onto MHC class I molecules [11]. This DALIS formation was also found to occur independently of HSP70 and Hsf-1 [11]. Interestingly, in lymphocytes it has been found that mild hyperthermia can induce an activation phenotype that involves the aggregation of spectrin with PKC, RACK-1 and HSP70 [12,33]. Thus it is intriguing to speculate that similar alterations in cytoskeletal associated aggregates might be involved in directing the activation associated migration and/or maturation of DCs upon mild thermal stress.

There has been some evidence that membrane associated events such as that involving the regulation of Ca^{2+} channels or lipid fluidity are involved in the ability of immune cells to sense changes in their thermal microenvironment. Interestingly, TRPV receptors, which are Ca^{2+} -

permeant ion channels that have been identified as heat receptors on sensory neurons [8], were also reported to be expressed on DC in data not shown [3]. Heat-induced changes in intracellular free Ca^{2+}, which correlated with the activation of phosphoinositide turnover, led Calderwood et al to speculate over 15 years ago that this class of lipids as well as Ca^{2+} homeostatis may be involved in the cellular response to heat [7]. Furthermore, the expression of several genes that respond to changes in temperature appear to be influenced or controlled by the membrane's physical state [32]. Thus, temperature sensitive biophysical properties of lipid membranes may play a role in the ability of DCs to sense changes in environmental temperature.

Summary Discussion

Overall, mild thermal stress appears to prime DCs in a manner that could enhance their ability to initiate and regulate immune responses. Several different thermally sensitive points of DC function have been identified beginning with antigen uptake and including T cell stimulation and cytokine production (**see** Table 1). Physiological increases in temperature might thus be used as an exogenous 'danger signal' to help mature DCs to a state where they activate antigen specific T cells, instead of deleting these cells and reinforcing peripheral tolerance as immature DCs are presently thought to do [25,28]. This highlights the potential use of elevated temperature to help overcome immune tolerance to tumors. Indeed, the studies outlined here support various clinical uses of hyperthermia as part of an immunotherapeutic strategy in treating cancer. However, to maximize the use of thermal stress in the clinic, various questions must still be answered. For example, what are the mechanisms by which elevations in temperature regulate DC function? While heat shock associated Hsf-1 and extracellular HSPs do not appear to be involved [11,35], the role of other stress associated signaling molecules, such as NFkB [3] and the stress associated protein kinase (SAPK) warrant further investigation. Future studies defining the role of protein aggregate structures such as DALIS [11] and spectrin associated aggregates [12,33], as well as TRPV receptors [3] or other membrane associated events [7,32] in regulating DC activation upon mild thermal stress will also be crucial to this field of study.

Other questions must be explored before we can develop rational approaches to using hyperthermia to enhance dendritic cell function in response to immunotherapy. When is the best time to apply hyperthermia to DCs and at what temperature will maximal results be obtained? Will systemic (whole body treatments) be better than local heating protocols? Will multiple *in vivo* heat treatments be more effective than a single one? Based on the studies summarized here, there are several thermally sensitive points that could be approached sequentially or by long duration, systemic treatments. Since DCs are known to play a role at the initiation of the immune response (i.e., following vaccination in the skin) and later, at the effector stage in the tumor, there could be merit in applying hyperthermic treatment at both of these timepoints. No data currently exists that indicates which protocol will be better. The studes by Zheng *et al* [35] indicated that DC might even be heated in vitro while loading them with antigen and then administering the cells as a DC vaccine to patients **(see** Fig. 1A). Alternatively, some of the studies described above support the idea that thermal therapy might be most effective when administered *in vivo* immediately after vaccination [3,20] (**see** Fig. 1B), either locally at the site of vaccine injection or systemically as a whole body hyperthermia, which would also affect the immune activities occurring in the draining lymph nodes or at the site of tumor (**see** Fig. 1C and D). These different strategies need to be directly compared for their efficacy.

In summary, while there remain multiple questions with regard to molecular mechanisms, there is growing evidence that critical cells in the immune response, such as antigen presenting cells, are highly sensitive to their thermal microenvironment. This relationship may have evolved as

a component of the febrile response in vertebrates upon infection. Full understanding of this intriguing relationship will provide critical information needed for the development of new clinical trials utilizing fever-like hyperthermia in combination with immunotherapy.

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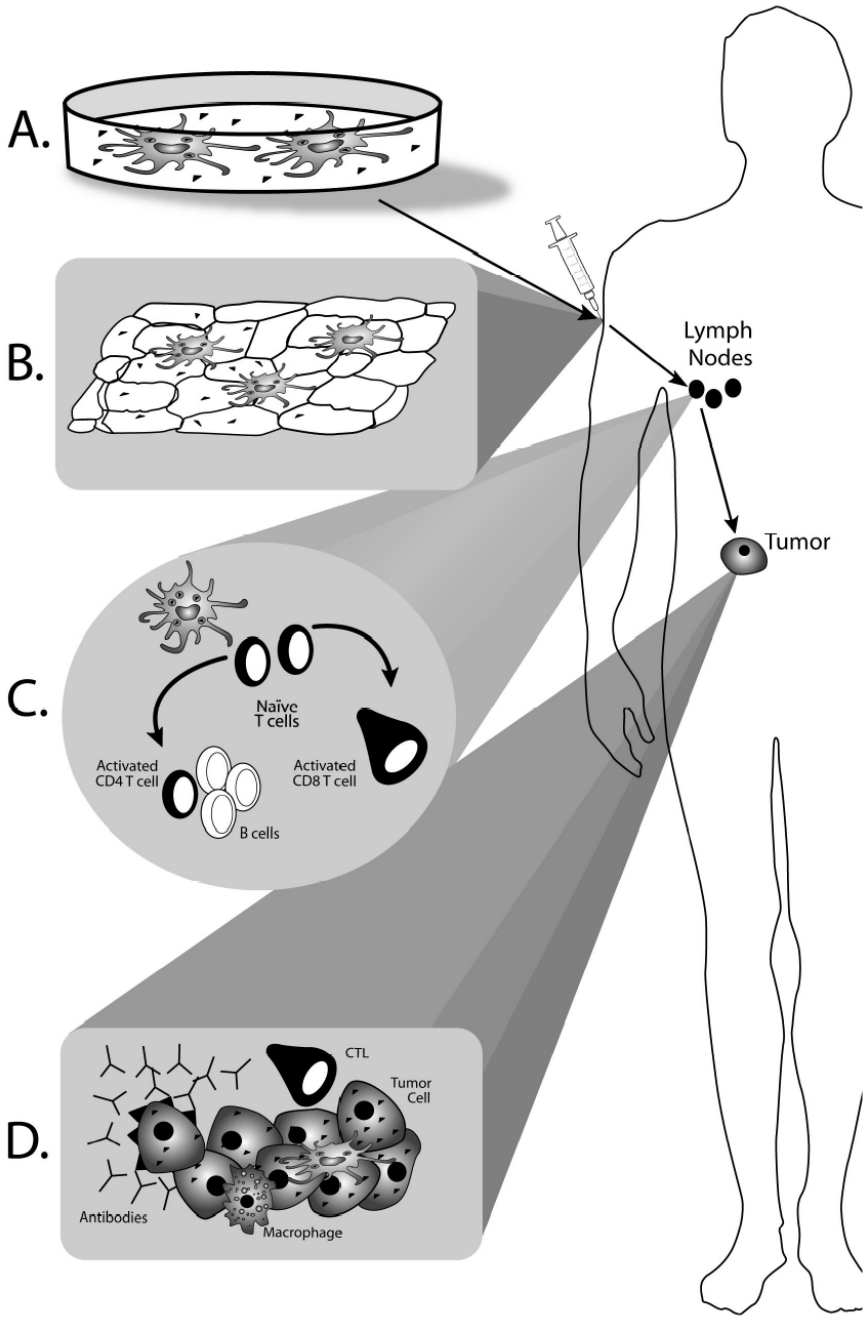

Figure 1. Several timepoints at which application of mild thermal stress might enhance the anti-tumor immune response.

Application of mild thermal stress *in vitro* (A) might assist in enhancing the loading of DCs with antigen and the resultant DC maturation prior to their use as DC based vaccines. Alternatively, application of mild thermal stress *in vivo* directly after administering an antigen based vaccine (B) might enhance the *in vivo* antigen uptake and activation associated migration of peripheral DCs to the draining lymph nodes (C), where they can then activate naïve T cells. Thus the application of heat would have the potential to enhance both antibody and cytotoxic T cell (CTL) mediated immune responses against tumor cells (D). Either local heating of the skin or systemic whole body hyperthermia could be used to upregulate the DC activity directly

after vaccination, as shown in B, but systemic hyperthermia might have the added advantage of helping enhance immune responses at other sites besides the skin, such as the lymph nodes (as in C) and the tumor site (as in D).

Table 1

Summary of APC activities known to be affected by mild thermal stress.

Activity	APC source [references]	Effect of hyperthermia	Experimental design
Antigen uptake	Murine resident peritoneal macrophage [30,34]	Enhanced phagocytosis	2h + cultures up to 40°C
Activation associated migration	Murine epidermal DCs (LCs) [21,23]	Enhanced migration out of epidermis	WBH and ear skin culture @ 40°C
		Enhanced localization to draining lymph nodes	WBH following FITC application
Activation associated maturation	Murine epidermal DCs [21]	Enhanced MHC II and CD86 surface expression	6.5h ear skin culture @ 40°C, 2–3 day recovery
	Human monocyte derived DCs	No effect	7h culture @ 40°C ± LPS
	8 day culture derived murine BMDCs [29]	No effect	3h culture @ 39 or 40°C ± LPS, 15h recovery
	5 or 6 day culture derived murine BMDCs [3,35]	Enhanced MHC I, MHC II, CD40, CD80 and CD86 surface expression	12–24h culture@39.5 or 40°C, 12h recovery
	Murine CD11c+ splenic DCs [35]	Enhanced MHC II and CD86 surface expression	WBH, 18h recovery
Cytokine release	8 day culture derived murine BMDCs [29]	Enhanced IL-12p70; reduced IL- 10 and TNF-α	3h culture @ 39 or 40°C + LPS or bacteria, 15h recovery
		No effect on IL-12p70, IL-10 or TNF-α	3h culture @ 39 or 40°C, 15h recovery
	5 day culture derived murine BMDCs [35]	No effect on TNF-α, IL-1β or IL-12	24h culture @ 40°C, 12h recovery
T cell stimulation	Murine epidermal DCs [21]	Enhanced allostimulation	6.5h ear skin culture @ 40°C, 3 day recovery
	8 day culture derived murine BMDCs [29]	Enhanced allostimulation	3h culture @ 39°C + LPS
	6 day culture derived murine BMDCs [3]	Enhanced allostimulation	6h culture @ 41°C
		Enhanced Ag specific T cell IFN-γ release	microinjection of Ag, 6h culture @ 41°C
	5 day culture derived murine BMDCs [35]	Enhanced allostimulation	24h culture @ 40°C, 12h recovery
		Enhanced Ag specific T cell proliferation in vivo	24h culture @ 40°C, 12h recovery, loaded with Ag and injected i.p.
	Murine epidermal DCs (LCs) [23]	Decreased proliferation of Ag specific draining LN T cells	WBH before Ag applied on skin, LN harvested 5 days later
		No effect on proliferation of Ag specific draining LN T cells	WBH after Ag applied on skin, LN harvested 5 days later
	Murine CD11c+ LN DCs [3]	Enhanced activation of Ag specific T cells in vitro	WBH after i.d. Ag injection, LN DCs harvested 1 day later

Ag, antigen; BMDC, bone marrow derived DCs; LN, lymph node; WBH, whole body hyperthermia