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**INFLUENCE OF HEAT STRESS ON HUMAN MONOCYTE-DERIVED DENDRITIC CELL
FUNCTIONS WITH IMMUNOTHERAPEUTIC POTENTIAL FOR ANTITUMOR
VACCINES**

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ABSTRACT: Mild heat stress can modulate the activities of immune cells, including dendritic cells (DC), and theoretically would constitute an innovative approach capable of enhancing the anti-tumor functions of DC. Therefore, we tested the effects of mild heat stress on the physiology and viability of human monocyte-derived DC, the major type of DC used in tumor immunotherapy trials. We first designed a heat stress protocol consisting of repetitive sub-lethal heat shocks throughout the generation of DC. Using this protocol, we observed that heat stress did not perturb either (1) the morphology and phenotype of immature or mature DC; or (2) the capacities of immature DC to uptake antigens efficiently. It is noteworthy that in response to heat stress, mature DC produced higher levels of IL-12p70 and TNF α , which are two cytokines involved in the stimulation of inflammatory reaction, whereas IL-10 production remained low. After heat stress exposure, mature DC have the full ability to stimulate naive T cells with Th1 response polarization (high IFN- γ and low IL-4 production) in a mixed allogeneic lymphocyte reaction. Interestingly, heat stress enhanced the migratory capacities of DC in response to MIP3 β /CCL19. Finally, heat stress partly protected DC from apoptosis induced by cytokine withdrawal. Overall, these findings validate the feasibility of improving immune response by heating human monocyte-derived DC and provide a strong rationale for using mild heat stress in combination with DC vaccination to increase antitumor response.

Key-words: heat shock, immunotherapy, temperature, hyperthermia, apoptosis, dendritic cells

INTRODUCTION

A growing body of evidence has demonstrated that dendritic cells (DC) are key actors in the diverse facets of immune regulation, including the induction of antitumor immune response (1, 2). Indeed, DC are the most potent antigen-presenting cells. In an immature state, DC exhibit the capacities to capture and process tumor antigens with high efficiency. Subsequently, DC undergo maturation and migrate to lymphoid organs in order to stimulate T cells against specific antigens, e.g., tumor antigens, thus allowing the development of cell-mediated cytotoxicity towards tumor cells. During the last decade, the use of DC has been tested in clinical immunotherapy against cancer, and preliminary positive results of vaccine therapy have already been reported. To perform cancer immunotherapy programs, high numbers of DC are required. For this purpose, methods have been developed to produce DC *in vitro* from precursor cells, including bone marrow, cord blood stem cells and easily accessible blood monocytes. Monocyte-derived DC generated *in vitro* in the presence of IL-4 and GM-CSF are the major type of DC currently being used in tumor immunotherapy trials.

It has been shown that fever-like temperature changes might activate innate and also adaptive immunity (3, 4). Hyperthermia regulates L-selectin-dependent adhesion of lymphocytes to endothelium (5) and T cell proliferation and activation (6). Moreover, mild heat stress can also modify DC immune functions (for review (7)). For instance, heat shock primes and matures mice DC in a heat shock protein (HSP) independent manner (8) and enhances the migration of Langerhans cells from the epidermis to lymph nodes. The aim of this work was to characterize the consequences of heat stress exposure on the phenotype, immune functions and viability of human monocyte-derived DC. As monocyte-derived DC play a pivotal role in immunotherapy against tumors, enhancing their function through heat stress might be a key mechanism for the development of new cancer vaccines.

MATERIALS AND METHODS

Reagents

FITC-conjugated monoclonal antibodies (mAbs) used were anti-HLA-DR, anti-CD86, anti-CD14, and anti-CD1a (Becton Dickinson, Le Pont de Claix, France), as well as anti-HSP70 and anti-HSC70 (Tebu, Le Perray-en Yveline, France). PE-conjugated mAbs included anti-CD80 and anti-IFN γ (Becton Dickinson), in addition to anti-CD83 (Beckman Coulter, Villepinte, France). Isotype control mAbs included FITC-, PE-labeled mouse IgG1 or IgG2a (Becton Dickinson). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 and propidium iodide (PI) was obtained from Sigma (Saint Quentin Fallavier, France). ProSpec-Tany TechnoGene LTD (Le Perray en Yvelines, France) provided GM-CSF and IL-4, while R&D Systems (Abington, UK) provided MIP-3 β /CCL19. 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Invitrogen (Cergy Pontoise, France) FITC-Annexin kit from Becton Dickinson. Ionomycin and Phorbol-12-myristate-13-acetate (PMA) were provided by Calbiochem (Fontenay sous bois, France). Finally, eBioscience (Montrouge, France) was the source of Brefeldin A.

Monocyte-derived dendritic cell generation

Blood monocytes from healthy donors were isolated from buffy coat. The buffy coat product was diluted in RPMI 1640 (Life Technologies, Paisley, Scotland) and layered over Ficoll gradient (Pharmacia, Upsalla, Sweden). After centrifugation (400g, 30 min.), peripheral blood mononuclear cells (PBMC) were harvested and washed. The PBMC were re-suspended in 100 ml in 0.9 mg/ml NaCl. Monocytes were purified by elutriation using an Avanti J-20 XP elutriation centrifuge from Beckman Coulter. The elutriation process was performed at 2500 rpm rotor speed, and cells were loaded in the elutriation chamber at a counterflow rate of 11 ml/min. Then, the flow rate was increased to 20 ml/min in order to eliminate T cells. The monocyte-enriched fraction was harvested at a flow rate of 30 ml/min. To obtain a monocyte

purity of > 95 %, elutriation was followed by an adhesion step: $10\text{-}25 \times 10^6$ monocytes were cultured in a 75cm^2 flask for 2 h at 37°C in RPMI 10 % fetal calf serum (FCS) supplemented with antibiotics, non essential amino acids and $5 \times 10^{-5}\text{M}$ 2-mercaptoethanol (complete RPMI). After intensive washes of the flask, monocytes were cultured in complete RPMI in the presence of GM-CSF (800 U/ml) and IL-4 (100 U/ml). On day 3, GM-CSF and IL-4 were added and further cultured until day 5. The dendritic cells then were harvested, washed and counted, and subjected to maturation in the presence of $1\mu\text{g/ml}$ of LPS for 18-24 h in 6-well plates at 10^6 cells/ml. On day 6, the cells were washed and further cultured for 4 h, or 24 h until analysis.

Heat treatment

The DC heat shock protocol consists of sequential short heat shocks during the differentiation and maturation of DC (Figure 1B). On day 0 and day 3, the culture flasks were dipped for 15 min in a fine-regulated water bath set up at 41°C . Then GM-CSF and IL-4 were added to the cells. DC generated on day 5 were heated at 41.5°C for 30 min and cultured in a 6-well plate in the presence of LPS. After 8 hours (day 5.5), the cells were re-heated at 41.5°C for 30 min. On day 6, the cells were washed and heated at 41.5°C for 30 min and further cultured until analysis. The control plates contained non heat stressed cells.

Immunocytochemistry

The DC underwent a cytopspin in adhesion slides and were frozen until staining. After thawing, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing in PBS, cells were permeabilized in 0.1% SDS at room temperature. After three washing in PBS, the cells were pre-incubated for 30 min at room temperature in a saturation buffer containing 10% FCS. Then cells were incubated with an adequate first antibody for 2 h at 37°C . After washing, the cells were exposed to an appropriate FITC-coupled secondary

antibody 1 h at room temperature. Finally, after rinsing and embedding in Vectashield with DAPI, they were examined using a fluorescence microscope (Leica, x630).

Cell surface immunophenotype

For two-color immunolabeling, the cells were washed twice in ice cold PBS and incubated in 100 μ l PBS containing appropriate fluorochrome-labeled mAbs for 30 min on ice. The cells were washed twice with ice-cold PBS. At least 5,000 cells were analyzed by flow cytometry (Beckman Coulter).

Evaluation of cytokine secretion

IL-12 (p70), TNF α and IL-10 production by mature DC was measured in supernatants by ELISA (Diacclone, Besançon, France). IFN- γ , IL-4, and IL-10 production by T cells co-cultured with DC were measured in supernatants by ELISA (Diacclone) after 5 days of co-culture followed by a 24 hour stimulation with PMA (50 ng/ml) and ionomycin (2 μ g/ml).

Cytokine intracellular staining

After 5 days of co-culture of DC with allogeneic T cells, the cells were harvested, washed and stimulated with PMA (50 ng/ml), ionomycin (2 μ g/ml), and Brefeldin A (3 μ g/ml) for 5 h. Then the cells were fixed and permeabilized using the cytofix/cytoperm kit from Becton Dickinson (France) according to the manufacturer's recommendations. Finally, the cells were stained with appropriate fluorochrome-labeled mAbs for 30 min on ice and washed, and then 5,000 events were analyzed by flow cytometry (Beckman Coulter).

Migration Assay

Four hours after the last heat shock, the DC were washed and re-suspended in migration buffer Hank's balanced salt solution (HBSS), 1mM CaCl₂, 0.5 mM MgCl₂, 1 mg/ml BSA (Sigma) at a density of 2-3 x 10⁶ cells/ml. 600 μ l of chemokine solution or buffer alone was added to individual wells of 24-well plates (Corning Life Science, Croissy, France) on ice. Immediately thereafter, Costar transwell devices with 5- μ m pore size, polyvinylpyrrolidone-

free polycarbonate membranes were inserted into the wells, and 100 μ l cell suspension was layered on top of the membrane. Cells were allowed to attach to and transmigrate through the membrane for 2 h at 37°C. The fluid phase above the membrane was then removed, and transwell inserts were taken out of the wells. Non-migrated cells were removed by scraping the filter, and the migrated filter-bound cells were fixed with 40 mg/ml paraformaldehyde for 30 min. at 4°C. The filters were mounted onto slides, and embedded in a VectaShield medium containing DAPI (Vector Laboratories, Paris, France) for staining cells in order to enumerate the cells that had migrated through the filter. Migrated cells were counted on the bottom side of the filter in 10 randomly selected high power fields (hpf) (magnification x 630).

Allogenic T cell activation

Naive CD4⁺ T cells were purified using the CD4⁺ T Cell Isolation Kit II from Miltenyi Biotec (Paris, France) and CD45RO beads Miltenyi Biotec. Briefly, the PBMC were incubated with CD45RO microbeads and a cocktail of biotin-conjugated mAbs against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and Glycophorin A for 10 min at 4°C and then incubated with anti-biotin microbeads for 15 min at 4°C. After washing the cells, the magnetically labeled non-CD4⁺ T cells were depleted by retaining them on a MACS column in a magnetic field, and the CD4⁺ CD45RA⁺ naive T cells were recovered. After washing, the cells were frozen until use. Once thawed, CD4⁺ CD45RA⁺ naive T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). The cells were washed in RPMI without FCS, and incubated with CFSE (2.5 μ M) at 37°C for 10 min. Reaction was stopped by adding FCS. Then the cells were washed extensively and re-suspended at 10⁶ cells/ml in a complete medium. In parallel, DC obtained 4 h after the end of the maturation were diluted at 100,000 cells/ml. A graded numbers of DC were dispensed in individual wells of 96-well round-bottom plates and co-cultured with 3 x 10⁵ allogeneic naïve CD4⁺ T cells for 5 days. The cells were then harvested and T cell proliferation was analyzed immediately by flow

cytometry measuring the CFSE fluorescence intensity. In parallel, cells were restimulated with phorbol-myristate-acetate (PMA) 50 ng/ml and ionomycin 2 µg/ml for 24 h. Then, supernatants were collected in order to measure secreted cytokines. Cells were also restimulated with PMA (50 ng/ml), ionomycin (2 µg/ml), and Brefeldin A (3 µg/ml) for 5 h in order to analyze cytokine expression intracytoplasmically.

Induction and evaluation of apoptosis

DC were cultured and heated as described above. To induce apoptosis, DC were harvested on day 5, extensively washed and further cultured for 28 h in medium without GM-CSF and IL-4 (GM/IL-4). Apoptotic DC were detected using the FITC-Annexin kit (BD Pharmingen) according to the manufacturer's instructions .

FITC-dextran assay

To evaluate the capacities for uptake of soluble antigens from the culture medium DC were incubated with 1mg/ml FITC-dextran (Sigma) at 37°C for 1 h. As a negative control, cells were incubated under the same conditions at 4°C. After incubation, the DC were washed twice in PBS and then analyzed by flow cytometry.

Statistical analysis

The results were analyzed using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA, USA). For the analysis of cytokine production and migration capacities in non heated and heated DC, a Wilcoxon matched rank test for paired data was employed. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of heat stress on the viability of DC

In a first series of experiments, we determined the conditions of exposure to heat that were just below a level that promoted DC death. Monocyte-derived DC on day 5 of culture were exposed to temperatures of 40°, 41°, 41.5°, 42° or 43°C for 15 to 90 min, followed by recovery at 37°C for 24 hours. We observed that the impact of heat stress on the viability of DC was dependent upon the temperature and duration of exposure: the DC did not lose viability upon exposure to 41.5 °C for up to 30 min. However, exposure to 42° or 43° C for 15 min was slightly lethal (Figure 1A); and beyond 60 min was highly toxic for DC. Extended incubation periods (24h and 48h), even at lower temperatures (i.e., 41° and 41.5°C), dramatically decreased the viability of DC (data not shown).

Since heat stress-induced HSP could play a crucial role in DC function, we also evaluated the impact of heat stress on HSPs expression by immunofluorescence (Figure 1B). Short incubation periods of 15 min at 41°C allowed an higher expression of HSP 27, 60, 70, 90 on heated DC (data not shown). Nevertheless, to maintain a high level of HSP expression along the culture, repeated heat shocks were needed. Indeed, cells heated once on day 0 or on day 3 of the culture either did not express or only partially expressed HSP70 on day 6, respectively. Conversely, cells heated once on day 5 strongly maintained high levels of HSP70 (Figure 1B). No significant differences in the expression levels of HSC 70, used as a control, were found after heat stress (Figure 1B). Based on these results, we designed a heat stress protocol consisting of repetitive sub-lethal heat shocks throughout *ex vivo* generation of DC (Figure 1C). This protocol was not toxic and allowed a high expression level of HSP 70 on DC (data not shown). Thus, under the optimal conditions of heating defined above, we evaluated the effects of heat stress on the physiology of monocyte-derived DC.

Effect of heat stress on the morphology and phenotype of DC

Adherent monocytes cultured *ex vivo* with GM-CSF and IL-4 for 6 days differentiated from non-adherent cells, which displayed typical morphological features of DC, i.e., the presence of cytoplasmic extensions on the periphery and irregularly shaped nuclei (Figure 2A). Heat stress did not modify the morphological aspects of DC when compared with control DC (Figure 2A). The expression of DC surface antigens was investigated by flow cytometry on immature (Figure 2B) and mature (Figure 2C) monocyte-derived DC exposed to heat stress or not (control). More than 80 % of nonadherent cells generated *ex vivo* with GM-CSF and IL-4 exhibited CD1a, a specific marker of dendritic cells, were negative for the monocyte-macrophage marker CD14, and exhibited a phenotype of immature DC (low expression or absence of HLA-DR, CD80, CD86 and CD83). Heat stress did not modify the expression of cell-surface molecules of immature DC (Figure 2B), nor did it result in the maturation of DC or in the modification of the maturation-associated phenotypical changes induced by LPS (Figure 2C). Next, the endocytic capacities of immature monocyte-derived DC was assessed by their ability to endocytose FITC-dextran. Immature heated DC internalized a comparable amount of FITC-dextran particles ($85 \% \pm 10$; 360 ± 66 MFI) as control immature DC ($88 \% \pm 10$; 349 ± 60 MFI) ($P=0.37$). Overall, these results indicate that heat stress modifies neither the phenotype of DC nor the capacities of immature DC to uptake antigens efficiently.

Heat stress regulated IL-12p70 and TNF α secretion

We then investigated whether heat stress could induce mature monocyte-derived DC to modulate the level of cytokine production. We focused on three major cytokines produced by DC: IL-12 p70, which plays a key role in the modulation of the T cell response toward a Th1 profile; TNF α , a pro-inflammatory cytokine; and IL-10, a cytokine that down-regulates DC functions. Immature DC did not produce these cytokines, and heat stress did not modulate the

level of cytokines in the supernatant (not shown). Mature DC produced only low levels of IL-10, and differences between heated and unheated DC were undetectable (Figure 3). In contrast, mature DC produced a significant amount of IL-12 p70 and TNF- α (Figure 3) and, upon heat stress, the production of IL-12 p70 and TNF- α increased significantly in the DC supernatants (Figure 3). TNF- α showed the most important increase with an average of 2.5 fold increase in secretion from DC cultures after heat stress. Thus, it appears that heat stress significantly enhances the production of cytokines by mature monocyte-derived DC.

Migration of DC in response to CCL19/MIP-3 β is enhanced by heat stress

To assess whether heat stress affects monocyte-derived DC migration, heated and control DC were evaluated for their migratory activity in a transwell system (Figure 4), as previously described (9). After 2 h of incubation in the presence of recombinant human CCL19/MIP-3 β , the migration of mature DC was significantly increased after heat stress. These results clearly demonstrate that heat stress can enhance the migratory capacities of mature monocyte-derived DC. In contrast, heat stress did not alter the response of immature DC against either of the chemokines CCL3 and CCL5, known to attract immature DC (data not shown).

Heat stress increases the capacities of LPS-stimulated DC to activate allogeneic naive T cells

We further assessed the impact of heat stress on the capacities of mature monocyte-derived DC to induce proliferation of T cells in a mixed allogeneic leukocyte reaction using CFSE labeling. CFSE is a stable cytoplasmic fluorescent dye that segregates equally between daughter cells upon cell division, enabling fine analysis of T cell proliferation both *in vitro* (10) as well as *in vivo* (11). Upon cell division, the relative intensity of the dye is decreased and dividing cells are characterized by low CFSE content (CFSE^{low} cells). On day 6, mature

control and heated DC were co-cultured with allogeneic naive CFSE-labelled CD4⁺ T cells for 5 days, and the percentage of CFSE^{low} T cells was estimated by flow cytometry as shown in Figure 5A. After the co-culture with control DC, alloactivation of CD4⁺ T cell was evident, since a significant proportion of CD4⁺ T cells were CFSE^{low}, indicating that they had undergone multiple cell divisions (Figure 5A). In addition, heated DC were significantly more potent in inducing T cell proliferation than control DC (Figure 5A and 5B).

We next evaluated the ability of heated DC to polarize naive T cells toward a Th1 or a Th2 phenotype in a mixed allogeneic leukocyte reaction. Heated and control DC were seeded together with purified CD4⁺ CD45RA⁺ T cells for 5 days, and the supernatants were analyzed for IL-4 and IFN- γ production. Consistent with their ability to release a higher amount of IL-12 p70, a strong inducer of Th1 differentiation (12), heated DC significantly induced a higher amount of IFN- γ in comparison to control DC (Figure 5C, left panel). Moreover, the difference was clearly sustained through the re-stimulation of T cells with PMA/ionomycin (Figure 5C, right panel). It is worth noting that heated DC mainly increased the proportion of IFN- γ secreting CFSE^{low} T cells, indicating that heated DC favors IFN- γ production by dividing T cells (Figure 5D). In contrast, only a small amount of IL-4, a Th2 cytokine, was detected in supernatant, and the level was not significantly modified by heat stress of DC (0.19 +/- 0.12 pg/ml for supernatant of control DC vs. 0.43 +/- 0.25 pg/ml for supernatant of heated DC; P = 0.25). Taken together, although expressing same levels of co-stimulatory molecules, heat stress enhances the capacities of LPS-pulsed DC to activate T cells and to promote a Th1 dominated response.

Heat stress reduced DC apoptosis

Since it has been described that repetitive exposure to heat shock protects cells from apoptosis (13), we tested the effect of heat stress on the apoptosis of DC induced by GM-CSF and IL-4

deprivation (Figure 6). GM-CSF/IL4 withdrawal induced a significant increase of early apoptosis (Annexin V+ PI-). The number of necrotic cells (AnnexinV + PI +) was only slightly increased. Heat stress had no toxic effect on DC, and significantly reduced the number of early apoptotic cells in response to GM-CSF/IL4 withdrawal (Figure 6).

DISCUSSION

The specific aim of the present study was to investigate whether the immunological function of human monocyte-derived DC generated *ex vivo* are potentiated by heat stress. We have established a heat shock protocol consisting of successive sub-lethal heat shocks throughout monocyte-derived DC culture. Our data indicate for the first time that human monocyte-derived DC are sensitive to heat stress and that the application of heat might constitute an alternative strategy to initiating a stronger immune response following DC vaccination for cancer therapy.

DC are antigen-presenting cells that play a critical role both in stimulating and silencing immune responses. Thus, it is tempting to control the direction of immune responses by manipulating DC. The differentiation and function of DC are largely regulated by exogenous factors such as UV- or γ -irradiation, microbial molecules and heat shock (14). γ -irradiation can result in DC maturation, but γ -irradiated DC reduced IL-12 production and were less effective at naïve T cell priming (15). UV radiation delays migration of Langerhans cells into the draining lymph nodes (16). Mildly elevated temperatures associated with inflammation or fever have been linked to enhanced immunological functions and might constitute a promising means to enhance the potency of DC-based anti-tumor vaccines (for review (7)). Indeed, we have demonstrated that heat stress can significantly improve the immune response of human mature monocyte-derived DC acting on several DC functions.

First, we observed that heat stress modulates cytokine secretion by monocyte-derived DC. During maturation, heated DC favored TNF α production, a proinflammatory cytokine with antitumor effect, although the level of IL-10 remained low. It is well-known that IL-10 producing DC are considered to be tolerogenic DC involved in the establishment of tolerance favoring the growth of the tumor (17). The secretion of these both cytokines suggests that heated DC should favor anti-tumor response. In parallel, mature DC secrete cytokines that

contribute to the polarization of helper T cells toward Th1 or Th2, depending on the type of the secreted cytokines. Interestingly, we observed a higher production of IL-12 p70 in heated monocyte-derived DC than in control cells. These data confirm those previously obtained with murine DC (18). Since it is known that IL-12 favors differentiation of T cells into Th1 cells (19), we further explored the effects of heat exposure on the Th1-polarizing capacities of monocyte-derived DC. We demonstrated that when exposed to heat stress, mature DC activated naïve T cells with a high capacity to induce T cell proliferation and a Th1 response (high level of IFN- γ , trace of IL-4). Thus, heat stress exposure favors the Th1-polarizing capacities of human monocyte-derived DC and TNF- α -secreting DC, both important effects for the development of immunity against tumor cells in animal (20) and human studies (21). In mice, the same effect has been described, i.e., a higher capacity of heated bone marrow expanded DC (BM-DC) to induce a mixed lymphocyte reaction (MLR) (8, 18, 22). Specifically, data suggests that it is a HSP90-dependent mechanism (22). In addition, HSP60 was described as inducing the maturation of murine BM-DC *in vitro* and eliciting a Th1 promoting phenotype(23). Our heating protocol strongly induces the expression of HSP90 and HSP60 in cytoplasm (data not shown), suggesting the same role of these molecules in human DC as in mouse DC. However, these immunological effects could be related to the overexpression of HSP70 on heated DC. It has been recently reported that in humans, unlike mice, HSP70 co-localized with MHC class I molecules, suggesting a role for HSP70 in class 1 antigen presentation (24). In addition, an other recent study shows that the increase in IL-12 production could be induced by HSP70, which binds and activates CD40 (25).

Second, in our study mature DC exposed to heat stress displayed a higher capacity to migrate in response to CCL19/MIP3 β in comparison to non heated cells. CCL19/ MIP3 β is a chemokine playing a pivotal role in the migration of DC into the lymph nodes (9). These results are consistent with the observation that, in mice, fever-range whole body hyperthermia

enhances the migration of Langerhans cells from the epidermis to the lymph node (26). These cells also display a higher capacity to stimulate T cell proliferation in a mixed leukocyte reaction (27).

Despite these immunostimulating effects, it must be noted that our heat shock protocol, consisting of several instances of successive mild heat stress, did not allow human monocyte-derived DC to significantly mature (Figure 2). In contrast, longer exposure to heat leads to the maturation of mice DC with a substantial increase in MHC I, II and co-stimulatory molecules such as CD80, CD86, CD40, (8, 22), which were not noted in humans (data not shown, (24, 25)). Interestingly, we have not observed any effect on immature DC in terms of phenotype (Figure 2), antigen uptake capacities, or migration against CCL3 and CCL5 (data not shown). This suggests that our heating procedure should not affect precursors and their capacities to differentiate into DC in the presence of IL-4 and GM-CSF, but rather differentiated DC.

Very recently, others have described that heat shock induces an increase in the expression of the Toll Like Receptor-4 (TLR-4) on monocyte-derived DC (24). TLR-4 recognizes microorganism-associated molecular patterns such as LPS. On DC, TLR-4 is required for LPS signaling (28). In our study, DC underwent maturation with LPS stimulation. We can hypothesize that DC exposed to heat stress might express more TLR-4 on their surface, which might induce a higher response to LPS (i.e., increase in (i) IL-12 and TNF α secretion, (ii) T cell activation and (iii) capacities to migrate in response to CCL19).

Since the longevity of DC may also play a role in regulating the strength of immune responses, we studied the effect of heat stress on apoptosis. Growth factor deprivation induced the aberrant exposure of phosphatidylserine residues on the outer plasma membrane leaflet of DC, a typical hallmark of apoptosis. We demonstrated that heat stress mediated significant protection, albeit transient (< 24h), against DC apoptosis (not shown). Recently it has been shown that IL-12 and TNF- α protect DC against apoptosis through an increase in anti-

apoptotic factors of the Bcl-2 family (29-32). However, we were unable to confirm this effect in our model since no changes in Bcl-2 or Bclxl expression in heated DC were observed (data not shown).

For over the last decade, numerous pre-clinical and clinical trials (Phase I and II) have demonstrated that the injection of human monocyte-derived DC pulsed with relevant antigen can induce a specific immune response in cancer patients and occasionally lead to the regression of metastatic tumors. However, the immune reaction is often transient, with limited efficacy (33, 34). A number of mechanisms have been suggested for the lack of a sufficient immune response to control cancer growth *in vivo*, leading to escape of cancer cells from immunosurveillance and thus limiting the efficacy of DC-based vaccines. Innovative approaches capable of enhancing anti-tumor functions of DC are therefore needed to overcome the limitations of current DC-based immunotherapy for cancer. Recently, it has been suggested that mild heat stress has the potential to improve the immune function of DC (for review (7)). Our work offers new experimental evidence supporting this concept and thus provides the rationale to improve the efficacy of DC-based immunotherapy in humans. The advantages of this approach are obvious in terms of cost and safety, given that exogenous material is injected together with DC. However, despite the obvious impact of repetitive heat shock on monocyte-derived DC functions and viability, it is very difficult to speculate how these results can be extrapolated to the *in vivo* context. Consequently, we assume that modifications of procedure are needed to further increase immune induction strongly enough to effectively employ DC for cancer immunotherapy. In order to further optimize our protocol, it will be very important to control the initial population of monocytes used to generate DC. Indeed, it has been recently described an heterogeneity in monocyte population directly purified from buffy coat in terms of the number of apoptotic cells (35). In this case,

heat stress might offer a promising way to improve the efficacy of monocyte-derived DC and could be beneficial for immunotherapy trials in cancer patients.

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FIGURE LEGEND

Figure 1. Effect of heat stress on DC viability and optimized heat stress protocol used in this study. (A) Influence of heating on death induction of monocyte-derived DC. On day 6 of the culture, monocyte-derived DC were heated at the indicated temperatures. At the indicated time points after heating, cells were recovered at 37°C for 24 hours and cell death was determined by Trypan Blue assay. Values represent the mean of 3 independent experiments. (B) Expression of HSP70 and HSC70 on mature DC obtained on day 6. Cells were heated for 15 min at 41°C on the indicated day of the culture or not heated (control). On day 5, cells underwent maturation for 24 h; then the cells were seeded on slides and were subjected to an indirect anti-HSP70 (upper panels) or anti-HSC70 (lower panels) immunostaining. The pictures shown were taken with a fluorescent microscope (X 630). (C) Diagram of the experimental protocol of repetitive short heat shocks used in this study.

Figure 2. Effect of heat stress on the morphology and phenotype of DC. (A) Microscopic analysis of DC following heat stress (right, HS) or kept untreated (left, control). After the last heat shock (on day 6), cells were further cultured for 4 h and stained with May Grünwald Giemsa (MGG) coloration. Original magnification x 630; Immunophenotypic analysis of immature (B) and mature (C) monocyte-derived DC heated (HS) or left unheated (Control). Monocyte-derived DC were generated and heated as described in the Material and Methods section. Cell samples were immunostained and analyzed by flow cytometry before (B, on day 5 of the culture) or after maturation with LPS (C, on day 6 of the culture). Red histograms represent the reactivity of a specific antibody; white histograms represent the reactivity of an isotype-matched control antibody. **Markers indicate the population of positive cells and numbers refer to the percentage of positive cells.** Results are representative for seven different experiments.

Figure 3. Regulation by heat stress of cytokine secretions of mature DC. Monocyte-derived, non heated (Co: white bars) or heated (HS: black bars) DC underwent maturation in the presence of LPS for 24 hours. Then supernatants were collected and IL-12 p70, TNF α and IL-10 were measured. The data show mean cytokine levels of culture supernatants (\pm SEM) representing 10 independent experiments. *, $p < 0.05$ as compared with control.

Figure 4. LPS-induced DC migration toward CCL19/MIP 3 β is increased by heat stress.

On day 6 of the culture, migration toward CCL19/MIP 3 β (100ng/ml) of mature heated DC (\bullet) or mature control DC (\circ) was analyzed using transwell chambers. 3×10^5 DC were seeded in the upper chamber in triplicate, and the number of migrated DC was analyzed by counting in 10 randomly selected high power fields on the bottom side of the filter after 2 h. Data are from 8 separate donors; circles represent single individuals, and horizontal bars represent group mean values *, $p < 0.05$ between the two groups.

Figure 5. Heated DC stimulate the capacities to induce a Th-1 response in a mixed allogeneic reaction. (A,B) On day 6 of the culture, a graded number of control and heated DC were co-cultured with allogeneic naive CD4 $^+$ T cells stained with CFSE. Then, after 5 days of co-culture, T cell proliferation was determined by the percentage of CFSE $^{\text{low}}$ cells: **(A)** represents typical FACS histograms of CFSE staining of T cells after 5 days of co-culture with DC at a ratio of 1:10 (DC:T) (full lines). Dotted lines represent control T cells cultured for 5 days without DC. The bar represent cells considered as CFSE $^{\text{low}}$. **(B)** The left panel is a typical representation of proliferation curves determined at a different stimulator-responder ratio ranging from 1:3000 to 1:10. **(B)** the right panel represents the percentage of CFSE $^{\text{low}}$ T cells after 5 days of co-culture at a ratio 1:10 (DC:T). Data are from 7 separate donors; circles represent single individuals, and horizontal bars represent group mean values (*, $p < 0.05$

between the two groups). **(C)** Left panel: the secretion of IFN- γ was measured by ELISA in the supernatant of T cells cultured for 5 days with heated DC (HS, black bar) or untreated DC (control, white bar). Results represent the mean \pm SD of 8 independent experiments ($p < 0.05$ was observed between two groups). **(C)** Right panel: on day 5, T cells were washed and restimulated with PMA/ionomycin for 24 h. The secretion of IFN- γ was measured by ELISA in the supernatant. The results represent the mean \pm SD of 3 independent experiments (*, $p < 0.05$ was observed between two groups). **(D)** On day 5, T cells were washed and restimulated with PMA/Ionomycin/Brefeldin A for 5 h, then IFN γ -secreting cells were analyzed by an intracytoplasmic immunostaining of IFN- γ . Double staining CFSE/IFN- γ were shown by the dot plot (left panel). Right histograms are IFN- γ staining on gated cells (square) corresponding to CFSE^{low} T cells. Black lines represent the reactivity of IFN- γ ; dotted lines represent the reactivity of the isotype-matched control antibody. Results are representative for 4 different experiments .

Figure 6. Heat stress reduced apoptosis induced by cytokine withdrawal. Heated (HS) or unheated (Control) DC were incubated in a medium with or without GM-CSF and IL-4 (-GM/IL-4) for 28 h, then tested for phosphatidylserine exposure and plasma membrane permeability by flow cytometry after staining with annexin V-FITC and PI. (A) Typical Annexin-V-FITC and PI-staining profiles are shown. (B) Early apoptotic (annexin V + PI-) and necrotic cells (annexin V + PI +) were scored. Results represent the mean of 8 independent experiments (SD are less than 10 %) * $p < 0.05$ was observed between heated and control DC.

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