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Dendritic cell function at low physiological temperature

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ABSTRACT

Compared with the stable core temperature, the skin temperature is lower and varies depending on ambient temperature and convection conditions. The function of DCs, which are plentiful in the skin at lower physiological temperatures, has not been reported. We show that DC performed some functions normally at 28°C, including phagocytosis and macropinocytosis. TLR-4 signaling via MAPK pathways was delayed at 28°C but reached normal levels, which may explain the observed slower kinetics of stimulated macropinocytosis and TNF production. TLR-4-induced NO production was compromised severely at 28°C. Collagen degradation and migration through matrigel-coated transwell inserts were decreased, but no effect on podosome number or DC migration through noncoated transwell filters was seen. Lowering the temperature differentially regulated functions associated with the role of DCs in adaptive immunity. LPS-induced up-regulation of CD86 was normal; however, CD40 up-regulation was suppressed after TLR-4 stimulation at 28°C. Nonactivated DC processed and presented antigen on MHC class II equally well at 28°C and 37°C. However, DCs that were loaded with antigens and stimulated with TLR ligand at 28°C were poor at activating T cells at 37°C compared with DCs that were activated and loaded with antigen at 37°C.
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Introduction

Temperature varies between different anatomical locations of the body [1–3]. The skin temperature of the body is on average 31°C (when the ambient temperature is 20°C), but at the same ambient temperature, the skin temperature may be as low as 26°C on the limb extremities [1–3]. As innate immune cells, DCs sample the antigenic milieu of body surface organs, i.e., the skin and mucosa, to maintain peripheral tolerance under noninfection circumstances and to induce immunity when the organism is challenged with a pathogen. To perform these duties, DCs must function under the various conditions present at different tissue locations. Although many DCs in the body function at the low physiological temperatures described above, little is known about how such temperatures affect their function, and publications about DC biology have thus far reported studies at 37°C or at hyperthermal temperatures [4–7]. DCs have different functions during different phases of their life cycle. As immature cells, they take up antigens via endocytosis and phagocytosis and monitor their environment for invading pathogens with PRRs, such as TLR family members [8]. One endocytic process that DCs use to sample their environment continuously is macropinocytosis, which is constitutively active in DCs in comparison with many other cells, such as macrophages [9, 10]. Using macropinocytosis, DCs internalize considerable volumes of extracellular fluid containing proteins and small particles, such as viruses and fragments from bacteria and apoptotic cells [9, 11–14]. The extracellular material that is taken up by DCs is then degraded and loaded onto MHC class II molecules in specialized lysosomal compartments [15–18]. Antigens taken up by DCs may also enter into the MHC class I presentation pathway through a process known as cross-presentation [9, 19].

When DCs recognize pathogen components with TLRs, they respond initially by boosting antigen uptake by the macropinocytic process [20]. After activation, DCs produce inflammatory cytokines, such as TNF, and participate in recruiting other immune cells to the site of infection by producing chemokines [21]. DCs also participate in the defense against pathogens using phagocytosis and the production of oxygen radicals, such as NO, to kill bacteria and other parasites [22, 23]. TLR signaling also prepares DCs for their role in adaptive immunity by inducing the up-regulation of the costimulatory molecules CD80 and CD86, which are important for the activation of naïve T cells [24]. Interactions between the CD40R on DCs and CD40L on Th cells are also important for the activation of naïve T cells. CD40 is induced on DCs triggered with TLR ligands, and subsequent CD40-CD40L interaction leads to fur-

Abbreviations: BMDC=bone marrow-derived DC, CD40L=CD40 ligand, HEL=hen egg lysozyme, MFI=mean fluorescence intensity, SRBC=sheep RBC

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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ther up-regulation of costimulatory molecules and the production of cytokines, such as IL-12 and IFN-γ by DCs, which play a central role in the induction of adaptive immunity [25, 26].

During the maturation process, DCs differentiate into migrating cells that home to draining LNs, where they initiate and shape the adaptive immune response [27]. DCs, like some other migratory cells, form actin-dense structures at their adhesion surfaces that are called podosomes [28, 29], which are sites of matrix metalloproteinase activity and are rich in adhesion molecules. Podosomes appear to be the major site where matrix degradation occurs, enabling DCs to cross matrix barriers [30].

As DCs seem more effective than other APCs in activating naïve T cells, DCs have for some time been considered good candidates for the development of new immunotherapy regimens [31]. Despite the fact that intradermal and s.c. injections are used commonly as administration routes for different types of vaccines that likely require the involvement of DCs, no studies have considered the parameter of low physiological temperatures of the skin when studying the biological functions of DCs. With this in mind, and as DCs have important functions in innate and adaptive immunity, we chose to study some key DC functions at the low physiological temperature of 28°C. For these studies, we use murine BMDCs generated as described previously by Inaba and co-workers [32]. These DCs are possible to generate in sufficient numbers to perform cell biological and biochemical assays and have been used for most studies about cellular processes in murine DCs. Here, we show that some DC functions were somewhat delayed but were otherwise relatively unaffected by this low temperature, whereas other functions were impaired. Our data indicate that temperature is a relevant factor for understanding DC biology and for developing vaccines targeting DCs or immunotherapy using DCs.

MATERIALS AND METHODS

Mice and bone BMDCs

Six- to 10-week-old female C57BL/6 and C3H/HeN mice were housed under specific pathogen-free conditions at the Department of Microbiology Tumor Biology and Cell Biology Animal Facility, Karolinska Institutet (Stockholm, Sweden), and used with the approval of the local ethics committee. The mice were killed by cervical dislocation. Murine BMDCs were generated from femurs and tibia at 37°C [32]. Briefly, cells from the marrow of C57BL/6 and C3H mice were grown in DC culture medium [DMEM containing 4.5 g/l glucose, L-glutamine, pyruvate, streptomycin (Invitrogen, Carlsbad, CA, USA), 10% FBS (HyClone, Logan, UT, USA), and 10 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ, USA)]. On Day 3, 1 ml fresh medium with GM-CSF was added to a final volume of 3 ml. On Day 6, the cells were harvested for use. The DC cultures were maintained at 37°C until the start of the experiments.

Analysis of cytokine production

DCs were stimulated with LPS for 4–18 h at 28°C or 37°C, and cell-free supernatant was harvested. TNF production was analyzed in triplicate using an OPTEIA kit (BD Biosciences, San Jose, CA, USA). Color conversion of the ELISA substrate K-blue (Neogen, Lansing, MI, USA) was quantified on a HIDEX (Finland) spectrophotometer using MikroWin software.

NO production

DCs were incubated at 37°C or 28°C with or without LPS. Small samples of the supernatants were harvested 0, 4, 20, and 40 h later. Using the Griess assay, NO concentrations were determined by measuring the amount of nitrite, a stable end-product of NO oxidation that is released into the culture medium. Supernatant (20 μl) was collected from each well at the end of the experiment and transferred to a new 96-well plate. An equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The samples were mixed gently and incubated for 3 min at room temperature. The absorbance was read with a microplate reader (Bio-Rad, Hercules, CA, USA) using Microplate Manager software (Bio-Rad). The concentration of nitrite in each sample was calculated from a sodium nitrite standard curve.

Phagocytosis assay

SRBCs were labeled with PKH26 dye (Invitrogen) and were then antibody-opsonized with anti–SRBC IgG (Sigma-Aldrich) for 30 min, followed by extensive washing. DCs were harvested and transferred to a 96-well plate at a density of 0.5–1 × 10^6/well in DC medium at 37°C or 28°C. The prepared SRBCs were added at a ratio of 25:30 SRBC:DC to the wells and were incubated together for 30 min at 28°C or 37°C, followed by the lysis of extracellular SRBC. The cells were washed 2 times before staining with Alexa 647–labeled anti-CD11c mAb (eBioscience, San Diego, CA, USA) on ice for 30 min, followed by 2 washes with PBS containing 1% FBS and 0.1% NaN_3. SRBC uptake by DC was quantified with a FACSCalibur cytometer using CellQuest software (BD Biosciences).

FITC-dextran uptake assay

DCs were harvested and transferred to a 96-well plate as described above. After 2 h of incubation at the different temperatures, cells were activated with 100 ng/ml LPS (Sigma-Aldrich). After LPS activation for the indicated times, 70 kDa FITC-dextran (Sigma-Aldrich) was added to each well and incubated for 20 min. The cells were washed 2 times before staining with Alexa 647-labeled anti-CD11c mAb on ice for 30 min, followed by 2 washes with PBS containing 1% FBS and 0.1% NaN_3. FITC-dextran uptake by DCs was quantified with a FACSCalibur cytometer using CellQuest software.

Immunoblotting to detect the phosphorylation state of ERK and MAPKAPK2

To assess the activation status of the ERK and P38 MAPK pathways, DCs were activated with LPS for 10–120 min at 37°C or 28°C and then spun down and resuspended immediately in SDS sample buffer (Invitrogen) containing 50 μM DTT. The samples were heated to 90°C and then frozen and thawed twice to degrade DNA. The proteins in the samples were separated on a 4–20% NOVEX SDS-polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membranes, which were blocked with 5% BSA in TBS and incubated with rabbit anti-phospho-ERK or rabbit anti-phospho-MAPKAPK2 antisera (Cell Signaling Technologies, Beverly, MA, USA) at 4°C overnight. After washing, the blot was incubated with biotinylated goat anti-rabbit antisera (Vector Laboratories, Burlingame, CA, USA), followed by streptavidin Qdot 605 (Invitrogen), or with HRP-coupled goat anti-rabbit antisera (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and was developed with Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). The chemiluminescent signal and the Qdot 605 signal (excited with blue epi-illumination) were captured with a digital LAS 4000 system (Fujifilm Life Science, Valhalla, NY, USA), and the signal intensity was analyzed with ImageJ software (http://rsbweb.nih.gov/ij/).

Evaluation of actin cytoskeleton morphology

Sterile coverslips were placed in 24-well plates, and 500 μl cell suspension containing DCs at a concentration of 0.5 × 10^6 cells/ml was added. The

cells were then incubated at 37°C or 28°C before LPS activation. Thirty minutes postactivation, the cells were fixed for 15 min with 4% PFA that was prewarmed to 37°C. The actin cytoskeleton was stained with phalloidin (Alexa Fluor 488, Invitrogen), and the coverslips were mounted with ProLong Gold antifade (Invitrogen) containing DAPI. The actin cytoskeleton morphology and podosome structures were observed with a Delta Vision Spectris microscope (Applied Precision, Issaquah, WA, USA) and analyzed with ImageJ software. The percentage of DCs that exhibited podosome structures was calculated.

ECM-degrading capacity

Oregon green gelatin (Molecular Probes, Eugene, OR, USA) was added to sterile coverslips in 24-well plates and incubated for 30 min. The gelatin-coated coverslips were then stabilized with 4% PFA for 15 min and washed extensively with PBS. After a last wash with DC medium, the coverslips were incubated in complete medium for 30 min to block remaining reactive PFA. After another wash, 500 μl DC cell suspension at a concentration of 0.5×10^6 cells/ml was added. The cells were then incubated overnight at 37°C or 28°C before they were fixed with 4% PFA and stained with phalloidin (Alexa Fluor 555, Invitrogen). The coverslips were mounted with ProLong Gold antifade containing DAPI. Images were acquired with a Delta Vision Spectris microscope (Applied Precision), and ECM-degrading capacity, i.e., podosome function, was analyzed with ImageJ software. The percentage of the image area that lacked a fluorescent signal from the Oregon green gelatin was measured, and an average of at least 25 fields was analyzed.

In vitro migration assay

Transwell filters were coated with a thin layer just covering the filter area of matrigel (BD Biosciences) and left to stabilize for 30 min at 37°C. DCs were harvested and seeded on matrigel-coated or uncoated transwell filters (6-well format; 3 μm pore size, BD Labware, Bedford, MA, USA) at a density of $2–3 \times 10^6$ cells/well and incubated for the indicated times at 37°C or 28°C. Cells that had migrated to the bottom compartment were counted in Bürker chambers using trypan blue exclusion.

Evaluation of cell surface expression of DC maturation markers

The DCs were activated with LPS as described above and incubated overnight at 37°C or 28°C. Before staining for FACS analysis, the FcRs were blocked using 2.4G2 Fc blocking antibody (BD PharMingen, San Diego, CA, USA). The DCs were then stained with anti-CD40 PE (eBioscience), anti-CD86 FITC (eBioscience), and anti-CD11c Alexa 647 (eBioscience). All staining was performed on ice. Cells were washed 3 times with 1% FBS and 0.1% NaN_3 in PBS. Cells were analyzed on a FACSCalibur cytometer using CellQuest software.

Antigen processing and presentation assay

HEL was added to 10^6 DCs from C3H/HeN mice (I-A^k) at different concentrations, with or without 100 ng/ml LPS. After overnight incubation at 37°C or 28°C, the cell surface expression of the I-A^k/HEL peptide (aa 46–61) was measured by staining with Alexa 647-conjugated C4H3 mAb that recognizes this peptide–MHC class II complex [33]. The DCs were analyzed with a FACSCalibur cytometer using CellQuest software.

T cell proliferation assay

DCs were loaded with 3 mg/ml chicken OVA in the presence of 100 ng/ml LPS at 28°C or 37°C for 4 h. The DCs were then washed and seeded into 96-well plates in triplicate at 40,000, 13,333, and 4444 cells/well. TCR-transgenic OT-II T cells (20,000) were added to the well, and the cells were incubated at 37°C for 72 h. Allogenic stimulation was performed using BMDCs from C57BL/6 mice and C3H/HeN spleen $CD4^+$ T cells. The allogenic DCs were seeded into 96-well plates in triplicate at 10,000, 3333, 1111, and 366 cells/well. They were incubated in the presence or absence of 100 ng/ml LPS at 28°C or 37°C for 4 h. The DC were then washed extensively, 100,000 $CD4^+$ T cells were added to each well, and the cells were incubated at 37°C for 72 h. [^{3}H]Thymidine (0.5 μCi) was added to each well of the T cell cultures during the last 16 h. The cells were harvested with a TOMTEC cell harvester onto filters (Printed Filtermat A, PerkinElmer, Wellesley, MA USA), and scintillation sheets (MeltLex A, PerkinElmer) were added. The proliferation was then measured using a β-counter (1450 Microbeta, PerkinElmer).

Statistical analysis

Statistical analysis was performed using the single-factor ANOVA test plug-in in Microsoft Excel. Results were considered significant at a $P < 0.05$, $^{**}P < 0.01$, and $^{*}P < 0.05$. SD and SE bars are used as noted in the figure legends.

Online Supplemental material

Supplemental Fig. 1 shows a longer time-course experiment of LPS-stimulated FITC dextran uptake.

RESULTS

Normal constitutive macropinocytosis and phagocytosis but delayed TLR-induced macropinocytosis at 28°C

As residents of peripheral tissues such as skin, DCs continuously sample the extracellular antigen environment by macropinocytosis and phagocytosis. To investigate the effect of a low physiological temperature on the antigen acquisition of DCs, we studied whether macropinocytosis and phagocytosis are affected by a temperature of 28°C, which is common in the skin of peripheral limbs. FITC-dextran uptake has been used extensively as a marker of macropinocytosis in mouse BMDCs [34–36]. In this and all of the following experiments, we used immature DCs that were differentiated at 37°C, as described in Materials and Methods. Constitutive FITC-dextran uptake was not affected at 28°C as compared with that at 37°C (**Fig. 1A**). To address whether phagocytosis was affected at 28°C, DCs were incubated with PKH26 dye and antibody-opsonized SRBC for 30 min at 28°C or 37°C, followed by the lysis of extracellular SRBC and analysis by flow cytometry. We found that phagocytosis, similar to macropinocytosis, was unaffected by lowering the temperature to 28°C (Fig. 1B). DCs sense the presence of microbial pathogens using TLR. One of the early events following TLR engagement is increased uptake of antigens via macropinocytosis [20]. This prompted us to investigate the effect of low temperature on stimulated macropinocytosis. TLR stimulation of FITC-dextran uptake was reduced after 30 min at 28°C as compared with stimulation at 37°C (Fig. 1C). Analysis of the kinetics of TLR-stimulated macropinocytosis showed that the peak level of TLR-4-stimulated FITC-dextran uptake was, however, not significantly lower at 28°C; instead, the stimulation was delayed and reached its

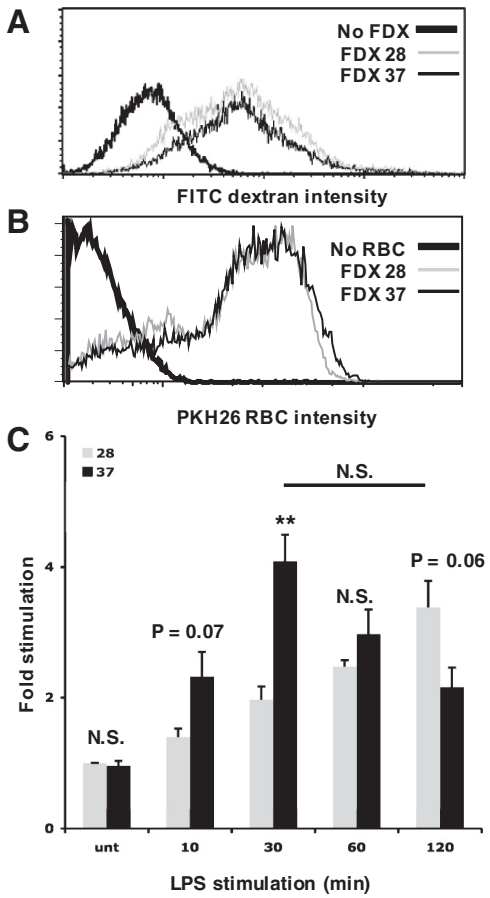

Figure 1. DC macropinocytosis and phagocytosis at 28°C and 37°C. (A) Nonstimulated FITC-dextran (FDX) uptake by DCs at 28°C and 37°C was quantified using flow cytometry. (B) Phagocytosis of PKH26 dye-labeled, opsonized SRBC by DCs at 28°C and 37°C was quantified using flow cytometry. (C) Kinetics of LPS-stimulated FDX uptake for 0–120 min (5 experiments). Data acquired by flow cytometry (in triplicate) were normalized to the FDX uptake of nonstimulated cells and expressed as fold induction. The error bars show SE of all of the normalized data points. unt, Untreated; $^{**}P < 0.01$.

highest level at the end of the experiment at 2 h of LPS stimulation (Fig. 1C). In addition, the kinetic experiment was extended to 240 min in control experiments, which showed that the peak of FITC-dextran uptake was approximately 2 h (Supplemental Fig. 1).

Delayed activation of MAPK signaling pathways downstream of TLR-4 activation at 28°C

The MAPK pathways are important signaling pathways downstream of TLR and are essential for stimulated macropinocytosis, DC maturation, and cytokine production [20, 37–39]. To investigate whether TLR signaling via these pathways was affected by low temperature and whether this may be a reason for the delay in TLR-stimulated macropinocytosis, we studied MAPK activation after TLR stimulation at 28°C and 37°C. We found that MAPKAPK2, as a measure of P38 activity, and ERK activation-associated phosphorylation were delayed at 28°C and reached a peak around 40 min after TLR-4 stimulation as compared with 10–20 min at 37°C (**Fig. 2A–D**). Although delayed, the duration of MAPK signaling was similar at 28°C. The activation of the ERK pathway had nearly returned to baseline after 60 min at 37°C; however, phosphorylation levels were still high at 60 min and still measurable at 90 min after TLR-4 stimulation at 28°C (Fig. 2B and D).

Delayed production of TNF and abolished NO production after TLR-4 stimulation at 28°C

Another important consequence of TLR activation is the production of proinflammatory cytokines. TNF is a critical member of the acute reaction of immune cells in response to infection, and production of TNF depends on MAPK activation [37]. To determine whether the slower kinetics of MAPK activation affect the production of TNF, TNF was measured at different time-points after LPS stimulation. TNF production, in an overnight stimulation assay, was similar at 28°C and 37°C (**Fig. 3A**). A reduction was seen, however, in the early response at 4 h, but at 8 h, there was no difference in the amount of TNF measured in the supernatant of DCs at the 2 different temperatures (Fig. 3B). DCs also produce NO during the early defense against pathogens after TLR activation [23]. Production of the NO metabolite nitrite after TLR stimulation was measured using the Griess reaction, and we found that its production was impaired severely at 28°C (Fig. 3C).

Normal podosome formation at 28°C but impaired podosome-mediated degradation and migration through matrix

Under steady-state and inflammatory conditions, DCs adopt a migratory phenotype and home to a draining LN, where they induce tolerance under steady-state conditions or elicit adaptive immune responses to the antigens that they have acquired under inflammatory ones [40]. First, we examined whether podosomes were present in the DCs and functional at low temperature. Podosomes are actin-rich foci of metalloprotease activity, which is important for the degradation of ECM and thus, DC migration. We found that podosomes formed normally in DCs at 28°C (**Fig. 4A** and **B**). However, when the ability to degrade a fluorescently labeled gelatin matrix on a coated glass coverslip was tested, we observed impairment in the capacity to degrade the matrix at 28°C (Fig. 4C and D). To investigate whether lower physiological temperatures also affect the capacity of DCs to migrate, we used a transwell migration assay. An ambient temperature of 28°C reduced the ability of DCs to migrate through matrigel-coated transwell inserts; however, this low temperature did not affect the ability of DCs to migrate through the 3-μm pores of uncoated tissue-culture inserts (Fig. 4E–G).

TLR activation at 28°C affects up-regulation of CD40 but not up-regulation of CD86

DCs are a link between the innate and adaptive immune responses. Innate recognition of pathogens by DCs with TLR leads to up-regulation of costimulatory molecules, enabling

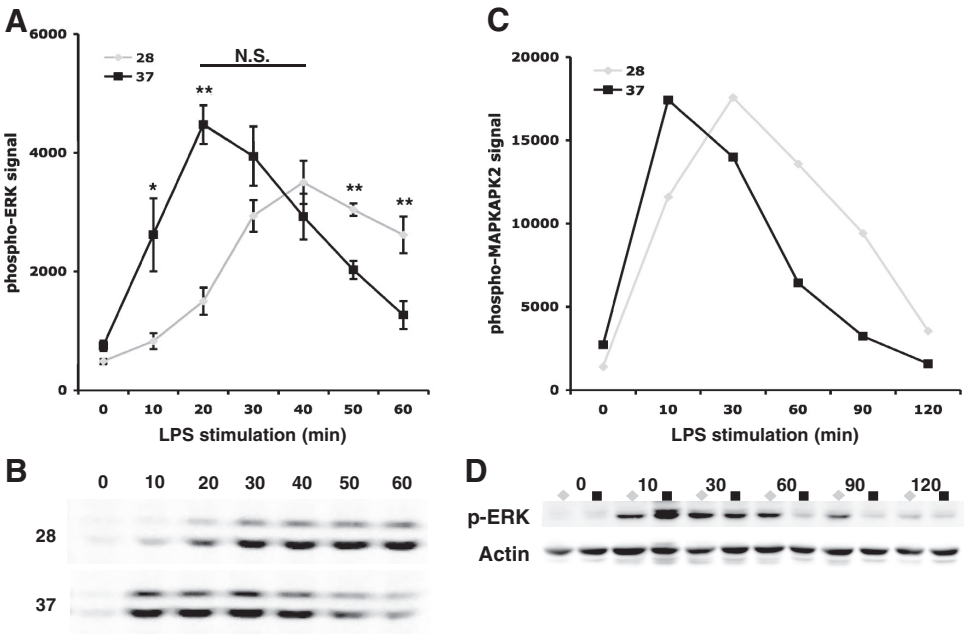

Figure 2. Kinetics of the ERK and P38 MAPK signaling pathways downstream of TLR activation. Proteins from cell lysates of LPS-stimulated DCs were separated with SDS-PAGE and analyzed by Western blotting to detect the kinetics of activation-associated phosphorylation of ERK and MAPKAPK2 (p38 substrate) after stimulation at 28°C and 37°C; data were digitally acquired, and the signal intensities of the phospho-ERK or MAPKAPK2 bands were measured using ImageJ software. (A) Phospho-ERK data from 4 experiments. $*P < 0.05$; $**P < 0.01$. N.S., Nonsignificant differences between 37°C at 20 min and 28°C at 40 min. (B) One representative phospho-ERK Western blot. (C) A kinetic experiment of MAPKAPK2 phosphorylation (p38 activation) and (D) ERK phosphorylation (p-ERK) in the same experiment. ■, DCs incubated at 37°C; shaded ◇, DCs incubated at 28°C. Error bars represent SE.

them to activate naïve T cells. To assess how the delayed TLR signaling at 28°C affects this aspect of DC maturation, we studied cell surface expression of one costimulatory molecule, CD86, and also surface expression of the CD40R, which enables DCs to respond to Th cell input that is important for naïve T cell activation. We found that up-regulation of CD86 after TLR-4 stimulation did not differ significantly at 28°C as compared with 37°C (**Fig. 5A**). Surprisingly, however, up-regulation of CD40 was strongly reduced at 28°C (Fig. 5B). We also investigated the effect on CD86 and CD40 expression after TLR stimulation at 28°C for 4 h, followed by increasing the temperature to 37°C for the remainder of the overnight incubation time. This experiment mimics, to some degree, the physiological condition in which initial stimulation of DCs occurs at low temperature, and then the temperature rises because of inflammation or DC migration to warmer tissue on the way to the LNs. We found that elevating the temperature after 4 h of TLR-4 stimulation, following washing out of the LPS, rescued the up-regulation of CD40 expression on DCs but that there was no effect on CD86 expression (Fig. 5A and B).

Lower stimulation of CD4 T cell proliferation by activated DCs that have taken up antigen at 28°C

DCs take up antigen at sites, e.g., the skin, where the temperature may be lower than the body core temperature. Our data

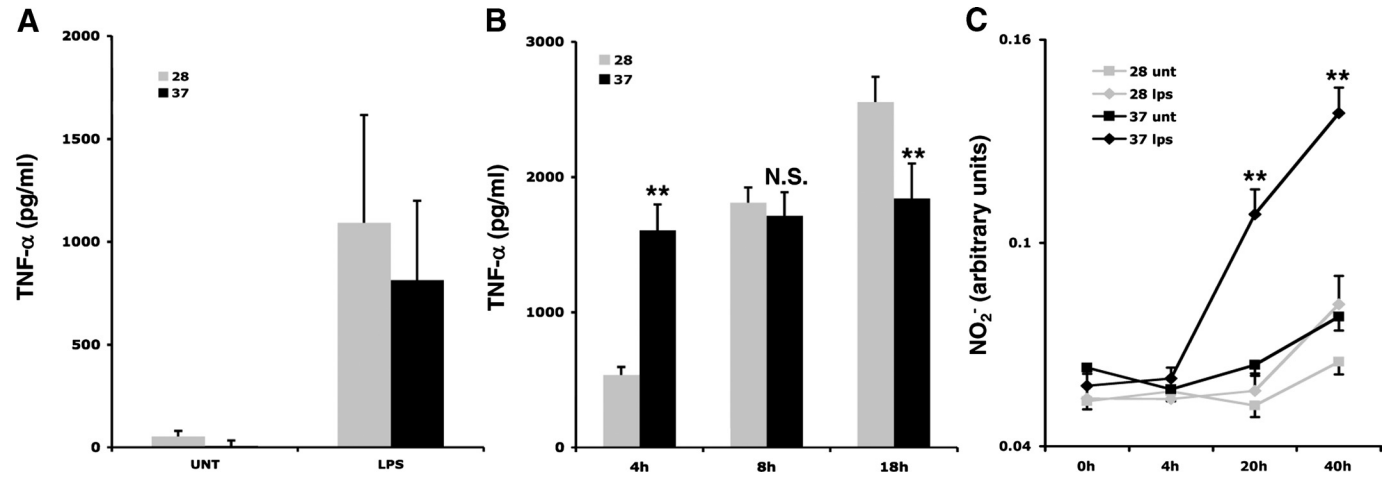

Figure 3. LPS-stimulated TNF-α and NO production. (A) The TNF-α concentration in the supernatant of unstimulated or LPS-stimulated DCs at 28°C and 37°C as measured by ELISA (3 experiments performed in triplicate). (B) Kinetics of TNF-α production after TLR activation at 28°C and 37°C. (C) Kinetics of NO production by DCs after TLR stimulation, as measured by the Griess reaction. All TNF-α ELISA measurements were done in duplicate. Measurement of the Griess reaction was done in triplicate. (A and B) Error bars show SD and (C) SE. $**P < 0.01$.

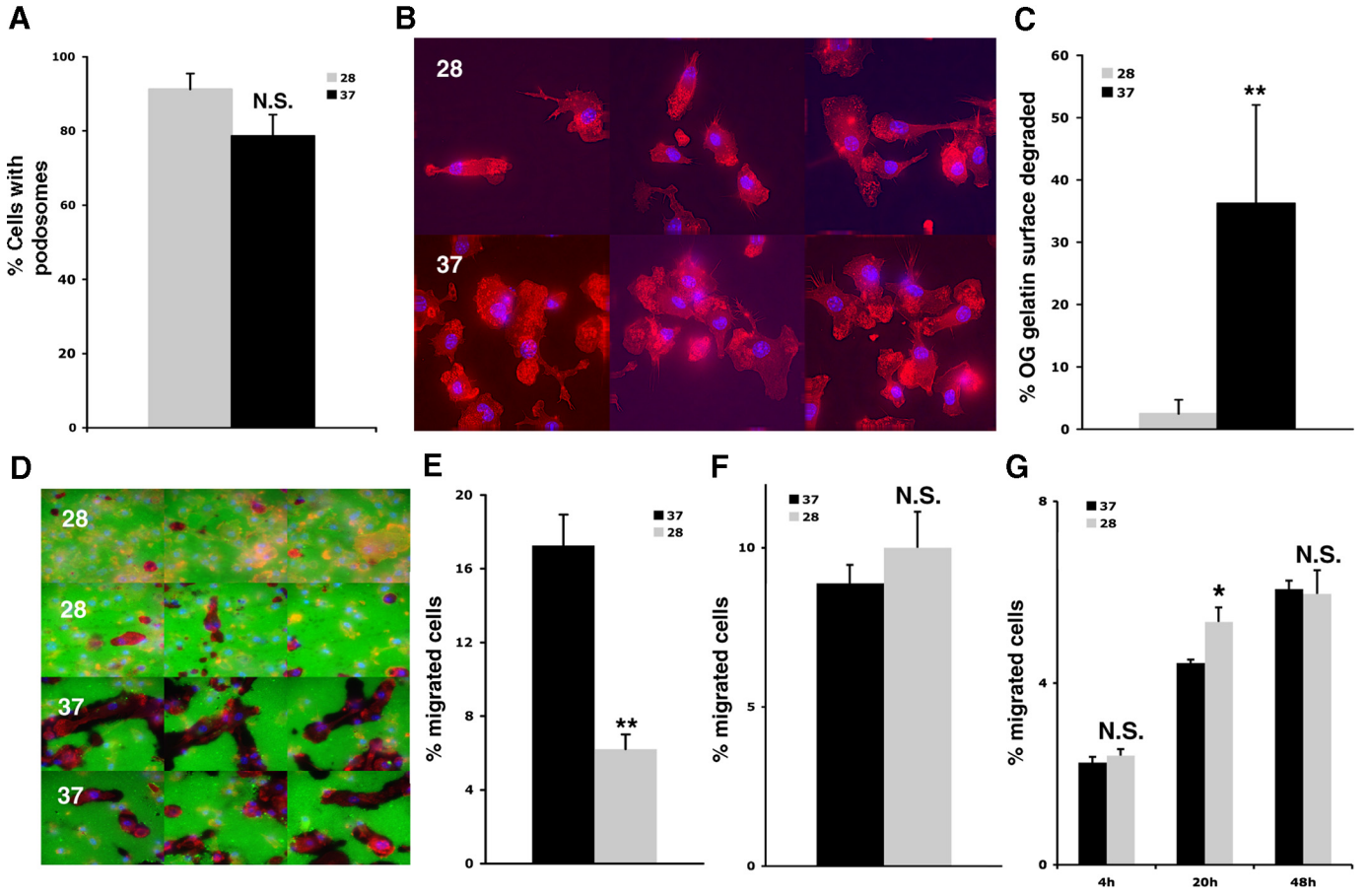

Figure 4. Podosome formation, function, and DC migration at 28°C and 37°C. (A) The number of DCs exhibiting podosomes at the 2 indicated temperatures. (B) Three representative images of phalloidin-stained DCs at the 2 temperatures. (C and D) The capacity of DCs to degrade Oregon green (OG)-labeled gelatin. (E) DCs were plated in matrigel-coated transwells or (F) uncoated transwells at 28°C and 37°C, and the average percentage of cells that migrated to the bottom well overnight (20 h) is shown (4 experiments). (G) Kinetics of DC migration measured by counting percent migrated cells after 4, 20, and 48 h at 28°C and 37°C (2 experiments). Migration experiments were performed in triplicate. Error bars indicate SE except in C and E, where error bars show SD. $*P < 0.05$; $**P < 0.01$.

suggest that uptake of antigen does not differ at 28°C and 37°C in unstimulated DCs. In LPS-stimulated DCs at 37°C, the boost in antigen acquisition occurs earlier; however, over a longer time, the amount of antigen taken up via TLR-stimulated macropinocytosis at 28°C is not expected to be much different. Thus, we next tested whether antigen processing and presentation were affected by incubating DCs at 28°C. DCs were incubated with titrated amounts of HEL at the 2 different temperatures overnight, and DC2 were then stained with the mAb C4H3 to evaluate the production of I-A^k/HEL peptide (aa 46–61) complexes on the surface of the cells. The production of HEL–MHC class II peptide complexes under non-TLR-stimulated conditions was unaffected by the lower temperature (**Fig. 6A**). When DCs were stimulated with LPS, however, we observed a trend toward somewhat more efficient antigen presentation at 37°C than at 28°C (Fig. 6B). Next, we studied antigen presentation and activation of $CD4^+$ T cells. First, DCs were loaded with OVA at 28°C in the presence of LPS for 4 h. Then, DCs were transferred to 37°C and cocultured together with TCR-transgenic OT-II T cells for 4 days. Proliferation, [^{3}H]thymidine incorporation, was then measured on Day 4. This revealed that DCs pulsed with antigen and LPS stimulated at 28°C were less efficient in activating OT-II T cells as compared with DCs pulsed with antigen and LPS stimulated at 37°C (Fig. 6C and D). An allogenic T cell stimulation experiment was also conducted to address whether the observed deficiency in T cell activation was a result of processing and presentation of the OVA antigen or a result of lower T cell stimulatory capacity of the DCs in general. C57BL/6 DCs were activated at 28°C or 37°C with LPS for 4 h, and the cells were then washed and cocultured at 37°C with purified $CD4^+$ T cells from a C3H/HeN spleen. Proliferation, [^{3}H]thymidine incorporation, was then measured on Day 4. Similar to OVA-pulsed DCs, allogenic DCs that were LPS-activated at 28°C were impaired in their capacity to stimulate T cell proliferation even in this setting, which is independent of antigen processing and presentation (Fig. 6D). Controls showed that MHC class II cell surface expression levels are not affected at 28°C (data not shown).

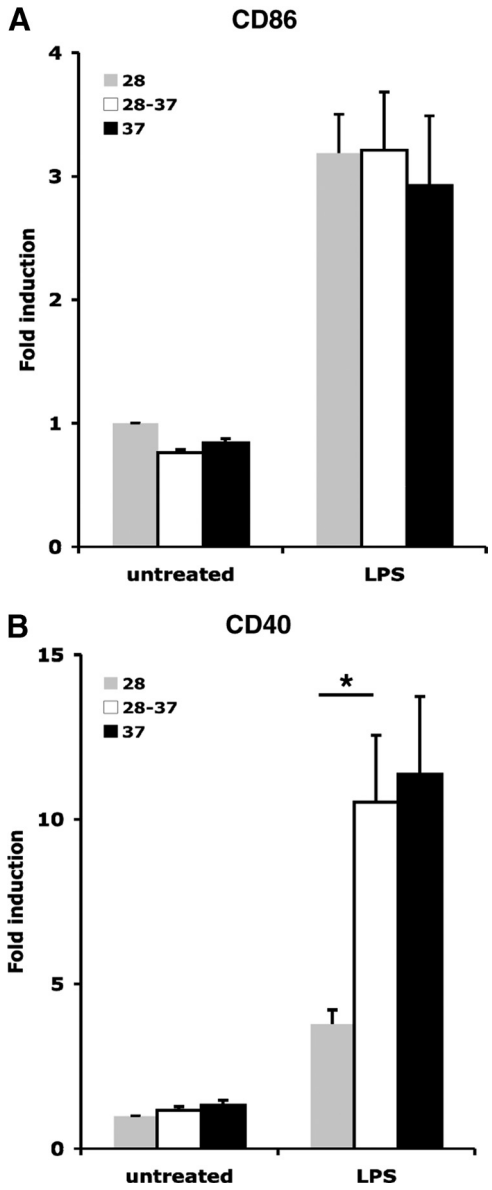

Figure 5. Up-regulation of DC maturation markers. Maturation of DCs was analyzed by measuring the expression of the CD40R and the co-stimulatory molecule CD86. (A) Up-regulation of CD86 18 h after LPS stimulation. (B) Up-regulation of CD40 18 h after LPS stimulation. Data from DCs incubated at 28°C are shown in gray bars. Open bars show data from DCs incubated with or without LPS at 28°C for 4 h and then shifted to 37°C for 14 h. Data from DCs incubated at 37°C are shown in black bars. The average of 3 experiments is shown (2 triplicate DC cultures and 1 duplicate DC culture) as fold up-regulation in relation to untreated DCs at 28°C (CD86 MFI=140; CD40 MFI=42). Error bars indicate SE.

DISCUSSION

In this study, we describe the effect of a low physiological temperature that is often found in the skin, especially in the extremities, on some important DC functions. We show that some functions of DCs, i.e., constitutive macropinocytosis, phagocytosis, podosome formation, migration, and antigen processing (without TLR stimulation), were similar at 28°C and 37°C. This may be a bit surprising from a biochemical point of view, as enzyme kinetics and membrane dynamics could be expected to be affected. From a physiological and evolutionary perspective, however, our results may be less surprising, as 28°C is a common temperature in parts of the skin of many animals, and many organisms live in colder temperatures. The kinetics of some stimulated functions were somewhat delayed, i.e., TLR-stimulated macropinocytosis and TNF production. This delay is likely explained by the delayed kinetics of TLR signaling, e.g., via the MAPK signaling pathway at 28°C. Other functions of DCs were affected more severely by the low temperature, such as TLR-stimulated NO production, CD40R up-regulation, the degradation of the ECM by podosomes, and thus, migration of DC through matrix. In addition, T cell activation by DCs, which were activated by TLR at 28°C, was reduced. DCs purified from the skin have been described as highly immunostimulatory, and also, skin is known to be a good site for eliciting strong responses to vaccine antigens. It may thus seem paradoxical and unphysiological that we find a strong effect of temperature on the immunostimulatory function of DCs. However, DC isolation protocols, e.g., skin-explant, DC-emigration experiments, are normally done at 37°C, and injections of vaccines are likely accompanied by inflammation as a result of adjuvants or endotoxin in the vaccine preparation. Pure proteins give weak immune responses. One of the hallmarks of inflammation is the heating of the inflamed area by increased blood-flow, which may have the effect of making DCs more immunostimulatory and enabling them to migrate more efficiently to the LNs.

Interestingly, up-regulation of CD40 was reduced when DCs were activated and incubated at 28°C, but the up-regulation was rescued when the DCs were moved to 37°C after 4 h of activation at 28°C. As mentioned above, the rationale for this experimental setup was to mimic the local increase in temperature during inflammation or when DCs migrate and encounter a higher temperature in the draining LNs. As signaling from TLR is down-regulated at this time, this may be a result of an effect on the transcription/translation machinery producing the CD40 protein. As the CD40-CD40L interaction is believed to be important in the activation of naïve T cells, the lack of up-regulation of CD40 at low temperature may prevent inappropriate, naïve T cell activation by DCs in the skin. Even if these temperature-shift data imply that the inhibitory effect of low temperature can be reversed if the temperature is increased, the capacity to activate T cells by DCs that had taken up antigen and had been activated by TLR at 28°C and then shifted to 37°C at 4 h was diminished. This observation was also confirmed in an allogenic setting, suggesting that temperature modulation of the ability of DCs to activate T cells is not dependent only on antigen processing and presentation. Thus, another unknown factor involved in the T cell stimulatory function of activated DCs is suppressed when DCs are activated at 28°C.

Phagocytosis is temperature-dependent in splenic phagocytes [41]; however, we show here that phagocytosis of opsonized sheep erythrocytes by DCs was unaffected by temperature. In this study, we also show that constitutive macropinocytosis at

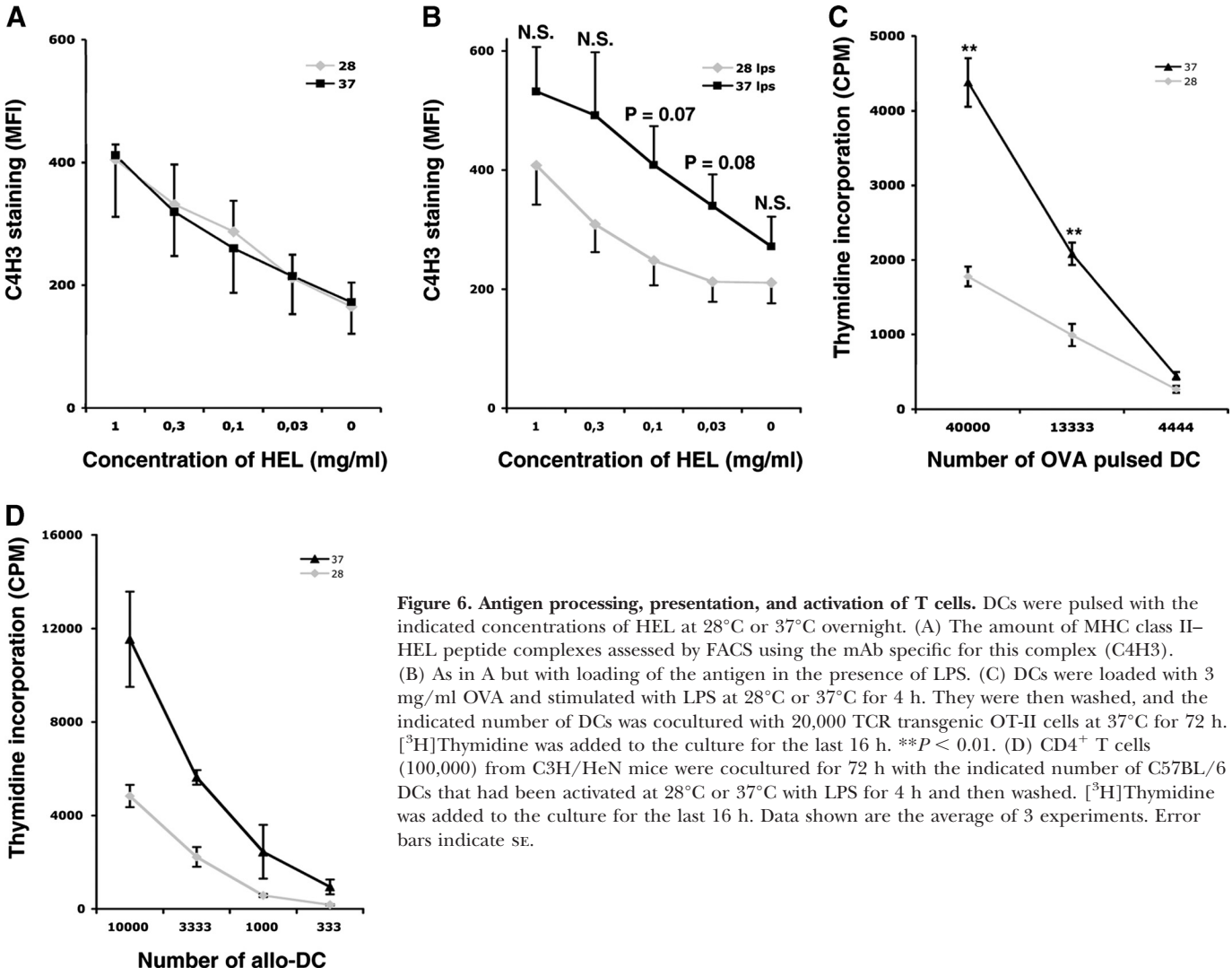

Figure 6. Antigen processing, presentation, and activation of T cells. DCs were pulsed with the indicated concentrations of HEL at 28°C or 37°C overnight. (A) The amount of MHC class II–HEL peptide complexes assessed by FACS using the mAb specific for this complex (C4H3). (B) As in A but with loading of the antigen in the presence of LPS. (C) DCs were loaded with 3 mg/ml OVA and stimulated with LPS at 28°C or 37°C for 4 h. They were then washed, and the indicated number of DCs was cocultured with 20,000 TCR transgenic OT-II cells at 37°C for 72 h. [^{3}H]Thymidine was added to the culture for the last 16 h. $^{**}P < 0.01$. (D) CD4$^+$ T cells (100,000) from C3H/HeN mice were cocultured for 72 h with the indicated number of C57BL/6 DCs that had been activated at 28°C or 37°C with LPS for 4 h and then washed. [^{3}H]Thymidine was added to the culture for the last 16 h. Data shown are the average of 3 experiments. Error bars indicate SE.

28°C was not affected and that stimulated macropinocytosis in response to LPS at 28°C was delayed but reached about the same levels as that at 37°C. MAPK activity is in part responsible and necessary for TLR-stimulated macropinocytosis [20]. A probable explanation for the delayed stimulation of macropinocytosis is the observed delay in the activation of the ERK and p38 MAPK pathways. As DCs exposed to infection probably encounter activating signals and antigens for longer than a few hours, we speculate that the delay in stimulation of antigen uptake by macropinocytosis would not, by itself, lead to reduced antigen presentation. We also addressed the effect of exposure to 28°C on the combined antigen uptake, processing, and presentation by studying the presentation of a HEL epitope on H-2 I-A^k with the specific antibody C4H3 [33]. We found no inhibitory effect of low temperature on the generation of this epitope in unstimulated DCs, suggesting that steady-state antigen processing and presentation, which are important for tolerogenic responses to self-antigens, were not affected by this temperature. A consistently higher generation of the HEL epitope was, however, observed at 37°C after LPS stimulation in 5 experiments, although the differences did not reach statistical significance.

DC migration is stimulated by fever-range hyperthermia [4]. However, the migratory capacity of immune cells at low physiological temperatures has been assessed only for neutrophils, in which a significant impairment of migration is seen at 29°C [42]. We show that an ambient temperature of 28°C did not affect DC migration through uncoated transwell filters. We also show that podosome formation was not affected, yet we detected a decrease in the ability of podosomes to degrade the matrix at 28°C. Podosomes are actin-rich foci that are important for the degradation of ECM [43] and thus, for DC migration. Consistent with this, we also observe a decreased ability of the DCs to migrate through Matrigel-coated transwell filters. Our data allow speculation that an increase in peripheral tissue temperature, as a consequence of inflammation, may enhance the ability of DCs to break through the surrounding tissue and migrate toward draining LNs.

In this study, immature BMDCs, differentiated with GM-CSF at 37°C, were used. This model was described in the early

1990s by Inaba and co-workers [32] and is one of the most well-established methods for generating DCs to study DC biology. The BMDCs probably resemble immature, interstitial dermal DC subsets more than the Langerhans cells, as the Langerhans cells may originate from a progenitor cell distinct from the ones giving rise to other DC subsets [44]. It would, of course, be interesting to study the effects of temperature on skin DC subsets isolated from mice; however, it has not been possible to get DCs in sufficient numbers and in an immature state to be able to perform such studies. When using BMDCs, the question of how the results obtained translate into the in vivo situation has to be considered. We believe that the DC functions that we have studied here are general in their nature and regulated in a similar manner in most DC subsets. To our knowledge, there are no published data that suggest that the functions that we have studied would be different in dermal DCs compared with BMDCs.

In summary, we speculate that DCs at low temperature, normal to the skin of the extremities, have the capacity to take up antigen, mature by up-regulation of some costimulatory molecules, and migrate slowly to the LNs to participate in maintaining peripheral tolerance during noninflammatory conditions. In addition, restricting the up-regulation of CD40 may serve to reduce CD40–CD40L interactions important for T cell activation and thereby, prevent "accidental triggering" of tissue resident memory T cells. Inhibition of NO production by low temperature may also serve to protect the skin somewhat from inflammation as a result of accidental triggering of DCs. During inflammation triggered by TLR signals, 1 rapid response is the development of the classical signs of inflammation: rubor (redness), tumor (swelling), and calor (heat). These symptoms are caused by increased blood-flow to the inflamed area. One of the important functions of this reaction, in addition to attracting more inflammatory cells and mediators to the area, we speculate, may be to enhance DC function. This could possibly affect antigen-processing capacities in the presence of stimulation and would "license" DCs to produce NO and participate in the inflammatory and antimicrobial responses. DCs would also gain higher efficiency in migrating through the ECM and thus, reach the LNs more rapidly and in greater numbers. Finally, we speculate that DCs activated in the tissue after the temperature has been raised as a result of, e.g., inflammation, may be more capable of activating T cells when they reach the LNs or in the periphery when encountering effector T cells at the site of inflammation. It has been suggested previously that mild thermal stimuli may be important when optimizing settings for DC-based vaccination protocols [4], and the data in this paper may strengthen this notion.

AUTHORSHIP

R.P.A.W. participated in the conception, design, and performance of the study. O.H. participated in the design and performance of the study.

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