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Dendritic epidermal T cells: Their role in the early phase of ectromelia virus infection*

Dendrytyczne epidermalne limfocyty T – ich rola we wczesnej fazie zakażenia wirusem ospy myszy

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Summary

Introduction: Dendritic epidermal T cells (DETCs) are bone marrow-derived T lymphocytes that express a canonical γδ TCR and form a dense network in the murine skin. Here, we sought to determine their role during the early phase of ectromelia virus (ECTV) infection.

Materials/Methods: *In vivo* and *in vitro* models were established for this purpose. In the first model, C57BL/6 mice were intradermally infected into the central part of the ear pinnas with 10^5 PFU ECTV-Mos strain per ear. At indicated time-points, the total pinna cell population was isolated to determine the presence of DETCs and the enumeration of DETCs secreting IFN-γ under *in vitro* stimulation. Purified DETCs were also analyzed for certain gene expressions by RT-PCR. In the second model, purified DETCs isolated from pinnas of uninfected C57BL/6 mice were stimulated in vitro with 5 MOI of UV-inactivated ECTV-Mos. Total RNA was isolated at indicated time-points for RT-PCR gene expression evaluation.

Results: A rapid increase in DETCs number in the pinnas was observed for 24 hours post-infection. During the next 24 h the DETCs number decreased, reaching control values. Rapid but short-lasting INF-γ secretion by purified DETCs *in vitro* was observed and correlated well with the expression of the β chemokine CCL5 gene responsible for macrophage and neutrophil attraction. It was also accompanied by DETCs expression of the immunoregulatory factors TGF-β, GM-CSF, and KGF genes important for maintaining skin integrity.

Conclusions: DETCs from mice infected with ECTV-Mos were rapidly induced to cascade the secretion of mediators that contribute to both immune protection and the control of skin integrity.

Key words: **mice • dendritic epidermal T cells • cytokine • skin • ectromelia virus**

Streszczenie

Wstęp: Dendrytyczne epidermalne limfocyty T (DETC) prezentują na powierzchni niezmienny receptor γδTCR i tworzą gęstą sieć w dolnej warstwie naskórka u myszy. Celem badań było ustalenie roli DETC we wczesnej fazie zakażenia wirusem ospy myszy (ECTV).

Materiały/Metody: Rolę DETC badano na modelu *in vivo* i *in vitro*. W pierwszym z nich myszy C57BL/6 zakażano dawką 10^5 PFU ECTV-Mos/ucho, podając ją śródskórnie w centralnej części małżowiny usznej. W różnym czasie po zakażeniu określano obecność DETC w całkowitej populacji komórek izo-

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lowanych ze skóry małżowin. Oczyszczoną populację DETC stymulowano *in vitro* celem ustalenia ich zdolności do wytwarzania IFN-γ. Ponadto oceniano u nich ekspresję genów wybranych cytokin metodą RT-PCR. W drugim modelu doświadczalnym izolowano populację DETCs ze skóry małżowin myszy kontrolnych, niezakażonych i pobudzano komórki *in vitro* dawką 5 MOI ECTV-Mos inaktywowanego UV. W określonym czasie po stymulacji oznaczano w DETC ekspresję genów dla cytokin metodą RT-PCR.

Wyniki: W ciągu 24 godzin po zakażeniu, nastąpił szybki wzrost liczby DETCs w skórze małżowin usznych. Po kolejnych 24 godzinach wartości te zmniejszały się do stwierdzanych u zwierząt kontrolnych. Wzrostowi liczby towarzyszyło gwałtowne, ale krótkotrwałe wydzielanie IFN-γ przez DETC po stymulacji *in vitro*, które było skorelowane ze wzrostem ekspresji genu dla β chemokiny CCL5, będącej chemoatraktantem makrofagów i neutrofilów. Prócz tego, stwierdzono w DETC wzrost ekspresji genów dla czynników regulatorowych, TGFβ, GM-CSF, KGF, kluczowych dla zachowania integralności skóry.

Wnioski: U myszy zakażonych ECTV-Mos DETC były pobudzane do kaskadowej sekrecji mediatorów, biorących udział zarówno w odpowiedzi immunologicznej jak i w ochronie integralności skóry we wczesnej fazie ektromelii.

Słowa kluczowe: myszy • dendrytyczne epidermalne limfocyty T • cytokiny • skóra • wirus ospy myszy

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Introduction

Dendritic epidermal T cells (DETCs) are bone marrow-derived T lymphocytes that form a dense network in murine skin. They express a skin-specific invariant γδT-cell receptor (TCR) composed of Vγ3Jγ1Cγ1 and Vδ1Dδ 2Jδ 2Cδ chains [1,15]. This cell population was initially determined by the expression of Thy1 and CD3 markers [4,25,26]. DETCs express several activating receptors other than the TCR, including 2(B)4 (CD244), Toll-like receptor 4 (TLR4), and NKG2D, but they lack mature T-cell markers such as CD4 and CD8. It has been reported that DETCs are not activated in the context of MHC class I or II as αβT cells are. Although the ligands for DETCs have not yet been identified, these cells can respond to autoantigens and are stimulated through the NKG2D surface receptor, associated with the DAP10 and DAP12 adaptor proteins. NKG2D recognizes self-ligands expressed on stressed, damaged, transformed, or infected cells (MICA or MICB [MHC class I chain-related proteins A or B] in humans or RAE 1 [retinoic acid early inducible 1 protein] in mice) [10,22,23,39,47]. Since DETCs are located in the skin, they provide not only a front line of defense against microbial invasion, but also take part in immune regulation. These cells are in intimate contact with neighboring epidermal cells, including Langerhans cells and keratinocytes. Through their unique antigen-recognition pathway, DETCs can immediately react to incoming signals and secrete a variety of cytokines, growth factors, and chemokines that have been implicated in tissue repair, cell survival, proliferation, migration, and recruitment [28,30,46].

Although the existence of γδT cells has been known for over 20 years, their role in protective infectious immunity is still being determined. The roles of various γδT cell populations in host immunity against viral infection have been demonstrated, but is still rudimentary. Increased numbers of γδT cells have been observed in patients with HIV and HSV-1 infections. This was associated with their potent cytotoxic response [44,45]. However, we still lack information about the role of DETCs during viral infection, especially during its early phase. To determine the events associated with viral infection, we chose ectromelia virus (ECTV), which belongs to the *Poxviridae* family. ECTV, the agent of mouspox, is closely related to variola virus (VARV), the agent of smallpox. ECTV has been used for years as an excellent model for studying the immune mechanisms involved during generalized viral infections. The natural route of infection is believed to be through skin abrasion, and viral transmission may occur directly from animal to animal. Replication in the epidermis and release of viral progeny from the initial site of infection results in spread to the regional lymph nodes, blood stream, and internal organs. The mechanisms involved in the clearance of ECTV usually engage T cell-mediated immunity and IFN-γ production and, in the late phase of infection, also humoral immunity [13,14,21,36,37]. Although later events during mousepox are well known, the early phase of infection and the induction of an immune response, especially on the molecular level, are less recognized. Studies by the Raymond

Welsh group shed some light on the role of γδT cells during viral infection vaccinia virus (VACV) and their engagement in clearance of the virus, but the analyzed cells belonged to the circulating γδT-cell population [45].

Mimicking the natural route of infection by introducing the virus intradermally to the ear pinna, we showed a rapid involvement of DETCs in response to ECTV infection and their simultaneous immunoregulatory role in maintaining skin homeostasis. Secretion of IFN-γ by DETCs in the hours post-challenge delivered signal to professional antigen-presenting cells, stimulating an immune response. At the same time, by releasing immunoregulatory mediators, DETCs could maintain integrity of the skin.

Materials and methods

Animals

Male C57BL/6 mice (H-2^{β}), 8 weeks old, were purchased from the animal facility of the Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology in Warsaw, Poland. In conducting the research the investigators adhered to the Guide for the Care and Use of Laboratory Animals. The 3rd Ethics Committee for Animal Experimentation at the Warsaw University of Life Sciences – SGGW issued the permission to perform the experiments on the laboratory animals. The animal facilities of the Faculty Veterinary Medicine (no. 0077) are fully approved by the district veterinary inspector.

Reagents

The ECTV Moscow strain (ECTV-Mos) was grown on Vero monolayers, then titrated and stored in aliquots at –80°C until used. GolgiStop reagent (containing brefeldin A) and reagents for cell fixation and permeabilization (Cytofix/Cytoperm and Perm/Wash, respectively) were purchased from Pharmingen/Becton Dickinson. Phorbol 12 myristate 13-acetate and ionomycin (PMA/Iono; Sigma) were used for nonspecific stimulation of interferon gamma (IFN-γ) production. Anti-TCRγδ and anti-IFN-γ monoclonal antibodies (mAbs), conjugated either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and purchased from BD Pharmingen, were used for fluorescence-activated cell sorter (FACS) staining. Freshly isolated DETCs for *in vitro* stimulation were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) hi-FBS, 1.5 mM L-glutamine, 50 μM 2-mercaptoethanol, and antimycotic antibiotic. The γ/δ^+ TCR T-cell isolation kit for γδT cell purification was purchased from Miltenyi Biotec. A total RNA isolation kit (A&A Biotechnology) was used for RNA isolation from purified DETCs.

Dendritic epidermal T-cell preparation and purification

For total cell isolation, the pinnas were separated into ventral and dorsal aspects, then floated with the dermal side up in 1% trypsin in phosphate-buffered saline (PBS) solution for 45 min at 37°C. The predigested ear skin was cut into small pieces and suspended in a solution of 0.2 mg/ml collagenase IV (Sigma), 0.2 mg/ml collagenase/dispase (Roche Diagnostics), and 50 μg/ml DNase I (Sigma) in RPMI 1640 supplemented with 10% (vol/vol) hi-FBS, 1.5 mM L-glutamine, 50 μM 2-mercaptoethanol, and c antimycotic antibiotic, then incubated in an orbital shaker for 20 min at 37°C in three subsequent cycles [27]. Every 20 min the released cells were collected and kept on ice, then a new solution of enzymes was added for the subsequent 20 min. Finally, after the last cycle of digestion, the remaining tissue was meshed through a metal sieve and all the harvested cells were washed twice in RPMI 1640. For the purification of γδ T cells, a TCRγ/δ^+ T-cell isolation kit was used and the isolation was performed according the manufacturer's protocols. For determining the kinetics of γδT cells at indicated times post-infection, the pinnas were separated into ventral and dorsal aspects, cut into small pieces, and suspended in a solution of collagenase D (Sigma) in RPMI 1640 medium at a concentration of 1 mg/ml and incubated in an orbital shaker for 1 h at 37°C. After incubation, resident tissue and harvested cells were meshed through a metal sieve and the obtained suspension was washed once in RPMI 1640 mixed with HBSS-EDTA at a 1:1 ratio. Finally, the cells were passed through a 70-μm nylon mesh. At each step of isolation the number of cells and their viability were assessed by trypan blue exclusion.

Flow cytometry and intracellular staining

To enumerate the TCRγδ^+ T cells at the indicated time points, surface marker staining with anti-TCRγδ mAb was performed according to Gieryńska et al. and assayed using a FACScan flow cytometer and CellQuest software [16]. Intracellular staining was performed to enumerate the number of IFN-γ-producing cells. A total of 10^4 freshly expanded pinna cells purified with MACS® Technology were cultured in U-bottom 96-well plates. The cells were stimulated with PMA/Iono (50 ng/ml and 500 ng/ml, respectively) in the presence of 1 μg of brefeldin A (to facilitate intracellular accumulation) for 4 h at 37°C in 5% CO_2. Surface staining was then performed using anti-TCRγδ mAb followed by intracellular IFN-γ staining with a Cytofix/Cytoperm kit (BD Pharmingen, USA) in accordance with the manufacturer's instructions. Two-color fluorescence analysis was performed using a FACScan flow cytometer and CellQuest software.

Semi-quantitative RT-PCR of cytokine and growth factor gene expression

Total RNA was extracted from 10^4 freshly isolated or cultured purified γδ T cells using a Total RNA isolation kit (A&A Biotechnology). The isolated RNA was analyzed for cytokine, chemokine, and growth factor mRNA expression using semi-quantitative RT-PCR. One μg of total RNA was used in the RT reaction in a 25-μl reaction volume containing 50 mM Tris-HCl buffer (pH 8.3) supplemented with 75 mM KCl, 3 mM $MgCl_2$, 1.6 mM of each deoxynucleotide triphosphate, 100 pmol of oligo (dT15) primer, 40 U RNase inhibitor, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega Corp.). The cDNA mixture was incubated as follows: 10 min at 25°C, 50 min at 37°C, 11 min at 42°C, 11 min at 50°C, 10 min at 72°C, and finally 4°C. The transcribed cDNA fragments were amplified in a total volume of 25 μl containing with final concentrations of 0.2 mM of each dNTP, 0.5 μM upstream primer, 0.5 μM downstream primer, 1.25 U of GoTaq® DNA Polymerase,

Table 1. Primers used in RT-PCR

Target	Forward (5′→3′)	Reverse 3′ (5′→3′)
β-actin	GTG GGG CGC CCC AGG CAC CA	CTC CTT AAT GTC ACG CAC GAT
IL-2	ATG AGA AGG ATG CTT CTG CAC	AGG GCT TGT TGA GAT GAT GCT
IFN-γ	ATG AAC GCT ACA CAC TGC ATC	GCA GCG ACT CCT TTT CCG CTT
KGF	AATCCAACTGCCACGGTCC	CGGAGCAAACGGCTACGAGTG
GM-CSF	TGTGGTCTACAGCCTCTCAGCAC	CAAAGGGGATATCAGTCAGAAAGGT
TGFβ	ACCGCAACAACGCCATCTAT	GTAACGCCAGGAATTGTTTGC
RANTES	CCA CGT CAA GGA GTA TTT CTA CAC C	TCT TCT CTG GGT TGG CAC ACA C

2 mM $MgCl_2$ solution, 1X Colorless GoTaq® Flexi Buffer (Promega Corp.), and nuclease-free water. The primer sequences are shown in Table 1. The amount of cDNA was normalized according to β-actin level. The PCR conditions were 35 cycles with each cycle consisting of three steps: denaturation at 94°C for 1 min 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Before the first cycle, a denaturing step at 94°C for 5 min was included and PCR was finished at 4°C. The amplified products were separated by electrophoresis on a 2% agarose gel, visualized by ethidium bromide staining, and analyzed with a VersaDoc Imaging System Model 1000 (Bio-Rad) and Quantity One 4.4.0 software. A 100-bp ladder (Gibco BRL Life Technologies Inc.) was used to verify the sizes of the PCR products.

Functional analysis of DETCs

To determine the contribution of DETCs during viral infection, C57BL/6 mice were intradermally infected into the central part of the pinna with 10^5 PFU/ear ECTV-Mos cells in a total volume of 10 µl. At indicated time-points (24, 48, 72, and 96 h) post-infection, the mice were sacrificed and the pinnas were collected for determining the presence of DETCs and enumerating IFN-γ-producing cells. DETCs from skin isolated from uninjured and uninfected C57BL/6 mice served as a control.

In vivo model

In parallel experiments, C57BL/6 mice were infected intradermally with 10^5 PFU/ear ECTV-Mos 10 and 20 h before DETCs isolation. In these experiments, cells isolated from C57BL/6 mice injected with 10 µl/ear sterile PBS into the central part of the pinna served as controls. The isolated and purified DETCs population was divided for enumerating IFN-γ-producing cells and for total RNA isolation, and these cells were immediately suspended in Trisol for total RNA isolation.

In vitro model

For the functional analysis of DETCs, a purified population from uninfected C57BL/6 mice was stimulated *in vitro* with 5 MOI (multiplicity of infection) of a UV-inactivated ECTV-Mos strain (UV-ECTV-Mos). The UV-ECTV-Mos-stimulated DETCs were collected and suspended in Trisol for total RNA isolation 1, 3, and 6 h after stimulation. Non-stimulated purified DETCs served as a control.

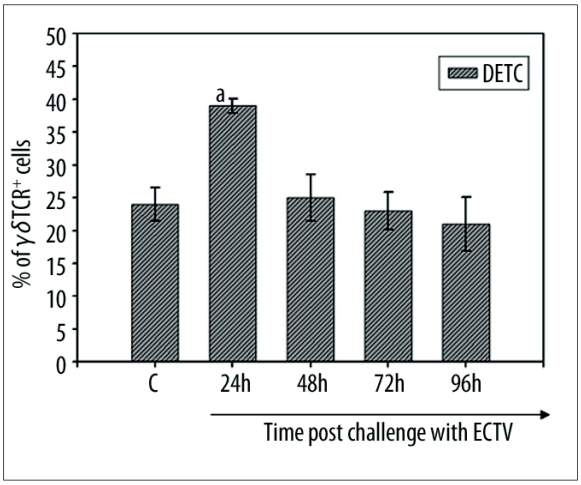

Figure 1. The presence of DETCs during ECTV-Mos infection. C57BL/6 mice were intradermally infected in the central part of the pinna with 10^5 PFU ECTV-Mos/ear in a total volume of 10 µl. After 24, 48, 72, or 96 h post-infection, the mice were sacrificed and the skin from the ears was collected to determine the presence of DETCs during viral infection. Uninjured and uninfected C57BL/6 mice served as controls. Harvested cells were counted, stained for surface markers, and analyzed by flow cytometry. The figure shows the kinetics of DETCs in skin after ECTV-Mos infection. *a* – value statistically significant different from that obtained for DETCs isolated from control mice. Statistically significant difference ($p \leq 0.05$ by the Mann-Whitney *U* test) is shown above the bar. The results are representative of three independent experiments.

Statistical analysis

Statistica 6.0 for Windows was used to evaluate difference in the profiles of γδT cells. The difference between groups was tested for statistical significance using the Mann-Whitney U test and $p \leq 0.05$ was considered significant.

Results and Discussion

Determination of the profile of γδT cells in murine skin after challenge with ECTV-Mos

To determine the role of DETCs during the early phase of ectromelia, C57BL/6 mice were intradermally challenged into central part of the pinna with 10^5 PFU ECTV-Mos/ear.

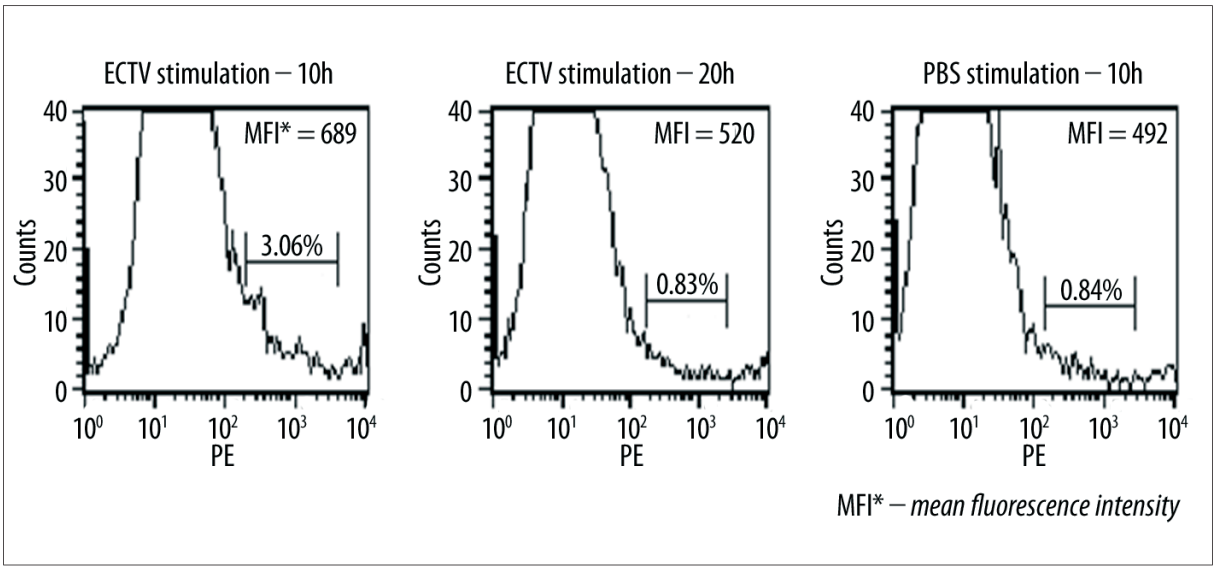

Figure 2. Functional analysis of DETCs isolated from C57BL/6 mice post challenge with ECTV-Mos strain.
C57BL/6 mice were intradermally infected in the central part of the pinna with 10^5 PFU ECTV-Mos/ear in a total volume of 10 µl for 10 or 20 h prior to DETC isolation. C57BL/6 mice injected with sterile PBS into the pinna 10 h prior to DETC isolation served as controls. DETC populations from all groups of mice were purified with the MACS® Technique as described in Materials and Methods. They were then stimulated with PMA/Iono (50 and 500 ng/ml, respectively) in the presence of 1 µg of brefeldin A to facilitate intracellular accumulation for 4 h at 37°C in 5% CO_2. After that time the production of IFN-γ was determined by intracellular staining. The figure shows an analysis of enriched populations of DETCs producing IFN-γ. The results are representative of three separate experiments.

The mice were sacrificed by cervical dislocation at indicated intervals (24, 48, 72, and 96) to quantify the number of DETCs present in the skin post-challenge as well as to evaluate stimulation of an immune response by measuring IFN-γ production. IFN-γ is one of the major cytokines released during viral infection and is involved in viral clearance by triggering the intracellular signaling pathway that stimulates an antiviral immune response. The results shown in Figure 1 indicate that intradermal ECTV-Mos challenge caused a rapid increase in DETCs number in the skin (39%) during first 24 h post-infection. The number of DETCs decreased during the next 24 h, reaching the values obtained in control mice (24%). The raise in DETC number did not correlate with IFN-γ production by these cells. In fact, we were unable to detect any cells producing IFN-γ under *in vitro* stimulation. Bacterial and viral infection as well as injury result in dermal γδT-cell activation. The result of this activation is the production of soluble factors such as cytokines, chemokines, and growth factors. It has been established that DETCs are able to lyse transformed cancer cells and the cytolytic activity of effector cells is associated with the ability of DETCs to produce IFN-γ [3,31,33]. Therefore our negative results concerning IFN-γ production evoked doubts about the correctness of the technique of isolating DETCs. To solve this problem we decided to change the isolation and purification methods. Two models (*in vivo* and *in vitro*) were also established to assess DETC involvement during the early phase of ECTV infection.

Evaluation of DETCs activity under stimulation

In vivo model

Since we did not detect γδT cells secreting IFN-γ 24 h after viral infection, we decided to challenge C57BL/6 mice intradermally with 10^5 PFU/ear ECTV-Mos 10 h and 20 h before DETC isolation. Animals injected with 10 µl of sterile PBS 10 h before DETCs isolation served as controls. In this model, DETCs were isolated according to Hommel et al. and purified with MACS® Technology [27]. The purity of the isolated DETCs was 96% (as assessed by flow cytometry) with 99% viability (as assessed by trypan blue exclusion, data not shown). To evaluate DETC activation after ECTV infection, we assessed their ability to secrete IFN-γ by intracellular production and by IFN-γ gene expression using semi-quantitative RT-PCR in freshly expanded DETCs. The results are presented in Figures 2 and 3. A positive DETC response measured by intracellular IFN-γ was evident only in the mice challenged with ECTV-Mos 10 h before cell isolation (3.06%), whereas in cells isolated 20 h after challenge the level of secreted IFN-γ was the same as in the control mice (0.83% and 0.84%, respectively). These results were confirmed by RT-PCR, in which IFN-γ gene expression was detected only in RNA isolated from freshly expanded DETCs 10 h after challenge. In this model we had two independent stimulators: skin injury (through injection) and the introduction of an infectious agent (ECTV-Mos), so DETCs isolated from the mice treated with sterile PBS 10 h before isolation served as a control. As injection of PBS did not trigger the production of IFN-γ the reliability of the control was confirmed.

Here we can only speculate on DETCs' ability to distinguish incoming signals. One of the possibilities of "danger signal" recognition is an involvement of heat-shock proteins (HSPs). HSPs are highly conserved among prokaryotes and eukaryotes and can be triggered by exposure to different environmental stress conditions, such as infection, inflammation, and hypoxia. They are also involved in evoking an immune response by binding antigen epitopes and

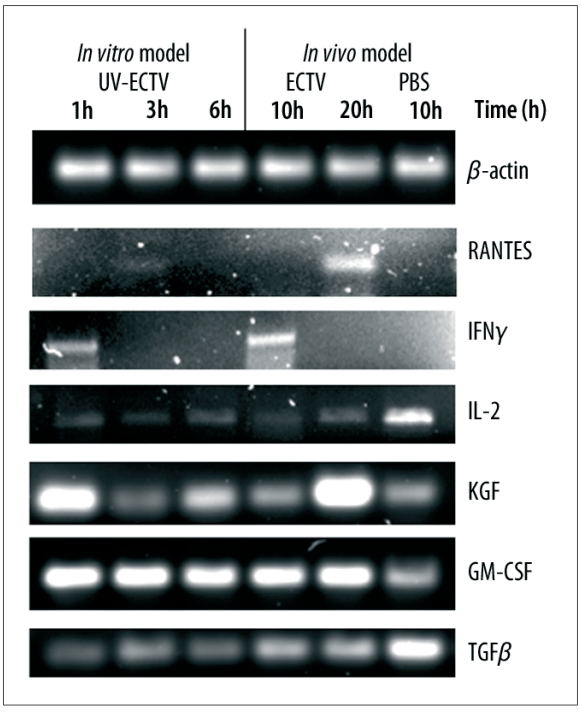

Figure 3. Analysis of cytokine, chemokine, and growth factor gene expression in DETCs stimulated with ECTV.
RT-PCR of total RNA from DETCs stimulated with ECTV-Mos (time-points above the lanes) isolated from the *in vitro* and *in vivo* models as described in Materials and Methods for the expressions of β-actin, RANTES, IFN-γ, IL-2, KGF, GM-CSF, TGF-β (indicated on the right).

stimulating immune cells and working as natural adjuvants [38]. ECTV infection could trigger the expression of certain HSPs that provide a "danger signal" for DETCs, which then secret IFN-γ. The participation of HSPs as a costimulatory signal in γδT-cell stimulation has been demonstrated in studies with γδT-cell hybridoma [9,20,40,41]. An evaluation of difference in HSPs levels and their possible role in DETCs stimulation in mice infected with ECTV is required and should bring some answers.

In vitro model

To eliminate nonspecific stimulation (injury) and to determine IFN-γ synthesis, we designed an *in vitro* model. Purified DETCs from uninfected and uninjured C57BL/6 mice were stimulated *in vitro* with 5 MOI of UV-ECTV-Mos for 1, 3, and 6 h. Then the cells were collected for total RNA isolation. Using RT-PCR, the expression of IFN-γ gene was detected 1 h after the stimulation with UV-ECTV-Mos, whereas cells stimulated for 3 and 6 h (Figure 3) and control cells (data not shown) did not reveal expression of this gene. These data confirmed our results from the *in vivo* model concerning the secretion of IFN-γ during ECTV infection, indicating this cytokine to be a very early released mediator.

DETCs in maintaining skin homeostasis during ECTV infection

Since DETCs are in intimate contact with neighboring epidermal cells, they are activated through their receptors at signs of damage or disease. As a result, they are able to secret soluble factors that may stimulate an immune response. It was shown that IFN-γ is a very early mediator with antiviral properties secreted by ECTV-activated DETCs. At the same time DETCs are responsible for maintaining skin homeostasis by preserving epithelia integrity from local damage or disruptive effects of systemic infiltration [17,18,28,29,30]. To assess DETCs' involvement in skin homeostasis during the early events of ECTV infection, the gene expressions of certain cytokines, chemokines, and growth factors were evaluated by RT-PCR in RNA obtained from purified DETCs in the established models. The expressions of interleukin (IL)-2, IFN-γ, the β chemokine CCL5 (RANTES), keratinocyte growth factor (KGF), granulocyte macrophage colony-stimulating factor (GM-CSF), and transforming growth factor β (TGFβ) were determined and the results are summarized in Figure 3.

Studies by others have shown that TGF-β is secreted constitutively [29]. Our data confirmed this and also showed expression of this gene in cells from both models, but the level of expression depended on the activation stage of the DETCs. However, contrary to others [7,24], the highest expression of the gene encoding IL-2 was observed in DETCs from mice treated with sterile PBS, whereas after viral stimulation the levels of IL-2 gene expression in both models were much lower. These findings differ from the results obtained by others and could be related to the specific ECTV stimulation; confirmation of our data thus requires further evaluation. RANTES, which plays an active role in recruiting leukocytes to an inflammatory site and supports the migration of monocytes [34], was detected in the DETCs of both models. Its expression correlated with IFN-γ presence. Early detection of this chemokine (3 h after UV-ECTV-Mos stimulation in the *in vitro* model and 20 h after ECTV challenge in the *in vivo* model) and its lack in the control cells may confirm DETC engagement in cell recruitment to the site of infection. However, the exact timetable of events concerning the production of IFN-γ and, subsequently, RANTES requires further evaluation. Such studies are currently underway in our laboratory. There was notable correlation between TGF-β and GM-CSF gene expressions. ECTV-stimulated DETCs (in the *in vitro* and *in vivo* models) and control cells synthesized both factors, which were secreted constitutively, but the level of expression correlated with the state of their activation. DETCs stimulated with ECTV showed an elevated level of GM-CSF expression and a lower level of TGF-β. A higher level of TGF-β was detected in non-stimulated DETCs (*in vitro* model, data not shown) and in cells derived from mice injected with PBS. One of the crucial factors involved in maintaining skin function is constitutively secreted KGF. The data in Figure 3 show that the highest expression of KGF gene resulted from ECTV activation: 1 h after UV-ECTV-Mos stimulation in the *in vitro* model and 20 h after ECTV challenge in the *in vivo* model.

Our data clearly show DETCs' association with immune response during ECTV infection. The host response to viral infection is biphasic, with innate effectors such as IFNs, NK cells, and macrophages that are critical in the early phase of infection and T cells and B cells engaged in viral clearance during the adaptive immune response. DETCs that are engaged in both protection and regulation

of skin homeostasis may be considered as the most important skin immune cells. Studies by others shed some light on the role of γδT cells during viral infection, but most of these studies were performed on circulating and organ-connected γδT cells or γδT-cell hybridomas [9,11,12,45]. Here we show the engagement of DETCs during the early phase of poxvirus infection and their simultaneous immunoregulatory role. Our data show that during the first hours of ECTV infection, DETCs provided a rapid but short response, as measured by IFN-γ production. This cytokine, together with its antiviral properties, is also known as an inducer of Langerhans cells, which modulate their adhesion molecule expression and their function [2,19,32]. At the same time, DETCs secreted mediator (TGFβ), which is responsible for decreasing their proliferation [35]. ECTV infection which caused local tissue damage resulted in RANTES secretion which, together with macrophage inflammatory protein (MIP) 1α, MIP1β and lymphotactin, is responsible for maintaining skin integrity and, as an early inflammatory stimulator, attracts monocytes and neutrophils to the damaged tissue and provides a proper microenvironment for skin repair [5,42,43]. Strong correlation between the levels of the gene expressions of GM-CSF and TGF-β was observed during ECTV infection. Both mediators are constitutively secreted and both are important in mediating skin homeostasis and skin immune defense [7,8,35]. Local synthesis of GM-CSF by activated DETCs might result in upregulation of phagocytic abilities of macrophages and neutrophils and, consequently, the secretion of proinflammatory cytokines by these cell populations. We determined TGF-β gene expression only up to 20 h after ECTV infection, but it would be worthwhile to determine it up to 96 h. Now we can speculate that this factor is responsible for diminishing early DETCs proliferation. The role of TGF-β in protecting proper skin function is unquestionable, since it participates in the inhibition of many cell types, including keratinocytes, but is also able to stimulate their migration and is crucial for Langerhans cell development which, together with IFN-γ, allows the induction of a proper immune response [8,35]. The detection of KGF gene expression in ECTV-activated DETCs confirmed the role of these cells during viral infection. In the skin, KGF is secreted constitutively only by DETCs and is crucial for keratinocyte survival and proliferation, the most important cells in tissue repair [6,30]. In our models, the highest level of this gene expression was detected in cells stimulated for 1 h with UV-ECTV-Mos, the same time as for IFN-γ production (*in vitro* model) and 20 h after ECTV-Mos challenge (*in vivo* model). The delay in KGF expression in this model could be related to the tissue damage caused by the replicating virus and antigen presentation by professional antigen-presenting cells.

Based on our data we can conclude that ECTV infection stimulates a cascade of events involving primarily DETCs. IFN-γ secreted by them provides a crucial role in viral clearance. It also influences Langerhans cells for immune response stimulation. At the same time, when the integrity of the epidermis is compromised, chemokines responsible for the macrophage and neutrophil chemoattraction required for proper tissue repair are released. This is accompanied by increased GM-CSF expression, resulting in a considerable increase in phagocytosis. The production of KGF, which is responsible for keratinocyte proliferation, is upregulated in ECTV-activated DETCs. KGF is required for keratinocyte functions since they synthesize and secrete hyaluronan, a non-sulfated glycosaminoglycan essential for proper macrophage migration to the site of damage [28].

Further studies should focus on evaluating the sequence of events that leads to releasing mediators that contribute to both immune protection and control of skin integrity in both models, *in vivo* and *in vitro*. The considered time should be longer for both models. Moreover, the influence of live ECTV-Mos on DETCs should be assessed in an *in vitro* model. This study did not resolve many questions concerning poxvirus infection and the early events of immune response stimulation, but the results presented here represent the first demonstration of an involvement skin γδT cells during the early phase of ECTV infection.

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