

Detection of local inflammation induced by repeated exposure to contact allergens by use of IVIS SpectrumCT analyses

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doi:10.1111/cod.12746

Summary

Background. Contact allergy is characterized by local skin inflammation that, in some cases, can result in systemic immune activation.

Objectives. To investigate whether IVIS SpectrumCT analyses can be used to detect the immune response induced by contact allergens.

Methods. Mice were repeatedly exposed to vehicle or allergens on the ears. The local and systemic responses were analysed at different times with the ProSense 750 FAST probe in IVIS SpectrumCT measurements. In addition, changes in ear thickness, cytokine profile in the skin and immunological phenotype in the draining lymph nodes and spleen were determined.

Results. Local inflammation was detected by ProSense 750 FAST and correlated with changes in ear thickness, cytokine profile and immunological phenotype following exposure to the strong contact allergen 2,4-dinitrofluorobenzene. Analysis of the systemic response with ProSense 750 FAST did not show any difference between allergen-exposed and control mice, although fluorescence-activated cell sorting analysis of the spleen showed increased numbers of $\gamma\delta$ T cells and CD11b⁺CD11c⁺MHCII⁺ cells in allergen-treated mice.

Conclusions. IVIS SpectrumCT analyses with ProSense 750 FAST as the probe can be used to detect local immune responses induced by contact allergens.

Key words: IVIS SpectrumCT; local inflammation; Prosense 750 FAST; systemic inflammation.

Contact hypersensitivity (CHS) is classically described as a T cell-mediated disease caused by the development of allergen-specific memory T cells (1). CHS can be divided into two phases, namely the sensitization phase and the elicitation phase (2). The sensitization phase occurs upon initial exposure to a specific contact allergen, and

results in the activation, differentiation and development of allergen-specific memory T cells (3). The elicitation phase is initiated when the skin is re-exposed to the same allergen, which reactivates the allergen-specific memory T cells, ultimately leading to skin inflammation (4). Several other cell types also seem to play an important role in the pathogenesis of CHS. These include B cells (5–8), $\gamma\delta$ T cells (9–13), including dendritic epidermal T cells (DETCs) (14, 15), natural killer cells (16), natural killer T cells (17, 18), and neutrophils (19). In addition to the local skin manifestations, exposure to contact allergens can lead to a systemic inflammatory reaction (17, 18, 20, 21).

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Conflicts of interest: The authors declare no conflict of interests.

Accepted for publication 15 November 2016

In the present study, we wanted to investigate whether *in vivo* imaging could be used to track the inflammatory response induced by contact allergens. We used the IVIS SpectrumCT, which performs both epi-illumination and trans-illumination, thereby allowing three-dimensional (3D) localization of sites with inflammation within the animal. To our knowledge, only two studies have previously used the IVIS platform during CHS investigations (22, 23). One study used the IVIS platform to validate involvement of the purinergic receptor P₂X₇ during CHS (23), and the other study identified the alarmin S100A8/S100A9 as a marker of local inflammation in several diseases, including CHS (22).

To evaluate the potential of the IVIS SpectrumCT to investigate the inflammatory response induced by contact allergens, we used a CHS protocol in which mice were repeatedly exposed to a contact allergen. Two different contact allergens were used: methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), and 2,4-dinitrofluorobenzene (DNFB). MCI/MI is a preservative used in industrial products, for example paint, ink, and cooling fluids, and many cosmetic products, for example shampoos. The use of MI as a stand-alone preservative has recently caused unprecedented significant increases in the numbers of MI-allergic and MCI/MI-allergic individuals (24, 25). The use of DNFB ensured that we would obtain strong CHS responses, and MCI/MI would provide data on a more clinically relevant allergen. After every second exposure, the mice were analysed for allergen-induced inflammation. To investigate this, we chose ProSense 750 FAST from PerkinElmer. We found that local inflammation could be tracked by injections with ProSense 750 FAST followed by analysis on the IVIS SpectrumCT, which correlated with both immunological phenotype of the skin draining lymph nodes (dLNs) and the cytokine profile in the skin. On the other hand, ProSense 750 FAST failed to detect any difference in the systemic response, although increased numbers of $\gamma\delta$ T cells and antigen-presenting cells (APCs) were identified in the spleens of allergen-exposed mice by flow cytometry.

Materials and Methods

Mice

Female C57BL/6NTac-*Tyr^{tm1Arte}* (11971-F) mice were purchased from Taconic Biosciences, Silkeborg, Denmark, and housed at the University of Copenhagen in accordance with national animal protection guidelines (licence number: 2012-15-2454-00663). In each experiment, groups of four mice were used at the age of 8 weeks. Initial

experiments with conventional C57BL/6 mice showed little to no fluorescent signal in ears following allergen treatment. We therefore used C57BL/6NTac-*Tyr^{tm1Arte}* (11971-F) mice because of their white fur, which allowed for much better detection of the fluorescent signal.

Chemicals

The following allergens were used: DNFB (CAS no. 70-43-8; Sigma, Brøndby, Denmark) and MI in a 1:3 combination with MCI, that is, MCI/MI (CAS no. 55965-84-9; Sigma).

Induction of contact hypersensitivity by repeated exposure to contact allergen

For study of the effect of repeated exposure to contact allergens, mice were painted on the dorsal sides of both ears once every second week for a total of six times. Mice were painted with 25 μ l of either 0.15% DNFB (vol/vol) or 0.0082% MCI/MI (vol/vol) in a 1:4 olive oil/acetone (OOA) mixture. Vehicle-treated mice were painted with OOA. Twenty-four hours after each exposure, ear thickness was measured to evaluate inflammation. Twenty-four hours after the sixth and final exposure, mice were killed, and dLNs and spleens were collected into complete RPMI medium for further analysis. Moreover, ears were snap-frozen and stored at -80°C until further analysis.

Analysis of local and systemic inflammation by use of the IVIS SpectrumCT and ProSense 750 FAST

Mice were intravenously injected with 100 μ l of ProSense 750 FAST (NEV11171) (PerkinElmer, Waltham, MA, USA), prepared according to the manufacturer's instructions 6 h after the second, fourth and sixth exposures to contact allergen or vehicle. Mice were anaesthetized with isoflurane 6 h post injection, before being transferred to the IVIS SpectrumCT (PerkinElmer) for imaging. Epi-illumination imaging was performed on each group of mice, before analysis of a single mouse at a time by fluorescence imaging tomography (FLIT). FLIT utilizes a trans-illumination light source to project emission light from below the mouse through the tissue. To evaluate potential systemic inflammation in the stomach region, a total of 12 trans-illumination points were chosen. Mice were held under isoflurane inhalation anaesthesia for the duration of the scan, and the imaging chamber was heated to 32°C . Data obtained by IVIS analysis were subsequently analysed with LIVING IMAGE software (PerkinElmer).

Flow cytometry

Single-cell suspensions were prepared manually from the dLN and spleen, and the total cell number was determined. To determine the distribution of T cells and CD11c⁺CD11b⁺MHC-II⁺ APCs, single-cell suspensions were incubated with fluorochrome-conjugated anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti- $\gamma\delta$ (GL3), anti-CD11c (HL3), anti-CD11b (M1/70), and anti-MHC-II (9), all purchased from Biolegend (San Diego, CA, USA). Cells were incubated on ice for 30 min with the indicated monoclonal antibodies diluted in cold phosphate-buffered saline containing 2% fetal bovine serum and 0.1% NaN₃. Subsequently, samples were run on the LSRFortessa (BD Biosciences, Denmark, Kgs. Lyngby), and data analysis was performed with FLOWJO (FlowJo, Ashland, OR, USA).

Cytokine detection with the MSD platform

For cytokine analysis, ears were homogenized and lysed individually by the use of Precellys technology (Bertin Technologies, Montigny-le-Bretonneux, France). Homogenized ear tissue samples were adjusted to 3 $\mu\text{g}/\mu\text{l}$ protein for the Bradford assay. Samples of 50 μl were used to determine cytokine levels with mouse V-PLEX pro-inflammatory Panel 1 (Meso Scale Diagnostics, Baltimore, MD, USA).

Statistical analysis

Statistically significant differences between control and experimental groups were determined with two-sample equal-variance *t*-tests. The analysis was performed on pooled data from two individual experiments with four mice per group in each experiment, that is, with eight mice per analysis group. Analysis was performed by comparing mice painted with contact allergen with control mice.

Results

ProSense 750 FAST as a tracker of local inflammation during CHS

ProSense 750 FAST is an *in vivo* imaging agent activated by the proteolytic function of cathepsins. Cathepsins are primarily found in the lysosomes, and thus ProSense 750 FAST primarily tracks lysosome-rich cells, for example macrophages, eosinophils, neutrophils, and dendritic cells. To evaluate ProSense 750 FAST as a tracker of inflammation during CHS, mice were repeatedly exposed to either DNFB, MCI/MI or OOA on the dorsal sides of both ears every second week for a total of six times (Fig. 1a).

Six hours after the second, fourth and sixth exposures to contact allergen or vehicle, mice were intravenously injected with 100 μl of ProSense 750 FAST (Fig. 1a). To monitor the fluorescence intensity at the point of allergen exposure, we performed epi-illumination on the dorsal sides of the ears of each group of mice (Fig. 1d). Subsequently, we performed region of interest (ROI) analysis on the ear region in order to compare the values with ear thickness data (Fig. 1b,c). After both the fourth and sixth exposures to DNFB, cells rich in lysosomes clearly accumulated in the area of exposure at a higher degree than in OOA-treated mice (Fig. 1c,d). We were unable to detect any difference between MCI/MI-treated and OOA-treated mice when they were analysed by epi-illumination (Fig. 1c,d). The response seen in the OOA-treated group is mostly mediated by the low grade of inflammation induced by OOA exposure.

In order to compare IVIS SpectrumCT measurements with the most common measurement of inflammation during CHS, ear thickness was measured 24 h after each exposure to contact allergen (Fig. 1b). DNFB-treated mice showed a significant increase in ear thickness as compared with OOA-treated mice (Fig. 1b). Interestingly, whereas IVIS SpectrumCT measurements showed no difference between MCI/MI-treated and OOA-treated mice, a small but significant increase in ear thickness was seen in MCI/MI-treated mice as compared with OOA-treated mice, although not after the fifth exposure (Fig. 1b). To investigate how ProSense 750 FAST signal and ear thickness data correlated with the cytokine profile in allergen-exposed skin, we purified protein from ears and measured cytokine levels by MSD. In agreement with both ear thickness and ProSense 750 FAST signal, we found significantly elevated levels of the proinflammatory cytokines tumour necrosis factor- α , interleukin (IL)-1 β and IL-6 in DNFB-exposed skin as compared with OOA-exposed skin (Fig. 1e). Also, the highly potent neutrophil attractant KC was significantly upregulated in the DNFB-treated group (Fig. 1e). It is well established that T cells are important for the development of CHS (2, 3, 26). Accordingly, DNFB-treated mice showed significantly elevated levels of the T cell cytokines interferon- γ , IL-4, IL-5, IL-2, and IL-10 (Fig. 1e). Taken together, the ProSense 750 FAST signal correlated well with both changes in ear thickness and levels of proinflammatory cytokines in DNFB-exposed skin. This indicates that ProSense 750 FAST could be a good tracker of local inflammation during the CHS response analysed with the IVIS SpectrumCT.

T cell and APC numbers in the dLNs

To further investigate the local response, the numbers of different T cell subsets and APCs in the dLNs were

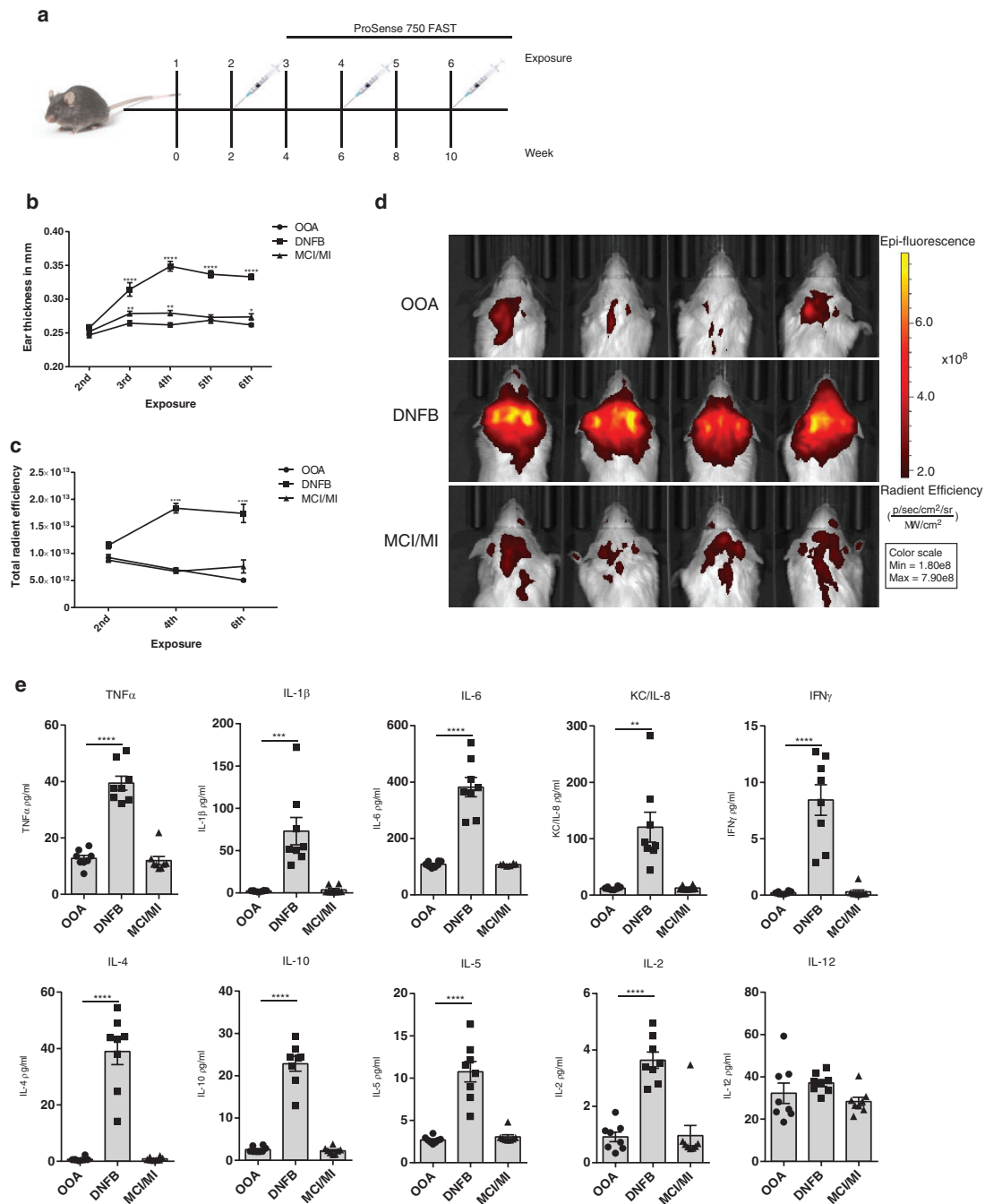


Fig. 1. Evaluating ProSense 750 FAST as a tracker of local inflammation during contact hypersensitivity analysed with the IVIS SpectrumCT. (a) Illustration of the experimental setup. Groups of mice were repeatedly exposed to 2,4-dinitrofluorobenzene (DNFB), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) or olive oil/acetone (OOA) once every second week for a total of 10 weeks. Six hours after the second, fourth and sixth exposures, mice were injected intravenously with 100 µl of ProSense 750 FAST to allow IVIS SpectrumCT analyses of local inflammation. (b) Local inflammation determined by ear thickness measured 24 h after allergen exposure. (c, d) Local inflammation determined by IVIS SpectrumCT measurements with epi-illumination obtained 6 h after injection of ProSense 750 FAST. (e) Twenty-four hours after the final allergen exposure, the mice were killed, and ear biopsies were harvested for detection of proinflammatory cytokines with the MSD platform. Results are presented as mean ± standard error of the mean. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Figures represent pooled data from two individual experiments with four mice per group per experiment. IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

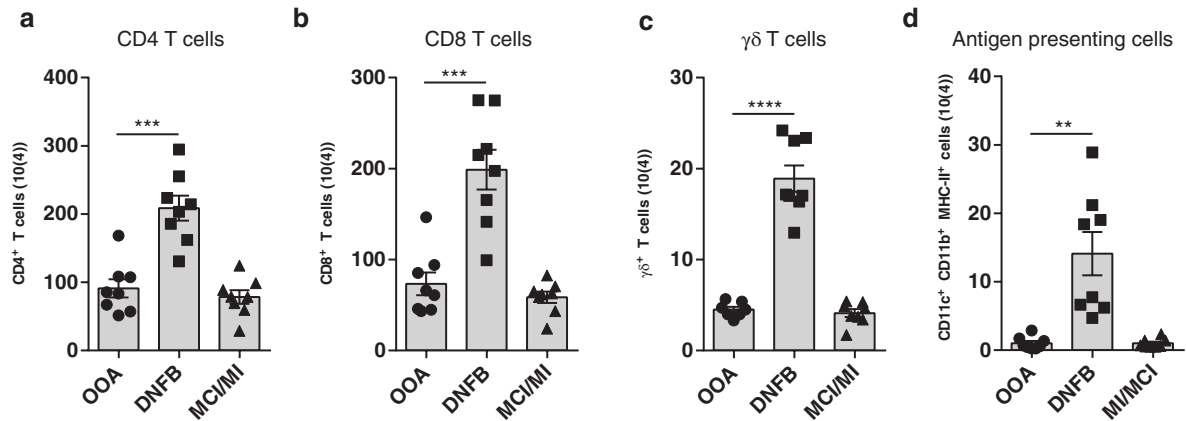


Fig. 2. T cell and antigen-presenting cell (APC) numbers in the draining lymph nodes (dLNs). Groups of mice were repeatedly exposed to 2,4-dinitrofluorobenzene (DNFB), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) or olive oil/acetone (OOA) once every second week for a total of 10 weeks. Twenty-four hours after the final allergen exposure, the mice were killed. The dLNs were collected to determine the number of (a) CD4⁺ T cells, (b) CD8⁺ T cells, (c) γδ⁺ T cells (GL3⁺) and (d) APCs (CD11c⁺CD11b⁺MHC-II⁺) by flow cytometry. Results are presented as mean ± standard error of the mean. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Figures represent pooled data from two individual experiments with four mice per group and experiment.

determined following repeated exposure to either DNFB or MCI/MI. The dLNs were collected 24 h after the sixth and final allergen exposures, and cell numbers were analysed by flow cytometry. Significant increases in the numbers of CD4⁺, CD8⁺ and γδ⁺ T cells were found in the DNFB-treated group as compared with control mice (Fig. 2a–c). However, no difference was found between MCI/MI-treated and control mice (Fig. 2a–c). Given that ProSense 750 FAST will locate to cells with a high number of lysosomes, for example macrophages and dendritic cells, which are both APCs, we further determined the number of APCs in the dLN defined as CD11c⁺CD11b⁺MHC-II⁺ cells (Fig. 2d). A significant increase in the number of APCs was observed in the DNFB-treated group, but not in the MCI/MI-treated group, as compared with control mice (Fig. 2d). Thus, these results correlate well with the inflammatory response detected in skin.

Determining systemic inflammation by flow cytometry and IVIS SpectrumCT analyses with ProSense 750 FAST

To identify possible systemic effects of repeated exposure to DNFB or MCI/MI, the spleen was collected 24 h after final allergen exposure, and the numbers of CD4⁺ cells, CD8⁺ cells, γδ⁺ T cells and APCs were determined by flow cytometry. We observed no difference in the number of either CD4⁺ or CD8⁺ T cells between the three groups of mice (Fig. 3a,b). Interestingly, however, we did find significant increases in the numbers of γδ T cells and APCs in both DNFB-treated and MCI/MI-treated mice

(Fig. 3c,d). To evaluate ProSense 750 FAST as a tracker of systemic inflammation in our model, FLIT analysis and 3D ROI measurements of the splenic area were performed after the second, fourth and sixth exposures (Fig. 3f–h). However, when comparing the quantification values for the fluorescent signal within the 3D ROI, we did not find a significant difference between the three groups (Fig. 3e–h). Although the difference was not significant, we observed a tendency for there to be slightly higher values in both allergen-treated groups after the second exposure (Fig. 3f). Taken together, the significantly increased numbers of γδ T cells and APCs in the spleen indicate that repeated exposure to contact allergens does lead to a systemic immune response. However, we were unable to detect this response with ProSense 750 FAST and FLIT analysis in the spleen.

Discussion

In the present study, we investigated whether IVIS SpectrumCT analyses with ProSense 750 FAST as the tracker could be used to determine allergen-induced inflammation during CHS. We found that detection of the ProSense 750 FAST signal correlated with DNFB-induced changes in ear thickness, cytokine profile, and the numbers of different T cell subsets and APCs in the dLNs. In contrast, the slightly increased ear thickness induced by exposure to MCI/MI could not be detected by ProSense 750 FAST. Interestingly, analysis of splenic cells showed significant increases in the number of APCs and γδ T cells in both the DNFB-treated and MCI/MI-treated groups,

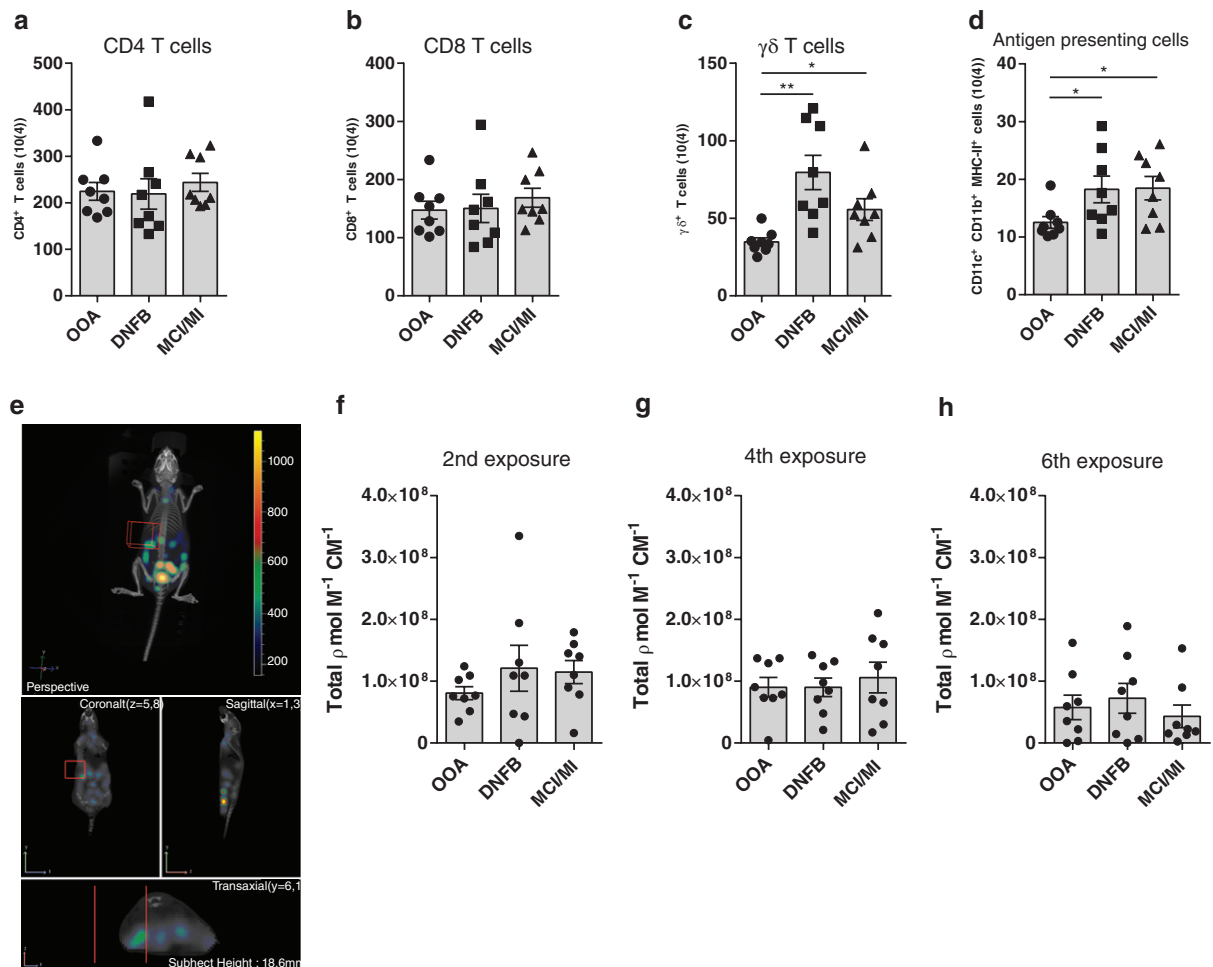


Fig. 3. Analyses of potential systemic inflammation by flow cytometry and IVIS SpectrumCT analyses with ProSense 750 FAST as a tracker. Groups of mice were repeatedly exposed to 2,4-dinitrofluorobenzene (DNFB), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) and olive oil/acetone (OOA) once every second week for a total of 10 weeks. Six hours after the second, fourth and sixth exposures, mice were injected intravenously with 100 µl of ProSense 750 FAST to allow IVIS SpectrumCT analyses of systemic inflammation. Twenty-four hours after the final allergen exposure, the mice were killed, and the spleens were collected to determine the number of (a) CD4⁺ T cells, (b) CD8⁺ T cells, (c) γδ⁺ T cells (GL3⁺) and (d) antigen-presenting cells (CD11c⁺CD11b⁺MHC-II⁺) by flow cytometry. Six hours after injection with ProSense 750 FAST, mice were individually analysed in the IVIS SpectrumCT by fluorescence imaging tomography (FLIT). (e) A representative three-dimensional (3D) reconstruction of FLIT data and the 3D region of interest (ROI) in which the fluorescence intensity of the splenic region is measured in a DNFB-treated mouse after the second exposure. (f–h) FLIT analysis and 3D ROI measurements were performed after the second, fourth and sixth exposures, with the value for quantification of the 3D ROI being total pmol/M per cm. Results are presented as mean ± standard error of the mean. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001. Figures represent pooled data from two individual experiments with four mice per group and experiment.

indicating that repeated exposure to contact allergens could have systemic effects. However, no evidence of systemic responses was found by IVIS SpectrumCT analyses with ProSense 750 FAST as the tracker.

The discrepancy between detection of local inflammation with ProSense 750 FAST signal by the use of IVIS SpectrumCT measurement and changes in ear thickness in mice exposed to MCI/MI might be explained by several factors. One possible explanation could be that the two

measurements were made at different time-points. Owing to the half-life of ProSense 750 FAST, we performed IVIS analyses 12 h after allergen exposure, whereas we measured ear thickness 24 h after allergen exposure. However, as we found that the ProSense 750 FAST signal correlates with cellular dynamics in the dLNs and the cytokine profile found in allergen-exposed skin, which were also analysed 24 h after allergen exposure, we do not believe that the difference in time could explain the

lack of detection of inflammation in the IVIS analyses. The change in ear thickness induced by MCI/MI was minor, and the lack of ProSense 750 FAST signal might therefore more easily be explained by the fact that the MCI/MI-induced local inflammation was below the threshold of detection by the probe.

Furthermore, when investigating the local response, we observed that fluorescent signal was not exclusively confined to the ears, but could also, to a lesser extent, be detected between the ears, in the snout, and in the paws. The lack of completely confined signal is most likely attributable to the way in which the allergen is applied. The allergen is dissolved and applied in the vehicle and pipetted onto the tip of the ear; from there, it disperses downwards towards the base of each ear. When allergen is applied in this way, it is very likely that the area between the ears will be exposed, and this could account for the signal observed in this area. Likewise, the signal detected on the paws and snouts of mice is likely to be attributable to the way in which we exposed the mice to allergen in our setup. When mice are exposed to allergen or vehicle, they are not sedated, but simply restrained. After exposure to allergen, mice are returned to their cage and are free to move around. Post-exposure, it is observed that mice groom themselves by using the front legs to groom the ears in a forward motion. By doing this directly after exposure, it is likely that mice are exposing both the snout and the paws to allergen, which would explain the appearance of signal at these locations.

Finally, we found elevated levels of $\gamma\delta$ T cells in the spleens of both allergen-treated groups. CHS is classi-

cally described as a T cell-mediated disease primarily dominated by $\alpha\beta$ T cells, but several studies have shown that $\gamma\delta$ T cells also play important roles in CHS development (9–15, 27–31). Different subsets of $\gamma\delta$ T cells are found in different anatomical sites of the mice, with DETCs being located in the epidermis, $V\gamma 5^+$ intraepithelial lymphocytes in the intestines, and $V\gamma 2^+$, $V\gamma 4^+$ or $V\gamma 1.1^+$ (Garman nomenclature) in the circulation and lymphoid tissue (27). In this study, we have not determined which subsets of $\gamma\delta$ T cells are increased in number in the spleen after repeated allergen exposure. However, this would be interesting to examine in future studies, as it would be helpful in understanding the mechanisms involved in systemic responses induced by contact allergens. Furthermore, the fact that we detected increased numbers of both APCs and $\gamma\delta$ T cells in the spleens of MCI/MI-treated mice, while finding no difference in the dLNs and the cytokine profile in the skin, is very interesting. This may indicate that exposure to weak or dilute allergens, which might not cause skin inflammation, could still induce a systemic immunological response. However, this needs further investigation.

In conclusion, our data show that IVIS SpectrumCT analysis with ProSense 750 FAST as the probe can be used to determine the local skin inflammation in CHS.

Acknowledgements

The study was supported by The Danish Environmental Protection Agency, Aage Bangs Fond and Aase og Ejnar Danielsen Fond.

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