

Systemic immunogenicity of para-Phenylenediamine and Diphenylcyclopropenone: two potent contact allergy-inducing haptens

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Published online: 3 January 2014
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Abstract *p*-Phenylenediamine (PPD) and Diphenylcyclopropenone (DPCP) are two potent haptens. Both haptens are known to cause delayed-type hypersensitivity, involving a cytokine response and local infiltration of T-cell subpopulations, resulting in contact dermatitis. We investigated the systemic immune effects of PPD and DPCP, two relatively unexplored skin allergens. The dorsal sides of the ears of BALB/c mice were exposed to PPD or DPCP (0.1 % w/v or 0.01 % w/v), or vehicle alone. Mice were treated once daily for 3 days (induction period) and subsequently twice per week for 8 weeks. Local and systemic immune responses in the auricular and pancreatic lymph nodes, spleen, liver, serum, and ears were analyzed with cytokine profiling MSD, flow cytometry, and qPCR. Ear swelling increased significantly in mice treated with 1 % PPD, 0.01 % DPCP or 0.1 % DPCP, compared with vehicle treatment, indicating that the mice were sensitized and that there was a local inflammation. Auricular lymph

nodes, pancreatic lymph nodes, spleen, and liver showed changes in regulatory T-cell, B-cell, and NKT-cell frequencies, and increased activation of CD8⁺ T cells and B cells. Intracellular cytokine profiling revealed an increase in the IFN- γ - and IL-4-positive NKT cells present in the liver following treatment with both haptens. Moreover, we saw a tendency toward a systemic increase in IL-17A. We observed systemic immunological effects of PPD and DPCP. Furthermore, concentrations too low to increase ear thickness and cause clinical symptoms may still prime the immune system. These systemic immunological effects may potentially predispose individuals to certain diseases.

Keywords *p*-Phenylenediamine ·
Diphenylcyclopropenone · Contact hypersensitivity ·
Contact allergy · Invariant NKT cells · Foxp3

Abbreviations

CHS	Contact hypersensitivity
DTH	Delayed-type hypersensitivity
PPD	<i>p</i> -Phenylenediamine
DPCP	Diphenylcyclopropenone
AL-N	Auricular lymph nodes
PL-N	Pancreatic lymph nodes

Electronic supplementary material The online version of this article (doi:10.1007/s12026-013-8482-z) contains supplementary material, which is available to authorized users.

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Introduction

Contact allergy, also known as contact hypersensitivity (CHS) in the animal setting, is a delayed-type hypersensitivity reaction in which cutaneous T cells mediate an inflammatory immune response. CHS can be divided into sensitization and elicitation phases. Both phases require interaction between innate and adaptive immune systems. In the sensitization phase, topical skin exposure to small

reactive chemicals (haptens) triggers dendritic cell activation and migration to the local lymph nodes, where naïve T cells are primed specifically for hapten-modified protein bound to class I or II MHC molecules [1, 2]. When re-exposed to the hapten, hapten-specific memory T cells are recruited to the site of contact, where they interact with the hapten–MHC complex and mediate the characteristic delayed inflammatory response seen 24–48 h following challenge [3]. Hapten-primed CD8⁺ T cells are the main effector cells in oxazolone, urushiol (poison ivy), and 2,4-dinitro-1-fluorobenzene (DNFB)-mediated CHS [3–6].

Recent studies have shown that a CD8-positive IL-17-producing T-cell subpopulation influences effector functions during the sensitization and elicitation phases [7–9]. Results indicate that hapten-primed T cells produce IL-17 and that it is possible to inhibit hapten-primed T-cell-mediated transfer of CHS, by neutralizing IL-17 [7]. Studies show that IL-17 regulates the production of several chemokines and cytokines, and the expression of adhesion molecules, enabling early infiltration of neutrophils into challenged skin, followed by the infiltration of effector CD8⁺ T cells [10–14].

Natural killer T cells expressing the invariant V α 14-J α 18 T-cell receptor (iNKT) can recognize lipids presented by CD1d [15, 16]. They contain stores of Th1 and Th2 cytokines, e.g., IFN- γ and IL-4, which can be released rapidly upon activation [17, 18]. NKT cells have previously been shown to play an important role in events taking place as early as 7 min following immunization [19]. Evidence suggests that hepatic iNKT cells secrete IL-4 to activate IgM antibody production by B-1 B cells for the recruitment of effector cells [19, 20]. Mice exposed to a hapten show increased presence of B-1 B cells in the draining lymph nodes and spleen during elicitation [3, 21].

p-Phenylenediamine (PPD) is a frequent contact sensitizer in hair dye formulas [22]. A study of Danish eczema patients in the period from 1985 to 2007 showed that 2 % were sensitized to PPD [23]. Diphenylcyclopropanone (DPCP) is a topically administered drug used in treating alopecia areata, an autoimmune disease causing spot baldness on the scalp. Topical immunotherapy using DPCP may also be an effective treatment option for recalcitrant warts and a potential immunotherapeutic for cutaneous metastatic melanoma [24, 25]. DPCP is not used in consumer products and is thus considered an experimental hapten.

This study was initiated to investigate, if repetitive cutaneous exposure to contact haptens will induce a systemic immune response in other organs remote to the skin, which could potentially influence the development of other diseases.

Materials and methods

Mice

Female BALB/c mice were used at 6 weeks of age (Taconic). Forty mice were split into five groups (eight in each group). Animal experiments were conducted at Copenhagen Biocenter, University of Copenhagen, Denmark, in accordance with the revised Council of Europe Convention ETS 123, the revised EU directive 2010/63 and the Danish Proclamation of law on animal experimentation 1306 from 23.11.2007, and were approved specifically by the Danish Animal Experiments Inspectorate and the Animal Ethics Council.

Hapten treatment for mice and measurement of ear thickness

The mice were sedated with hypnorne/midazolam (0.01 μ l per gram), and weight and ear thickness were measured prior to the first application of hapten. The haptens were dissolved in the vehicle acetone/olive oil 4:1 (AOO), and the mice were exposed on the dorsal sides of both ears to 25 μ l of the respective hapten, *p*-Phenylenediamine (#P6001, Sigma-Aldrich, Brøndby, Denmark), Diphenylcyclopropanone (#177377, Sigma-Aldrich, Brøndby, Denmark), or vehicle. For the PPD groups, the concentrations were 1.0 or 0.1 % (w/v). For the DPCP groups, the concentrations were 0.1 or 0.01 %. Sensitization was achieved by treatment once daily for 3 days followed by challenge twice per week for 8 weeks. The mice were euthanized by cervical dislocation 24 h after the last treatment, and the draining auricular lymph nodes (ALN), pancreatic lymph nodes (PLN), spleen, liver, ears, and pancreas were removed and used for flow cytometry and quantitative PCR. Serum samples were collected by jaw puncture prior to euthanization. Ear thickness was measured using an electronic digital micrometer (BP Technologies).

Antibodies

The following antibodies were purchased from BioLegend: FC-block anti-CD16/CD32 mAb (clone 93), BV650-conjugated anti-CD19 mAb (clone 6D5), PerCP-Cy5.5-conjugated anti-TCR β mAb (clone H57-597), APC-AF780-conjugated anti-CD11c mAb (clone N418), AF700-conjugated anti-CD8a mAb (clone 53-6.7), BV570-conjugated anti-CD4 mAb (clone RM4-5) and BV605-conjugated anti-CD69 mAb (clone H1.2F3). The following antibodies were used for intracellular staining: PE-conjugated anti-Foxp3 mAb (clone 150D), PE-conjugated anti-IL-4 mAb (clone 11B11), PE-conjugated anti-IL-10 mAb (clone JES5-16E3), PE-conjugated anti-IL-17A mAb (clone TC11-18H10.1), and PE-conjugated anti-IFN- γ

mAb (clone XMG1.2). AF647-conjugated PBS-57-loaded CD1d tetramers were kindly donated by the NIH Tetramer Core Facility.

Cell purification and flow cytometric analysis

Single-cell suspensions were prepared from A-LN, P-LN, spleen, and liver by passing the organs through 70- μ m cell strainers (BD Biosciences). Spleen red blood cells were lysed with the red blood cell lysis buffer (#00-4300-54, eBioscience). Lymphocytes were purified from whole liver cell extract by Lympholyte-M density centrifugation (#CL5035, Tebu-Bio). Cells from two mice were pooled to obtain a sufficient number of cells. The pooled cell suspensions were divided into two groups. The first group was stimulated by PMA (50 ng/ml) and ionomycin (2 μ g/ml) for 30 min, followed by the addition of Golgi-stop (final concentration of 10 μ g/ml for IL-4, IL-17A, IFN- γ , and Foxp3) or a mix of Golgi-stop and Golgi-plug (final concentration of 5 μ g/ml of each for IL-10). The cells were then incubated for an additional 3.5 h. The second group was analyzed unstimulated. To distinguish live cells from dead cells, the Live/Dead fixable aqua dead cell stain kit (Invitrogen) was used, followed by incubation with CD16/CD32 antibody and a mixture of pre-titrated cell surface-specific antibodies. Subsequently, the cells were fixed and permeabilized, according to the manufacturer's protocol, using the Mouse regulatory T cell staining kit (eBioscience, San Diego, CA). Intracellular cytokine staining was carried out on stimulated cells (group one). Samples were run on an LSR II flow cytometer (BD) and analyzed with FlowJo software (Treestar, Ashland, OR). Fluorescence minus one (FMO) controls were used for gating.

Quantitative PCR

Tissue samples from the ear, pancreas, and liver (approximately 20 mg) were homogenized by sonication (Branson sonifier 250). Total RNA was isolated using Maxwell[®] 16 LEV simplyRNA Purification Kits (Promega, AS1270). Approximately 0.5 μ g of RNA was reverse-transcribed into cDNA using the iScript kit (Bio-Rad) following manufacturer's guidelines. Specific mRNA levels were quantified on a Lightcycler II (Roche, Penzberg, Germany) using SYBR II qPCR mixture (Takara, Otsu, Japan) and normalized to the housekeeping gene β -Actin. Primers (see supplemental materials) were designed using the Primer3 software [26] and synthesized by TAGCopenhagen (Copenhagen). Purified and diluted PCR products were used to create a standard curve for each primer pair.

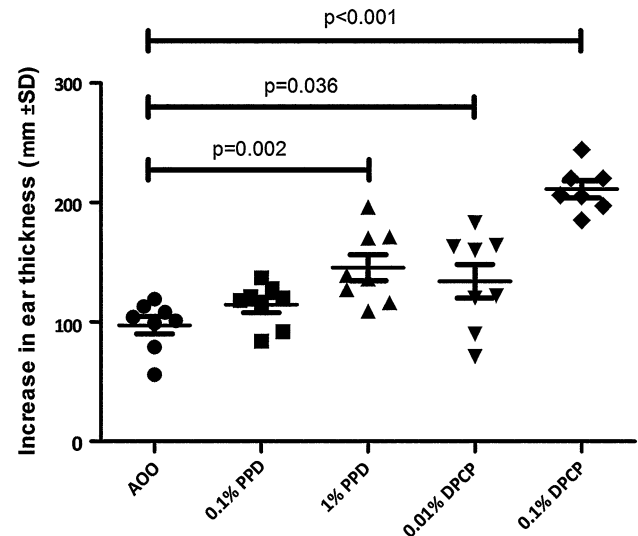


Fig. 1 PPD and DPCP treatment increases average ear thickness. BALB/c mice were contact sensitized with 0.1 % PPD, 1 % PPD, 0.01 % DPCP, 0.1 % DPCP, or 4:1 acetone/live oil (AAO). Eight weeks later, at 24 h following the last treatment, ear thickness was measured. We found a significant increase in the ear thicknesses of animals treated with 1 % PPD, 0.01 % DPCP, or 0.1 % DPCP, as compared to AAO-treated mice. Data represent mean increase in ear thickness $\pm$ SD from eight mice in each group

MSD ELISA assay

Blood samples were collected by jaw puncture and incubated for 2 h at room temperature to allow clotting. Samples were centrifuged for 5 min at 1,300 g and serum was collected.

Interferon (IFN)- γ , interleukin (IL)-1 β , interleukin (IL)-2, interleukin (IL)-4, interleukin (IL)-5, interleukin (IL)-10, interleukin (IL)-12, interleukin (IL)-17A, tumor necrosis factor (TNF)- α , and mouse keratinocyte chemoattractant (mKC) were quantified using a mouse TH1/TH2 9-Plex Assay Ultra-Sensitive Kit and a IL-17 Ultra-Sensitive Kit (Meso Scale Discovery), according to the manufacturers' instructions. They were then analyzed in a SECTOR imager 2400.

Data analysis

Student's *t* tests were performed to determine whether the changes in cell frequencies observed in the organs (ALN, PLN, spleen, and liver) or ear thickness were significantly different from AAO. A Student's unpaired *t* test was used to compare surface CD69 expression, depicted by geometrical mean values. Cells from two mice were pooled, so that each column showing flow cytometric results is based on 4 data points for each of the five groups. The graphic presentation of the results was created using GraphPad

Prism version 5 software (GraphPad Software, La Jolla, CA). Differences at $P < 0.05$ were considered significant.

Results

Changes in ear thickness following PPD and DPCP treatment

Ear thickness was measured both before sensitization and 8 weeks later to indicate whether PPD and DPCP doses were able to induce inflammation (Fig. 1). There was a dose-dependent increase in average ear thickness in animals treated with PPD or DPCP. About 0.1 % PPD was the only concentration that did not result in a significant increase in ear thickness compared to AOO treatment.

PPD and DPCP modulate local and systemic activation of T-cell and B-cell population frequencies

CD8⁺ T cells and B cells are required for the development of CHS; analyses of these cell populations were carried out using flow cytometry and qPCR.

There was no difference in the overall CD4⁺ or CD8⁺ T-cell frequencies in AL-N, PL-N, or spleen (data not shown). However, PPD and DPCP treatment increased the proportion of CD19⁺ B cells in AL-N and spleen (Fig. 2c).

DPCP significantly increased CD69 expression on CD19⁺ B cells, as well as CD69 expression on CD8⁺ T cells in AL-N, PL-N, and spleen (Fig. 2a), indicating an increased activation of these cell populations. PPD did not increase the expression of CD69. The proportion of CD8⁺ T cells gated as CD69^{High} increased significantly in AL-N, PL-N, and spleen in mice treated with DPDP, as compared to AAO treatment (Fig. 2b). The opposite effect was seen for PPD, which reduced the proportion of CD8⁺ T cells gated as CD69^{High}. The frequencies of CD69^{High} CD19 B cells increased significantly with both concentrations of DPCP and PPD. The increase in CD8⁺ T-cell activation correlates well with qPCR data showing increased levels of CD8a mRNA in the ears of mice treated with DPCP (Fig. 2d). The mRNA levels of the adhesion molecule ICAM-1 increased with 1 % PPD and both concentrations of DPCP (Fig. 2d).

PPD and DPCP induce changes in the cytokine profile

To address changes in cytokine production by T-cell and B-cell subpopulations, we used flow cytometry to look for alterations in intracellular IL-4, IL-10, IL-17A, and IFN- γ . Additionally, we investigated cytokine mRNA levels in tissue using qPCR and in serum using MSD ELISA.

Intracellular staining showed that PPD significantly increased the frequency of IL-17A-positive CD8⁺ T cells

Fig. 2 *p*-Phenylenediamine (PPD) and Diphenylcyclopropanone (DPCP) modulate B-cell and T-cell activations. Flow cytometric analysis showing CD69 expression, an activation marker for B and T cells and qPCR on total RNA from ear tissue. Cells were isolated from auricular (AL-N), pancreatic lymph nodes (PLN), and spleen. **a** B and T cells isolated from mice treated with DPCP showed an increased CD69 expression (geometric mean). PPD did not increase CD69 expression. **b** The proportion of B cells gated as CD69^{High} increased in AL-N, PL-N, and spleen with both PPD and DPCP. The proportion of CD69^{High} CD8⁺ T cells increased in AL-N, PL-N, and spleen in mice treated with both concentrations of DPCP, but decreased in mice treated with both concentrations of PPD, as compared to AOO. **c** PPD and DPCP increased the frequency of B cells in AL-N and spleen. **d** DPCP and the high concentration of PPD increased ICAM-1, whereas only both concentrations of DPCP increased CD8a mRNA levels in ears. T cells were gated as TCRbeta⁺CD8⁺ and B cells were gated as CD19⁺CD11c⁻. Pools of lymphocytes from eight mice in each group were used ($n = 4$)

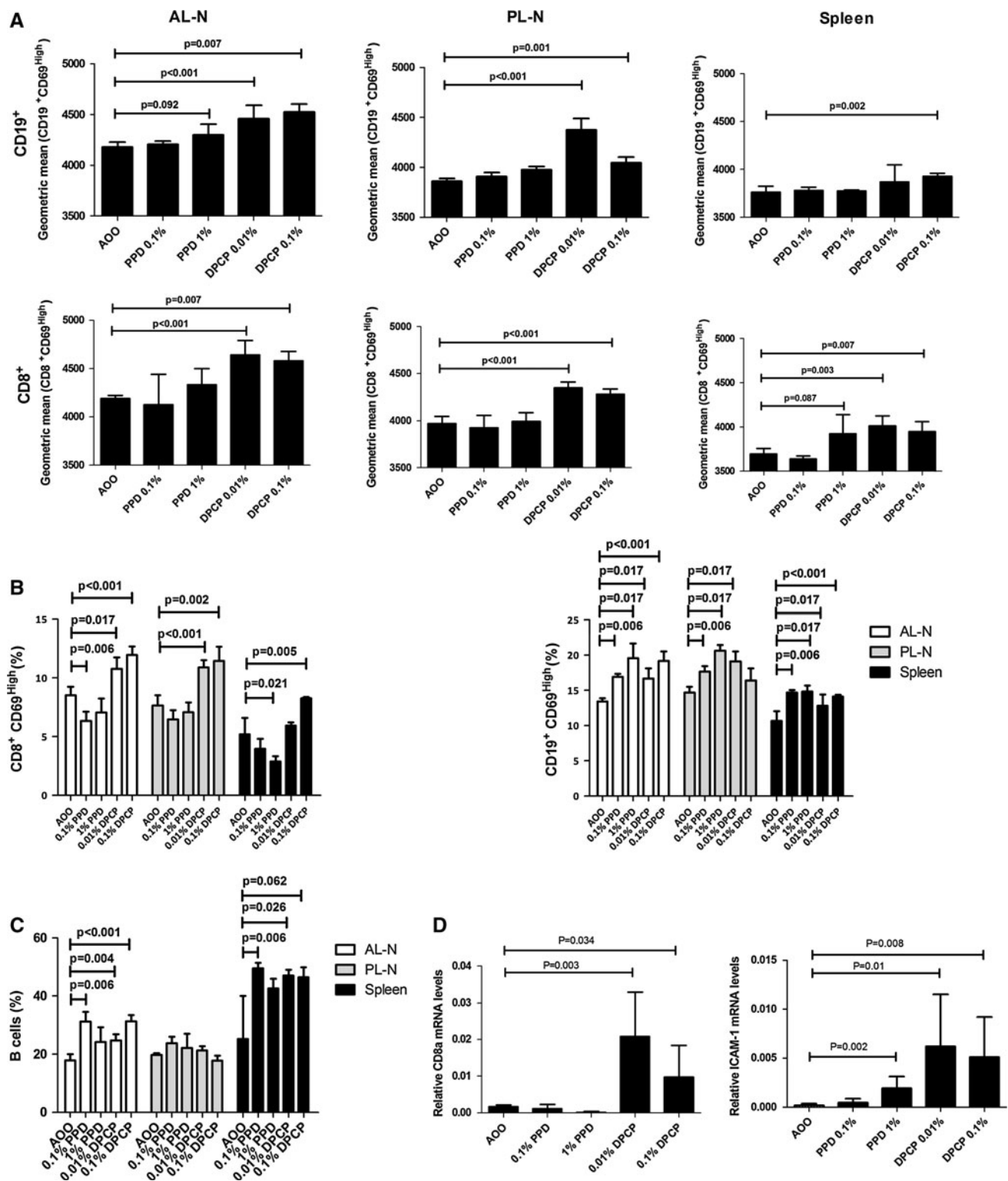
in AL-N, PL-N, and spleen (Fig. 3a). No effect was seen for DPCP treatment. The frequency of IL-4-producing CD8⁺ T cells increased in spleen when mice were treated with the low concentrations of the haptens (Fig. 3b). The high concentration of PPD significantly increased IL-10-positive CD8⁺ T cells in PL-N (Fig. 3c). DPCP increased the proportion of IFN- γ -positive CD8⁺ cells in PL-N (Fig. 3d). No differences in cytokine production by CD4⁺ T cells were observed (data not shown).

Quantitative PCR data show an increase in IL-17A mRNA levels in DPCP-treated ears, but a decrease in ears treated with PPD (Fig. 3a). Pancreatic IL-17A mRNA levels increased significantly in the mice exposed to 0.1 % PPD or either concentration of DPCP. Liver IL-17A mRNA levels increased with both haptens. We observed the same pattern for IL-4 mRNA levels in pancreatic and liver tissue (Fig. 3b). IL-10 mRNA levels significantly increased in the ears of mice treated with 1 % PPD, 0.01 % DPCP, or 0.1 % DPCP (Fig. 3c). The same overall increase was observed for IFN- γ in mice treated with PPD or DPCP (Fig. 3d).

Cytokine levels in serum were quantified by MSD ELISA (Figure E1 in the Online Repository). IFN- γ was significantly increased by 0.1 % DPCP. IL-1 β levels were significantly decreased by PPD. IL-4 levels in serum decreased significantly following treatment by 0.1 % DPCP. We observed a small but significant decrease in IL-5 in serum from mice treated with 0.1 % PPD. IL-10 levels significantly decreased with 0.1 % PPD treatment. IL-12 levels in serum significantly decreased following 1 % PPD or 0.1 % DPCP treatment.

PPD and DPCP change the frequency of Foxp3-positive T cells

To investigate whether PPD and DPCP were able to affect regulatory T-cell populations, we looked at Foxp3-positive cell frequencies and Foxp3 expression.



Low doses of both haptens significantly increased the frequency of CD8⁺ Foxp3⁺ T cells in AL-N (Fig. 4b). DPCP decreased the proportion of CD4⁺ Foxp3⁺ T cells in

PL-N, but increased CD4⁺ Foxp3⁺ T cells in spleen. When investigating Foxp3 mRNA levels in ear, pancreas, and liver tissues, we found increased mRNA levels in ears

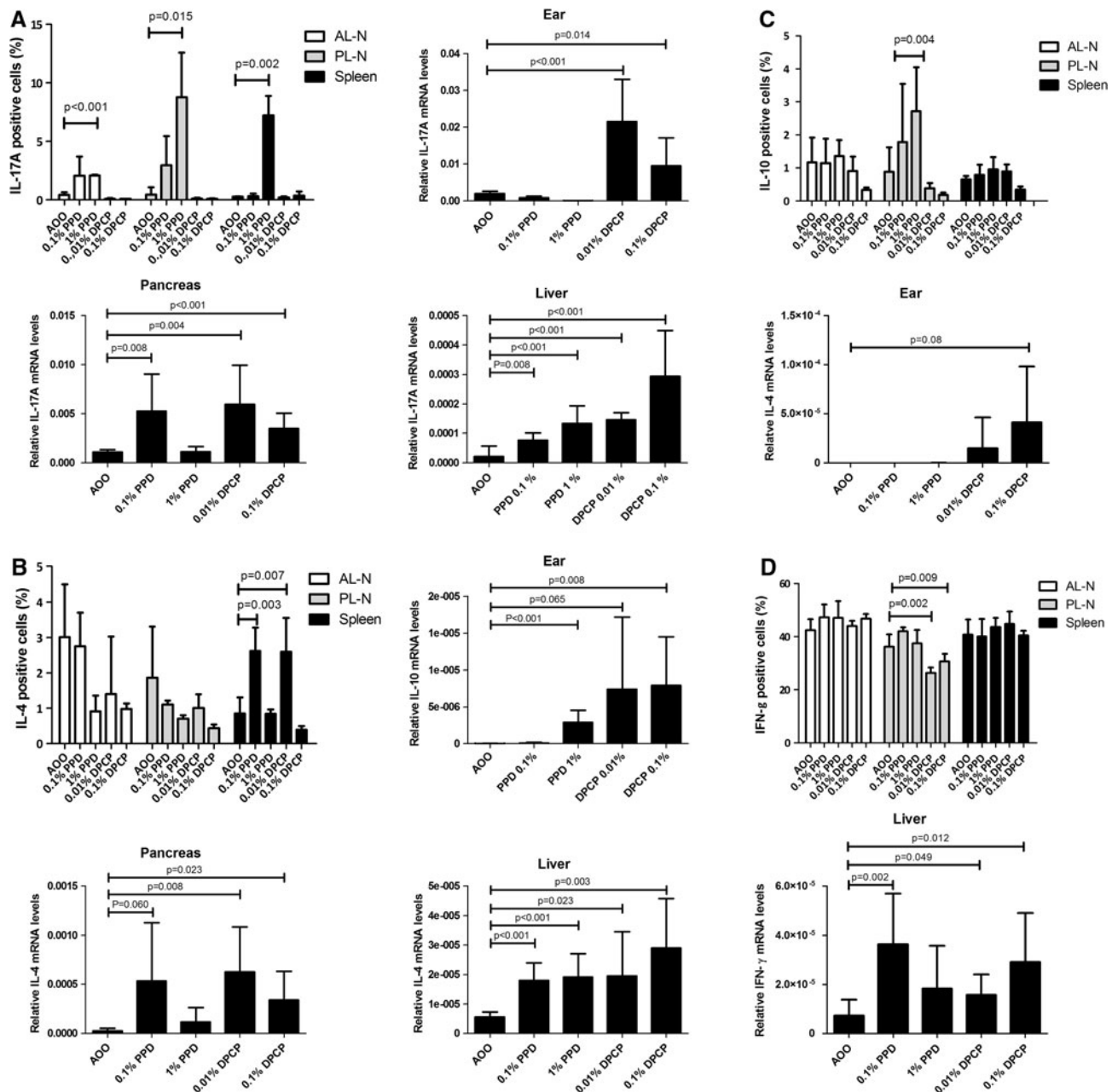


Fig. 3 Cytokine profile in AL-N, PL-N, spleen, pancreas, and liver from PPD- and DPCP-treated mice. Cells were isolated and stimulated with PMA and ionomycin in the presence of Golgi-stop (IL-4, IL-17A, and IFN- γ) or a mixture of Golgi-stop/Golgi-plug (IL-10). Stimulated cells were analyzed by flow cytometry, and qPCR was performed on ear, pancreatic, and liver tissue. **a** PPD increased the frequency of IL-17A-positive CD8 $^{+}$ T cells in AL-N, PL-N, and spleen. Neither PPD nor DPCP changed the proportion of IL-17A-positive cells in the CD4 $^{+}$ subpopulation. **b** Low doses of PPD and DPCP (0.1 and 0.01 %, respectively) significantly increased the percentage of IL-17A-positive CD8 $^{+}$ T cells in spleen. Both haptens

increased IL-4 mRNA levels in pancreatic and liver tissue, whereas only DPCP increased expression in ears. **c** No significant changes to IL-10-positive subpopulations were observed, but there was a tendency toward an increase in the proportion of IL-10-positive CD8 $^{+}$ T cells. IL-10 mRNA levels in ears increased with 1 % PPD and with both concentrations of DPCP. **d** DPCP reduced the proportion of IFN- γ -positive CD8 $^{+}$ T cells in PL-N. PPD and DPCP increased IFN- γ mRNA levels in liver. T cells were gated as TCRbeta $^{+}$ CD8 $^{+}$ and B cells were gated as CD19 $^{+}$ CD11c $^{-}$. Pools of lymphocytes from eight mice in each group were used ($n = 4$)

following PPD and DPCP treatment (Fig. 4c). We also saw an increase in pancreatic Foxp3 mRNA levels in mice treated with the low concentration of PPD or either

concentration of DPCP. Finally, we observed a slight tendency toward an increase in liver Foxp3 mRNA levels, with increasing hapten concentrations.

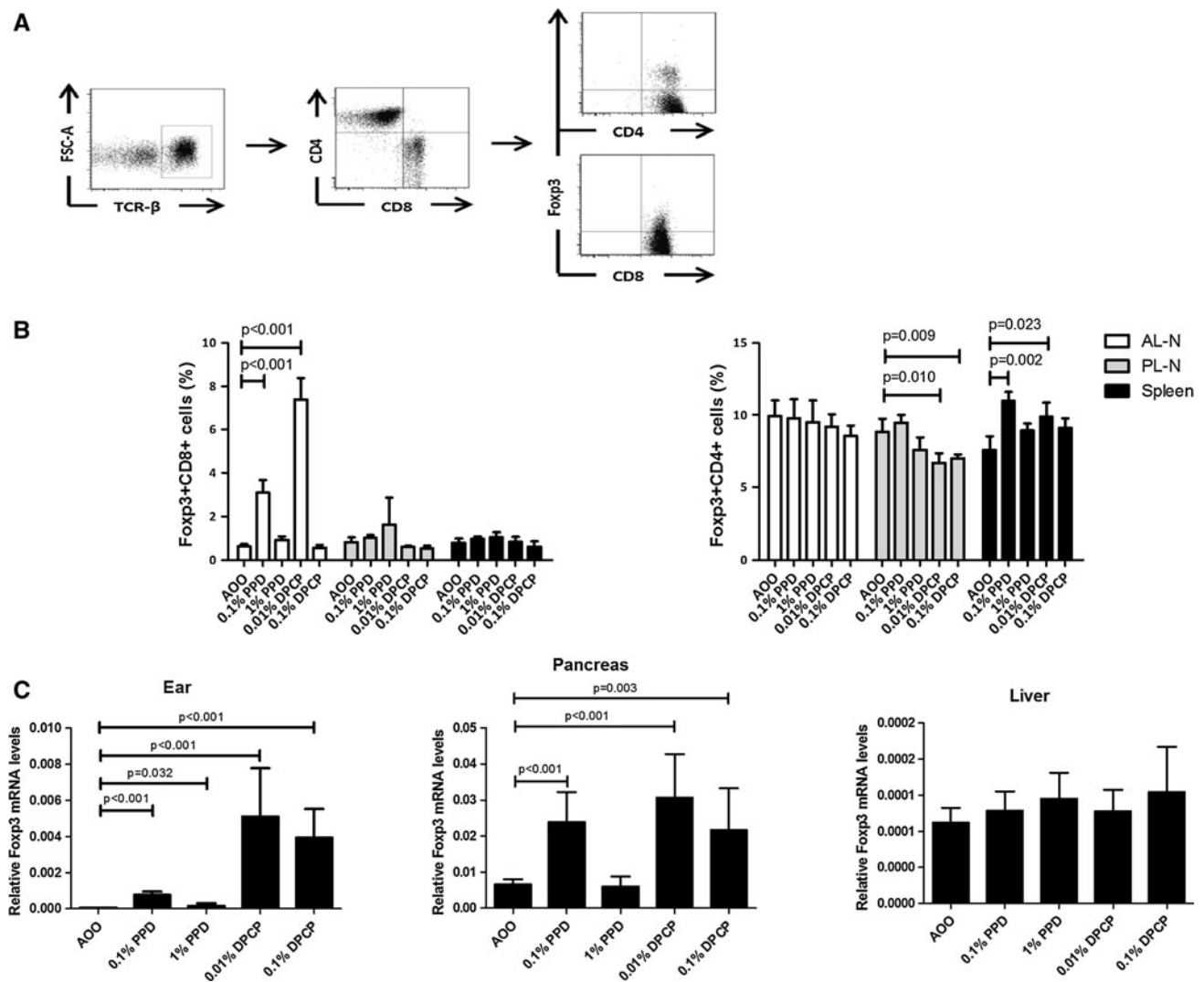


Fig. 4 *p*-Phenylenediamine (PPD) and Diphenylcyclopropanone (DPCP) induce regulatory T cells. Flow cytometric analysis was carried out on auricular (AL-N), pancreatic lymph nodes (PLN), and spleen cells, isolated from BALB/c mice. qPCR was performed on total RNA isolated from ear, pancreatic, and liver tissue. **a** Scatter plots showing gating of Foxp3-positive T cells. **b** dot plot showing TCR-β⁺, CD4⁺, CD8⁺ gate, and the proportion of foxp3-positive cells. CD8⁺ Foxp3 cells frequencies in AL-N increased by 0.1 % PPD and 0.01 % DPCP, when compared to the vehicle solution (AOO).

PPD and DPCP increase iNKT-cell frequencies and cytokine production in liver

As liver iNKT-cell frequencies have previously been shown to increase following hapten treatment, we wanted to investigate how 8 weeks of PPD and DPCP treatment would affect iNKT-cell populations.

iNKT-cell frequencies were increased 24 h following the last treatments of PPD or DPCP (Fig. 5a). PPD significantly increased the proportion of IL-4-positive iNKT cells compared to AOO treatment (Fig. 5b). The same tendency was seen for mice treated with DPCP. PPD and

The proportion of CD4⁺Foxp3 cells decreased in the PL-N of mice treated with both concentrations of DPCP, but increased in the spleen at low hapten concentrations. **c** DPCP and the low concentration of PPD increased Foxp3 mRNA levels in ear and pancreatic tissue, when compared to AOO. Cytotoxic T cells were gated as TCRβ⁺ and CD8⁺ cells. Regulatory T cells were gated as being either TCRβ⁺CD4⁺Foxp3⁺ or TCRβ⁺CD8⁺Foxp3⁺. Pools of lymphocytes from eight mice in each group were used (*n* = 4)

DPCP both increased the frequency of IFN-γ-positive liver iNKT cells (Fig. 5c). As for IL-4, the effect seemed to increase in a dose-dependent manner.

Discussion

Repeated application of the haptens PPD and DPCD leads to sensitization and elicitation of CHS through a complex series of inflammatory events that culminate in the characteristic epidermal edema. These events are the result of the interplay between keratinocytes, endothelium, and cells

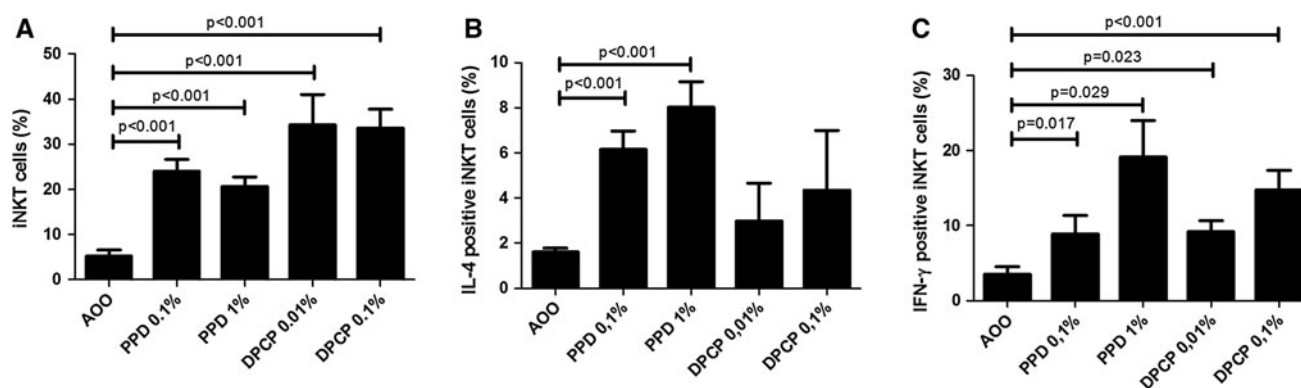


Fig. 5 Changes in invariant NKT-cell frequency and cytokine production. Lymphocytes were isolated from the liver and stimulated with PMA and ionomycin in the presence of Golgi-stop (IL-4 and IFN- γ). The cells were then analyzed by flow cytometry, and qPCR was carried out on total RNA isolated from liver tissue. **a** PPD and DPCP increase the frequency of liver iNKT cells. qPCR showed a dose-dependent up-regulation of V α -14 mRNA. There is a tendency toward an increase in V α -14 mRNA levels with increasing hapten

concentrations. **b** PPD was found to significantly increase the proportion of iNKT cells positive for IL-4. qPCR data indicate an increase in IL-4 mRNA with both haptens. **c** Both PPD and DPCP increased the percentage of IFN- γ -positive iNKT cells. iNKT cells were gated as TCR β ⁺CD4⁺ and able to bind PBS-57-loaded CD1d tetramers. Pools of lymphocytes from eight mice in each group were used ($n = 4$)

in the innate and adaptive immune system responses, leading to increased production of chemokines, cytokines, and heightened expression of adhesion molecules [27]. The local immune response in the skin and local lymph nodes have been the focus of most studies, but less is known about the systemic response.

Studies have shown a correlation between CHS and diseases like cancer, rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes, suggesting that systemic effects of CHS are enough to prime the immune system and increasing the risk of other diseases [28–31]. The goal of the current study was to investigate the systemic effects of the two potent haptens PPD and DPCP.

Our results show that DPCP is a more potent hapten than PPD in terms of activating T and B cells, even at lower applied doses. Increased ear thickness, activation of T and B cells in AL-N, and higher levels of CD8a mRNA in ear tissue all indicate infiltration of effector cells, a hallmark of CHS. PPD treatment increases B-cell frequencies and activation in AL-N, but it does not appear to increase activation and infiltration of CD8⁺ T cells in hapten-exposed tissue. The findings suggest that both haptens are able to induce an immunological response, but that PPD treatment for some reason fails to induce effector T-cell infiltration. This is supported by an increased expression of ICAM-1, the adhesion molecules required for CD8⁺ T-cell infiltration in the ears following DPCP treatment. A lesser increase is found following PPD treatment.

The increased levels of IL-17 mRNA in the ears of DPCP-treated mice suggest infiltration. Again, this is not seen in PPD-treated mice, where we instead observe an increased frequency of IL-17A-positive CD8⁺ T cells in AL-N, a response not seen following DPCP treatment. It

has previously been shown that neutralizing ICAM-1 results in a reduction in IL-17A production at the challenged site [14]. The number of IL-17A-positive cells correlates well with the ICAM-1 mRNA levels we observed. Whether ear IL-17A mRNA levels reflect local tissue infiltration of Tc17 or Th17 cells remains to be investigated, but it is yet another indication that both haptens induce an immunological response; the effect of PPD, however, appears to be confined to the local lymph nodes. Evidence indicates that IL-17A is required to maintain inflammation and the production of pro-inflammatory cytokines and chemokines [7]. IL-17A production is restricted to AL-N and is not present in the skin, as is seen with DPCP. This result could explain the lack of CD8⁺ cell migration to ear tissue seen in PPD-treated mice. The increase in ear thickness observed in 1 % PPD-treated mice could be caused by inflammation produced by non-CD8⁺ T-cell leukocytes. This raises the question whether increased ear thickness is sufficient as a sole indicator of, at the very least, CD8⁺ T-cell-induced CHS. By only using ear thickness for an indicator, there is a risk of drawing incomplete, or even inaccurate, conclusions about molecules for which the mechanism is insufficiently investigated.

Regulatory T cells are historically defined within the CD4⁺ T-cell compartment, but recent studies have revealed the presence of naturally occurring CD8⁺ Foxp3 Treg populations [32]. However, the relevance of these cells representing a minor population in both the thymus and secondary lymphoid organs of non-manipulated mice remains unclear. There are contradictory opinions on whether CD8⁺ Foxp3-positive T cells confer the same potent immune-regulatory potential as CD4⁺ Foxp3 T cells

[32, 33]. We show that CD8⁺ Foxp3⁺ T cells increase in local lymphoid organs following PPD and DPCP treatments of mice. It is not possible to say whether the increase in Foxp3 mRNA observed in the ears reflects infiltration of CD4⁺ Foxp3⁺ or CD8⁺ Foxp3⁺ Treg cells, nor whether these cells are expressing the IL-10 mRNA levels observed in ear tissue. Foxp3 expression in ear tissue indicates infiltration of Treg cells trying to control the inflammatory response, whereas PPD fails to induce the same degree or intra-tissue migration of Tregs. It has previously been shown that low-dose antigens promote induction of Foxp3-positive CD4⁺ T cells, with an inverse relationship between antigen dose and Foxp3 [34]. We observe the same response in CD8⁺ Foxp3⁺ cells, where frequencies increase with low doses of haptens. Whether these cells act to dampen the inflammatory response by suppressing T-cell proliferation and IFN- γ production needs further investigation.

Little is known about the effects of skin-applied haptens outside the local environment of the treated skin and lymph nodes. Our data show that DPCP increases the activation of CD8⁺ T cells in AL-N, PL-N, and spleen. PPD on the other hand appears to decrease the frequency of CD69^{high} CD8⁺ T cells. The same tendency was seen for B cells. Both haptens increased the proportion of B cells gated as CD69^{high}. This indicates systemic effects of DPCP and PPD influencing T-cell and B-cell activations beyond the local site of application.

The systemic cytokine profiles reveal changes in cytokine production and cytokine levels. The effects seem mainly to be limited to organs and lymph nodes, with a less pronounced effect in serum from treated animals (Fig. 1 in the electronic supplementary material).

PPD significantly increases IL-17A-positive CD8⁺ T cells in PL-N. DPCP treatment increased intra-tissue expression of IL-17A mRNA levels, suggesting a similar response to what was observed in the ears. However, PPD also appears to increase pancreatic and liver transcription of IL-17A. Increased pancreatic IL-17A levels could indicate a pancreatic inflammation, caused by epidermal hapten application. The spleen also shows changes in the frequency of cytokine production by CD8⁺ T cells. The highest concentration of PPD increases IL-17A-positive cells, while the low concentrations of both haptens induce IL-4-positive CD8⁺ T cells. No similar effects were found for CD4 T cells and B cells.

We also observed systemic changes in regulatory T-cell populations. At low doses, DPCP appears to decrease the frequency of Foxp3-positive CD4⁺ T cells in PL-N, while increasing this cell population in spleen. We also observed that the levels of Foxp3 mRNA in the pancreas increased with the low concentration of PPD and with both concentrations of DPCP. The same tendency was observed for

Foxp3-positive CD8⁺ T cells in AL-N. Both haptens appear to induce migration of Treg cells into pancreatic tissue. The observed decrease in CD4⁺ Treg cells present in PL-N suggests that CD4⁺ Foxp3⁺ T cells migrated from PL-N to pancreatic tissue. The low concentration of PPD induced high levels of Foxp3 mRNA. This result might explain the previous findings that low-dose application of PPD reduces diabetes in non-obese diabetic (NOD) mice [35]. Moreover, there is a tendency for an increase in IL-10-positive CD8⁺ T cells in PL-N, further supporting this previous finding.

iNKT cells expressing a CD1d-restricted invariant TCR have key regulatory functions in autoimmunity, pathogen immunity, and tumor surveillance, but their role in controlling allergic skin diseases needs further investigation [16, 36]. Using this model of CHS, we show that iNKT cells seem to play a role in the elicitation phase of DTH. iNKT frequencies in the liver were heightened 24 h after challenged with PPD and DPCP, 8 weeks following the sensitization. This finding is consistent with earlier studies showing the accumulation of iNKT stimulatory lipids as soon as 30 min following sensitization and increased cell numbers 24 h after hapten challenge [35, 37, 38]. This clearly shows that iNKT cells play a continuous role in DTH, even after an adaptive immune response has been established. An increase in IL-4-positive iNKT cells points toward a possible activation of B-1 cells, as iNKT cells have been shown to be important for the activation of B-1 cells producing IgM, which helps local T-cell recruitment [19]. This finding could be interesting to investigate further. The cytokine production by hepatic iNKT cells is, however, not restricted to IL-4, as the proportion of IFN- γ -positive iNKT cells is found to be higher than that of IL-4-positive.

In conclusion, our study shows that epicutaneous application of PPD and DPCP affects the systemic immune balance. The organs and lymph nodes investigated all showed changes in cytokine profile and immune cell subpopulations. These findings support previous studies showing a correlation between CHS and a number of immune diseases [28–31]. Furthermore, we show that concentrations too low to cause clinical symptoms may still be enough to prime the immune system. This could mean that we are constantly changing the immune balance through daily use of perfumes, hair products, and jewelry containing haptens like nickel, even when there is no allergic response. Such effects could be further explored for their potential to predict, treat, or prevent other diseases.

Acknowledgments The authors would like to thank NIH Tetramer Core Facility for providing CD1d tetramers. This study was financed by the Capital Region Research Fund and the Beckett Foundation.

Conflict of interest The authors declare that they have no conflict of interest.

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