Exposure to the immunomodulatory chemical triclosan differentially impacts immune cell populations in the skin of haired (BALB/c) and hairless (SKH1) mice

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ARTICLE INFO
Handling Editor: Lawrence Lash

Keywords:
Triclosan
Skin
Neutrophils
Dendritic cells
ILC2s

ABSTRACT
Workers across every occupational sector have the potential to be exposed to a wide variety of chemicals, and the skin is a primary route of exposure. Furthermore, exposure to certain chemicals has been linked to inflammatory and allergic diseases. Thus, understanding the immune responses to chemical exposures on the skin and the potential for inflammation and sensitization is needed to improve worker safety and health. Responses in the skin microenvironment impact the potential for sensitization; these responses may include proinflammatory cytokines, inflammasome activation, barrier integrity, skin microbiota, and the presence of immune cells. Selection of specific mouse strains to evaluate skin effects, such as haired (BALB/c) or hairless (SKH1) mice, varies dependent on experimental design and needs of a study. However, dermal chemical exposure may impact reactions in the skin differently depending on the strain of mouse. Additionally, there is a need for established methods to evaluate immune responses in the skin. In this study, exposure to the immunomodulatory chemical triclosan was evaluated in two mouse models using immunophenotyping by flow cytometry and gene expression analysis. BALB/c mice exposed to triclosan (2%) had a higher number and frequency of neutrophils and lower number and frequency of dendritic cells in the skin compared to controls. Although these changes were not observed in SKH1 mice, SKH1 mice exposed to triclosan had a higher number and frequency of type 2 innate lymphoid cells in the skin. Taken together, these results demonstrate that exposure to an immunomodulatory chemical, triclosan, differentially impacts immune cell populations in the skin of haired and hairless mice. Additionally, the flow cytometry panel reported in this manuscript, in combination with gene expression analysis, may be useful in future studies to better evaluate the effect of chemical exposures on the skin immune response. These findings may be important to consider during strain selection, experimental design, and result interpretation of chemical exposures on the skin.

1. Introduction

The CDC estimates that over 32 million workers in the United States are employed in industries with potential chemical exposures and are at risk of occupational skin diseases [8,31]. Furthermore, over 42,000 chemicals are currently in use and more are being added each year [12]. The skin is one of the most common occupational routes of chemical exposure and adverse reactions may occur, depending on the type of chemical [32]. Exposure to chemicals can result in the increased incidence of inflammatory and allergic diseases such as irritant contact dermatitis (ICD), allergic contact dermatitis (ACD), urticaria, and asthma [3]. The microenvironment of the barrier site can influence the development of disease and a variety of events; these events may be involved in inflammation and sensitization, including release of danger signals, inflammasome signaling, upregulation of proinflammatory cytokines, alterations in the microbiota, decreases in skin integrity, and changes in immune cell populations [39].

Hundreds of chemicals have been associated with inflammatory and allergic diseases [18,3,4]. However, the mechanism of how chemical exposure induces these immune responses is an area of active
investigation. Immune cells play an essential role in the development of inflammatory and allergic responses in both the skin and draining lymph nodes (dLNs); signals and mediators in the microenvironment of the skin are more recently being recognized as key mediators in these processes. For example, type 2 innate lymphoid cells (ILC2s) have emerged as important players in the type 2 helper T cell (Th2)-skewing response and the development of allergic disease [16]. Dermal exposure to the antimicrobial chemical didecyldimethylammonium chloride (DDAC) has been demonstrated to activate ILC2s; these cells have been shown to be involved in Th2 cytokine production in mice [40]. Dendritic cells (DCs), basophils, and eosinophils are also recruited through Th2 responses and chemokin signaling [16]. Neutrophils are involved in inflammation and are thought to be important in sensitization by promoting a proinflammatory response and the activation and migration of DCs to the dLNs [41,44]. Taken together, these findings demonstrate that changes in immune cells in the skin influence the inflammatory and sensitization process and development of disease.

BALB/c mice have commonly been used in investigations of asthma and allergic disease due to their Th2 bias [5,45]. In addition to activation of the innate immune system and proinflammatory cytokines, the microbiome and skin barrier integrity have been demonstrated to influence the immune microenvironment following chemical exposure [6,39]. SKH1 hairless mice are a common model for studies investigating the skin microbiome and skin barrier integrity [14,35,6]. The SKH1 mouse strain has also been used in studies investigating dermal and immunological responses following exposure to sulfur mustard on the skin [28]. SKH1 hairless mice are immunocompetent and have similar immune responses compared to C57BL/6 mice, which have a Th1 bias [38]. There are several advantages of using hairless mice in skin microbiome and skin integrity research including the larger surface area available for microbial assessment and ability to measure skin integrity without interference of hair. Previous investigations in our lab have shown differences between BALB/c ear skin and SKH1 dorsal skin in expression of immune-related genes (e.g., Tslp) following dermal chemical exposure [1,6]. However, the immune cell differences between BALB/c mice and SKH1 mice in response to chemical exposures on the dorsal skin have not been determined.

Triclosan is an immunomodulatory, antimicrobial chemical that is associated with adverse health effects, including food allergy, aero-allergy, and asthma exacerbation [36,37]. Although triclosan has not been identified as a sensitizer in mice [5], dermal exposure to triclosan has been shown to augment the allergic response in a mouse model of asthma [2]. Additionally, studies have demonstrated the involvement of the Th2-skewing thymic stromal lymphopoietin (TSLP) pathway [26] and the S100 calcium-binding protein A8 and A9 (S100A8/S100A9)/Toll-like receptor (TLR4) signaling pathway [27] following triclosan exposure on mice. The initiating factors involved in these Th2-skewing pathways are an area of interest, including the potential involvement of the inflammasome, skin microbiome, and skin barrier [39]. Recently, we have demonstrated that dermal exposure to triclosan on mice activates the NLRP3 inflammasome [43], decreases the integrity of the skin barrier, and alters the skin microbiota composition [6]. Together, these studies demonstrate a change in responses within the skin microenvironment that correlate with changes in the Th2-skewing immune responses. Historically, our lab has used BALB/c mice in evaluations of immune responses following triclosan exposure on the skin [2,26,27,5]. However, more recently, our lab used SKH1 hairless mice to investigate the skin barrier and skin microbiota [6]. Additionally, while the contribution of the skin in allergic respiratory reactions is an active area of research, established methods to evaluate the immune effects in the skin are lacking. Here we report the use of an extensive immunophenotyping flow cytometry panel in addition to gene expression analysis to better characterize immunological effects in the skin following dermal exposure to a representative immunomodulatory chemical.

2. Materials and methods

2.1. Animals

Female SKH1 mice (6–8 weeks old, Charles River) and BALB/c mice (8 weeks old, Taconic) were purchased and acclimated for at least one week. Mice were randomly assigned to an exposure group and identified with tail markings made with a permanent marker. Mice were housed (5/cage; same exposure group; 5 mice/exposure group) in ventilated plastic shoebox cages with autoclaved bedding and crinkle nest material. Harlan NIH-31 modified 6% irritated rodent diet and tap water were available ad libitum. Housing facilities were maintained with a 12-hour light/dark cycle. All animal experiments were performed in the AAALAC International accredited National Institute for Occupational Safety and Health (NIOSH) animal facility in accordance with an animal protocol (19-003) approved by the CDC-Morgantown Institutional Animal Care and Use Committee.

2.2. Triclosan exposures

Triclosan (CAS# 3380–34–5) was purchased from Calbiochem (EMD Millipore Corp.) and acetone (CAS# 67–41–1) was purchased from Sigma-Aldrich. Dorsal hair on BALB/c mice was shaved using electric clippers one time, prior to the first exposure. Mice (5/group) were exposed once per day for 2, 4, and 7 consecutive days to acetone (0%, vehicle control) or to triclosan (2%) dissolved in acetone (w/v) on the entire dorsal back skin (100 µL/mouse). An additional experiment was included to mock shave SKH1 mice (5/group) prior to 4 days of triclosan (2%) exposure to evaluate the effects of shaving. The concentrations were selected based on previous study findings where immune changes were observed following 2% triclosan exposure on SKH1 dorsal skin [6]. Acetone was selected as the vehicle based on solubility and historical control data for triclosan studies [2,26,5]. Endpoints were evaluated following 7-day exposures because previous kinetic studies have demonstrated that multiple immune changes occurred during this triclosan exposure duration in mice [1].

2.3. Euthanasia and skin collection

Animals were euthanized by CO₂ inhalation 24 h after the final exposure. Back skin (~1 cm²) was collected, fat removed, and weighed. For immune phenotyping analysis, skin was placed into tubes containing 2 mL RPMI and kept on ice. For gene expression analysis, skin was placed into tubes containing 500 µL RNAlater (Invitrogen) and frozen at −80 °C until processed.

2.4. Immune phenotyping analysis

Skin was minced and then digested with 0.5 mg/mL Liberase TL (Roche) in RPMI containing 100 µg/mL DNase I (STEMCELL Technologies) and for 2 h at 37 °C in a shaking water bath. Following incubation, samples were transferred to ice and 2 mL RPMI with 10% FBS was added to each tube to stop digestion. Cells were passed through a 70 µm cell strainer and washed with RPMI with 10% FBS. Live cells were counted on a Cellometer using acridine orange and propidium iodide solution (Nexcelom). Cells were incubated with anti-mouse CD16/32 anti-FcγII and FcγIII Fc Block (Invitrogen) for 10 min on ice and then washed. For staining, cells were incubated with a cocktail of fluorochrome-conjugated mouse antibodies. For the innate/DC panel: Superbright 780 CD45 (30-F11), PerCP-Cy5.5 CD11b (M1/70), PE-Cy7 F4/80 (BM8) (Invitrogen), BV510 Ly6G (1A8), PE-Dazzle594 CD207 (4C7), BV711 CD103 (2E7), BV605 CD11c (N418), APC-Fire750 CD24 (M1/69), AF488 SIRP-α (P84) (BioLegend), PE SiglecF (E50–2440) (BD Pharmingen), AF700 MCHC1 (M5/114.15.2) (ebioscience). For the lymphocyte panel: Superbright-780 CD45 (30-F11), APC KL1G1 (2F1), PE-eFluor610 CD25 (PC61.5) (Invitrogen), FITC CD3 (145–2C11) (BD
Neutrophils
- Involved in inflammation and sensitization
- CD45+, CD11b+, Ly6G+, SiglecF
- CD45+, CD11b+, Ly6G

Eosinophils
- Promote type 2 allergic responses
- CD45+, CD11b+, Ly6G, SiglecF

DCs
- Migrate to dLNs and present antigen
- CD45+, CD11c+, MHCII

CD207+CD11b+ DCs
- Double negative dermal DCs, minor population, no defined function
- CD45+, CD11c+, MHCII, CD11b, CD207, CD24, SIRP-a, CD103

CD207+CD11b+ DCs
- Type 2 conventional DCs (cDC2s), most abundant type
- CD45+, CD11c+, MHCII, CD11b, CD207, CD24, F4/80, SIRP-a, CD103

CD207+CD103+ DCs
- Langerin+ dermal DCs, may be involved in sensitization
- CD45+, CD11c+, MHCII, CD11b, CD207, CD24, CD103

CD207+CD103+ DCs
- Type 1 conventional DCs (cDC1s), may be involved in sensitization
- CD45+, CD11c+, MHCII, CD11b, CD207, CD24, CD103

Epidermal Langerhans cells (LCs)
- Reside in epidermis, crosstalk with keratinocytes
- CD45+, CD11c+, CD103, CD207, CD24, F4/80, SIRP-a, CD103

Lymphocytes
- Various functions, includes CD4+ T cells, CD8+ T cells, γδ T cells, IL-22, NK cells

CD4+ T cells
- Helper T cells, mediate type 2 allergic responses
- Cytokine functions
- CD45+, CD25+, CD4, CD69, CD103, Gr1

CD8+ T cells
- γδ T cells
- Involved in skin homeostasis, role not well-defined

IL-22
- Promote type 2 allergic responses, early responders
- CD45+, CD25+, CD69, NKp46, ICOS

NK cells
- Innate immune cell, involved in inflammation, wound healing, and Th1 immunity

2.5. Gene expression analysis

Total RNA was isolated from the skin using the RNeasy kit per manufacturer’s instructions (Qiagen). A QIAcube (Qiagen) automated RNA isolation machine was utilized in conjunction with the RNA isolation kit. The concentration and purity of the RNA were determined using a NanoDrop Spectrophotometer (Thermo Scientific). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per manufacturer’s instructions. TaqMan Fast Universal PCR Master Mix (Applied Biosystems), cDNA, and gene-specific primers (TaqMan Gene Expression Assays) were combined, and real-time quantitative PCR (qPCR) was performed per manufacturer’s instructions. MicroAmp Fast Optical 96-Well Reaction Plates were analyzed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using cycling conditions per manufacturer’s instructions. Actb (Mm01265647_g1) was used as the reference gene. Data was collected and relative fold change compared to acetone (vehicle control) was calculated using the cycle threshold (Ct) and the 2−ΔΔCt method. Genes involved in neutrophil responses were evaluated and include: Cxcl1 (Mm00446366_m1), Cxcl2 (Mm00436450_m1), Tslp (Mm01579588_m1), Il10a (Mm00496696_g1), and Il4 (Mm00445259_m1).

2.6. Statistical analysis

Data from each strain were analyzed independently from one another. All data were analyzed using JMP version 16.4 (SAS Institute, Cary, NC). Two-way analyses of variance (Exposure by Time) were conducted for each dependent measure, and relevant post-hoc pairwise comparisons were calculated using Fishers LSD test. When examination of the residuals indicated that the assumptions of the analysis were not met, particularly heterogeneous variance among the groups, a log transformation was performed and the analysis was repeated. All differences were considered significant at p < 0.05.

3. Results

3.1. Dermal exposure to triclosan increased neutrophils in BALB/c mouse skin

Neutrophils were significantly higher in BALB/c mouse dorsal skin exposed to triclosan for 4 (number and frequency (% of CD45+ cells)) and 7 (number and frequency (% of CD45+ cells)) days (Fig. 1A-B, Table 2) compared to vehicle control exposed BALB/c mice, but no significant difference was observed between triclosan and vehicle exposed SKH1 mouse skin (Fig. 1C-D, Table 3). To further investigate the impact of triclosan on neutrophil function, select chemokines were examined following exposure. Triclosan-exposed skin had higher gene expression levels of a chemokine involved in neutrophil recruitment, Cxcl2, compared to vehicle control exposed mice in BALB/c mouse skin but not SKH1 mouse skin (Fig. 2B and D). Expression of another neutrophil recruiting chemokine, Cxcl1, was significantly higher in BALB/c mouse skin following 2 and 7 days of triclosan exposure (Fig. 2A) and SKH1 mouse skin following 7 days of exposure to triclosan (Fig. 2C) compared to vehicle control exposed mouse skin. To evaluate the potential effects due to shaving, SKH1 mice were shaved or not shaved, and both groups were dermally exposed to 2% triclosan for 4 days. The number and frequency of neutrophils in the SKH1 shaved skin was not significantly different compared to unshaved SKH1 skin (Fig. 1E-F). Additionally, no changes in expression of Cxcl1 or Cxcl2 were identified between the SKH1 mice that were shaved or not shaved (data not shown). Triclosan-exposed BALB/c mouse skin (2 and 7 days) had a significantly lower frequency and number of eosinophils compared to vehicle control-exposed BALB/c mice (Table 2), but no significant difference was observed between SKH1 mouse skin exposed to triclosan or vehicle (Table 3). Together this data demonstrates that the skin response to triclosan is different in SKH1 and BALB/c strains.
dermal exposure to triclosan increases the number and frequency of neutrophils in BALB/c mouse skin and suggests that this occurs through CXCL2 signaling. However, these effects were absent in the SKH1 mice.

3.2. Dermal exposure to triclosan decreased DCs in BALB/c mouse skin

DCs (frequency and number) were significantly lower in BALB/c mouse skin exposed to triclosan for 2, 4, and 7 days compared to vehicle
control exposed mice (Table 2). SKH1 mice had a higher frequency of DCs (% of CD45+ cells) in the skin following 4 days of triclosan exposure and a lower number of DCs in the skin following 7 days of triclosan exposure compared to vehicle exposure (Table 3). CD207+ dermal DCs, CD207+CD103+ dermal DCs, and CD207+CD11b+ dermal DCs were all significantly decreased in both number and frequency in BALB/c dorsal skin following 2, 4, and 7 days of triclosan exposure compared to vehicle-exposed SKH1 mice (Table 3). Together these results show that exposure to triclosan decreased the frequency of dermal DCs and epidermal LCs after 7 days of triclosan exposure and increase in frequency of epidermal LCs after 2 days (Table 2). The small population of CD207+CD11b+ dermal DCs was significantly increased (frequency) following 4 days of triclosan exposure on BALB/c mouse skin compared to vehicle control (Table 2), but this subset of cells was unchanged in triclosan-exposed SKH1 mice compared to vehicle-exposed SKH1 mice (Table 3). Together these results show that exposure to triclosan on the skin decreases the number and frequency of dermal DCs in BALB/c mouse dorsal skin with minimal effects in SKH1 mice.

3.3. Dermal exposure to triclosan increased ILC2s in SKH1 mouse skin

Dermal exposure to triclosan significantly decreased lymphocyte frequency (% of CD45+ cells) in BALB/c mouse skin after 2, 4, and 7 days (Table 4) and SKH1 mouse skin after 4 days (Table 5) compared to vehicle exposure on each corresponding day. Additionally, triclosan exposure on the skin decreased the frequency of CD4+ T cells in BALB/c mouse skin after 2 and 7 days compared to vehicle exposure (Table 4). Furthermore, dermal exposure to triclosan decreased the frequency of γδ T cells in BALB/c mouse skin after 4 and 7 days compared to vehicle exposure (Table 4), although no change was observed in SKH1 mouse skin between exposure groups (Table 5). No significant differences in CD8+ T cells were identified following triclosan exposure in BALB/c mouse skin (Table 4) and in SKH1 mouse skin (Table 5) compared to controls.

#### Table 2

| Exposure Duration | 2 Days | 4 Days | 7 Days |
|-------------------|--------|--------|--------|
| Triclosan (w/ y)  | 0%     | 2%     | 2%     |
| Neutrophils       | 82 ±   | 349 ±  | 198 ±  |
| (%)               | 198 ±  | 3089 ± | 76 ±   |
| Neutrophils (%)   | 0.04   | 1.04 ± | ± 0.45 |
| (%)               | ± 0.45 | 4.53 ± | ± 6.04 |
| Eosinophils       | 1303 ± | 597 ±  | 791 ±  |
| (%)               | ± 193  | 791 ±  | ± 313  |
| Eosinophils (%)   | 0.61   | 0.29 ± | ± 0.41 |
| (%)               | ± 0.41 | 0.62 ± | ± 0.27 |
| DCs (#)           | 4134   | 1603   | 4242   |
| (%)               | ± 559  | ± 211  | ± 587  |
| DCs (%)           | 18.18  | 10.26  | 16.78  |
| CD207-CD11b DCs   | 7 ±    | 6 ±    | 5 ±    |
| (%)               | ± 14   | ± 1.34 | ± 6.98 |
| CD207-CD11b DCs (%)| 0.03  | 0.04 ± | 0.02   |
| (%)               | ± 0.02 | ± 0.02 | ± 0.02 |
| CD207-CD11b DCs (%)| 0.03  | 0.01   | 1.14   |
| (%)               | ± 0.03 | ± 0.03 | ± 0.03 |

Immune phenotyping of the skin following 2, 4, and 7 days of triclosan exposure. Numbers represent the mean (± SEM) of 5 mice/group. * p < 0.05 vs. 0% on corresponding day.

#### Table 3

| Exposure Duration | 2 Days | 4 Days | 7 Days |
|-------------------|--------|--------|--------|
| Triclosan (w/ y)  | 0%     | 2%     | 2%     |
| Neutrophils       | 466 ±  | 474 ±  | 552 ±  |
| (%)               | 552 ±  | 352 ±  | 377 ±  |
| Neutrophils (%)   | 1.77   | 1.90   | 2.10   |
| (%)               | 2.10   | 2.80   | 1.27   |
| Eosinophils       | 969 ±  | 1258   | 620 ±  |
| (%)               | 1258   | 523 ±  | 287 ±  |
| Eosinophils (%)   | 185    | 138 ±  | 164 ±  |
| (%)               | ± 138  | ± 91   | ± 43   |
| DCs (#)           | 4221   | 4209   | 4854   |
| (%)               | ± 243  | ± 259  | ± 328  |
| DCs (%)           | 15.50  | 16.62  | 19.34  |
| CD207-CD11b DCs   | 16 ±   | 3 ±    | 12 ±   |
| (%)               | 12 ±   | ± 1 ±  | ± 1    |
| CD11b DCs (%)     | 0.17   | 0.77   | 0.53   |
| (%)               | 2.20   | 2250   | 466    |
| CD11b DCs (#)     | 0.48   | ± 1.34 | ± 1.17 |
| (%)               | ± 1.17 | ± 0.39 | ± 0.95 |
| CD207+CD103 DCs (%)| 62   | 47 ±   | 39 ±   |
| (%)               | ± 39   | ± 41   | ± 48   |

Immune phenotyping of the skin following 2, 4, and 7 days of triclosan exposure. Numbers represent the mean (± SEM) of 5 mice/group. * p < 0.05 vs. 0% on corresponding day.

ILC2s in SKH1 mouse skin were significantly increased following 4 days (frequency and number) and 7 days (frequency) of triclosan exposure compared to vehicle exposure (Table 5), but not in BALB/c mouse skin following triclosan exposure (Table 4). Additionally, ICOS (days 2, 4, and 7) and CD25 (days 4 and 7) were elevated on ILC2s and CD127 was decreased (days 2, 4, and 7) on ILC2s in SKH1 dorsal skin exposed to triclosan compared to vehicle exposure (Fig. 4D, F, H). In BALB/c dorsal skin, KLRF1 (day 7), CD25 (day 2), and CD127 (day 7) were increased on ILC2s following triclosan exposure compared to vehicle (Fig. 4A, E, G). The frequency (days 2, 4, and 7) and number (day 2) of natural killer (NK) cells in triclosan-exposed BALB/c mouse skin was lower compared to vehicle-exposed BALB/c mouse skin (Table 4), but the frequency (day 7) of NK cells in triclosan-exposed SKH1 mouse skin was higher compared to vehicle-exposed SKH1 mouse skin (Table 5), although NK cells are a small population. Genes known to be involved in Th2 responses were also evaluated in the dorsal skin following triclosan exposure. BALB/c mice exposed to triclosan for 2, 4, and 7 days had higher expression of S100A8 and Tslp in their skin compared to vehicle exposed mice and BALB/c mice exposed to triclosan for 4 and 7 days had higher Il4 in their skin compared to vehicle exposed mice (Fig. 5A-C). SKH1 mice exposed to triclosan for 7 days also had
higher expression of S100a8 and Il4 in their skin compared to vehicle exposed mice, although mice exposed to triclosan for 4 days had higher Tslp expression in their skin compared to vehicle exposed mouse skin (Fig. 5 D-F). No changes in expression of S100a8, Tslp, or Il4 were identified between SKH1 shaved and unshaved mice (data not shown). Together these results show that exposure to triclosan alters lymphocyte populations in mouse skin and increases ILC2s in SKH1 mouse skin.

3.4. Impact of shaving on immune cell populations

To evaluate the potential effects of shaving in combination with chemical exposure, SKH1 mice were mock shaved and exposed to triclosan for 4 days. Mice in the shaved group had a lower number of CD4+ T cells and ILC2s in their dorsal skin (Table 6). Furthermore, these mice had a lower number and frequency of NK cells in the dorsal skin (Table 6). Shaving did not alter lymphocytes, CD8+ T cells, and γδ T cells (Table 6) or number or frequency of neutrophils, eosinophils, or DCs (data not shown). Together these results suggest that shaving in combination with chemical exposure may impact select immune cell populations in the skin, notably NK cells.

4. Discussion

Although the skin provides a physical barrier to pathogens and certain environmental exposures, some chemicals can be absorbed through the skin and result in adverse outcomes including allergic disease [13,24]. Immune cells play an important role in transporting agents and communicating messages from barrier sites to dLNs. Additionally, recruitment of immune cells to the barrier site is critical for activities such as crosstalk to other immune cells, elimination of the foreign molecule, and tissue healing. In immunotoxicological studies, animal models are important to evaluate the movement of immune cells to and from the barrier sites and throughout the body. In this study, flow cytometry and gene expression analysis were used to evaluate immune responses in the skin between two mouse strains (BALB/c and SKH1) following dermal exposure to a representative immunomodulatory chemical, triclosan.

Interestingly, in this study, neutrophils were significantly increased in BALB/c skin but not SKH1 skin following exposure to triclosan. Additionally, following exposure to triclosan, the chemokines Cxcl1 and Cxcl2 were increased in BALB/c skin compared to vehicle exposure. These results align with previous findings showing that myeloid cells and chemokines (Cxcl1, Cxcl2) are increased in BALB/c ears following triclosan exposure [43]. CXCL1 and CXCL2 are involved in neutrophil recruitment, are produced by tissue resident macrophages, and are dependent on TLR signaling [11]. The increase in chemokines in BALB/c mouse skin aligns with the finding that neutrophils are recruited to the skin. Neutrophils have been shown to be involved in migration of DCs to the dLNs [44], and this result aligns with the observation that DCs are decreased in BALB/c mouse skin following triclosan exposure. Migration of DCs to the dLN is a critical step in chemical sensitization and the development of subsequent allergic responses. Exposure to triclosan had minimal impact on the neutrophils and DCs in SKH1 mouse skin. SKH1 mice have been demonstrated to have elevated numbers of neutrophils following exposure to sulfur mustard [19], suggesting that neutrophils are not defective in hairless mice. Although the exact mechanism behind differences in neutrophils and DCs in these mouse strains is unknown, these results are important to consider when selecting a mouse strain and interpreting results.

Triclosan-exposed BALB/c mouse skin had a lower number and frequency of DCs compared to vehicle-exposed BALB/c mouse skin, but no change was observed between triclosan and vehicle exposed SKH1
mouse skin. Specifically, triclosan exposure decreased CD207$^{-}$CD11b$^{+}$ DCs, CD207$^{+}$CD103$^{-}$ DCs, CD207$^{+}$CD103$^{+}$ DCs, and epidermal LCs in BALB/c skin. These DC subsets may be involved in the process of sensitization (Table 1). Although triclosan itself is not a sensitizer, exposure to triclosan may increase the trafficking of DCs from skin to dLNs and increase the risk of sensitization through the increase of Th2 mediators. Additionally, our lab has previously shown that 4 days of dermal triclosan exposure increases DCs in the dLNs [26,27]. Although in the studies described in this manuscript BALB/c and SKH1 mice have been shown to have similar levels of CD103$^{-}$ and CD11b$^{+}$ DCs in the

Fig. 3. Dermal exposure to triclosan decreased DC subsets in BALB/c mouse skin. Number (A) and frequency (% of CD45$^{+}$ cells) (B) of CD207CD11b$^{+}$ DCs, number (C) and frequency (% of CD45$^{+}$ cells) (D) of CD207$^{+}$CD103$^{-}$ DCs, number (E) and frequency (% of CD45$^{+}$ cells) (F) of CD207$^{+}$CD103$^{+}$ DCs, and number (G) and frequency (% of CD45$^{+}$ cells) (H) of epidermal LCs in BALB/c mouse skin following dermal exposure to 0% or 2% triclosan. Bars represent the mean (±SEM) of 5 mice/group. *p < 0.05 vs. 0% triclosan on each corresponding day.

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BALB/c mice may be a more useful strain compared to SKH1 in the absorption of triclosan and subsequently less migration of DCs out of the skin, potentially due to the increased passage of this chemical through hair follicles in haired mice. Hair follicles are also a site of absorption of ovalbumin to dLNs via DCs compared to SKH1 mice [42]. Due to their abnormalities in hair follicles, SKH1 mice may have reduced uptake of molecules through their skin [7], which presents a potential explanation for the current findings. Hair follicles are also a site of absorption of chemicals [29] and hairless mice have been shown to have reduced permeation of benzo[a]pyrene compared to haired mice, potentially due to the increased passage of this chemical through hair follicles in hairless mice [20]. Together this suggests that SKH1 mice may have reduced absorption of triclosan and subsequently less migration of DCs out of the skin. Collectively, these results demonstrate that dermal triclosan exposure decreases DCs in BALB/c skin specifically and suggest that BALB/c mice may be a more useful strain compared to SKH1 in the investigation of DCs and their trafficking patterns in the context of chemical exposures on the skin.

Lymphocytes play a role in skin homeostasis, although their mechanism of action is not well understood [10]. Certain subsets, such as Th2 cells and ILC2s, play a central role in allergic responses [16]. BALB/c mouse skin exposed to triclosan had a lower frequency (but not number) of lymphocytes, specifically CD4+ T cells and γδ T cells, compared to vehicle-exposed skin. Because the ratio and not number was decreased, the large influx of neutrophils into the skin could be a potential explanation for this finding. In contrast, triclosan exposure increased the number and frequency of ILC2s in the skin of SKH1 mice, but not BALB/c mice. ILC2s are thought to play an important role in allergic disease because of their Th2-skewing properties and are influenced by the cytokines IL-25, IL-33, and TSLP [16]. Interestingly, dermal exposure to triclosan only significantly increased Tslp gene expression in SKH1 mice on day 4, but not day 2 or 7 (Fig. 5). The lack of change on day 7 is in agreement with a previous study by our lab that showed that 7 days of dermal exposure to triclosan did not change Tslp gene expression in SKH1 dorsal skin [6]. In contrast, although in the absence of increases in ILC2s, triclosan exposure has been demonstrated to increase Tslp in BALB/c mouse dorsal skin at multiple timepoints in this study and in the ears of BALB/c mice as shown previously by our lab [1,27]. Although ILC2s have been shown to be activated following dermal exposure to the antimicrobial chemical DDAC by measuring the expression of KLRG1, ICOS, CD25, and CD127, the number of ILC2s was unchanged with exposure [40]. In the present study, total ILC2 numbers were increased and the expression of ICOS and CD25 activation markers on ILC2s was increased. CD127 expression on ILC2s was decreased in SKH1 dorsal skin at all timepoints and indicates activation of ILC2s [34]. Further investigations are needed to explore the biological significance of increased ILC2 numbers. These results demonstrate that exposure to triclosan increases ILC2s in the skin of SKH1 mice, but not BALB/c mice. ILC2s in SKH1 mice are not well understood; this strain has previously been compared to the Th1-biased C57BL/6 strain and studies directly comparing with the Th2-biased BALB/c strain are limited. The effects of dermal triclosan exposure on the immune response of C57BL/6 mice has not been evaluated. The difference between SKH1 and BALB/c responses following chemical exposure could be due to the hairless gene or differences in immune bias. However, the underlying mechanism of this differential finding is not yet understood. Future research could explore the role of the hairless gene on a BALB/c background to better control for immune differences.

Barrier-disruptive activities such as scratching and tape stripping of the skin have been shown to be involved in neutrophil recruitment to the skin [33]. Shaving may also have a disruptive and inflammatory effect in the skin. Although mock shaving in combination with triclosan exposure did not impact the number of neutrophils in the skin, mock shaving SKH1 mouse dorsal skin significantly decreased the number and frequency of NK cells in the skin. This result is surprising, because NK cells have been shown to increase in inflammatory skin diseases [41] and

Table 4
BALB/c phenotyping (lymphocytes) following dermal exposure to triclosan.

| Exposure Duration | 2 Days | 4 Days | 7 Days |
|-------------------|--------|--------|--------|
| Triclosan (w/v)   | 0%     | 2%     | 2%     | 0%     | 2% |
| Lymphocytes (%)   | 38.08 ± 1.21 | 31.84 ± 2.80 | 29.98 ± 0.76 | 23.08 ± 0.85 | 39.82 ± 1.19 | 25.12 ± 0.76 |
| CD4+ T cells (%)  | 87.2 ± 0.30 | 51.2 ± 1.10 | 5.84 ± 0.25 | 5.87 ± 0.51 | 12.68 ± 0.71 | 6.21 ± 1.17 |
| CD8+ T cells (%)  | 1 ± 4e-001 | 0 ± 0 | 4e-001 ± 2e-001 | 0 ± 0 | 1 ± 2e-001 | 2e-001 ± 2e-001 |
| CD6+ T cells (%)  | 0.06 ± 0.02 | 0.06 ± 0.03 | 0.06 ± 0.01 | 0.03 ± 4.5e-003 | 0.17 ± 0.05 | 0.11 ± 0.01 |
| γδ T cells (%)    | 30.8 ± 17 | 17 ± 7 | 15 ± 5 | 6 ± 1 | 14 ± 3 | 6 ± 5 |
| ILCs (%)          | 6 ± 1 | 5 ± 2 | 2 ± 1 | 64 ± 14 | 22 ± 3 | 21 ± 4 |
| ILC2s (%)         | 0.57 ± 0.02 | 0.55 ± 0.14 | 0.29 ± 0.02 | 0.17 ± 0.02 | 0.25 ± 0.02 | 0.19 ± 0.02 |
| NK cells (%)      | 7 ± 2 | 2 ± 1 | 4 ± 1 | 2 ± 1e-001 | 2 ± 1 | 1 ± 1 |
| NK cells (%)      | 0.61 ± 0.09 | 0.24 ± 0.08 * | 0.45 ± 0.04 | 0.26 ± 0.03 * | 0.56 ± 0.07 | 0.25 ± 0.04 * |

Immune phenotyping of the skin following 2, 4, and 7 days of triclosan exposure. Numbers represent the mean (± SEM) of 5 mice/group. *p < 0.05 vs. 0% on corresponding day.

Table 5
SKH1 phenotyping (lymphocytes) following dermal exposure to triclosan.

| Exposure Duration | 2 Days | 4 Days | 7 Days |
|-------------------|--------|--------|--------|
| Triclosan (w/v)   | 0%     | 2%     | 2%     | 0%     | 2% |
| Lymphocytes (%)   | 572 ± 732 | 667 ± 493 | 612 ± 606 | 612 ± 606 | 612 ± 606 |
| CD4+ T cells (%)  | 87.8 ± 37.92 | 33.26 ± 26.08 | 40.44 ± 34.60 | 41.11 ± 22.27 | 21.7 ± 2.09 | 2.67 ± 1.18 |
| CD8+ T cells (%)  | 116 ± 116 | 129 ± 92.0 ± 20 | 151 ± 170 | 151 ± 170 | 151 ± 170 |
| CD6+ T cells (%)  | 8.23 ± 6.79 | 4.66 ± 4.91 | 9.76 ± 9.58 | 9.76 ± 9.58 | 9.76 ± 9.58 |
| γδ T cells (%)    | 0.97 ± 0.94 | 0.25 ± 0.49 | 0.79 ± 0.45 | 0.79 ± 0.45 | 0.79 ± 0.45 |
| ILC2s (%)         | 1 ± 4e-04 | 5 ± 3 | 5 ± 1 ± 3 | 1 ± 3 |
| NK cells (%)      | 0.07 ± 0.04 | 0.29 ± 0.34 | 11.0 ± 10.6 | 11.0 ± 10.6 | 11.0 ± 10.6 |
| NK cells (%)      | 0.03 ± 0.02 | 0.13 ± 0.06 | 0.13 ± 0.06 | 0.13 ± 0.06 | 0.13 ± 0.06 |
| γδ T cells (%)    | 176 ± 196 | 94 ± 110 | 106 ± 104 | 106 ± 104 | 106 ± 104 |
| ILC2s (%)         | 4 ± 1 | 7 ± 1 | 15 ± 5 | 15 ± 5 | 15 ± 5 |
| Tslp (%)          | 0.55 ± 0.32 | 0.24 ± 1.42 | 1.42 ± 3.53 | 1.42 ± 3.53 | 1.42 ± 3.53 |
| NK cells (%)      | 4 ± 1 | 5 ± 1 | 6 ± 1 | 6 ± 1 | 6 ± 1 |

Immune phenotyping of the skin following 2, 4, and 7 days of triclosan exposure. Numbers represent the mean (± SEM) of 4–5 mice/group. *p < 0.05 vs. 0% on corresponding day.
Fig. 4. Dermal exposure to triclosan altered the expression of activation markers on ILC2s in mouse skin. Relative median fluorescence intensity (MFI) of KLRG1 (A), ICOS (C), CD25 (E), and CD127 (G) on ILC2s in BALB/c mouse skin following dermal exposure to 0% or 2% triclosan. Relative MFI of KLRG1 (B), ICOS (D), CD25 (F), and CD127 (H) on ILC2s in SKH1 mouse skin following dermal exposure to 0% or 2% triclosan. Bars represent the mean (±SEM) of 4–5 mice/group. * p < 0.05 vs. 0% triclosan on each corresponding day.
Investigating the immune cell changes in response to chemical exposure and sensitization, contributing to the development of allergic diseases. The biological significance of this finding is unknown given the small size of this population. Described here can be utilized in future studies to better characterize immune flow cytometry panels in addition to gene expression analysis and the extensive mental design of skin chemical exposure studies. However, the extensive design of mouse strain is an important factor to consider during experimental design of skin chemical exposure studies. The extensive immune flow cytometry panels in addition to gene expression analysis described here can be utilized in future studies to better characterize immunological effects in the skin following dermal exposure.

Table 6  SKH1 phenotyping following mock shaving and dermal exposure to triclosan.

| Triclosan (w/v) | No Shave | Mock Shave |
|----------------|----------|------------|
| Lymphocytes (#) | 787 ± 139 | 457 ± 42 |
| Lymphocytes (%) | 23 ± 0.84 | 25 ± 0.73 |
| CD4+ T cells (#) | 221 ± 47 | 104 ± 11 |
| CD4+ T cells (%) | 6.3 ± 0.30 | 5.5 ± 0.26 |
| CD8+ T cells (#) | 0.4 ± 0.2 | 1 ± 0.5 |
| CD8+ T cells (%) | 0.015 ± 0.0028 | 0.076 ± 0.038 |
| γδ T cells (#) | 44 ± 16 | 35 ± 7 |
| γδ T cells (%) | 1.2 ± 0.42 | 1.8 ± 0.25 |
| ILC2s (#) | 5 ± 0.7 | 3 ± 0.2 |
| ILC2s (%) | 0.15 ± 0.020 | 0.14 ± 0.021 |
| NK cells (#) | 11 ± 2 | 3 ± 0.4 |
| NK cells (%) | 0.32 ± 0.026 | 0.15 ± 0.0073 |

Immune phenotyping of the skin following mock shaving and 4 days of triclosan exposure. Numbers represent the mean (±SEM) of 5 mice/group. *p < 0.05 vs. no shaving.

have been shown to be involved in wound healing [23]. However, the biological significance of this finding is unknown given the small size of this population.

Exposure to chemicals on the skin can lead to inflammatory reactions and sensitization, contributing to the development of allergic diseases. Investigating the immune cell changes in response to chemical exposures on the skin is important to improve understanding of mechanisms of allergic disease. Although haired mice (BALB/c) are typically used in studies evaluating allergic responses, hairless (SKH1) mice are useful in certain experimental designs such as investigations of the skin microbiota and skin integrity. In this study, exposure to the representative immunomodulatory chemical triclosan differentially impacted immune cell populations in haired and hairless mice, demonstrating that selection of mouse strain is an important factor to consider during experimental design of skin chemical exposure studies. However, the extensive immune flow cytometry panels in addition to gene expression analysis described here can be utilized in future studies to better characterize immunological effects in the skin following dermal exposure.

Funding

This work was supported by internal funds from the Health Effects Laboratory Division of the National Institute for Occupational Safety and Health.

Declaration of Competing Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.09.005.

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