Materials and Methods

Animals

Female SKH1 mice (6-8 weeks old, Charles River) and BALB/c mice (8 weeks old, Taconic) were purchased and allowed to acclimate 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) in ventilated plastic shoebox cages with autoclaved bedding and crinkle nest material. Harlan NIH-31 modified 6% irradiated 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.

Triclosan Exposures

Triclosan (CAS# 3380-34-5) was purchased from Calbiochem (EMD Millipore Corp.). Acetone (CAS# 67-41-1) was purchased from Sigma-Aldrich. Dorsal hair on BALB/c mice was shaved using electric clippers prior to exposures. 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 (Baur, et al. 2021). Acetone was selected as the vehicle based on solubility and historical control data for triclosan studies (Anderson et al. 2013; Anderson et al. 2016; Marshall et al. 2015). Endpoints were evaluated following exposures up to 7 days because previous kinetic studies have demonstrated

that multiple immune changes occurred during this triclosan exposure duration in mice (Anderson et al. 2020).

Euthanasia and Skin Collection

Animals were euthanized by CO_2 inhalation 24 hours after the final exposure. Back skin (~1 cm^2) 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.

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 hours 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 MHCII (M5/114.15.2) (eBioscience). For the lymphocyte panel: Superbright-780 CD45 (30-F11), APC KLRG1 (2F1), PE-eFluor610 CD25 (PC61.5) (Invitrogen), FITC CD3 (145-2C11) (BD Pharmingen), BV711 CD4 (RM4-5), V500 CD8 (53-6.7), BV421 TCR-γδ (GL3) (BD Horizon), BV605 NKp46 (29A1.4), PE-Cy7 ICOS (C398.4A)

(BioLegend), PerCP-Cy5.5 Lineage Gate (CD11b (M1/70) (Invitrogen), CD11c (N418), Ter119 (TER-119), CD19 (eBio1D3), Ly6G (RB6-8C5)), APC-eFluor780 CD90.2 (53-2.1), PE CD127 (A7R34) (eBioscience). Following incubation, cells were washed and then fixed in Cytofix buffer (BD Biosciences). Cells were resuspended in phosphate buffered saline containing 1% bovine serum albumin and 0.1% sodium azide and events were collected on an LSR II flow cytometer (BD Biosciences) within 24 hours. Compensation controls were prepared with UltraComp eBeads (Invitrogen). Data was analyzed using FlowJo v10. Cell populations were defined as shown in Table 1. DC subsets were determined based on expression of certain cell surface markers (https://www.rndsystems.com/resources/cell-markers/immune-cells/dendritic-cells/mouse-tissue--specific-dendritic-cell-subset-markers). Gating strategies for immune cell populations are shown in Supplemental Figures 1-3, gates were drawn based on FMO controls. Frequencies for all cell populations are shown as % of $CD45^{+}$ cells. Cell numbers were normalized to total cells/mg skin tissue for each animal.

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* (Mm01205647_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 $2^{-\Delta\Delta Ct}$ method. Genes involved in neutrophil responses were evaluated and include: *Cxcl1* (Mm04207460_m1), *Cxcl2* (Mm00436450_m1), *Tslp* (Mm01157588_m1), *S100a8* (Mm00496696_g1), and *Il4* (Mm00445259_m1).

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Table 1. Types of immune cells and their significance.

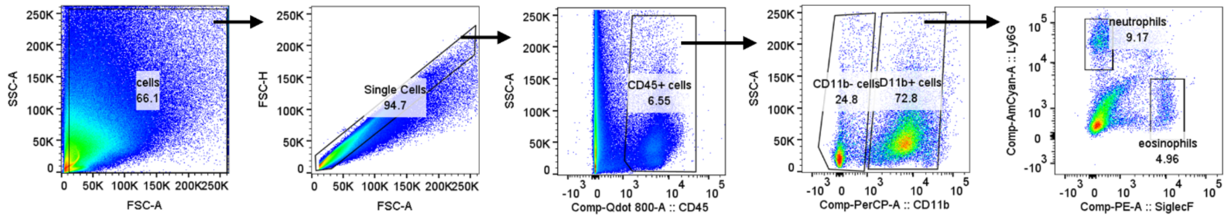
Immune Cell Type	Significance	Gating	Reference
Neutrophils	Involved in inflammation and sensitization	$CD45^+$, $CD11b^+$, $Ly6G^+$, $SiglecF^-$	(Silvestre et al. 2018; Weber et al. 2015)
Eosinophils	Promote type 2 allergic responses	$CD45^+$, $CD11b^+$, $Ly6G^{int}$, $SiglecF^+$	(Hammad and Lambrecht 2015)
DCs	Migrate to dLNs and present antigen	$CD45^+$, $CD11c^+$, $MHCII^+$	(Koppes et al. 2017)
$CD207^-CD11b^-$ DCs	Double negative dermal DCs, minor population, no defined function	$CD45^+$, $CD11c^+$, $MHCII^+$, $CD11b^-$, $CD207^-$, $CD24^-$, SIRP-α^-, $CD103^-$	(Malissen et al. 2014)
$CD207^-CD11b^+$ DCs	Type 2 conventional DCs (cDC2s), most abundant type	$CD45^+$, $CD11c^+$, $MHCII^+$, $CD11b^+$, $CD207^-$, $CD24^-$, $F4/80^+$, SIRP-α^+, $CD103^-$	(Guilliams et al. 2014; Malissen et al. 2014)
$CD207^+CD103^-$ DCs	Langerin$^+$ dermal DCs, may be involved in sensitization	$CD45^+$, $CD11c^+$, $MHCII^+$, $CD11b^-$, $CD207^+$, $CD24^+$, $CD103^-$	(Honda et al. 2013)
$CD207^+CD103^+$ DCs	Type 1 conventional DCs (cDC1s), may be involved in sensitization	$CD45^+$, $CD11c^+$, $MHCII^+$, $CD11b^-$, $CD207^+$, $CD24^+$, $CD103^+$	(Bursch et al. 2007; Guilliams et al. 2014; Honda et al. 2013)
Epidermal LCs	Reside in epidermis, crosstalk with keratinocytes	$CD45^+$, $CD11c^+$, $MHCII^+$, $CD11b^+$, $CD207^+$, $CD24^+$, $F4/80^+$, SIRP-α^+, $CD103^-$	(Malissen et al. 2014)
$CD4^+$ T cells	Helper T cells, mediate type 2 allergic responses	$CD45^+$, SSC^{low}, Lin^-, $CD3^+$, $CD4^+$	(Hammad and Lambrecht 2015)
$CD8^+$ T cells	Cytotoxic functions	$CD45^+$, SSC^{low}, Lin^-, $CD3^+$, $CD8^+$	(Niec et al. 2021)
$\gamma\delta^+$ T cells	Involved in skin homeostasis, role not well-defined	$CD45^+$, SSC^{low}, Lin^-, $CD3^+$, TCR-$\gamma\delta^+$	(Cruz et al. 2018)
ILC2s	Promote type 2 allergic responses, early responders	$CD45^+$, SSC^{low}, Lin^-, $CD90^+$, $CD3^-$, $NKp46^-$, $ICOS^+$, $CD127^+$, $CD25^+$	(Hammad and Lambrecht 2015)

NK cells	Innate immune cell, involved in inflammation, wound healing, and Th1 immunity	CD45^{+}, SSC^{low}, Lin^{-}, CD90^{+}, CD3^{-}, NKp46^{+}	(Kobayashi et al. 2020; Liu et al. 2012; Silvestre et al. 2018)

Supplemental Information

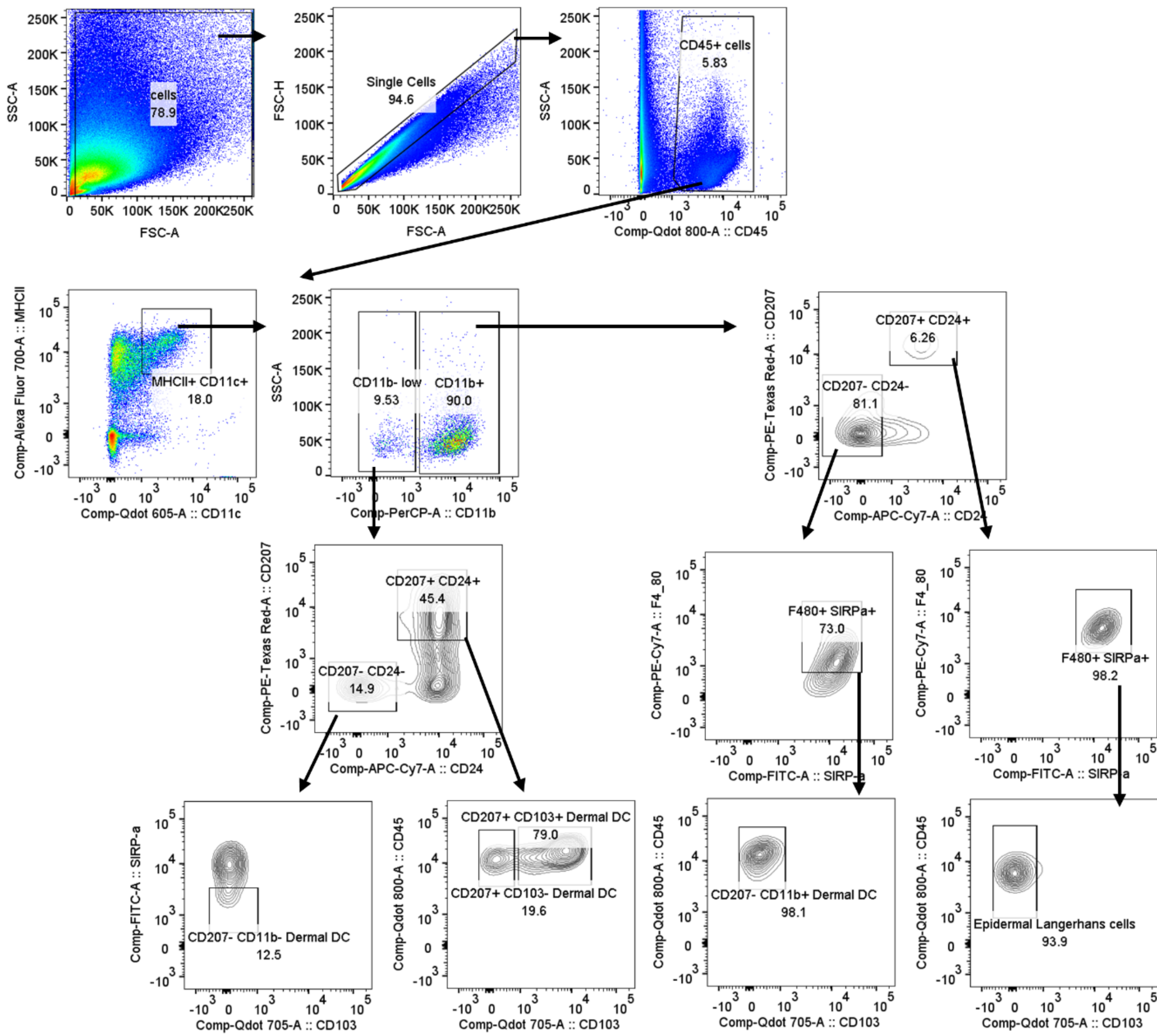
Supplemental Figure 1

Neutrophils & Eosinophils



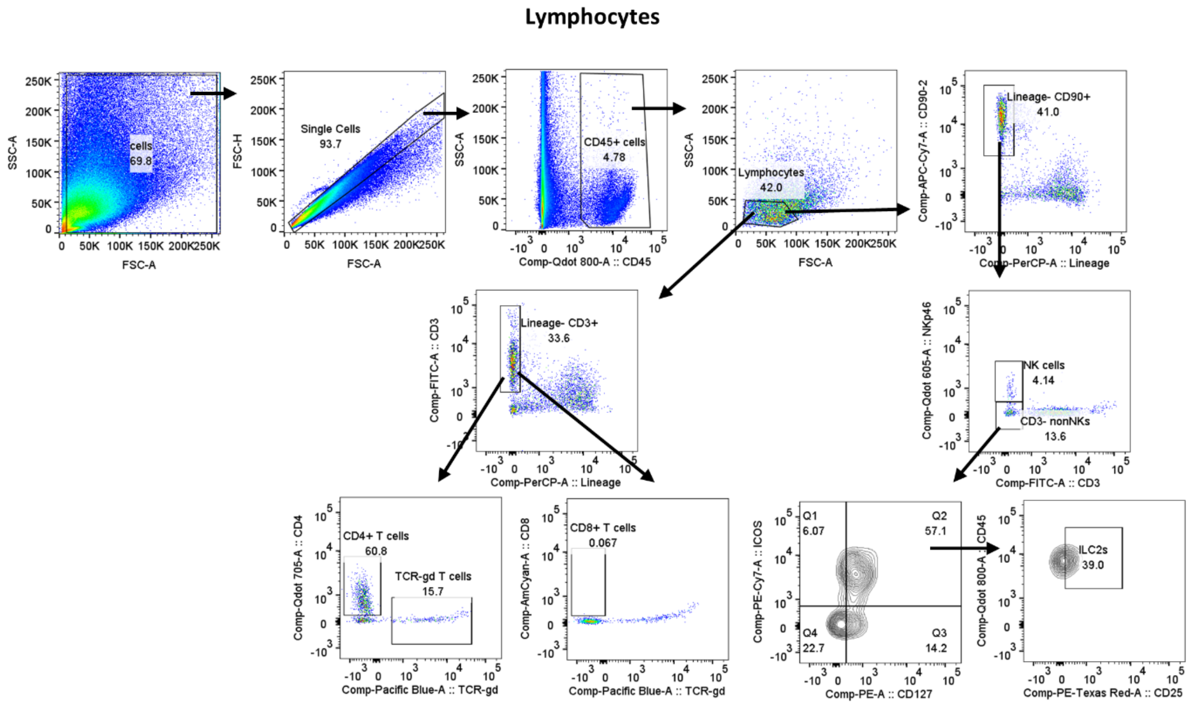
Supplemental Figure 1. Gating strategy for neutrophils and eosinophils.

Supplemental Figure 2



Supplemental Figure 2. Gating strategy for subsets of DCs.

Supplemental Figure 3



Supplemental Figure 3. Gating strategy for lymphocytes.