Toluene Diisocyanate (TDI) Disposition and Co-Localization of Immune Cells in Hair Follicles

Ajay P. Nayak,^{*,1} Justin M. Hettick,^{*} Paul D. Siegel,^{*} Stacey E. Anderson,^{*} Carrie M. Long,^{*,†} Brett J. Green,^{*} and Donald H. Beezhold^{*}

^{*}Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, West Virginia 26505; and [†]Department of Microbiology, Immunology and Cell Biology, School of Medicine, West Virginia University, Morgantown, West Virginia 26506

¹To whom correspondence should be addressed at Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 1095 Willowdale Road, Morgantown, WV 26505. Fax: (304) 285-6126. E-mail: fyg1@cdc.gov.

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Diisocyanates (dNCOs) are potent chemical allergens utilized in various industries. It has been proposed that skin exposure to dNCOs produces immune sensitization leading to work-related asthma and allergic disease. We examined dNCOs sensitization by using a dermal murine model of toluene diisocyanate (TDI) exposure to characterize the disposition of TDI in the skin, identify the predominant haptenated proteins, and discern the associated antigen uptake by dendritic cells. Ears of BALB/c mice were dosed once with TDI (0.1% or 4% v/v acetone). Ears and draining lymph nodes (DLNs) were excised at selected time points between 1 h and 15 days post-exposure and were processed for histological, immunohistochemical, and proteomic analyses. Monoclonal antibodies specific for TDI-haptenated protein (TDI-hp) and antibodies to various cell markers were utilized with confocal microscopy to determine co-localization patterns. Histopathological changes were observed following exposure in ear tissue of mice dosed with 4% TDI/acetone. Immunohistochemical staining demonstrated TDI-hp localization in the stratum corneum, hair follicles, and sebaceous glands. TDI-hp were co-localized with CD11b⁺ (integrin α M/Mac-1), CD207⁺ (langerin), and CD103⁺ (integrin α E) cells in the hair follicles and in sebaceous glands. TDI-hp were also identified in the DLN 1 h post-exposure. Cytoskeletal and cuticular keratins along with mouse serum albumin were identified as major haptenated species in the skin. The results of this study demonstrate that the stratum corneum, hair follicles, and associated sebaceous glands in mice are dendritic cell accessible reservoirs for TDI-hp and thus identify a mechanism for immune recognition following epicutaneous exposure to TDI.

Key words: diisocyanates; langerin; dendritic cells; allergy.

Diisocyanates (dNCOs) are highly reactive low molecular weight (LMW) chemicals employed as precursors in polyurethane-based products, such as foams, elastomers, insulations, varnishes, paints, adhesives, and protective coatings for automobiles (Allport *et al.*, 2003). Workplace exposure to dNCOs is one of the most reported causes of occu-

pational asthma in industrialized countries and an estimated 280,000 workers have occupational exposures to dNCOs (Bernstein, 1996; Liu and Wisnewski, 2003). The incidence of non-occupational exposures to dNCOs is unknown; however, a few reports have documented environmental exposures to toluene diisocyanate (TDI) (Darcey *et al.*, 2002; De Zotti *et al.*, 2000).

Exposure to dNCOs is the commonly reported cause of LMW occupational asthma and is known to cause other hypersensitivity diseases such as allergic rhinitis, asthma, hypersensitivity pneumonitis, and allergic contact dermatitis (Hur *et al.*, 2008; Johnson *et al.*, 2007; Karol, 1986; Vandenplas, 2011). The pathogenesis and the underlying immunological mechanisms of dNCO-induced asthma remain poorly understood, as the presence of specific-IgE or atopic status is only associated with a fraction of dNCO-induced asthmatics. Studies in humans and experimental animals have highlighted a mixed Th₁/Th₂ immune response following exposure to dNCOs (Maestrelli *et al.*, 1995, 1997; Redlich and Karol, 2002). Previously, inhalation was identified as the most likely route of exposure, however, increasingly, the dermal route of dNCO sensitization has been of concern (Redlich, 2010). Using animal models, it has been demonstrated that dermal sensitization prior to intranasal challenge influences sensitization to TDI (De Vooght *et al.*, 2010; Nabe *et al.*, 2005; Tarkowski *et al.*, 2007; Vanoirbeek *et al.*, 2003, 2004), and may induce a Th₂ immune response (Ban *et al.*, 2006).

Understanding the distribution of dNCOs in various skin compartments following exposure and identification of the haptenated protein reservoirs and key immune cell populations responsible for dNCO-hapten recognition are important in identification of potential biomarkers of exposure and sensitization to dNCOs. In this study, we employed a murine model of dermal exposure to characterize the disposition of TDI in skin. Dose and time-dependent dermal inflammation and epidermal thickening of the exposed ears following a single application

of TDI were evaluated. Furthermore, we utilized an anti-TDI-haptenated protein (TDI-hp) monoclonal antibody to visualize entry into skin, to examine co-localization with specific dendritic cell (DC) populations in the skin, and to characterize localization of TDI in the draining lymph nodes (DLNs). We also identified major target proteins for TDI haptenation using western blot analysis and mass spectrometric analyses.

MATERIALS AND METHODS

Animals and exposure procedure. Female BALB/c mice (6–8 weeks of age) were purchased from Taconic (Germantown, NY) and were housed in ventilated cages with hardwood chip bedding. Animals were provided NIH-31-modified 6% irradiated rodent diet (Harlan Teklad) and filtered water *ad libitum*. Mice were given a single dermal dose (25 μ l/ear) of different concentrations (0.1% and 4%, v/v) of 2, 4-toluene diisocyanate (TDI) (Sigma Aldrich, St. Louis, MO) or acetone control using methodologies described previously (Anderson *et al.*, 2013). Ears and the superficial parotid lymph nodes (DLN) that drain the head and ears were collected at designated time points. All animal procedures were reviewed and approved by the NIOSH Animal Care and Use Committee (ACUC).

Histology and epidermal thickness measurement. Excised ears and DLNs were fixed in 10% formalin buffered saline prior to tissue processing for histology. Paraffin-embedded ears were sectioned and stained with Hematoxylin-Eosin (H&E) for histological analysis and assessed to evaluate extent of inflammation and determine epidermal thickening as a result of acute exposure to TDI. Sections were analyzed using an Olympus AX70 microscope (Olympus America Inc., Center Valley, PA); images were captured using an Olympus DP73 camera and processed using CELLSSENS imaging software (Olympus America Inc.). Epidermal thickness was measured by using CELLSSENS imaging software and based on a previously described methodology (Rizzo *et al.*, 2011). Measurements were obtained from each animal and reported as an average for the specific time point ($n = 3–5$).

Fluorescence immunohistochemistry. For confocal imaging, paraffin-embedded ear and DLN sections were de-paraffinized by heating at 60°C for 25 min. Prior to staining, some DLN sections were subjected to permeabilization with Triton X-100 (0.2%) in PBS for 10 min and washed thoroughly in PBS. Sections were blocked in phosphate buffered saline pH 7.4 (PBS) containing 5% bovine serum albumin and 10% goat serum (blocking buffer). Sections were incubated with anti-TDI-hp monoclonal antibody (mAb) 60G2 (IgG₁), previously developed (Ruwona *et al.*, 2011) and conjugated with AlexaFluor568 dye (Molecular Probes, Eugene, OR). mAb 15B5 (IgG₁) also previously developed in our laboratory (Nayak *et al.*, 2012) was labeled with AlexaFluor568 dye and served as an irrel-

evant and isotype control for these experiments. mAbs were incubated with either AlexaFluor488-conjugated anti-mouse CD207/Langerin antibody (eBioscience, San Diego, CA) or FITC-conjugated anti-mouse CD103 hamster mAb (clone 2E7), Invitrogen (Frederick, MD). For analysis of immunolocalization of CD11b expressing cells, sections were incubated with rabbit polyclonal anti-mouse CD11b antibody (Novus Biologicals, Littleton, CO), washed and incubated with AlexaFluor488-labeled goat anti-rabbit IgG (H+L) (Molecular Probes). Slides were rinsed with sterile PBS containing 0.02% Tween-20, dried and mounted with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Images were captured using a Zeiss LSM 510 Axioplan 2 imaging confocal microscope (Zeiss, Thornwood, NY) and processed using the Zen 2009 software.

Protein extraction from mouse ear tissues. Frozen ear tissues were immersed in liquid N₂ and processed by mechanical disruption. Total protein was extracted from the disrupted tissue using T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) containing EDTA (Sigma Aldrich) and Halt protease inhibitor cocktail (Thermo Scientific). The samples were centrifuged to separate the soluble and insoluble (pellet) fractions. Supernatant containing solubilized proteins was stored at 4°C and protein concentration determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE). The pellet material (insoluble fraction) was extracted in Laemmli sample buffer (Bio-Rad, Hercules, CA) to solubilize pelleted proteins.

Gel electrophoresis and proteomic analysis. Protein extracts from the soluble fraction (10 μ g/lane) and the insoluble fraction 1/50 (v/v) were diluted in Laemmli sample buffer and separated by SDS-polyacrylamide gel electrophoresis (PAGE) electrophoresis. Protein staining and western blot analysis were performed as previously described (Nayak *et al.*, 2011a). Sample preparation and proteomic analyses were performed as described (Hettick and Siegel, 2011; Nayak *et al.*, 2011b) and data were analyzed with ProteinLynx Global Server (Waters, Milford, MA), to query the entire non-redundant UniProtKB/Swiss-Prot database.

Statistical analysis. Data were analyzed using SAS/STAT software (Version 9.3). Two-way analysis of variance (Treatment by Time) was performed using Proc Mixed. Significant interactions were analyzed using the “slice” option. A p value < 0.05 was considered statistically significant.

RESULTS

Histological Changes with TDI Exposure

Inflammation, characterized by increased cellular infiltration, tissue damage, and interstitial edema were observed from his-

tological sections of murine ears exposed to 4% TDI and to a lesser extent in 0.1% TDI-treated animals (Fig. 1A). Edema was observed by 6 h post-4% TDI exposure and progressed with cellular infiltration in the dermis at early time points. Epithelial hyperplasia (acanthosis) and hyperkeratosis were evident from day 4 onward. Significant reconstitution of skin architecture with little residual inflammation was evident by day 15. In comparison, ear sections from the acetone control animals demonstrated no signs of inflammation (Fig. 1B). These observations were comparable for all the animals within the same group of exposure. Assessment of ears highlighted significant changes in epidermal thickening between 0.1% and 4% dosed ears at days 2, 4, and 9 ($p < 0.0001$) (Fig. 1C). Analysis of epidermal thickening between ears dosed with control and 0.1% and 4% TDI suggested no significant differences at 1 h post-dosing. However, at 4 days post-exposure, the differences were significant for 0.1% ($p < 0.05$) and 4% TDI ($p < 0.0001$) compared with the control group. At this time point, epidermal thickening was more in animals dosed with 4% TDI (>50%) when compared with animals dosed with 0.1% TDI and this difference was statistically significant ($p < 0.05$). Epidermal thickening resolved significantly in all animals by day 15 post-exposure.

TDI Localizes to the Stratum Corneum and the Hair Follicles on Topical Application

TDI-hp staining was observed in the stratum corneum, hair follicles, and the sebaceous glands (SGs) within 3 h post-dosing of animals (Fig. 2). By 15 days, ears receiving 4% TDI demonstrated no signs of TDI-hp staining. Differential interference contrast (DIC) imaging demonstrated localization of TDI-hp staining to the epidermal region and different compartments of hair follicles, including the hair shaft. TDI-hp staining appeared strongly in the SGs and throughout the hair bulb. TDI-hp staining was also observed in the dermis in the region around the hair bulb.

TDI-hp staining was observed in 100% of the animals treated with either concentration of TDI. Ear sections from mice exposed to 4% TDI demonstrated TDI-hp staining in a larger number of hair follicles as compared with those exposed to 0.1% TDI (data not shown). These differences were consistently noted between the animals dosed with either concentration of TDI. In animals dosed with 4% TDI, TDI-hp staining was evident in every single hair follicle and the stratum corneum of every animal. The intensity of TDI-hp was also stronger in animals dosed with 4% TDI compared with those treated with 0.1% TDI. The staining for TDI-hp was specific as sections stained with the IgG₁ isotype control did not demonstrate immunostaining. No staining for TDI-hp was seen in ear tissue sections from acetone (vehicle control) exposed mouse tissue sections (Supplementary fig. 1).

CD11b⁺ Cells Migrate to Hair Follicles and Co-Localize with TDI Staining

The kinetics of localization of cells expressing CD11b (integrin α M/Mac-1) to areas of TDI-hp localization in the ears was investigated. CD11b⁺ cells in the skin typically include macrophages and a large population of dermal dendritic cells (dDCs) that can potentially serve as professional antigen presenting cells. In control ears, sparse numbers of CD11b⁺ cells were observed in the hair follicle (Supplementary fig. 1). In ears dosed with 0.1% TDI, migration of CD11b expressing cells to the hair follicle regions was observed with maximal staining at 6 h post-dosing (Fig. 3). Staining was prominent in the hair follicles at the infundibulum, but not in the interfollicular epidermis (IFE). Minimal staining was observed in the dermis around the hair follicles.

Animals dosed with 4% TDI demonstrated intense staining for CD11b⁺ cells indicating localization to the hair follicle as early as 1 h post-exposure (Supplementary fig. 2). Staining was located throughout the hair follicle and in the SGs. At the 1-h time point, weak staining was also observed in the epidermis surrounding the follicles. Langerhans cells (LCs) residing in the epidermis express CD11b on their surface, albeit at lower levels, compared with CD11b⁺ dDCs. At later time points, staining was gradually diminished in the hair follicles and the stratum corneum. Sections stained with goat anti-rabbit IgG (H+L) alone as a secondary antibody control demonstrated no reactivity (Supplementary fig. 2).

Langerin (CD207) Expressing DCs Co-Localize to the Regions of TDI Staining

DCs residing within the epidermis (LCs) and the dermis (dDCs) of the skin play a significant role in antigen uptake and immune activation. Migration of langerin-expressing DCs was assessed in ear tissue from mice exposed to TDI. Immunohistochemical staining of the ear sections of control animals demonstrated sparse populations of CD207⁺ dermal and epidermal DCs (Supplementary fig. 1). Animals dosed with 0.1% TDI demonstrated increasing intensity of langerin staining over time in the hair follicles and epidermis (Fig. 4A). All animals ($n = 5/5$, 100%) dosed with 0.1% TDI demonstrated characteristic retention of TDI-hp in the stratum corneum up to 15 days, the final time point in these studies. At 1 h post-exposure, minimal staining for langerin-expressing DCs was observed in the hair follicles and with minimal co-localization with TDI-hp. Langerin staining was stronger in the hair follicles at 6 h post-exposure and was observed up to day 9. Interestingly, langerin-expressing DCs under the stratum corneum gradually increased, reaching a maximum by day 2. Langerin staining was not observed at day 15, suggesting egress of langerin-expressing DCs from the epidermis.

Assessment of CD207 expressing LCs and dDCs after dosing of animals with 4% TDI revealed extensive langerin staining in the hair follicles and in the epidermis (Supplementary fig. 3).

FIG. 1. Histopathological analysis of H&E stained sections of murine ears. (A) H&E staining of representative ear sections from mice exposed to 0.1% and 4% TDI. Animals dosed with 4% TDI demonstrate significant cellular infiltration and tissue repair. In comparison, animals dosed with 0.1% TDI show minor inflammatory changes. Representative images from three to five animals per group, per time point, per exposure. Scale bar: 100 μm . (B) H&E staining of representative ear sections from mice exposed to vehicle (acetone only). Animals dosed with the vehicle do not show any changes in murine skin architecture. Scale bar: 100 μm . (C) Mean values of epidermal thickness measurements from ear sections of animals exposed to single dose of TDI (0.1% and 4%). Standard deviation is representative of measurements from ears of three to five animals per group, per time point post-exposure.

FIG. 2. Confocal microscopy analysis TDI-hp staining in ears. Representative ear sections from an animal exposed to 4% TDI. Red (AlexaFluor568): TDI-hp proteins). mAb 15B5 (IgG₁) served as an isotype control for all time points. The micrograph emphasizes the specificity of the 60G2 antibody in detecting TDI-hp in murine skin. Scale bar: 100 μm . Representative images from three to five animals per group, per time point exposure. DIC: differential interference contrast imaging.

FIG. 3. Confocal microscopy of co-localization patterns of TDI and CD11b expressing cells in ears. Representative ear sections from animals exposed to 0.1% TDI. Blue (DAPI: nucleus), green (AlexaFluor488: CD11b), and red (AlexaFluor568: TDI-hp proteins). TDI-hp staining was observed in the hair follicles and on the surface of the skin (epidermis). CD11b expressing antigen presenting cells migrated to the hair follicles to sample the antigen as reflected by co-localization observed in the merged image. Representative images from three to five animals per group, per time point exposure. Scale bar: 20 μ m.

Detailed examination of a representative section revealed a strong co-localization pattern for CD207⁺ cells and TDI-hp in the IFE basal cell layer, SG, and the dermal papillae (DP) (Fig. 4B).

CD103⁺ Subset of Langerin (CD207⁺) Cells Migrate to Hair Follicles with Differential Kinetics

In the dermis, CD207⁺ cells are subdivided into three populations, including the CD207⁺CD103⁻ and CD207⁺CD103⁺ dDC populations and the DLN-destined migrating LCs (mLCs) (Henri *et al.*, 2010) which do not express CD103. The localization of CD103⁺ dDCs was further investigated because these cells have been identified as a key population of langerin-expressing cells implicated in contact hypersensitivity to chemical allergens (Bursch *et al.*, 2007). In animals dosed with 0.1% TDI, the kinetics of localization of CD103⁺ cells to the hair follicles demonstrated minimal staining and exhibited altered kinetics to localization of CD207⁺ cells (Figs. 4A and 5). Interestingly, CD103 staining was more pronounced at 9 days and was stronger in the outer root sheath (ORS) of the hair follicles in animals receiving a dose of 0.1% TDI, although this was observed inconsistently ($n = 3/5$, 60%). In animals dosed with 4% TDI, the kinetics was different and CD103 staining was more prominent at early time points and was consistently observed for all animals ($n = 5/5$, 100%) (Supplementary fig. 3). Staining was consistently observed in the infundibulum and the ORS of the hair follicle. In control animals, CD103⁺ staining was sparse and not focused around the hair follicles (Supplementary fig. 1).

Localization of TDI-hp Proteins in the DLNs

Regionalization and co-localization of TDI-hp with antigen presenting cells was evaluated in the DLN. TDI-hp was observed along the subscapular sinus of the DLNs and within the T-cell-rich area in the cortex from animals exposed to 4% TDI, at 1 h post-exposure (Fig. 6A). In order to ascertain localization of TDI-hp in the DLNs, TDI-hp was immunochemically stained in permeabilized tissue sections. The intensity of immunostaining for TDI-hp in permeabilized samples was significantly stronger. Based on the kinetics of migration of DCs and LCs which is extensively reviewed in the literature, these cells typically take many hours to migrate into DLNs after acquisition of the antigen. Our analysis of DLNs at 1 h post-exposure suggests that TDI-hp enter the draining lymphatics at early time points and enter the DLN in a soluble form as free TDI-hp and are rapidly acquired by DLN resident immune cells. CD11b⁺ cells are found adjacent to the reticular network and appear to play a role in antigen uptake. TDI-hp staining was also observed within a network of the DLN, which likely represents the reticular network, although this aspect requires further investigation (Fig. 6B). TDI-hp staining in the lymph nodes at time points later than 1 h was sparse and dispersed (data not shown). TDI-hp immunostaining could not be detected in the DLN from animals exposed to 0.1% TDI of either non-permeabilized or permeabilized sections.

FIG. 4. Confocal microscopy of co-localization patterns of TDI and CD207⁺ (langerin) DCs in ears. (A) Representative ear sections from animals exposed to 0.1% TDI. Blue (DAPI: nucleus), green (AlexaFluor488: CD207/langerin), and red (AlexaFluor568: TDI-hp proteins). TDI-hp staining was observed in the hair follicles and the surface of the epidermis. Langerin-expressing DCs migrated to the sites of TDI-deposition in skin tissue as evidenced by co-localization in merged figures. Representative images from three to five animals per group, per time point exposure. Scale bar: 100 µm. (B) Representative ear section from animal exposed to 4% TDI, 6 h post-exposure. Blue (DAPI-nucleus), green (AlexaFluor488: CD207/langerin), and red (AlexaFluor568: TDI-hp proteins). TDI-hp and langerin staining was noticed in various compartments of the hair follicle. Representative image from three to five animals per group, per time point exposure. Scale bar: 20 µm. IFE: interfollicular epidermis; SG: sebaceous glands; DP: dermal papillae.

Skin Keratins and Albumin are Putative TDI Targets

Western blot analysis was conducted on the soluble and pellet fractions collected from ears dosed with 0%, 0.1%, and 4% TDI to assess immunoreactivity and identify predominant protein targets of TDI haptenation, 1 h post-exposure (Fig. 7). No immunoreactivity was observed in the soluble fraction collected from 0.1% TDI-dosed animals. However, the insoluble fraction demonstrated TDI-haptenation to proteins at ~70 kDa and 55 kDa (Fig. 7 and Supplementary fig. 4). Proteomic mass spectral analyses identified keratin 2e (Krt 2e) and keratin 10 (Krt 10) from these bands, respectively. Stronger immunoreactivity was observed in samples from 4% TDI-dosed ears (Fig. 7). In the soluble fraction, the strongest immunoreactivity was observed to an ~70 kDa protein and this protein was identified as mouse serum albumin. In the same fraction, weak immunoreactivity

was observed to multiple protein bands including one at ~15 kDa. Proteomic analysis of this band identified hemoglobin (α and β -1) subunits. On analysis of the insoluble fraction, several cytoskeletal and cuticular keratins were identified in the TDI-hp-immunostained protein bands. Proteomic analysis also suggested potential binding of TDI to other cytoskeletal proteins including actin. A large number of peptides were recovered for type II keratins (Krt 2e and Krt5) and type I keratins (Ker 10 and Ker 14) during the proteomic analysis of these bands. Peptides were also recovered for Krt 2e and Krt10 at ~120 kDa. These two subunits combine to form a heterodimer and recovery of peptides for this band suggests intact dimer of Krt 2e/10. Protein fractions collected from vehicle-only ears did not demonstrate any immunoreactivity. Western blot analysis performed using the IgG₁ isotype control mAb 15B5 demonstrated no im-

FIG. 5. Confocal microscopy of co-localization patterns of TDI and CD103⁺ dDCs in ears. Representative ear sections from animals exposed to 0.1% TDI. Blue (DAPI-nucleus), green (FITC: CD103), and red (AlexaFluor568: TDI-hp proteins). Migration of CD103⁺ DCs was noticed at later time points with antigen sampling observed in the ORS compartment of the hair follicle. Representative images from three to five animals per group, per time point exposure. Scale bar: 20 µm. ORS: outer root sheath.

munoreactivity (data not shown). These experiments were repeated a total of three times.

DISCUSSION

Respiratory exposure has been described as a key route for dNCOs; however, concerns have been raised about skin exposure as a potential route of allergic sensitization that could possibly lead to asthma (Bello *et al.*, 2007; Redlich, 2010). Recent literature suggests frequent skin exposure to isocyanates can occur in occupational environments (Bello *et al.*, 2008; Bernstein *et al.*, 1993; Liljelind *et al.*, 2010; Petsonk *et al.*, 2000; Pronk *et al.*, 2006). The underlying immunological mechanisms of skin sensitization to TDI and how this drives respiratory complications are poorly understood.

The studies presented here indicate that the hair follicle and associated SG may be an important route of entry and may provide a reservoir of antigen and immune sampling, as previously proposed (Blume-Peytavi and Vogt, 2011; Heath and Mueller, 2012; Nagao *et al.*, 2012; Simonsson *et al.*, 2012). Furthermore, persistence of TDI in the stratum corneum demonstrates an additional source for immune sampling of applied hapten in the skin. During inflammation, various immune cell populations migrate to the site of disruption and play an essential role in antigen uptake and presentation. In skin, this role has been attributed to macrophages and pre-stationed DC populations (Henri *et al.*, 2010). Skin DCs have been shown to be capable of promoting hypersensitivity responses upon exposure to an inducing aller-

gen (Honda *et al.*, 2010; Kaplan *et al.*, 2008; Romani *et al.*, 2010). We investigated the co-localization of CD11b (integrin α M/Mac-1), CD207 (Langerin), and CD103 (integrin α E) to regions of TDI-hp accumulation. All antigen presenting cells investigated here localized to different regions of the skin with differing kinetics that appeared to be antigen concentration dependent.

CD11b expressing cells (macrophages and ~65% of dermal dendritic populations) are essential for stimulation of cytokine production by antigen-specific effector T cells in inflamed skin (Henri *et al.*, 2010; Iwasaki, 2003; McLachlan *et al.*, 2009). CD207⁺/langerin-expressing DCs (LCs and dDCs) have been reported to mediate allergic sensitization resulting in contact hypersensitivity and atopic dermatitis (Bennett *et al.*, 2007; Bursch *et al.*, 2007). These cells constitute ~12% of total DC populations in skin and consist of epidermal LCs, and dermal DCs (CD207⁺CD103⁺ and CD207⁺CD103⁻) populations (Henri *et al.*, 2010). CD103⁺ cells form a subset of CD207⁺ cells and are the only cell population in skin to express this marker (Henri *et al.*, 2010). CD103⁺ cells have been reported to exist adjacent to hair follicles and interlock with follicular epidermal cells and speculated to be involved in antigen uptake from the ORS of hair follicles (Bursch *et al.*, 2007; Henri *et al.*, 2010; Poulin *et al.*, 2007).

In the hair follicles, CD11b⁺ cells sample antigens from the hair follicles and the epidermis. Differential kinetics and dermal localization of CD207⁺ and CD103⁺ cells following epicutaneous application of TDI was observed. Migration of CD207⁺

FIG. 6. Confocal microscopy of co-localization patterns of TDI and CD11b⁺ DCs in lymph nodes. (A) Representative superficial parotid DLN section from animal exposed to 4% TDI, 1 h post-exposure. Sections were permeabilized with Triton X-100 and compared with non-permeabilized section from the same animal. Permeabilization of DLN sections showed that TDI-hp delivered to lymph nodes is rapidly acquired by lymph node resident cells. Scale bar: 50 µm. (B) Representative superficial parotid DLN section from animal exposed to 4% TDI, 1 h post-exposure. Blue (DAPI: nucleus), green (AlexaFluor488: CD11b), and red (AlexaFluor568: TDI-hp proteins). The micrograph shows the localization of CD11b⁺ antigen presenting cells in the lymph node and their putative acquisition of TDI-hp draining into the lymph nodes. Scale bar: 10 µm.

cells to hair follicles was within 6 h, however, the CD103⁺ cells that form a subset of CD207⁺ cells peaked at day 9. This suggests that the CD207⁺ cells localizing to the hair follicles likely represent the CD207⁺CD103[−] dDC and mLC populations. CD103⁺ cells localized exclusively to the infundibulum and the ORS of hair follicles compared with CD207⁺ cells that appeared in most compartments of the hair follicles. Based on langerin staining alone it is difficult to differentiate between LCs and dDCs; however, the hair follicles have been suggested as a likely site for egress and/or replenishment of epidermal LCs (Heath and Mueller, 2012; Nagao *et al.*, 2012).

Although LCs form a constitutive part of the normal skin, we observed weak staining in control animals. This can be partly explained by the fact that the results presented in our studies represent a transverse section and are likely to represent only a minor population of LCs due to the plane in which keratinocytes differentiate in the stratum corneum. It has been established that LCs residing in the epidermis function co-operatively with ker-

atinocytes in the reorganization of tight junctions for acquisition of antigens that have traversed the stratum corneum barrier (Kubo *et al.*, 2009; Simpson *et al.*, 2011). These studies demonstrated that penetrated dendrites of LCs uptake antigens from the tip region where it co-localizes with langerin staining. Because langerin is directly involved with antigen recognition, increased staining observed on exposure to TDI could be a result of “capping” of langerin to the sites of TDI localization.

Previously, studies in our laboratory demonstrated that a single dermal dose of 4% TDI is sufficient to sensitize animals and promote a mixed Th₁/Th₂ response highlighted by the cytokines IL-4, IL-5, IL-13, and IFNγ (Anderson *et al.*, 2013). Furthermore this dose was sufficient to promote increased serum IgE levels in these animals. In the present study, in animals receiving a single dose of 4% TDI, we observed that different populations of antigen presenting cells co-localized to the regions of TDI-hp immunostaining. Indeed, CD207⁺, CD103⁺, and CD11b⁺ cells migrating to the DLN from the site of inflammation have been suggested to play a vital role in the development of allergic diseases (Guilliams *et al.*, 2010). Furthermore, in the DLNs we observed expansion of the germinal centers in the B cell region when animals were dosed with 4% TDI, but not in animals dosed with 0.1% TDI (data not shown).

Antigen presentation to naïve T cells by skin-associated DCs is a prerequisite for allergic sensitization (Martin, 2004; Thierse *et al.*, 2005). Haptenated antigens can gain access to the DLN in two distinct “waves” (Itano *et al.*, 2003). First, the soluble form (free) of antigen drains into the DLN unidirectionally through the afferent lymphatic vessels. Later, DCs (LCs and dDCs) that have acquired antigens at the original site of antigen entry traverse through the draining lymphatics and migrate to the local DLN where they present antigen–carrier complexes to naïve T cells and initiate immune activation (Merad *et al.*, 2008; Nagao *et al.*, 2009). The uptake of these antigens arriving in the DLN in soluble form is reliant on antigen properties such as molecular size (Gretz *et al.*, 2000; Lammermann and Sixt, 2008; Swartz and Lund, 2012). Soluble antigens from the afferent lymphatics are typically directed into the cortex through narrow conduits that allow for transportation of unopsonized molecules smaller <70 kDa in molecular size. Rapid transportation of TDI-hp to the DLN, 1 h post-exposure is consistent with the established paradigm on the kinetics of hapten transportation through lymphatics from the site of inoculation (Simonsson *et al.*, 2012; Sixt *et al.*, 2005). At later time points, immunohistochemical signal for TDI-hp in lymph nodes was limited, even though staining was prominent in the skin. Our studies on DLN correlate with the proteomic findings that identified most targets of TDI-haptenation to be <70 kDa. TDI-hp was not observed in DLN at the 0.1% TDI dose. This may be due to sensitivity limitations for immunohistochemical detection of TDI-hp.

A key paradigm of skin sensitization to haptens is associated with the first contact of the hapten to the skin and its binding to endogenous proteins (Christensen and Haase, 2011). Previously, dNCOs have been demonstrated to bind various pro-

FIG. 7. Western blot analysis of TDI hp in fractions extracted from ears. Soluble and insoluble protein fractions from ears from animals, 1 h post-exposure to acetone only, 0.1% TDI, or 4% TDI. TDI binding was noted to proteins ranging from 15 kDa to 250 kDa. MW: molecular weight marker, Precision Plus Protein All blue standards.

teins *in vitro* and *in vivo* such as albumin, hemoglobin, tubulin, actin, glucose-regulated protein (GRP78), and others (Day *et al.*, 1996; Jin *et al.*, 1993; Lange *et al.*, 1999; Wisniewski *et al.*, 2000). Krt 10 has also been suggested as a target for dNCO (HDI) conjugation in the skin (Wisniewski *et al.*, 2000). Our results indicate that TDI likely binds to keratins in stratum corneum and the basal layers of the epidermis and the IFE. Cuticular keratins found in hair follicles were also identified by proteomics in this study and correlated with TDI-hp localization in the immunohistochemistry studies. It is important to note that the precise identification of some of these proteins, especially various keratins, is limited by the use of single dimension electrophoresis. Furthermore, the extensive sequence homology between keratins limits precise identification. It should also be noted that other proteins may also bind to TDI and may not have been identified in our analysis.

Based on our observations, we propose that hair follicles and their associated SGs may provide a route of entry and a reservoir for TDI-hp and antigen uptake by immune cells. Additionally, TDI-hp proteins persist for days in the stratum corneum. LCs, dDCs, and CD11b expressing cell populations appear to be essential in uptake of TDI-hp antigens. Preliminary studies suggest that mouse serum albumin, cytoskeletal, and cuticular keratins are primary targets for haptentation upon dermal exposure. Results from our previous study (Anderson *et al.*, 2013) and from the data presented here suggest that it is likely that the TDI exposure dose may play a critical role on the nature of allergic sensitization. The data from animals exposed to 4% TDI

also suggest that general irritation caused by the chemical reactivity with skin may also drive such a response. Further studies should provide insight into the pathology of TDI exposures and identify immunological mechanisms of TDI-induced contact hypersensitivity, allergy, and asthma. The dynamics related to dose of TDI exposure, molecular size of the TDI-hp proteins, and the migration kinetics of various immune cell populations to the draining DLN are likely to influence the overall response and are a subject of future investigations.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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