

Impact of Cutaneous IL-10 on Resident Epidermal Langerhans' Cells and the Development of Polarized Immune Responses

Marie Cumberbatch,¹ Kirsty Clelland, Rebecca J. Dearman, and Ian Kimber

Prolonged topical exposure of BALB/c mice to chemical contact and respiratory allergens stimulates, respectively, preferential Th1- and Th2-type responses with respect to serum Ab isotype and cytokine secretion phenotypes displayed by draining lymph node cells. We now report that differential cytokine secretion patterns are induced rapidly in the skin following first exposure to the contact allergen 2,4-dinitrochlorobenzene (DNCB) and the respiratory sensitizer trimellitic anhydride (TMA). TMA induced early expression of IL-10, a cytokine implicated in the negative regulation of Langerhans cell (LC) migration, whereas exposure to DNCB resulted in production of the proinflammatory cytokine IL-1 β . Associated with this, TMA provoked LC migration with delayed kinetics compared with DNCB, and local neutralization of IL-10 caused enhanced LC mobilization in response to TMA with concomitant up-regulation of cutaneous IL-1 β . We hypothesize that these differential epidermal cytokine profiles contribute to the polarization of immune responses to chemical allergens via effects on the phenotype of activated dendritic cells arriving in the draining lymph node. Thus, TMA-exposed dendritic cells that have been conditioned *in vivo* with IL-10 (a potent inhibitor of the type 1-polarizing cytokine IL-12) are effective APCs for the development of a Th2-type response. *The Journal of Immunology*, 2005, 175: 43–50.

The development of polarized immune responses is orchestrated by the activity of CD4⁺ Th cell subpopulations and their cytokine products, with Th1 and Th2 cells, in combination with their characteristic cytokine secretion profiles predominating in the mature immune response (1). There is increasing evidence that dendritic cells (DC),² a family of highly specialized bone marrow-derived APC that are potent activators of naive T cells and are regarded as important initiators of primary immune responses (2), may direct the selective induction of distinct T cell subpopulations. Experiments performed with cultured monocyte-derived DC showed that the presence of IFN- γ during activation of immature DC primes for mature cells that express high levels of IL-12 and, consequently, a Th1-polarizing capacity (3). In contrast, the presence of PGE₂ or histamine promotes monocyte-derived DC with low IL-12 expression that favors Th2 cell responses (4, 5). Other factors that prime DC for the induction of Th1 immune responses include pathogen products such as *Staphylococcus aureus* Cowan strain I, LPS, the toxin from the bacterium *Bordetella pertussis*, or poly(I:C) (a mimic of viral RNA) (3, 6, 7). Conversely, *Schistosoma* egg Ags and the toxin from the bacterium *Vibrio cholerae* each activate DC to drive Th2 cell responses (6). It is believed that the Th cell-polarizing effects of mature DC could arise from a combination of both direct effects of pathogen on DC and indirect influences of tissue mediators re-

leased by surrounding cells in response to the invading pathogen (7–9). Although it has been reported that immunization of mice with a naturally occurring, low pathogenic Theiler's murine encephalomyelitis virus variant results in DC that preferentially express IL-10 and induce Th2-type responses, whereas DC from the wild-type virus-infected mice express IL-12 (10), there are relatively few other reports that demonstrate DC with Th1- or Th2-polarizing activity *in vivo*.

Chemical allergens that cause contact sensitization, a delayed-type hypersensitivity reaction, and those that provoke sensitization of the respiratory tract and immediate-type hypersensitivity reactions induce in mice divergent cytokine secretion phenotypes consistent with the selective activation of Th1 and Th2 cell subsets, respectively. Thus, under conditions of exposure of equivalent immunogenicity with respect to lymphocyte proliferation, draining lymph node cells (LNC) excised from mice treated topically with the contact allergen dinitrochlorobenzene (DNCB) expressed high levels of the type 1 cytokines IFN- γ and IL-12, but relatively low levels of the type 2 cytokines IL-4, IL-5, IL-10, and IL-13 (11, 12). The converse type 2 cytokine secretion profile was provoked by the chemical respiratory allergen trimellitic anhydride (TMA) (11, 12). These polarized cytokine phenotypes take time to mature, with LNC isolated 3 days after initiation of exposure expressing both type 1 and type 2 cytokines following treatment with either chemical contact or respiratory allergens (13–15). These data suggest that the molecular signature of chemical allergens may be being misread by the immune system as pathogens, resulting in Th1- or Th2-type polarization.

In the current experiments, we have investigated the role of Langerhans' cells (LC), the DC of the epidermis, in the initiation of immune responses to topically applied chemical allergens. The kinetics of LC migration away from the epidermis and their subsequent accumulation in skin draining lymph nodes as DC have been monitored, as has allergen-induced cutaneous cytokine expression. We have also examined, using neutralizing Ab, the role of IL-10, a cytokine described originally as a Th2 cell product that

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² Abbreviations used in this paper: DC, dendritic cell; AOO, acetone:olive oil; DNCB, 2,4-dinitrochlorobenzene; LC, Langerhans cell; LNC, lymph node cell; TMA, trimellitic anhydride.

inhibited Th1 cell cytokine production (16) and that has been shown to be involved also in modulating cutaneous immune responses, including contact hypersensitivity (17, 18). Of particular interest are the observations that IL-10 can influence the Ag-presenting function of LC and DC and that IL-10 pretreatment results in APC that promote Th2, but not Th1, cell activation (19–21). We have now demonstrated that chemical contact and respiratory allergens exert differential effects on the kinetics of LC mobilization and migration as a result of differential induction of cutaneous IL-10. It appears therefore that different classes of chemical allergen stimulate polarized T cell responses secondary to the induction of a cytokine environment that promotes the activation of DC with the ability to drive Th1- or Th2-type immune responses.

Materials and Methods

Animals

Young adult (8–12 wk old) BALB/c strain mice, obtained from the Specific Pathogen Free Breeding Unit (Alderley Park), were used throughout these studies. Food (SDS PCD pelleted diet; Special Diets Services, U.K.) and water were available *ad libitum*. All procedures were approved by the U.K. Home Office and conducted in compliance with the Animals (Scientific Procedures) Act 1986 under a Home Office granted Project License.

Chemicals and exposure

TMA (99% pure) and DNCB (97% pure) were obtained from Sigma-Aldrich. Chemicals were dissolved in 4:1 v/v acetone:olive oil (AOO) immediately before dosing. For measurement of cytokine production by draining LNC, a prolonged exposure protocol was used (11, 12) in which groups of mice were exposed topically on both shaved flanks to 50 μ l of 25% TMA, or to 1% DNCB, on days 0 and 5. Five days later, animals received 25 μ l of the same concentration of chemical on the dorsum of both ears daily for 3 consecutive days. For LC and DC experiments, a single-dose exposure protocol was used with application of 25 μ l of test chemical (25% TMA, 1% DNCB, or 0.25% DNCB) to the dorsum of both ears.

Preparation of a single-cell suspension from lymph nodes

Draining auricular lymph nodes were excised and pooled for each experimental group. A single-cell suspension of LNC was prepared under aseptic conditions by mechanical disaggregation through sterile 200-mesh stainless steel gauze and resuspended in RPMI 1640 growth medium (Invitrogen Life Technologies) supplemented with 25 mM HEPES (pH 7.2–7.5), 400 μ g/ml streptomycin, 400 μ g/ml ampicillin, and 10% heat-inactivated FCS (RPMI 1640-FCS). Viable cell counts were performed by exclusion of trypan blue dye and the total cellularity per lymph node was recorded.

Culture of LNC for cytokine measurement

LNC suspensions were prepared 13 days following initiation of exposure (prolonged exposure protocol) and were seeded into 24-well cell culture plates at 1×10^7 cells/well. Cells were cultured in the presence or absence of 2 μ g/ml T cell mitogen Con A (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂ in air. Culture supernatants were collected by centrifugation at $150 \times g$ for 10 min and stored at –70°C. The IL-4 content of culture supernatants derived from Con A-stimulated LNC and IL-10, IL-12, and IFN- γ content of supernatants derived in the absence of Con A were measured by customized sandwich ELISA as described previously (11, 12).

Preparation and analysis of epidermal sheets

Epidermal sheets were prepared and stained for MHC class II (Ia) expression as described previously (22). Samples were examined in a blinded fashion with fluorescence microscopy, and the frequency of stained cells was assessed using an eyepiece with a calibrated grid (0.32×0.213 at $\times 40$ magnification). For each sample, 10 consecutive fields in the central portion of the ear were examined. In no instance was any fluorescence detected following treatment with isotype-matched control Ab. Images were acquired digitally using an Olympus BX50 fluorescence microscope coupled with an RS Photometrics Coolsnap color charge-coupled device camera (Princeton Instruments) and were processed equally for image sharpness and brightness using a MetaMorph Imaging System (version 4.01; Princeton Instruments). In one series of experiments, epidermal sheets were examined for CD86 expression (10 μ g/ml; clone GL1, rat IgG_{2a} κ ; BD Pharmingen).

Identification of lymph node DC following enrichment by gradient centrifugation

Draining auricular lymph nodes were excised 18 h after treatment. Nodes were pooled for each experimental group and single-cell suspensions of LNC were prepared. The cell concentration was adjusted to 5×10^6 cells/ml in RPMI 1640-FCS and DC-enriched populations were prepared as described previously by discontinuous gradient centrifugation on metrizamide (14.5% in RPMI 1640-FCS; Sigma-Aldrich) (22). The frequency of DC in such low buoyant density fractions was assessed routinely by direct morphological examination using phase-contrast microscopy. DC were identified on the basis of their dendritic/hairy morphology. Results are expressed as DC per node.

Identification of lymph node DC by flow cytometry

Draining auricular lymph nodes were excised and nodes were pooled for each experimental group. A single-cell suspension of LNC was prepared by digestion of lymph nodes for 25 min at room temperature in medium containing 0.5 mg/ml collagenase IV and 0.02 mg/ml DNase I (Sigma-Aldrich). The resulting cell suspension was filtered through 200-mesh stainless steel gauze, washed, and resuspended in 5% FCS/PBS. LNC suspensions were plated into 96-well round-bottom plates (5×10^5 /well) and were incubated with mouse Fc block (rat anti-mouse CD16/CD32; clone 2.4G2, rat IgG2b κ ; BD Pharmingen, San Diego, CA) for 10 min. To detect CD11c⁺/Ia⁺ DC in LNC suspensions, cells were incubated for 30 min with 10 μ g/ml R-PE-conjugated hamster anti-mouse CD11c (clone HL3; BD Pharmingen) and 2 μ g/ml FITC-conjugated rat anti-mouse I-A^d/I-E^d (clone 2G9; rat IgG2a κ ; BD Pharmingen). Control cells were treated with either 10 μ g/ml R-PE-conjugated hamster IgG (clone G235-2356; BD Pharmingen) in place of anti-CD11c or 2 μ g/ml FITC-conjugated rat IgG2a κ (clone R35-95; BD Pharmingen) in place of anti-I-A^d/I-E^d, or were exposed to both isotype controls together. All incubations and washes were performed in 5% FCS/PBS at 4°C. Cells were resuspended in 1% FCS/0.05% azide/PBS for analysis of 10^5 cells by two-color flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Phenotypic analysis of lymph node DC

Approximately 10^5 DC enriched by metrizamide centrifugation were incubated in round-bottom 96-well plates for 30–45 min on ice with mAbs directed against I-A^d/I-E^d (2 μ g/ml; clone 2G9, rat IgG2a κ ; BD Pharmingen), CD80 (5 μ g/ml; clone 1G10, rat IgG2a κ ; BD Pharmingen), or CD86 (5 μ g/ml; clone GL1, rat IgG2a κ ; BD Pharmingen) or with purified rat IgG2a κ (5 μ g/ml) followed by further incubation for 30–45 min on ice with FITC-conjugated F(ab')₂ goat anti-rat IgG (1/100; mouse adsorbed; Serotec). All incubations and washes were performed in 5% FCS/PBS at 4°C. Cells were washed and resuspended in 1% FCS/0.05% azide/PBS for analysis of 10^4 cells using a FACSCalibur flow cytometer.

Administration of Ab

Polyclonal goat anti-mouse IL-10 (R&D Systems) was supplied as a purified IgG fraction (endotoxin content, <0.04 ng/ μ g) and was diluted in sterile PBS containing 0.1% BSA as carrier protein. Mice received 30 μ l of intradermal injections containing 1 μ g of anti-IL-10 or an equivalent amount of normal goat IgG (endotoxin content, <0.01 ng/ μ g; R&D Systems) into both ear pinnae using 1-ml syringes with 30-gauge stainless steel needles.

Measurement of epidermal cytokine production

Ears were removed and prepared for explant culture under aseptic conditions. Ears were washed immediately in 70% ethanol, rinsed in PBS, and split with the aid of forceps into dorsal and ventral halves. Dorsal halves were floated on 250 μ l of RPMI 1640 medium in 24-well tissue culture plates (one dorsal ear half per well). Supernatants were collected after 16 h of culture, pooled for each mouse, and centrifuged at $150 \times g$ for 5 min before storage at –70°C. Concentrations of IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, and TNF- α were measured in supernatants using the Bio-Plex cytokine array system (Bio-Rad) according to the manufacturer's instructions. Cytokine content was measured using a Luminex 100 multiple analyte profiler (MiraiBio Hitachi Genetic Systems).

Statistical analyses

The statistical significance of differences in LNC cytokine secretion, LC frequencies, and lymph node DC counts between experimental and control groups was calculated using Student's two-sided *t* test. Statistical analysis of cutaneous cytokine production was performed using ANOVA following a logarithmic transformation, separately for each time point. Differences between individual means were compared using a two-sided Student's *t*

test based on the error mean square of the ANOVA. For the influence of anti-IL-10 Ab on TMA-induced cytokine expression, the Wilcoxon rank sum test was used.

Results

TMA and DNCB induce divergent cytokine secretion profiles

LNC were prepared following a prolonged (13-day) exposure protocol to TMA (25%) or to DNCB (1%) and cytokine secretion patterns were analyzed by ELISA for cytokine production. These concentrations of allergen were selected on the basis of inducing equivalent levels of immunogenicity following a single topical exposure, measured as a function of lymphocyte proliferation in the lymph node draining the site of exposure (data not shown). As reported previously (11, 12), LNC derived following treatment of mice with 1% DNCB produced high levels of the type 1 cytokines IFN- γ and IL-12, but relatively low levels of the type 2 cytokines IL-4 and IL-10 (Fig. 1). In contrast, identical treatment with 25% TMA provoked a type 2 cytokine secretion profile similar to that shown previously for 10% TMA, with vigorous IL-4 and IL-10 production being detected, but relatively low expression of IFN- γ and IL-12 (Fig. 1). These data confirm that at these doses, DNCB and TMA provoke in BALB/c strain mice qualitatively divergent immune responses.

TMA and DNCB provoke LC mobilization and migration with different tempos

To explore whether DC populations resident in skin play a role in regulating early Th1 or Th2 commitment to these allergens, the mobilization/activation of epidermal LC and the subsequent accumulation of DC in draining lymph nodes were investigated. Changes in the frequency of MHC class II (Ia)⁺ LC remaining in epidermal sheets prepared 4 and 17 h after treatment with allergen were examined in Fig. 2. Compared with naive or vehicle (AOO)-treated mice, TMA failed to stimulate a significant reduction in epidermal LC numbers within 4 h following exposure (mean reduction, 5.1%). In contrast, treatment of mice with DNCB caused a significant ($p < 0.005$) mean reduction in LC numbers of 30.1%. Measurement of LC frequencies after 17 h of exposure revealed a different pattern. Relative to naive or vehicle-treated mice, a significant decrease in LC numbers was observed for TMA (18.7%; $p < 0.005$). In the case of DNCB, a further decrease in the number of LC was recorded (39.4% reduction; $p < 0.005$, Fig. 2). These results demonstrate that TMA and DNCB each stimulate LC migration, but with different kinetics. Additional kinetic experiments

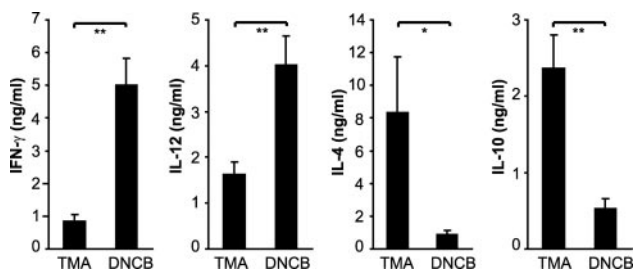


FIGURE 1. Cytokine secretion profiles induced by repeated exposure of BALB/c strain mice to TMA and DNCB. LNC were isolated 13 days after the initiation of repeated topical exposure of mice ($n = 3$ /group) to 25% TMA or to 1% DNCB. Concentrations of IFN- γ , IL-12, and IL-10 were measured by ELISA in supernatants prepared following culture for 120 h. The IL-4 concentration was analyzed by ELISA in supernatants prepared following culture for 24 h in the presence of 2 μ g/ml Con A. Results are mean $\pm$ SE of $n = 8$ experiments. *, $p < 0.05$; **, $p < 0.005$: significant difference in cytokine production for TMA vs DNCB.

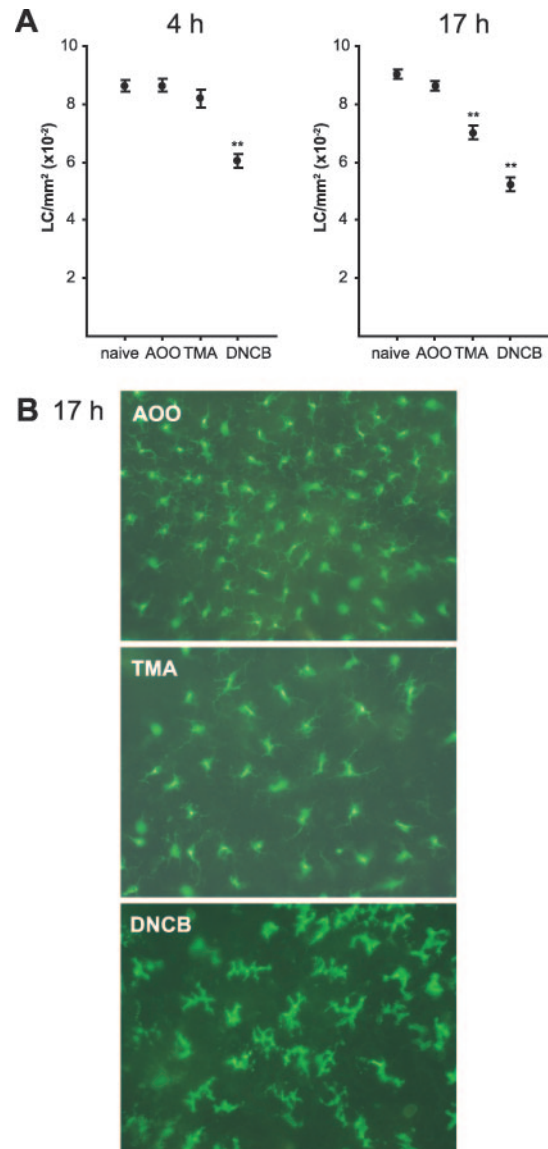


FIGURE 2. Differential effects of TMA and DNCB on LC migration and activation. Groups of mice ($n = 2$) were exposed topically on the dorsum of both ears to 25% TMA, 1% DNCB, or AOO. Control mice were untreated (naive). Epidermal sheets were prepared for analysis of MHC class II (Ia)⁺ LC frequencies 4 or 17 h later. A, LC numbers (mean $\pm$ SE; $n = 12$) are derived from analysis of three independent experiments, each comprising $n = 4$ epidermal sheets/treatment group/experiment. **, $p < 0.005$: significantly different from AOO-treated group. B, LC morphology 17 h after exposure to AOO, TMA, or DNCB. Original magnification, $\times 100$.

incorporating time points up to 72 h (four ears per treatment group per time point) revealed that differences in the kinetics of LC migration were maintained, with decreases in LC numbers of 46% ($p < 0.005$) and 69% ($p < 0.005$) being recorded for DNCB 48 and 72 h after exposure, compared with reductions of only 20% ($p < 0.005$) and 29% ($p < 0.005$) for TMA at the same times (data not shown). Clear morphological differences were also evident in epidermal sheets examined after application of chemical. As illustrated in Fig. 2, 17 h after exposure to DNCB, LC were activated with extended dendritic processes and enhanced MHC class II (Ia) expression, whereas similar morphological changes were not observed with LC from TMA-treated mice. Examination of epidermal sheets prepared and stained for Ia determinants at later time points revealed that although remaining LC continued to

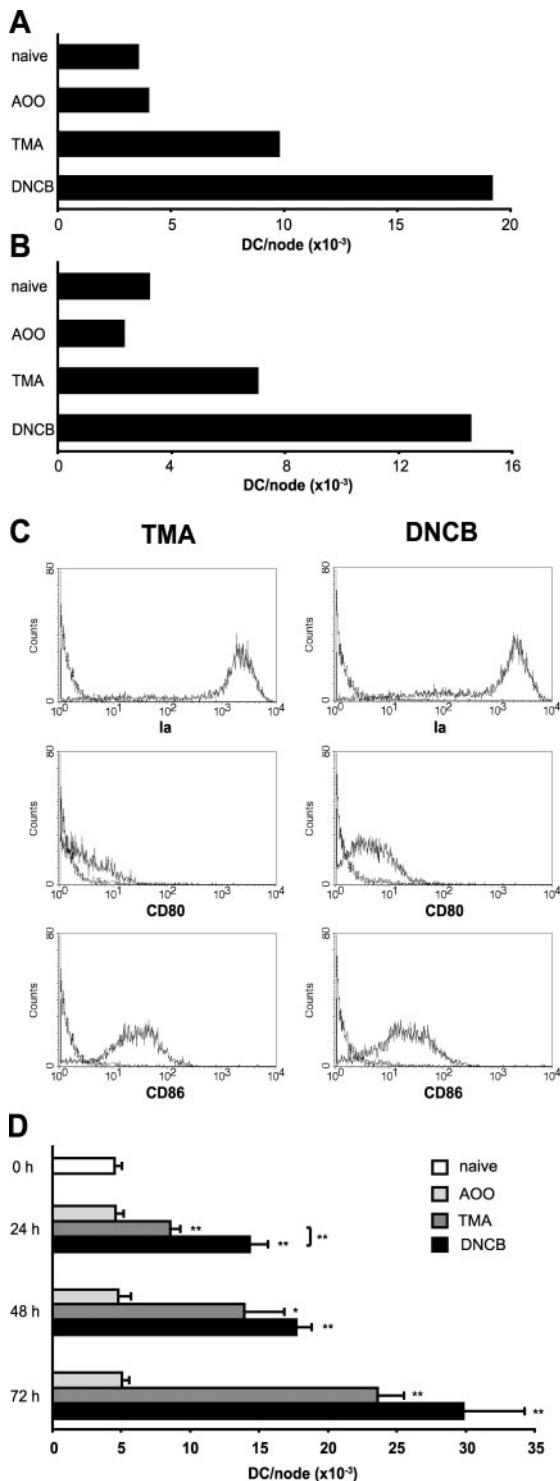


FIGURE 3. Differences in draining lymph node DC accumulation provoked by TMA and DNCB. Mice received a single topical application of either 25% TMA or 1% DNCB to the dorsum of both ears. **A**, Lymph nodes were excised 18 h after exposure ($n = 10$ mice/group) and DC were enriched by density gradient centrifugation. DC numbers were assessed following direct morphological examination of DC-enriched fractions. Data are representative of $n = 2$ independent experiments. **B**, Lymph nodes were removed 18 h after exposure ($n = 2$ mice/group) and DC numbers were determined following two-color flow cytometric analysis of LNC suspensions for CD11c/MHC class II expression. Results are representative of $n = 4$ experiments. **C**, LNC suspensions were prepared 18 h after exposure and enriched for DC by density gradient centrifugation. DC-enriched fractions were analyzed by flow cytometry for MHC class II (Ia), CD80, and CD86 expression. Data are representative of $n = 3$ experiments. **D**, Lymph nodes were excised at the times

display an activated phenotype in DNCB-treated mice, low level LC activation only became apparent for TMA 48 to 72 h after treatment (data not shown). Furthermore, in concurrent analyses of CD86 expression where epidermal sheets from naive and vehicle-treated mice failed to display detectable levels of this marker, both allergens caused up-regulated CD86 expression, but only by a proportion of LC (up to 15%). However, compared with TMA, DNCB-treated skin displayed higher levels of CD86 (data not shown). These results suggest that LC may experience different cytokine microenvironments during the first few hours of exposure to these allergens.

In subsequent experiments, allergen-induced accumulation of DC in draining lymph nodes was investigated. DC accumulation was monitored either following enrichment of DC from LNC suspensions by metrizamide gradient centrifugation (Fig. 3, **A** and **C**) or by two-color flow cytometric analysis of unfractionated LNC suspensions for CD11c/MHC class II (Ia) expression (Fig. 3, **B** and **D**). Consistent with the differential kinetics of LC migration, the accumulation of DC in draining lymph nodes analyzed 18 h after exposure to chemical was less vigorous for TMA than for DNCB, irrespective of the method used for enumeration of DC (Fig. 3, **A** and **B**). The phenotypic characteristics of DC arriving in draining lymph nodes at this time point (18 h) after treatment with DNCB or TMA were found to be similar with respect to expression of MHC class II (Ia), CD80, and CD86 (Fig. 3C). At time points beyond 24 h, proliferating T cells cofractionate with DC and contaminate metrizamide-enriched DC fractions. To assess accumulation of DC at time points beyond 24 h, lymph node DC numbers were assessed by two-color flow cytometry for CD11c⁺ and MHC class II⁺ expression (Fig. 3D). At 24 h the accumulation of DC provoked by TMA remained significantly lower than that observed for DNCB, similar to results obtained at 18 h (Fig. 3, **A** and **B**). In analyses performed 48 and 72 h after exposure, lymph node DC populations continued to increase for both chemicals such that the induced increases in DC frequencies were no longer significantly different from each other.

Delayed kinetics of LC mobilization and migration provoked by TMA is regulated by IL-10

We hypothesized that IL-10, a cytokine implicated in the negative regulation of LC function and the development of Th2-type responses (17–21), may be a key regulator of the delayed kinetics of LC mobilization observed for TMA. The influence of prior local (intradermal) administration of a neutralizing anti-IL-10 Ab on allergen-induced LC migration and on subsequent lymph node DC accumulation was therefore examined (Figs. 4 and 5). The influence of anti-IL-10 Ab on changes in epidermal LC frequencies 4 h after exposure to allergen was determined; a time when substantial emigration of LC is evident for DNCB, but not TMA (Fig. 2). LC values for control mice exposed intradermally to goat IgG before topical application of vehicle (AOO) were not significantly different from those derived from naive mice (Fig. 4, **A**, **C**, and **D**). Intradermal administration of anti-IL-10 Ab to AOO-treated sites was associated with a significant decrease in LC numbers (14–19%). Treatment with anti-IL-10 was also associated with a

indicated and DC numbers were measured by two-color flow cytometric analyses of LNC suspensions for CD11c/MHC class II expression. Results are expressed as mean $\pm$ SEM of $n = 4$ experiments for 0 h, $n = 4$ experiments for 24 h, $n = 3$ experiments for 48 h, and $n = 5$ experiments for 72 h. *, $p < 0.05$; **, $p < 0.005$: significant increase in DC numbers compared with concurrent AOO group. Double asterisks over bar, $p < 0.005$: significant difference between TMA and DNCB.

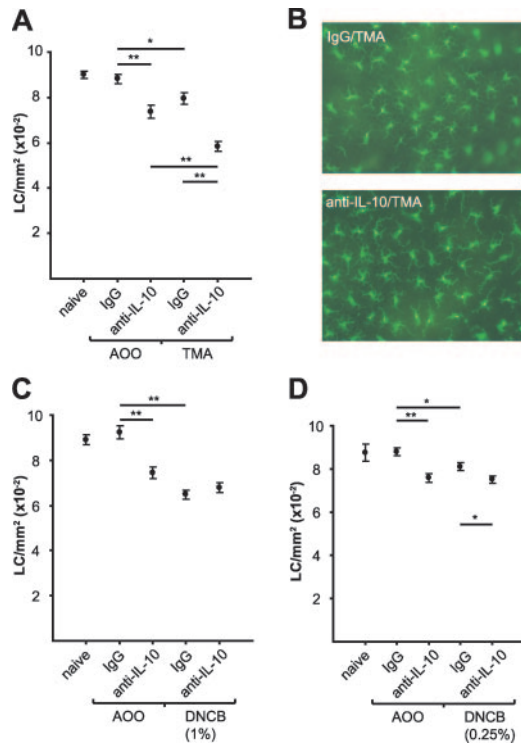


FIGURE 4. Influence of anti-IL-10 Ab on LC migration provoked by TMA and DNCB. Groups of mice ($n = 3$) received 30- μ l intradermal injections into both ear pinnae of 1 μ g of anti-IL-10 or goat IgG 30 min before exposure on the dorsum of both ears to 25 μ l of 25% TMA (A and B), 1% DNCB (C), or 0.25% DNCB (D). Control mice were untreated (naive). Ears were removed 4 h later and epidermal sheets were prepared for analysis of MHC class II (Ia)⁺ LC frequency. Results are expressed as the mean number of LC/mm² ($\pm$ SE) of $n = 12$ ears (6 ears/group for each of two independent experiments, A) and $n = 6$ ears (single experiments, C and D). *, $p < 0.05$; **, $p < 0.005$: significant differences between treatment groups. B, LC morphology 4 h after exposure to TMA in Ab-treated mice. Original magnification, $\times 50$.

significant increase in LC migration in response to TMA, 34% migration in the presence of anti-IL-10 compared with a 10% reduction in LC frequency in goat IgG-treated controls (Fig. 4A). In addition, Ab treatment was associated with effects on LC activation, including up-regulation of MHC class II (Ia) expression and increased cell size, for both TMA (Fig. 4B)- and vehicle-treated mice (data not shown). In contrast, anti-IL-10 Ab failed to enhance further the decrease in LC frequencies induced following exposure of mice to 1% DNCB (Fig. 4C). The lack of effect on DNCB-induced migration was not a consequence of the more vigorous response to this allergen. Identical exposure to a lower dose of DNCB (0.25%) that provokes relatively weak LC migration at 4 h (8% reduction in LC frequency; Fig. 4D) was unaffected by administration of anti-IL-10 Ab. The influence of anti-IL-10 Ab on allergen-induced lymph node DC accumulation was assessed also (Fig. 5). In the presence of TMA, pretreatment with anti-IL-10 Ab approximately doubled the accumulation of DC in draining lymph nodes (from 5,922 DC/node to 10,695 DC/node) in the experiment illustrated in Fig. 5A, but was without marked effect on the influx of DC caused by either 1% (Fig. 5B) or 0.25% DNCB (Fig. 5C).

TMA and DNCB provoke divergent cutaneous cytokine expression profiles

Having confirmed the potential of IL-10 to regulate the kinetics of LC mobilization and subsequent DC accumulation, we then inves-

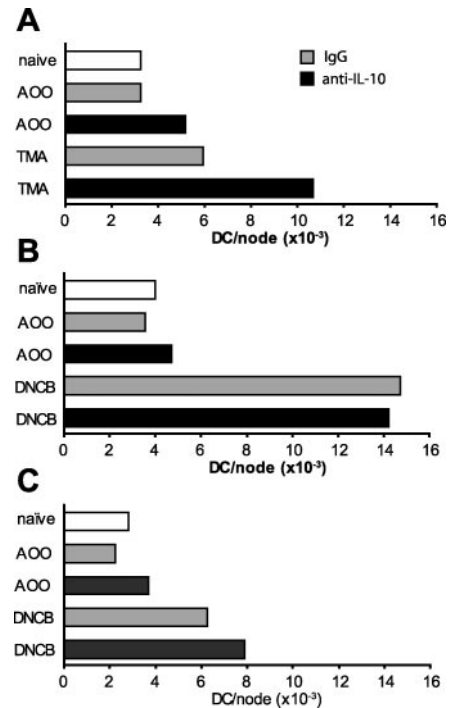


FIGURE 5. Influence of anti-IL-10 Ab on DC accumulation stimulated by TMA or DNCB. Groups of mice ($n = 10$) received 30- μ l intradermal injections into both ear pinnae of 1 μ g of anti-IL-10 or goat IgG 30 min before exposure on the dorsum of both ears to 25 μ l of 25% TMA (A), 1% DNCB (B), or 0.25% DNCB (C). Control mice were untreated (naive). Draining auricular lymph nodes were excised 18 h later and the number of DC per node was measured. Results are individual experiments in each case.

tigated cutaneous cytokine expression induced by allergen. Supernatants derived from dorsal ear explant cultures prepared from mice exposed in vivo to allergen for various periods were analyzed using the Bio-Plex cytokine array system for IL-1 α , IL-1 β , IL-6, IL-10, IL-12p40, IL-12p70, and TNF- α . Treatment of mice with vehicle (AOO) caused no change in cytokine expression throughout the course of these investigations (Fig. 6). However, significant changes in allergen-induced cytokine secretion were observed. Cutaneous exposure to TMA resulted in a very rapid (within 30 min) increase in IL-10 expression, which remained at relatively high levels compared with AOO- or DNCB-treated tissue for 1–3 h. By 4 h, IL-10 levels had decreased for TMA-treated tissue whereas expression of this cytokine by DNCB-exposed tissue had increased somewhat. In contrast, exposure to DNCB enhanced IL-1 β production, although with a more delayed time course than that observed for TMA-induced IL-10, reaching maximal levels after 4 h. No marked change in expression of this cytokine was noted for TMA. Raised IL-1 α levels were apparent as early as 30 min after exposure to DNCB; however, both TMA and DNCB were associated with variable expression of this cytokine at different times. IL-6 was expressed at high levels (range, 11–16 ng/ml) in all culture supernatants, with no allergen-associated alterations recorded at any time point investigated (data not shown). Similarly, no treatment-related changes in the low levels of IL-12p40 expression (range, 3–14 pg/ml) or presence of IL-12p70 (range, 89–130 pg/ml) were detected (data not shown). TNF- α concentrations remained low throughout (2–9 pg/ml; data not shown). To exclude the possibility that low-dose DNCB (0.25%; Figs. 4 and 5) may stimulate less vigorous LC migration through increased IL-10 production, the cytokine content of explant culture supernatants prepared 2 h after exposure to 0.25% DNCB was compared with that

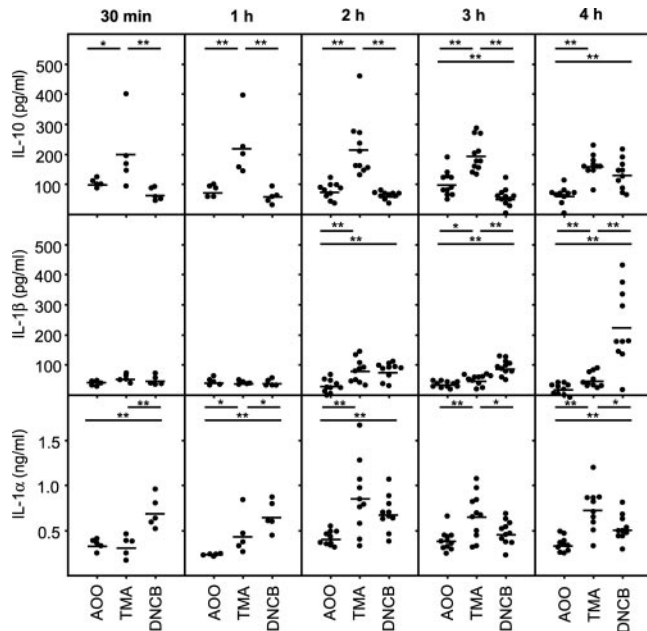


FIGURE 6. Cytokine responses in skin promoted by exposure of mice to TMA and DNCB. Mice were exposed topically on the dorsum of both ears to a single application of 25% TMA, 1% DNCB, or AOO alone. Ears were removed 30 min to 4 h later and dorsal ear halves were cultured separately (two per mouse) on medium for 16 h. Culture supernatants were pooled for each mouse and the cytokine content was measured by Luminex analysis. Results are plotted as cytokine content per mouse for experiments performed at 30 min ($n = 5$ mice), 1 h ($n = 5$ mice), 2 h ($n = 10$ mice), 3 h ($n = 11$ mice), and 4 h ($n = 10$ mice). *, $p < 0.05$; **, $p < 0.005$: significant differences between treatment groups.

provoked by TMA treatment (Fig. 7A). Following treatment with 0.25% DNCB, small, but nevertheless significant, increases in IL-1 α and IL-1 β release similar to those seen previously were recorded, but no marked changes in secretion of IL-6, IL-12, or TNF- α were observed. Importantly, however, exposure to 0.25% DNCB was not associated with elevated IL-10 release.

IL-10 inhibits TMA-induced cutaneous IL-1 β expression

Finally, we investigated whether one action of TMA-induced IL-10 was to lower expression of IL-1 β ; a cytokine known to be necessary for LC mobilization. For this purpose, responses were examined at 4 h following exposure, at which time elevated IL-1 β expression was found in response to treatment with DNCB, but not with TMA (Fig. 6). Treatment with anti-IL-10 Ab not only enhanced IL-1 β secretion in control skin, but resulted in a further elevation in the amount of IL-1 β produced in TMA-treated skin (Fig. 7B). IL-12 p40 concentrations were also raised significantly at sites pretreated with anti-IL-10 Ab, concomitant with complete inhibition of constitutive and induced IL-10 release. There was some increase, although statistical significance was not reached, in IL-1 α production (data not shown) and all other cytokines remained unchanged.

Discussion

Prolonged exposure to chemical contact and respiratory allergens promotes divergent immune responses in mice with contact allergens such as DNCB and respiratory allergens such as TMA stimulating the preferential activation of Th1 and Th2 cells, respectively. We report here that the polarization of immune responses commences within the first few hours of exposure to allergen, with differential cutaneous cytokine expression patterns induced with respect to IL-10 and IL-1 β production. In addition, although both

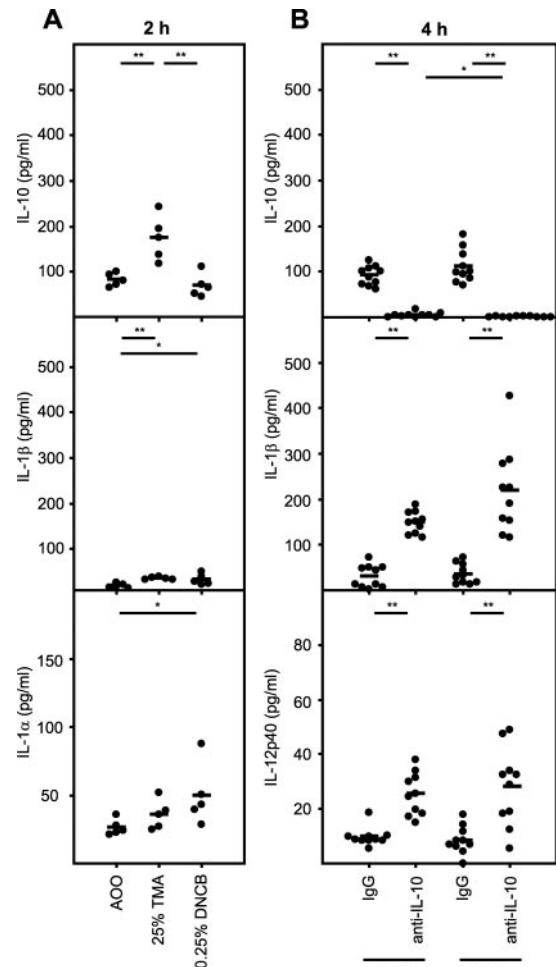


FIGURE 7. Cutaneous cytokine secretion in response to a lower dose of DNCB (0.25%, A) and TMA following pretreatment locally with anti-IL-10 Ab (B). A, Mice were exposed topically on the dorsum of both ears to a single application of 25% TMA, 0.25% DNCB, or AOO alone. B, Mice received 30- μ l intradermal injections into both ear pinnae of 1 μ g of anti-IL-10 or goat IgG 30 min before exposure on the dorsum of both ears to 25 μ l of 25% TMA or AOO alone. Ears were removed 2 h (A) or 4 h (B) later and dorsal ear halves were cultured separately (two per mouse) on medium for 16 h. Culture supernatants were pooled for each mouse and the cytokine content was measured by Luminex analysis. Results are plotted as cytokine content/mouse for a representative experiment ($n = 5$ mice, A) and pooled data from two experiments (B). *, $p < 0.05$; **, $p < 0.005$: significant differences between treatment groups.

TMA and DNCB induced the mobilization of epidermal LC and lymph node DC accumulation, the response to TMA was delayed compared with that observed for DNCB. A role for IL-10 in the regulation of LC migration in response to TMA was confirmed by treatment of mice with anti-IL-10 Ab, which enhanced LC migration and lymph node DC accumulation to TMA, but not to DNCB, with a concomitant up-regulation of cutaneous IL-1 β expression. We hypothesize that these differential epidermal cytokine profiles contribute to the polarization of immune responses to chemical allergens by influencing the phenotypic and functional characteristics of activated DC arriving in the draining lymph node. Thus, local IL-10 secretion is likely to be an early defining event in the development of the Th2-type immune responses to TMA.

We proposed that one of the actions of local IL-10 production induced by TMA may be to inhibit the expression of IL-1 β , a

cytokine known to be required for the mobilization of LC in response to chemical contact allergens and the development of contact hypersensitivity (23, 24). Exposure to the potent contact allergen trinitrochlorobenzene, but not to skin irritants, has been shown previously to enhance epidermal IL-1 β mRNA expression (25), which is consistent with the current observations that treatment with DNCB enhanced IL-1 β secretion by skin explants. In murine epidermis, IL-1 β is a product primarily of LC and not keratinocytes (26); although in explant culture supernatants comprising epidermal and dermal tissue, this cytokine could derive from dermal cells, such as dermal DC, also. Regardless of the cellular source of IL-1 β , it is clear that neutralization of IL-10 in both control (vehicle-treated) and TMA-treated skin resulted in enhanced expression of IL-1 β , and in both cases enhanced LC migration also. These data suggest that one function of IL-10 in the skin is to down-regulate cutaneous inflammatory responses via inhibition of proinflammatory cytokines such as IL-1 β , an activity of this cytokine that has been demonstrated previously in the skin (18, 27). Despite the lack of early IL-1 β expression in the immune response to TMA, this allergen is able to stimulate LC migration, DC accumulation, and lymph node activation, albeit with delayed kinetics compared with DNCB, and to stimulate IgE Ab production, Th2 cytokine expression, and challenge-induced cell-mediated cutaneous inflammatory responses (12, 28, 29). However, analogous with responses stimulated by another Th2-inducing chemical allergen, FITC, the skin reactions provoked by challenge of TMA-sensitized mice are likely to be mediated by Th2-type cells rather than by IFN- γ -expressing Th1-type cells, the cellular effectors of classical contact hypersensitivity reactions (30). Effects of IL-10 other than on IL-1 β expression have been reported that may contribute to the delayed LC migration provoked by TMA, including inhibition of the chemokine receptor switch (down-regulation of the inflammatory receptors CCR1, CCR2, and CCR5 and induction of CCR7) necessary for LC mobilization (31, 32).

The key event following treatment with the chemical respiratory allergen TMA is a rapid increase in the local availability of IL-10 (within 30 min of exposure), consistent with either a direct or indirect effect of this allergen on release of constitutively expressed cytokine. The fact that IL-10 production in response to TMA does not follow the same kinetic profile as IL-1 α , an intracellular cytokine that is released only in response to cellular stress or disruption (33, 34), argues against a direct traumatic effect of TMA mediating IL-10 release. One possible mechanism for the rapid induction of IL-10 by TMA might be through direct perturbation of the nervous system that innervates the epidermis and/or effects on receptors for neurotransmitters expressed by immune cells. For example, the sympathetic neurotransmitter norepinephrine stimulates bone marrow-derived DC to produce IL-10 through activation of β_2 adrenoreceptors (35). In the epidermis, β_2 adrenoreceptors are expressed by both LC and keratinocytes (36), raising the possibility that activation of these receptors may represent one mechanism for the rapid mobilization of cutaneous IL-10. In contrast, neuropeptides, such as pituitary adenylate cyclase-activating polypeptide and calcitonin gene-related peptide, inhibit cutaneous immune responses and are associated with the ability to enhance IL-10 production (37, 38). In addition, mediators such as PGE $_2$, histamine, and increased cAMP all favor IL-10 production and Th2 responses (4, 5, 39). Thus, activation of the cutaneous nervous system and neuropeptide release by TMA and/or release of other cutaneous mediators resulting in IL-10 release may contribute to the Th2-promoting effects of this allergen.

One important consideration relevant to the induction of cutaneous cytokine secretion by these chemical allergens is their in-

herent irritant potential. In common with other chemical allergens, DNCB and TMA both display irritant properties; however, 1% DNCB is somewhat more irritant than 25% TMA. Irritancy may therefore contribute to the more vigorous response stimulated by a single application of 1% DNCB compared with 25% TMA. However, 0.25% DNCB is of equivalent irritancy to 25% TMA. Despite this, we have demonstrated that only TMA provokes the rapid expression of IL-10 and that LC migration induced by TMA (but not DNCB) was enhanced by anti-IL-10 treatment. These results suggest that the irritant properties of these chemical allergens are not responsible for the differential cutaneous cytokine secretion profiles observed in these experiments.

Irrespective of the mechanism through which TMA promotes early IL-10 release in the skin, this cytokine has marked effects on LC function and the subsequent development of immune responses. Significantly, IL-10 influences the Ag-presenting function of DC (and LC) and it has been shown that IL-10-pretreated LC/DC are effective APC for Th2 cells, but not for Th1 cells (19–21). It has been suggested that one possible mechanism by which IL-10 may drive the development of Th2-promoting DC is by blocking selectively the ability of DC to produce IL-12 (40). The balance of IL-10 and IL-12 production by DC early in the generation of immune responses to a variety of pathogens has been shown to be critical for the development of a polarized immune response *in vivo* (40–42). In the current series of investigations, we failed to detect elevated IL-12 production in the skin during the first 4 h of exposure to DNCB. However, skin painting with TMA was shown to induce rapid IL-10 secretion before marked LC migration, such that epidermal LC will experience allergen in a different cytokine microenvironment to DNCB and the tempo of migration is delayed. It has been observed that DC activated with an appropriate stimulus (such as for instance LPS) produce IL-12, but for only a very limited period. After this IL-12 production ceases and DC fail to respond to further stimulation with IL-12 expression (43). The implication is that there might exist only a narrow window following activation of DC during which they are able to promote preferential Th1-type responses, after which in the absence of IL-12 polarization would cease or Th2-type responses would be favored. Although as yet unproven, our hypothesis is that DNCB-activated DC that arrive rapidly in draining lymph nodes would be producing relatively high levels of bioactive IL-12, whereas DC accumulating in lymph nodes with a delayed kinetics after exposure to TMA may be a source of high IL-10 production and/or lower IL-12. Alternatively, or in addition, early IL-10 release may prime DC for Th2-promoting activities via effects on cell surface molecule expression, although no phenotypic differences between TMA- and DNCB-activated DC were apparent in the somewhat limited range of markers examined in the current investigation.

A supplementary interesting observation was that anti-IL-10 Ab had a small, but reproducible, impact on normal resting skin, stimulating both increased LC migration and lymph node DC accumulation. Under steady-state conditions, epidermal LC have been shown to have a half-life in the skin of some 15 days, although any form of cutaneous trauma (allergen or irritant) causes them to be mobilized to the draining lymph node (44). These data suggest that low levels of constitutively expressed IL-10 in normal skin may act to constrain basal LC migration and are consistent with the observation that IL-10 knockout mice display enhanced allergen-induced LC migration (18).

In previous publications (45), it has been suggested that epidermal LC stimulate polarized type 2 responses following epicutaneous exposure to protein allergens compared with s.c. injection of

the same protein. In these experiments, protein was applied following tape stripping of the stratum corneum, a relatively traumatic procedure that will impact on the local cytokine environment. In contrast, in the current experiments in which chemical allergen was applied to intact skin, LC migration provoked by the chemical contact allergen DNCB leads to the development of a preferential type 1 immune response, suggesting that the intrinsic ability of DNCB to stimulate type 1 responses can override the propensity of LC to stimulate a type 2 response.

These current findings show that classes of chemical allergen that induce polarized T cell subsets following prolonged topical exposure induce characteristic profiles of cutaneous cytokine expression within hours of treatment. Exposure to TMA induces a local cytokine environment (IL-10) that provides for the development of DC with Th2-polarizing activity, whereas treatment with DNCB provokes IL-1 β expression, allowing rapid migration of LC and the development of a type 1 response.

Disclosures

The authors have no financial conflict of interest.

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