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Arsenic mobilizes Langerhans cell migration and induces Th1 response in epicutaneous protein sensitization *via* CCL21: A plausible cause of decreased Langerhans cells in arsenic-induced intraepithelial carcinoma

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ABSTRACT

Arsenic, still a significant environmental threat in several regions in the world, induces various cancers, including lungs, skin, and bladder. Arsenic-induced Bowen's disease (As-BD) is generally an indolent cutaneous intraepithelial carcinoma in susceptible people. Patients with As-BD have been found to have attenuated contact hypersensitivity. Skin samples collected from these patients have reduced numbers of Langerhans cells (LCs), the major epidermal antigen presenting cells expressing Langerins. This study uses an epicutaneous protein sensitization model to investigate the mechanism through which LCs are decreased in As-BD. It further investigates the possibility that arsenic alters LC migration and polarizes Th responses. To do this, we patch-sensitized Balb/c mice or DT-treated Langerin-DTR mice (conditional depletion of Langerin$^+$ cells) with OVA or PBS, and fed them water containing 300 ppb arsenic or regular water for 200 μl for five days. Ninety-six hours after OVA sensitization, Langerin$^+$EpCAM$^+$ cells in arsenic-treated WT mice were significantly increased in draining lymph nodes and decreased in epidermis without changes in the dermis. Lymph node cells from arsenic-treated WT mice were found to proliferate more than lymph node cells from control PBS-treated mice after OVA challenge *in vitro*. They also secreted more IFN-γ and IL-12, but not IL-4, IL-13, or IL-17. However, cell proliferation and the induction of IFN-γ by arsenic were found to be abolished in DT-treated Langerin-DTR mice. The expressions of CCL21 and CXCL12 were also increased in lymph nodes from arsenic-treated WT mice. The administration of a neutralizing antibody against CCL21, but not CXCL12, abolished the increase of LCs in lymph nodes *in vivo*. The results of this study, the first to study oral arsenic polarization of Th1 responses in epicutaneous protein sensitization through CCL21-mediated LC migration, suggest the chronicity of As-BD without invasion might result from enhanced Th1 responses and altered LC migrations by arsenic.
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1. Introduction

Arsenic, one of the twenty most abundant elements in the earth crust, is an important environmental human carcinogen that has been implicated in lung, bladder, skin, and liver cancers. It remains a significant environmental threat in India [1], Bangladesh [2], China [3], Chile, and Argentina, where exposure occurs through drinking contaminated water or burning contaminated coal. In Taiwan, early in sixties, Tseng et al. reported several cancers from people drinking contaminated deep well water in endemic southwestern part of the island [4]. While the consumption of

Abbreviations: As-BD, arsenic-induced Bowen's disease; DTH, delayed-type hypersensitivity; LNs, lymph nodes; DNFB, 2,4-dinitrofluorobenzene; LCs, Langerhans cells.

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contaminated drinking water is the major route of human exposure [4], its use in the production of high technology and industrial semiconductors poses further environmental health hazards [5]. Multiple cutaneous keratosis, Bowen's disease (an intraepithelial carcinoma of the skin), and invasive non-melanoma skin cancers can develop in the skin decades after exposure to arsenic [6].

The most common form of arsenic-induced cutaneous pathologies, arsenic-induced Bowen's disease (As-BD), which is frequently accompanied by inflammatory infiltrates in the underlying dermis, is often confined to the epidermis without penetrating the basement for several decades [7]. Only 1% of the population exposed to arsenic develops classic skin manifestations and internal cancers, while the other 99% of the people do not develop overt pathology even after years of arsenic exposure [8]. This difference in susceptibility suggests the existence of complex interplay between host immune responses and environmental factors in the development of As-BD. T cells have been studied extensively in arsenic-induced Bowen's disease (As-BD) where arsenic has been found to impair T cell proliferation [9,10] and induce apoptosis of CD4+ T cells *via* an autocrine TNF-alpha loop [9,11]. In addition, the Fas ligand expressed by cells from Bowen's disease can induce selective CD4+ T cell apoptosis through Fas–FasL interaction [12].

The role of the epidermal antigen-presenting cell, the Langerhans cell (LC), on As-BD has attracted less attention. Although Langerins can be expressed in both LCs and dermal dendritic cells (DDCs), LCs can be distinguished from DDCs by the presence of EpCAM. LCs are thought to play important roles in skin immunity against the progression of cancer, as studies found that when LCs are depleted *in vivo*, the protective effect of protein vaccinations against melanoma is reduced [13,14]. In DMBA- and UV-induced skin cancer tissues, LCs have found to be reduced in number due to their migration to lymph nodes [15]. In addition, in cervical cancer, a cancer arising from cervical keratinocytes, higher numbers of infiltrating LCs have been associated with longer survival and less dysplasia [16]. LCs and other skin dendritic cells are also important in tumor surveillance, since early in carcinogenesis, LC depletion and modification have been found to allow aberrant cells to proliferate [17,18].

A delayed-type hypersensitivity (DTH) response is a type IV hypersensitivity response that requires the presence of cytokines derived from LCs or other dermal dendrititc cells (DDCs) (such as IL-12 and IL-10) and from T cells (such as IFN-γ, IL-4, and IL-10). Patients with As-BD have impaired DTH response to 2,4-dinitrochlorobenzene (DNCB) as well as a quantitative loss and qualitative impairment of dendrite formation in LCs in the As-BD lesions [19]. Furthermore, we and others have found that, in patients with As-BD, the number of LCs decrease progressively from normal skin areas to lesions [20,21], though the exact role of this decrease in numbers and activation of LCs in As-BD remains elusive.

Analogous to the diminished DTH in humans with As-BD, Balb/c mice exposed to 50 mg/l arsenic in drinking water were found by one study to have a reduction in lymph node cell proliferation and ear swelling following sensitization with 2,4-dinitrofluorobenzene (DNFB) [22]. While that study found a decrease in the numbers of LCs in draining lymph nodes, they did not count the numbers of LCs in epidermis. DTH is the overall result of a dynamic process initiated by epicutaneous sensitization [23]. There may be a difference in immune responses to protein and hapten (ex. DNFB) with regard to intrinsic processing, effectiveness, and clinical implications in tumor immunity. Therefore, this study was designed to investigate whether arsenic affects the epicutaneous protein sensitization and dynamic LCs migration leading to polarized Th responses in the skin and to elucidate the role of LCs in this process. Using a protein epicutaneous patch model to study the dynamic process of epicutaneous sensitization in WT mice or in DT (diphtheria toxin)-treated Langerin-DTR mice in which the Langerin$^+$ cells were depleted, we measured the numbers of LCs in the skin and lymph nodes and the proliferation and polarization of lymph node cells.

2. Materials and methods

2.1. Immunofluorescence exam in arsenic-induced Bowen's disease and in mouse skin

The specimens of arsenic-induced Bowen's disease, all from non-sun-exposed skin, were obtained from 10 patients (5 men and 5 women; 73.5 ± 9.5 years old) with arsenic-induced Bowen's disease from endemic areas in southwestern Taiwan, where artesian water is contaminated by high concentrations of arsenic. The specimens for the non-As-BD group were obtained from patients with biopsy-proven BD who had never lived in the endemic areas (n = 8, 5 men, 3 women; 69.2 ± 8.3 years old). The specimens for the control group were obtained from the non-sun-exposed skin of 10 age-comparable patients (5 men and 5 women; 71.8 ± 9.4 years old) who did not live in the endemic areas and did not have a previous history of cancer. All patients and control subjects provided informed consent before receiving biopsies. This study was approved by the Institutional Review Board of Kaohsiung Medical University. For our animal study, mouse skin was obtained by 2-mm punch biopsy. The tissue sections from both humans or mice were fresh-frozen, fixed in acetone (Sigma, St Louis, MO) at 20 °C for 5 min, air-dried for 5 min, and washed in PBS for 15 min. They were incubated overnight at room temperature in a humidifier with or without the addition of a mouse monoclonal anti-Langerin (1:100) antibody (eBioscience, San Diego, CA) or a rat monoclonal anti-EpCAM (1:200, eBioscience, San Diego, CA). The slides were washed in PBS, and then incubated with either PE-goat anti-mouse IgG or FITC-goat anti-rat IgG (eBioscience, San Diego, CA) for 1 h at room temperature. Nuclei were counterstained with DAPI. Image analysis was performed by NIH Image J, an open image analysis software program (http://rsbweb.nih.gov/ij/) with a plugin that measures the fluorescent intensity score from 0 to 255. Numbers of positive-stained cells were measured and averaged in the epidermis from five random high power fields.

2.2. Mice and reagents

Female Balb/c mice (8–12 weeks old) were obtained from the National Laboratory Animal Center-Tainan Facility (Tainan, Taiwan). Langerin-DTR mice were described previously [24]. Langerin$^+$ cells were depleted in those mice following a previously described method [25]. Briefly, heterozygous Langerin-DTR mice received i.p. injections of 1 μg of diphtheria toxin in 200 μl of PBS 10 days before starting OVA patch. All mice were housed in a specific pathogen-free animal facility and treated following experimental protocols approved by the Animal Care and Use Committee of the Kaohsiung Medical University (Approval Number 96019). OVA (Grade V) was purchased from Sigma–Aldrich (St Louis, MO). Capture and biotin-conjugated detecting antibodies for IFN-γ and IL-5 used in the ELISA were purchased from Pharmingen (San Diego, CA), and streptavidin-alkaline phosphatase was purchased from Southern Biotechnology (Birmingham, AL). The murine IL-13, IL-17, and IL-12 ELISA kits were obtained from R&D Systems (Minneapolis, MN). Antibodies used for flow cytometry were purchased from either Pharmingen (San Diego, CA) or e-Bioscience (San Diego, CA). Antibody-conjugated microbeads used for isolating cells were all purchased from Miltenyi (Bergisch Gladbach, Germany).

2.3. Preparation of epidermal sheets

Back skin was depilated seven days before the attachment of OVA-patch to avoid excessive trapping of dyes in hair follicles. Skin was floated on 3.8% ammonium thiocyanate (Sigma, St Louis, MO) in 100 mM sodium phosphate/100 mM potassium phosphate (both from Sigma, St Louis, MO) for 20 min at 37 °C. Epidermal and dermal sheets were separated and fixed in acetone at −20 °C for 15 min. Epidermal sheets were then incubated overnight at room temperature in a humidifier with or without the addition of a mouse monoclonal anti-Langerin (1:100) antibody (eBioscience, San Diego, CA), followed by PE-goat anti-mouse IgG (1:2000, eBioscience, San Diego, CA) for 1 h at room temperature. Image analysis was performed to measure the numbers of positive-stained cells from five random high power fields by NIH Image J.

2.4. Preparation of single cell suspensions for flow cytometry

Epidermal cells were prepared as described previously with slight modifications [26]. Briefly, back skin was incubated in HBSS (Hanks' Balanced Salt Solution) containing 0.5% trypsin and 1 mM EDTA (all from Invitrogen, Carlsbad, CA) for 45 min at 37 °C. Epidermis was peeled from the dermis and dissociated into single cells using vigorous trituration. To prepare single cell suspensions from dermis, dermal sheets were minced and incubated for 2.5 h in collagenase D (Roche Applied Science, Mannheim, Germany). Single-cell suspensions were prepared from fragments of lymph nodes and spleens after incubation in collagenase D for 30 min at 37 °C. Low density cells were enriched using Histodenz® density gradients (Sigma, St Louis, MO). Single cell suspensions were then stained with antibodies against Langerin and EpCAM (eBioscience, San Diego, CA) for subsequent flow cytometry or immunofluorescence exams.

2.5. Arsenic treatment and immunization

Mice were immunized as previously described [27]. Briefly, 20 μl of OVA (100 mg ml^{-1}) was placed on the disc of a Finn chamber (Epitest, Tuusula, Finland). This disk was applied to an area of shaved skin on the back of a mouse. For each course of immunization, freshly prepared OVA patches were applied on five consecutive days. The arsenic-treated group was fed with 200 μl of arsenic water at 50 ppb or 300 ppb (roughly equal to 0.46 and 2.3 μM, respectively) immediately prior to receiving the patch on days 1–5. The dosages were determined according to previous studies in which high concentrations of arsenic were required to induce biological effects in mice [28,29]. Control mice received a similar treatment but were fed with regular water. For blocking experiments, neutralizing antibodies against CCL21 or CXCL12 (both from R&D, Minneapolis, MN) were administered intraperitoneally 1 μg every day for 5 days before OVA sensitization and arsenic treatment.

2.6. Cytokine determinations in supernatants of lymph node cell cultures

Ten days after the start of the immunization course, mice were sacrificed, and axillary, subscapular, and inguinal LNs were harvested. Pooled LN cells (1×10^6) were cultured in the presence or absence of 100 μg/ml OVA. Cell proliferation was measured using an automatic counter (Countess, Invitrogen, Carlsbad, CA) according to manufacturer's directions. Supernatants were harvested 48 h later and stored at −80 °C. The levels of IFN-γ, IL-5, IL-13, IL-12, and IL-17 were measured by standard sandwich ELISAs. The limit of detection for IL-5, IL-12, and IL-13 was 10 pg ml^{-1}. For IFN-γ and IL-17 it was 50 pg ml^{-1}.

2.7. Total RNA extraction, cDNA preparation, and quantitative real-time PCR

Draining LNs were obtained 96 h after patch immunization. Total RNA from lymph nodes was extracted and the purity of RNA samples with the ratios between the absorbance of 260 nm and 280 nm exceeding 1.7 were stored at −70 °C for further analysis. Using 5.0 μg of RNA to reverse-transcribe the cDNA with the Ready-to-Go reverse transcription quantitative polymerase chain reaction (RT-QPCR) kit (Amersham Biosciences, Uppsala, Sweden), we measured expressions of MCP-1, SDF-1, and CCL21 using a LightCycler TaqMan Master Kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's directions. Each sample was analyzed in triplicate. The relative cytokine expression level was normalized by the relative expression level of β-actin for each sample.

2.8. Flow cytometry analysis

Skin-draining LNs (axillary, subscapular, and inguinal) were excised 96 h after the start of the immunization course. LN cells were prepared by digestion of isolated LNs with 2.5 mg ml^{-1} collagenase for 30 min at 37 °C, and the CD11c+ cells were isolated by anti-CD11c microbeads (Miltenyi, Bergisch Gladbach, Germany). Cells were stained with various combinations of antibodies (CD11c-APC (HL3)), MHC class II-biotin (2G9) (both from e-Bioscience, San Diego, CA), and their isotype controls with or without subsequent addition of streptavidin-FITC. Intracellular staining of anti-Langerin (Langerin)-PE was also performed as previously described [27,30].

2.9. Statistical analysis

The numeric variables between two groups were compared by nonparametric Mann–Whitney *U* test, and ratio variables were compared by Chi-square test. All statistical operations were performed using SPSS version 14 (Chicago, IL). A p value of less than 0.05 was considered significant.

3. Results

3.1. Decreased numbers of Langerhans cells in As-BD

We stained lesions of As-BD with Langerin (CD207) and EpCAM, two better-defined LC markers, to identify LCs in epidermis by immunofluorescence exam. $Langerin^+EpCAM^+$ cells with dendrites in the epidermis of normal skin were clearly evident (14.3 ± 3.3 double positive cells per high power fields (HPF)), though there were significantly fewer numbers of double positive cells per HPF in As-BD (8.1 ± 3.9) than in normal control skin. In contrast, the numbers of LCs in non-As-BD (13.6 ± 4.2 double positive cells per HPF) appeared to be similar to those in normal skin (Fig. 1).

3.2. Reciprocal quantitative changes of Langerhans cells in the epidermal sheet and lymph nodes from mice treated with arsenic

To simulate the arsenic oral exposure in patients with arsenic-induced Bowen's disease, we fed Balb/c mice arsenic (200 μl) at 300 ppb per day for 5 days and then introduced OVA using an epicutaneous patch. After 96 h, CD11c+ cells were isolated from epidermal sheets, draining lymph nodes, and spleen. $Langerin^+$ LCs in the epidermal sheet were identified and measured by a fluorescence microscopy (Fig. 2A1–4). Mice fed with arsenic-containing water and patched with PBS had minimal changes in the numbers of $Langerin^+$ cells (113 ± 25 per HPF, Fig. 2A1) compared with those fed regular water and patched with PBS

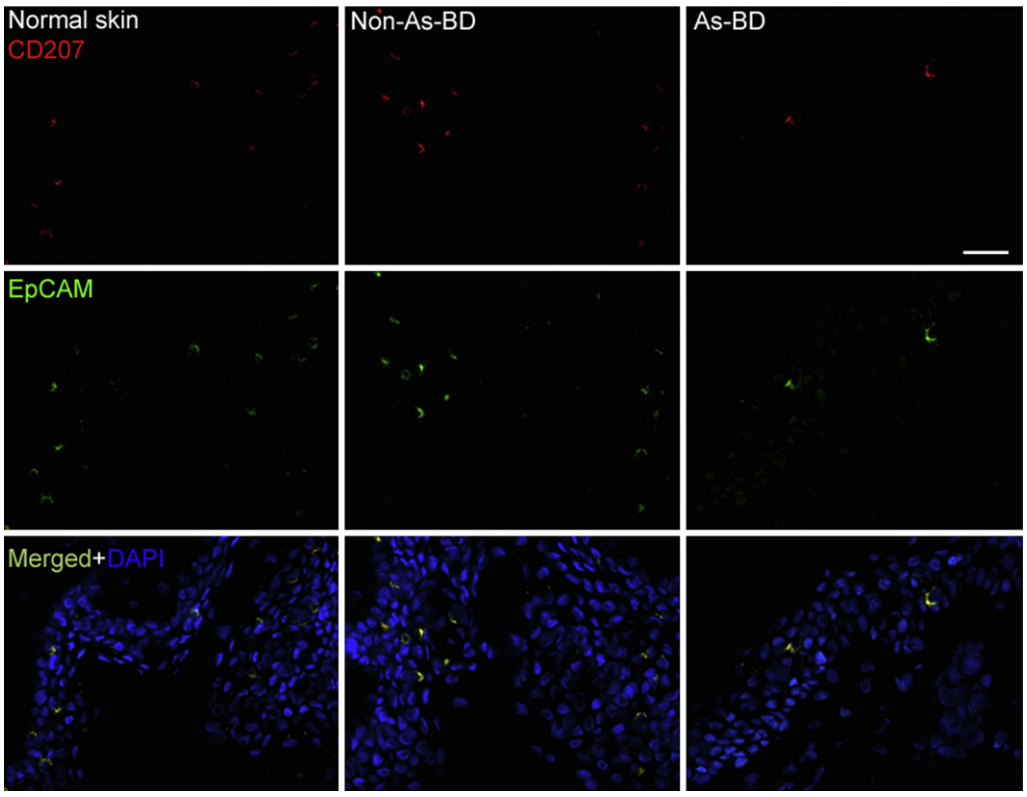

Fig. 1. Langerhans cells (LCs) are decreased in numbers in skin of arsenic-induced Bowen's disease. LCs were stained with (CD207/Langerin) (red), EpCAM (green), and DAPI for nucleus (blue). (n = 10, 10, and 8 in controls, As-BD, and non-As-BD, respectively; representative images are shown, scale bar = 20 μm.)

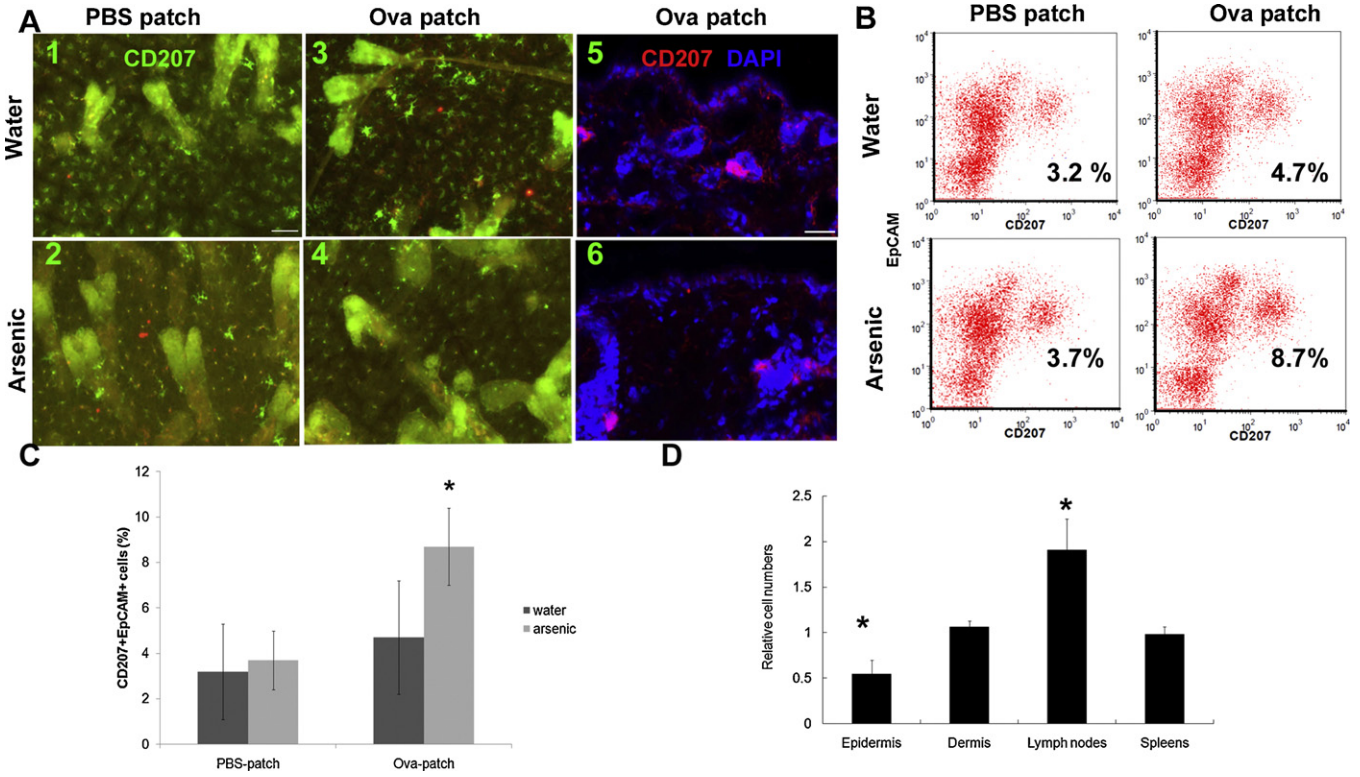

Fig. 2. Reciprocal changes in numbers of LCs in the epidermal sheet and lymph nodes from mice treated with arsenic. (A) Mice were fed with arsenic-containing water (2,4,6) or regular water (1,3,5) and patched with PBS (1,2) or OVA (3,4,5,6) on the back on 5 consecutive days. Epidermal sheets from patched skin on the back were stained by CD207 (green, in horizontal sections, 1,2,3,4, n = 5, representative data was shown, scale bar = 20 μm) and CD207 (red, in vertical sections, 5, 6, n = 5, representative data was shown, scale bar = 20 μm). Single cell suspensions were obtained in the lymph nodes 96 h after OVA sensitization. By flow cytometry, percentage of Langerin and EpCAM positive cells among CD11c-magnetic-sorted cells in lymph nodes is shown (B, representative data) and quantified (C) (n = 5, $^*p < 0.05$). (D) Quantified data from 5 experiments from arsenic-fed OVA-patch mice that was normalized with mice patched with OVA and fed with water (A). Y axis of relative cell numbers represents the ratio of percentages of positive cells from single cell suspensions from arsenic-fed OVA-patch mice to those from water-fed OVA-patch mice. *The significant differences compared to baseline level at $p < 0.05$.

(103 ± 36 per HPF, Fig. 2A2). Of note, while OVA patch alone substantially increased Langerin$^+$ cells in epidermal sheets (193 ± 12 per HPF, Fig. 2A3), arsenic feeding abolished the increase of Langerin$^+$ cells in mice fed arsenic and patched with OVA (121 ± 15 per HPF, Fig. 2A4). Vertical sections were used to confirm our findings that Langerin$^+$ cells were decreased in the epidermis (Fig. 2A5 and 6). Likewise, OVA-induced increases of Langerin$^+$ cells (18 ± 5 per HPF, Fig. 2A5) in the epidermis were abolished in mice fed with arsenic and patched with OVA (8 ± 2 per HPF, Fig. 2A6).

3.3. Increases of Langerin$^+$EpCAM$^+$ cells in draining lymph nodes but not in dermis

A reciprocal change in the numbers of LCs was found in skin draining lymph nodes (Fig. 2B). The mice fed with arsenic and patched with PBS showed minimal changes in the numbers of Langerin$^+$EpCAM$^+$ cells compared with those fed regular water and patched with PBS (3.2 ± 1.7% *vs.* 3.7 ± 1.2%, respectively; Fig. 2B and C). OVA patch alone significantly increased numbers of Langerin$^+$EpCAM$^+$ cells in lymph nodes (4.7 ± 0.6%, Fig. 2B and C). Of note, the lymph nodes of mice epicutaneously sensitized with OVA and fed with arsenic-containing water contained more Langerin$^+$EpCAM$^+$ cells in mice fed with arsenic-containing water than in those fed with regular water and patched with OVA (8.7 ± 0.8% *vs.* 4.7 ± 0.6%, $n = 5$, Fig. 2B and C). In contrast to Langerin$^+$EpCAM$^+$ cells, there were only a few Langerin$^+$EpCAM$^-$ cells (dermal dendritic cells) present in the lymph nodes in all the experimental groups (Fig. 2B). Cell numbers of positive cells from single cell suspensions in epidermis, dermis, lymph nodes, and spleens, from mice patched with OVA and fed with water were normalized with those from mice patched with OVA and fed with water (Fig. 2D). In the dermis, after OVA-patch sensitization, there was no difference between relative cell numbers of Langerin$^+$EpCAM$^+$ cells in arsenic-fed groups and those fed regular water (Fig. 2D). There was no difference in numbers of Langerin$^+$MHCII$^+$ cells in the spleen the arsenic-fed mice and those fed regular water (Fig. 2D). Therefore, the reciprocal changes in the numbers of Langerin$^+$EpCAM$^+$ cells in the skin and in the lymph nodes suggested that LCs migrated from skin to lymph nodes in arsenic-fed WT mice.

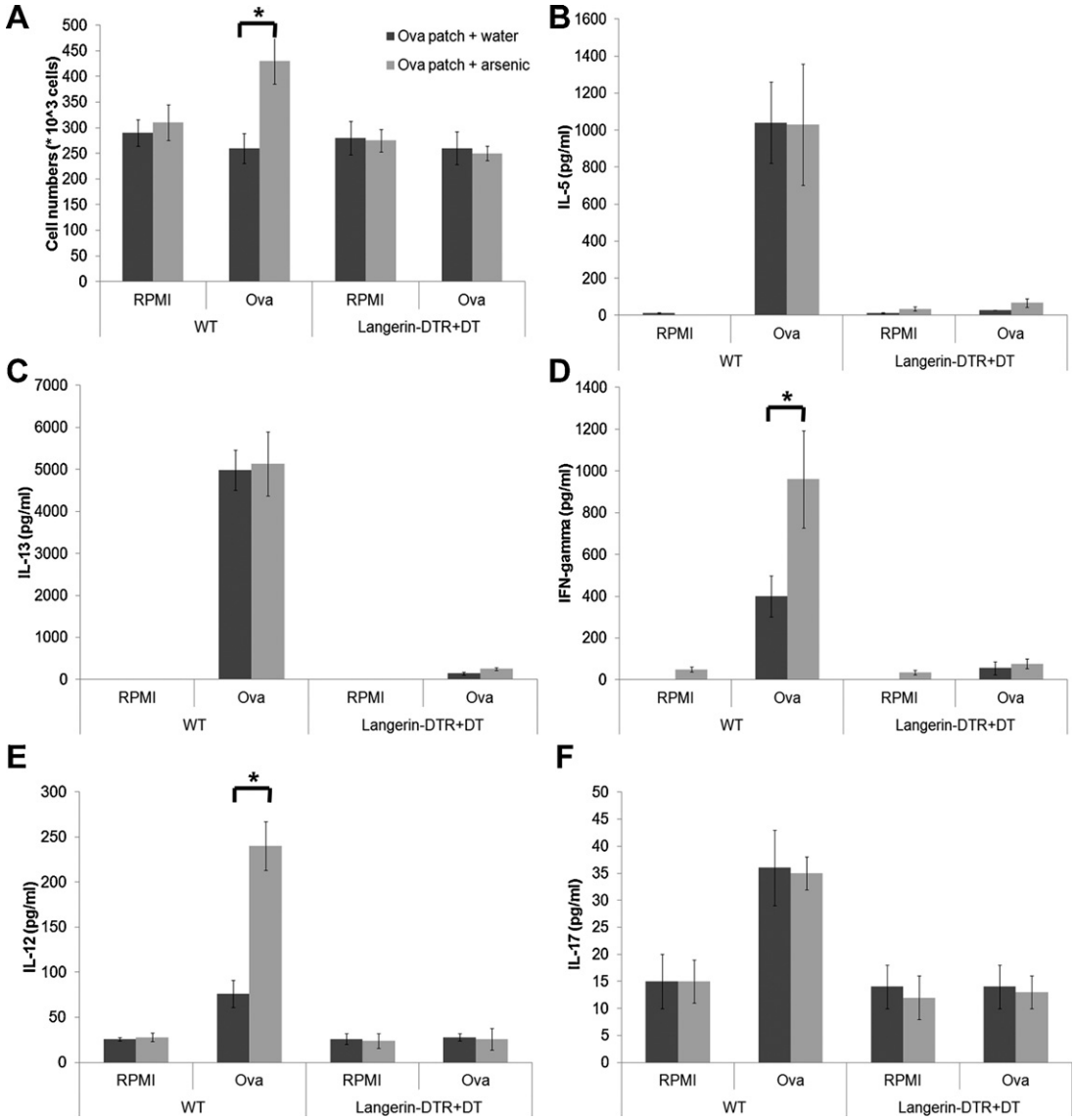

Fig. 3. Enhanced cell proliferation and IFN-γ production by OVA challenge from cells of draining lymph nodes from WT mice but not those from DT-treated Langerin-DTR mice. For conditional depletion of Langerin$^+$ cells, heterozygous Langerin-DTR mice received i.p. injections of 1 μg of diphtheria toxin in 200 μl of PBS ten days before OVA patch. Cells were isolated from mice patched with OVA with or without arsenic feeding. Those cells were challenged with OVA *in vitro* and total cell counts (A) were measured by Trypan blue-exclusion assays by an automatic counter. IL-5 (B), IL-13 (C), IFN-γ (D), IL-12 (E), and IL-17 (F), were measured from the supernatant by ELISA ($n = 3$, $^*p < 0.05$).

3.4. The enhancement of T cell proliferation and IFN-γ production in lymph node cells of OVA-sensitized and arsenic-fed mice was dependent on the presence of Langerin-expressing cells

To evaluate LC function and T cell proliferation *in vitro*, we divided Balb/c mice into two groups, one patched with OVA and fed arsenic for five successive days and the other also patched with OVA but fed regular water for the same amount of time. For the *in vitro* proliferation assay, lymph node cells were challenged by OVA and cell proliferation and cytokines were measured. We found an increased proliferation of lymph node cells in mice fed with arsenic (Fig. 3A). The cytokine contents of the lymph node cell supernatants after *in vitro* stimulation with OVA were chosen as indicators of Th immune response, with IL-5 and IL-13 representing the Th2 immune response, IFN-γ and IL-12 representing the Th1 immune response, and IL-17 representing the Th17 immune response. As expected, epicutaneous sensitization with OVA induced a predominant Th2 and a weak Th1 immune response in the control group, as indicated by high IL-5 and IL-13 levels as well as low IFN-γ contents in the culture supernatants from WT mice (Fig. 3B–D). The arsenic-treated group had significantly higher IFN-γ and IL-12 levels in the supernatant and higher cell counts than the control group (Fig. 3D and E). Arsenic treatment, however, did not affect Th2 or Th17 cytokines, as IL-5, IL-13, and IL-17 concentrations from the arsenic-treated group were similar to those of the control group (Fig. 3B, C, and F), suggesting that arsenic exposure may augment Th1 response induced by epicutaneous sensitization with protein antigen in Balb/c mice without modulating the Th2 or Th17 responses. Since DDCs (Langerin$^+$EpCAM$^-$ cells) were barely present in the lymph nodes in WT mice fed with OVA, we postulated the enhancement of cell proliferation and Th1 polarization might result largely from LCs. To find out, we treated some Langerin-DTR mice with diphtheria to deplete Langerin$^+$ cells. This would also deplete DDCs. Other mice were left untreated. We then repeated the same experiments that we performed using Balb/c WT mice. The untreated Langerin-DTR mice had similar cell proliferation and cytokine production patterns as the Balb/c WT mice in the previous experiment (data not shown), and the treated Langerin-DTR mice were found significantly diminished arsenic-induced cell proliferation and cytokine production (Fig. 3). These findings suggest that enhancement of T cell proliferation and IFN-γ production in the lymph node cells of OVA-sensitized and arsenic-fed mice was mediated, at least in part, by LCs.

3.5. Effects of LC depletion in arsenic-mediated reduction of LCs in epidermal sheets from OVA-patched mice

To confirm whether DT had adequately depleted LC in the Langerin-DTR mice, we stained the epidermal sheets collected from WT and Langerin-DTR mice treated or untreated with DT after OVA epicutaneous sensitization. Regardless of the presence of DT, Langerin$^+$ cells were confirmed to be reduced in WT mice fed with arsenic water (Figs. 2A3 and A4 and 4). Langerin-DTR mice that had not been treated with DT had the same staining pattern as the WT mice. However, in those treated with DT, there was about a 75% depletion of LCs in epidermal sheets collected from mice patched with OVA. Those treated with arsenic had a further depletion of Langerin$^+$ cells (Fig. 4B, quantified data from Fig. 4A). The data confirmed that the Langerin$^+$ cells had been adequately depleted.

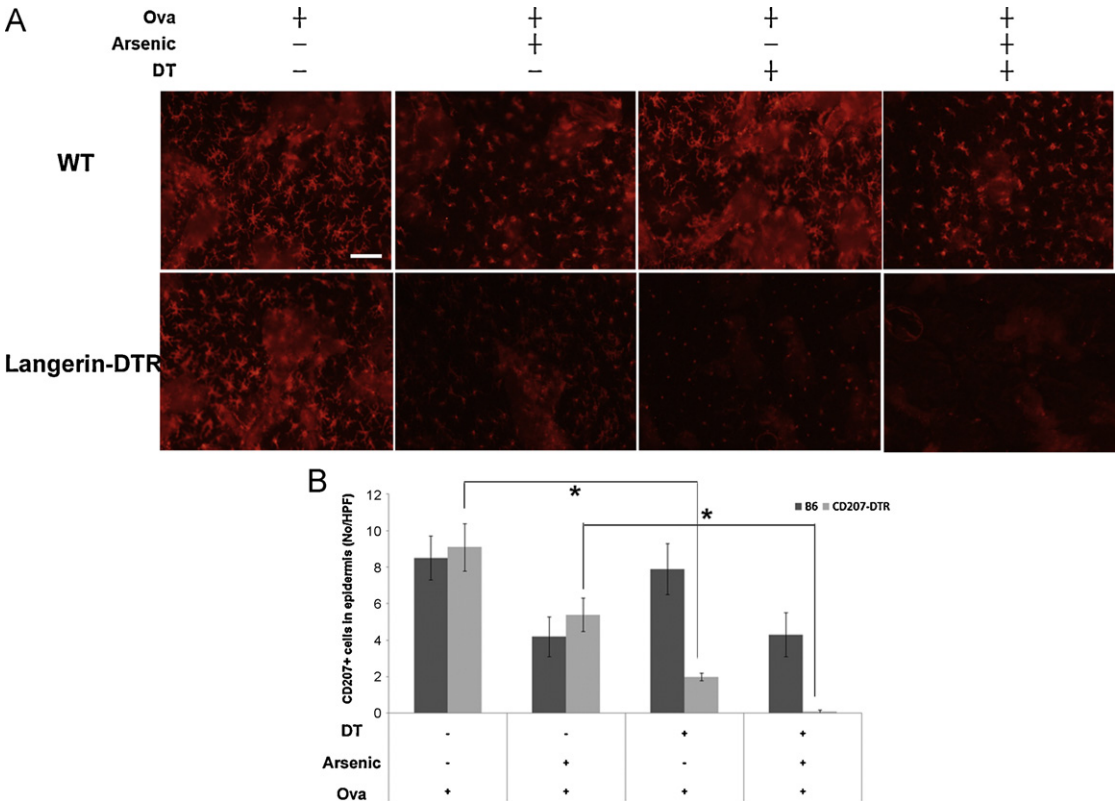

Fig. 4. Arsenic augmented the depletion of LCs in the epidermis from DT-treated Langerin-DTR mice in the process of OVA epicutaneous sensitization. Heterozygous epidermal sheets from patched skin on the back were stained by Langerin (red) (n = 5, representative data shown, A, scale bar = 20 μm). Numbers of Langerin$^+$ cells were measured and averaged per five high power fields (HPF). The quantifications are summarized (B).

3.6. Induction of CCL21 (SLC) and CXCL12 (SDF-1) chemokines but not MCP-1 (CCL2) in lymph nodes from arsenic-treated mice

We wanted to know if the arsenic-enhanced migration of LCs into the regional lymph nodes was facilitated through increased chemokine expression in the lymph nodes. To find out, we harvested the lymph nodes from arsenic-treated and control mice, and measured the expression of specific chemokines: CCL21 (SLC), the major chemokine involved in LC migration from skin to the lymph nodes; CXCL12 (SDF-1), a chemokine also able to attract LCs; and CCL2 (MCP-1), a strong chemokine for monocytes and macrophages, but not LCs or other dendritic cells. We treated the lymph nodes of control mice with 0.1 and 0.5 μM of arsenic *in vitro* for 96 h. We found an increase in CCL21, CCL2, and CXCL12 after treatment both concentrations 0.1 and 0.5 μM (Fig. 5A). *In vivo* experiments showed a significant induction of CCL21, CXCL12 and CCL2 in lymph nodes of the arsenic-fed mice. CCL21, in particular, was upregulated 2.5–3 fold in mice treated with arsenic at 300 ppb (Fig. 5B). These results suggested that enhancement of LC migration into regional lymph nodes may be mediated by CCL21 and CXCL12, at least in part, in the lymph nodes of arsenic-fed mice (Fig. 5B). Thus, we wanted to know whether the enhanced migration of LCs to lymph nodes was mediated by CCL21 or CXCL12. Neutralizing antibodies (1 μg) against CCL21 or CXCL12 were administered intraperitoneally to the mice every day before OVA patch attachment and arsenic treatment. Neutralizing antibody against CCL21 abolished the increase of LCs in the lymph nodes, but treatment with neutralizing antibody against CXCL12 did not (Fig. 5C), revealing that arsenic mobilized LC migration in OVA epicutaneous sensitization through CCL21.

4. Discussion

This study found that arsenic enhanced the cell proliferation and polarized Th1 immune response induced by epicutaneous sensitization with a protein antigen. This process was dependent, at least in part, on the presence of Langerhans cells (Fig. 6). Arsenic treatment increased LCs in the regional lymph nodes and decreased LCs in the epidermis in the OVA epicutaneous sensitization. Neutralizing antibody against CCL21 abolished the increase of LCs in draining lymph nodes, suggesting that arsenic-induced LC migration to lymph nodes is mediated by CCL21. Although one study has shown that arsenic enhances the generation of several chemokines related to monocyte/macrophage lineages, CXCL2, CCL22, and CCL2 [31], the current study is the first to show that arsenic can induce CCL21, a LC-relevant chemokine. To our knowledge, this effect of arsenic on LCs with distinct Th responses in protein allergen sensitization has not been reported previously.

Most investigations of arsenic effects on contact hypersensitivity have been conducted using chickens [32], rats [33], or B6 mice [34]. While some studies report a decrease in contact hypersensitivity reaction as a result of exposure to arsenic, others have found no appreciable decrease. The discrepancy among those studies might result from differences in species, contact sensitizers, routes of arsenic administration, and duration of exposure and dosage of arsenic. A contact hypersensitivity response requires cytokines derived from LCs and/or other skin dendritic cells (such as IL-12) and from T cells (such as IFN-γ, IL-4, and IL-10). A diminished hypersensitivity response in the arsenic-treated animal might result from the inhibition of any of the components involved in the initiation and progression of the contact hypersensitivity response. For example, patients with As-BD have been found to have impaired T cell activation and proliferation and increased T cell apoptosis [11,19]. In addition, Patterson et al. reported that arsenic is able to modulate DTH responses, which have been associated with reduced migration of LC to lymph nodes [22] in Balb/c mice. Our finding that it may be increased by arsenic might result from several factors. First, their study used MHCII positive cells as

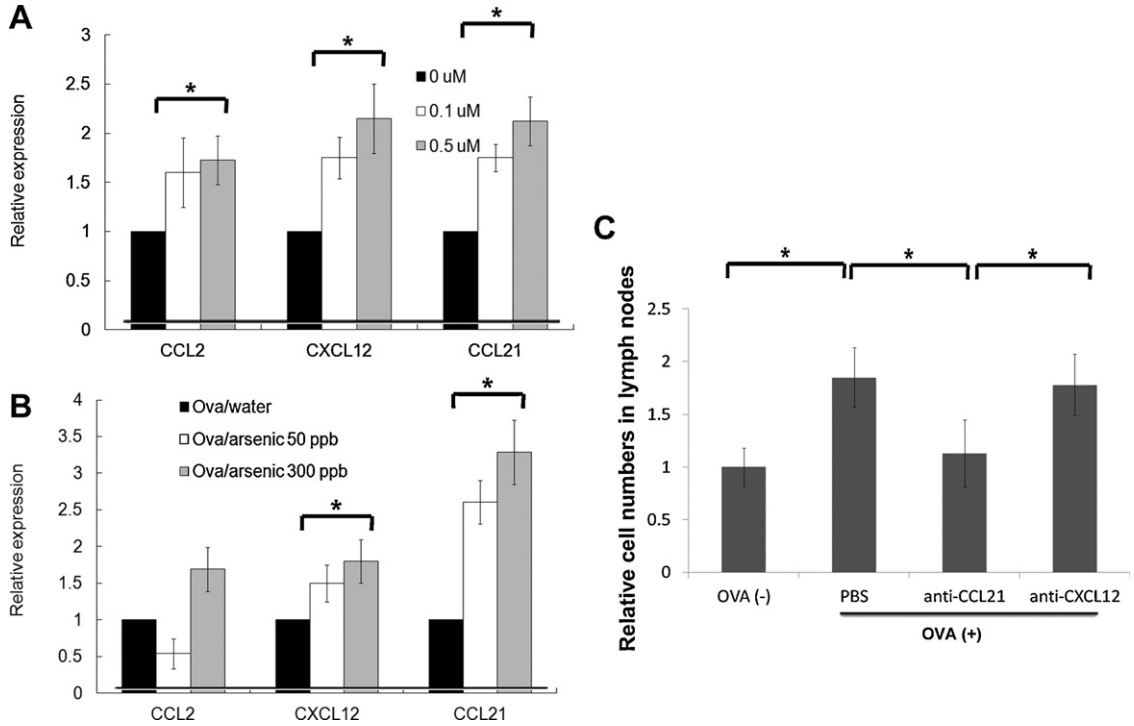

Fig. 5. CCL21 mediated the increase of LCs in lymph nodes from mice treated with arsenic. (A) Arsenic at 50 and 300 ppb in 200 μl was given to mice. Lymph nodes were isolated and real time PCR was run to compare the relative gene expressions of CCL2, CXCL12, and CCL21 ($n = 5$). The horizontal bar reflects the expression levels of the chemokines from mice without OVA patch with or without arsenic. (B) Cells from lymph nodes of control mice were treated with arsenic *in vitro* at 0.1 and 0.5 μM. Relative expression was normalized with baseline level by real time PCR ($n = 3$). The horizontal bar reflects the expression levels of the chemokines from mice without OVA patch with or without arsenic. (C) In mice treated with neutralizing antibody against CCL21 or against CXCL12 *via* intraperitoneal injection, the increase in percentage of LCs in the lymph nodes by arsenic was abrogated in the presence of anti-CCL21 ($n = 3$, $^*p < 0.05$).

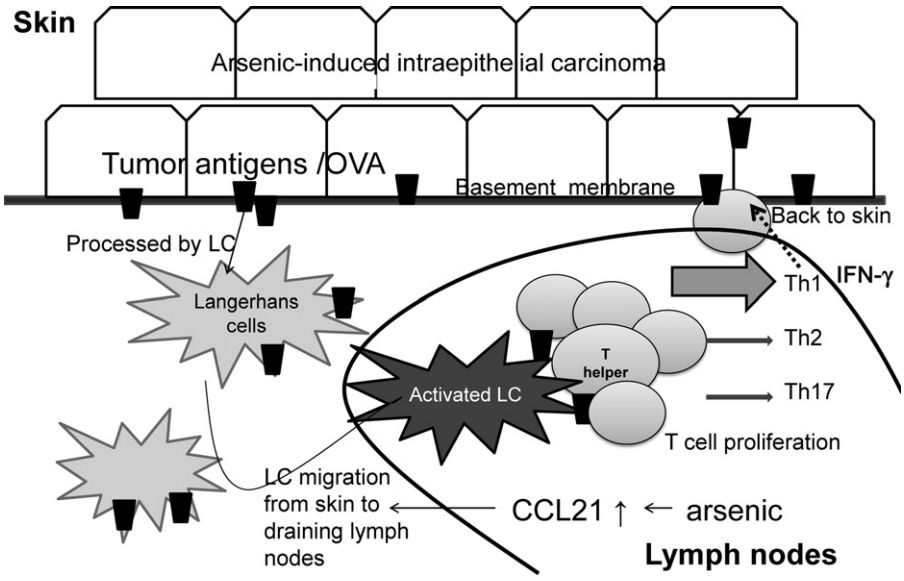

Fig. 6. Arsenic potentiated T cell proliferation and Th1 polarized response in epicutaneous protein sensitization through enhancement of Langerhans cell migration and CCL21 upregulation. This process was dependent at least in part on the presence of Langerhans cells. The epicutaneous sensitization by OVA served as a surrogate marker for tumor antigens. The increased IFN-gamma production in the OVA epicutaneous sensitization in As-treated mice might explain chronicity and indolent nature of As-BD without dermal invasion despite the moderate inflammatory infiltrate underneath.

indicators of LCs, while we used Langerin and EpCAM positive cells to indicate the presence of LCs. Langerin, which has been linked to the Birbeck granule, is a more specific marker of LCs than MHCII and CD3. EpCAM can also be used to distinguish Langerin$^+$ DDCs from LCs. Second, their study used hapten sensitization by DNFB painting and ours used OVA epicutaneous sensitization. Because tumors tend to express certain protein antigens, not haptens, epicutaneous sensitization by protein antigen may serve as a more relevant model in mice for patients with tumors and in arsenic-induced Bowen's disease.

In this study, Langerin$^+$ cells were identified as epidermal LCs in lymph nodes as commonly recognized [35]. However, recently several groups reported that a subset Langerin+ DC population located in the dermis had different migratory kinetics from those of epidermal LCs [36,37]. Dermal Langerin+ DCs arrive at draining nodes significantly earlier than epidermal LCs after epicutaneous sensitization (24 h *vs.* 96 h post-immunization) [30,38]. Because we measured Langerin$^+$ cells 96 h post epicutaneous sensitization with OVA, our measurement of Langerin$^+$ cells may better indicate epidermal LCs. Our study confirmed this by a finding of barely detectable Langerin$^+$EpCAM$^-$ cells harvested 96 h after OVA patch.

An important chemoattractant to LCs, CCL21 is produced by fibroblastic reticular cells of the T-cell-rich area and by high endothelial venules in the lymph nodes, which may explain the reason that arsenic induced a higher increase of CCL21 *in vivo* than in the isolated lymph node cells (preferentially lymphoid cells) *in vitro*. Arsenic is known to enhance the generation of several chemokines related to monocyte/macrophage lineages, CXCL2, CCL22, and CCL2 [31]. Until this study, arsenic had not been reported to induce the expression of CCL21, a LC-relevant chemokine.

In this study, the LCs were decreased in arsenic-induced intraepithelial carcinoma. Similarly, in vulva intraepithelial carcinoma, there is a reciprocal change in numbers of CD1a+ Langerhans cells and the grade of vulvar intraepithelial carcinoma [39]. In cervical intraepithelial carcinoma, infection with human immunodeficiency virus further decreases the numbers of LCs that inversely correlate with the cancer grade [40]. The association of S-100-positive LCs coupled with changes in cervical inflammation suggests that LCs play an important role in the development of an anti-HPV response and the progression of the intraepithelial carcinoma [41]. The similarity in LC shift found in the progression of HPV-induced and arsenic-induced intraepithelial carcinomas suggest that viral and chemical carcinogenesis are similar in that aberrant LC immune responses are involved in both.

T helper polarization is important to the determination of appropriate and effective immune responses. However, how arsenic affects T helper polarization is seldom addressed. In cardiac transplants, intraperitoneal arsenic treatment at 1 mg/kg causes an enhanced graft survival and upreguation of TGF-β, downregulation of TNF-alpha and interferon-γ without significant changes in IL-2 and IL-10 levels in Th1 prone C57BL mice [42]. In our study, when fed with oral arsenic at 300 ppb, Balb/c mice, which are Th2 prone, had an increase in IFN-γ production, but no changes in Th2 cytokines when cells from lymph nodes were re-challenged with OVA. The differences in polarization might result from different mouse strains used, route of administration, and/or the dosage given. In fact, the enhancement of T cell proliferation and Th1 polarization in arsenic-fed OVA-patched mice is dependent at least in part on the presence of LCs, since Langerin$^+$ cell depletion abolishes the two processes. This study is the first to address Th1 polarization by oral arsenic treatment in epicutaneous protein sensitization.

Arsenic can directly induce IFN-γ production in various cells [43], including peripheral lymphocytes [11]. In the lymph nodes, Th1 cells produce IFN-γ. The mechanisms through which arsenic induces Th1 cells to produce IFN-γ are unclear, though one study suggested that arsenic might induce the production of IFN-γ through enhancer-promoter of either metallothionein-I or heat shock proteins [43]. LCs can induce CD8 T cells to produce IFN-γ through a CD70-dependent pathway [44]. Thus, the increase of LCs in the lymph nodes mediated by arsenic may be also involved in the regulatory process leading to the increase of IFN-γ production in arsenic-treated Balb/c mice.

Enhanced IFN-γ production has been associated with the regression of basal cell carcinoma [45] and malignant melanomas [46]. IFN-γ induces the expression of HLA-DR in squamous cell carcinomas and keratinocyte cell lines [47]. In addition to enhancing Th1 responses, arsenic enhances immune responses by also deleting the precursors of suppressor T cells from normal spleen cells [48]. Thus, an enhancement of IFN-γby arsenic may

actually promote the regression of arsenic-induced Bowen's disease and explain its chronicity. Our data showed a reciprocal decrease in epidermal LCs and an increase of LCs in the lymph nodes but no appreciable changes of LCs in the dermis of arsenic-treated OVA-patched mice, suggesting that arsenic may alter the LC dynamics and trafficking from skin to cutaneous draining lymph nodes. UVB, another skin carcinogen, also depletes LCs from the epidermis while causing the LC migration to lymph nodes [18]. In UVB-induced lupus erythematous lesions, there is a skewing of cytokines toward Th1 [49]. Another skin carcinogen, human papilloma virus, also depletes LCs in the epidermis [50]. Interestingly, the density of LCs in the epidermis has been successfully restored with IFN-γ treatment [34]. Thus, a common early defense mechanism in skin carcinogenesis might involve the triggering of LC migration and appropriate Th1 cell responses.

5. Limitations

This study has some limitations. First, there is no known "one-hit" arsenical skin cancer mouse model to date. Thus, it is difficult to investigate if LCs indeed play a role in the tumor immunity of arsenical cancers based the current study design. Second, both LCs and DDCs share Langerin as a common marker, DT-treated-Langerin-DTR mice have depletions in both populations, making it difficult to distinguish the effects of LCs and DDCs in epicutaneous sensitization based on this conditional knock out model.

6. Conclusion

We concluded that arsenic induces a Th1 response in the epicutaneous protein sensitization process and enhances LC migration by upregulation of CCL21 in the skin draining lymph nodes. This process is dependent at least in part on the presence of LCs. These findings may explain the indolent course of arsenic-induced Bowen's disease by Th1 response and suggest Th1 responses may be targeted using such drugs as imiquimoid for the treatment of premalignant arsenical skin cancers.

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