Age-Related Skin Inflammation in A 2,4-Dintrochlorobenzene-Induced Atopic Dermatitis Mouse Model

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Abstract
One of the most affected aspects of the aging process is immunity, with age-related immune system decline being responsible for an increase in susceptibility to infectious diseases and cancer risk. On the other hand, the aging process is accompanied with low-grade pro-inflammatory status. This condition involves a persistent rise in cytokine levels that can activate both innate and adaptive immune systems. Finally, despite the fact that immunological responses to antigenic stimulations decrease with age, the incidence and prevalence of many common autoimmune diseases increase in the elderly population. Overall, the co-existence of a prolonged, low-grade inflammatory status and declining immune activity appears to be a paradoxical phenomenon. This study characterized skin inflammation in mouse dermatitis model of various ages to monitor possible changes of inflammatory responses during aging.

Keywords: Aging, Dermatitis, Immune System, Inflammation

Citation: Cho KA, Kwon JY, Kim HJ, Woo SY. Age-related skin inflammation in a 2,4-dintrochlorobenzene-induced atopic dermatitis mouse model. Cell J. 2023; 25(9): 660-664. doi: 10.22074/CELLJ.2023.2001403.1301
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The aging process is accompanied with low-grade pro-inflammatory status (1). Inflammation is essential to health, helping organisms fight with the invasion of pathogens and playing essential roles in organ repair and maintenance (2, 3). Transient inflammation that increases when needed and decreases when no longer necessary is not associated with long-term adverse consequences. However, prolonged inflammation due to the intrinsic immune system dysregulation or the presence of a persistent inflammatory reaction trigger can result in accumulated damage that eventually manifests as pathology (3). Inflammation is accompanied by elevated levels of cytokines that are capable of activating both the innate and adaptive immune systems. On the other hand, aging of the immune system is termed immunosenescence (4). This phenomenon results in the remodeling of lymphoid organs, leading to immune dysfunction among elderly (5). Moreover, this age-related immune system decline affects both innate and adaptive arms of the immune system (6) and increases susceptibility to infections as well as the risk of cancer (7). In addition, the incidence and prevalence of many common autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus increase among older population, despite declining immunologic responses to antigenic stimuli (8). This might be due to prolonged low grade inflammation that is a common characterization of aging. This state of chronic inflammation that correlates with aging is sometimes referred to as "inflamm-aging" and is a strong risk factor for the occurrence, progression, and complications of many chronic diseases (9). Overall, the co-existence of a prolonged, low-grade inflammation and weak immune activity appears to be a paradoxical phenomenon in elderly (10).

Our study is one of the first studies aimed to analyze immune characteristics associated with aging in a mouse model of dermatitis. We used antigenic stimulation to induce skin inflammation, mimicking atopic dermatitis (AD), and expanded our immunological analysis to the adaptive immunity. AD, a chronic and persistent inflammatory disease of the skin, which is characterized by eczema lesions and itching, has become a serious health problem (11-13).

To induce an AD-like condition in mice, we used the cutaneous application of 2,4-dinitrochlorobenzene (DNCB). DNCB is a chemical substance that causes chronic contact dermatitis and is widely used in human studies of AD (14-17). Mechanistically, it is generally thought that upon topical application, DNCB can complex with various skin proteins to form covalent conjugates and thereby function as immunogen(s) that activate local APCs, such as skin Langerhans cells, dermal dendritic cells, macrophages, and T cells (18-20). Approximately twenty-four hours after subsequent exposures to DNCB (often referred to as "challenges"), the visible inflammatory symptoms begin to appear (21). Thus, DNCB is a useful chemical to simply mimic AD-
like skin dermatitis.

Specifically, 7-, 25-, and 39-week-old C57BL/6 mice (n=5/each group) were sensitized by topical application of 1% DNCB dissolved in an acetone: olive oil mixture (4:1 vol/vol) on the shaved back skin for 3 consecutive days. After 5 days, 1% DNCB dissolved in a PBS:olive oil mixture (9:1 vol/vol) was applied to the back skin for 2 consecutive days. Normal control mice were treated with the vehicle. A variety of mouse ages were adopted to compare the responses based on aging. All procedures were approved by the Ewha Womans University College of Medicine Animal Care and Use Committee (Ewha MEDIACUC 22-008-t). The day after the last application of DNCB or vehicle, the mice were sacrificed and their skin and spleen tissues were collected. On day 9, the dorsal skin of the mice sensitized with DNCB showed prominent erythema, edema, excoriation, and scaling/dryness compared with the dorsal skin of the mice treated with vehicle, indicating that DNCB efficiently induced an AD-like phenotype (Fig.1A). Although the visible extent of skin inflammation appeared similar across the different age groups, the oldest DNCB-treated mice had a higher mortality rate than the younger DNCB-treated mice, 7 weeks old DNCB-treated mice and 25 weeks old DNCB-treated mice, which was correlated with the extent and duration of weight loss, as shown in Figure 1B, C. The oldest DNCB-treated group (n=5) experienced greater than 20% weight loss, whereas the other DNCB-treated mice (7 and 25 weeks of age, n=5/each group) experienced less than 10% weight loss. Differences in body weight recovery were also noted across age groups. The 7 weeks old DNCB-treated mice regained their previous body weight more rapidly than the 25 weeks old DNCB-treated mice and 39 weeks old DNCB-treated mice. 25 weeks old DNCB-treated mice and 39 weeks old DNCB-treated mice revealed similar rate of regaining their basal weights (Fig.1C).

Histological examination of hematoxylin and eosin (H&E)-stained sections from the AD-like skin lesions obtained from DNCB-treated mice revealed epidermal and dermal hyperplasia, reflecting a hyper proliferative state compared with skin sections obtained from vehicle-treated mice. Also, hyperkeratosis and parakeratosis were observed in skin sections from DNCB-treated mice regardless of their age (Fig.2A). We observed the dermal accumulation of mast cells via toluidine blue stain in DNCB-treated mice at all ages and compared them to the vehicle-treated control mice, with the oldest mice showing the highest level of accumulation (Fig.2B, C). We further observed CD3+ T cell infiltration in AD-like skin lesions by immunohistochemistry using anti-mouse CD3 antibody (Santa Cruz Biotechnology Inc., USA).

![Fig.1: Survival course and weight change in DNCB-induced AD mice model at different ages.](image-url)
Interestingly, the oldest DNCB-treated mice displayed the highest amount of accumulated CD3⁺ T cells in inflammatory skin lesions across all ages, particularly in areas characterized by hyperkeratosis and parakeratosis (Fig. 3A, B). Next, we measured the splenic expression of CD3 in each experimental mouse via quantitative reverse transcription-polymerase chain reaction using a StepOnePlus instrument (Applied Biosystems, USA) to investigate whether the increased expression of CD3 in the skin tissue is associated with an expression in CD3 in the spleen.

As shown in Figure 4, DNCB treatment led to decreased CD3 expression at all ages compared with that in the vehicle treatment group. Our results proposed age-dependent dynamics of immune cells, including T cells and mast cells, under certain inflammatory conditions. It is thought that the pathogenesis of DNCB-induced AD-like skin inflammation is predominantly the result of T cell-mediated immune responses (18). In our study, we also observed accumulation of T cells in the skin of mice treated with DNCB, and this phenomenon was particularly prominent in the oldest mice. Specifically, the decreased expression of CD3 in the spleen suggests that T cells may have migrated to the skin. These results suggest that DNCB treatment induces activation and migration of T cells, particularly eliciting a stronger response in the immune system of aged mice. Characterizing T cell infiltration in skin lesions and their effects on inflammatory processes may contribute toward identifying specific immune networks that are altered during aging. Although immunosenescence is thought to accompany aging, our results showed an active immune response in aged mice, which might be associated with mortality. If we can understand immune cell dynamics during aging, we may be able to support appropriate immune responses.

Fig. 2: Histological characteristics of DNCB-induced AD mice model at different ages. A. H&E-stained sections of the back skin from vehicle- or DNCB-treated mice at each age. B. Toluidine blue-stained sections of the back skin sections from vehicle- or DNCB-treated mice at each age showed different amounts of mast cells in the dermis, as indicated by the arrowheads. Fields were taken using the Olympus DP71 and the DP controller software (Tokyo, Japan). C. Dermal mast cells from each experimental mouse were counted in toluidine blue-stained sections. Statistical significance was determined by t test and data are presented as the mean ± SEM. DNCB; 2,4-dinitrochlorobenzene, AD; Atopic dermatitis, w; Week, *; P<0.05, and **; P<0.01.
Fig. 3: The skin expression of CD3 in DNCB-induced AD mice model at different ages. A. Immunohistochemical staining for CD3 was performed on the posterior skin of control vehicle- and DNCB-treated mice (n=5/each group) to compare the accumulation of T cells (scale bar: 200 µm). B. The quantitative analysis of CD3 staining was performed using ImageJ program and statistical significance was determined by t test. DNCB; 2,4-dinitrochlorobenzene, AD; Atopic dermatitis, w; Week, *; P<0.05, and **; P<0.01.

Fig. 4: The spleen tissue from each experimental mouse was collected and mRNA expression of CD3 was analyzed by real time-quantitative polymerase chain reaction (RT-qPCR). Statistical significance was determined by t test and data are presented as the mean ± SEM. DNCB; 2,4-dinitrochlorobenzene, w; Week, *; P<0.05, **; P<0.01, and ***; P<0.001.

Acknowledgments
This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (grant numbers NRF-2022R1I1A1A01066175 and NRF-2021R1A2C1012551). There is no conflict of interest in this study.

Authors’ Contributions
K.-A.C.; Performed the experiments and wrote the
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