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*J Immunol* 2002; 168:2020-2027; doi: 10.4049/jimmunol.168.4.2020

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Development of Atopic Dermatitis-Like Skin Lesions in STAT6-Deficient NC/Nga Mice

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Atopic dermatitis (AD) is a pruritic inflammatory skin disease characterized by elevation of plasma levels of total IgE, infiltration of mast cells and eosinophils, and the expression of cytokines by Th2 T cells. However, the role of Th2 cells in the pathogenesis of AD is not fully understood. In this study we examined the NC/Nga (NC) mouse model of AD and established STAT6-deficient (STAT6−/−) NC mice to investigate the relevance of IL-4-mediated immune responses. Surprisingly, these mice elicited AD-like skin lesions at equivalent frequency and time of onset compared with normal NC littermates. Histological features of the lesion in STAT6−/− NC mice fulfilled the criteria for the pathogenesis of AD, although these mice fail to produce IgE and Th2 cytokines. The lymph nodes proximal to the regions of skin that developed lesions exhibited massive enlargement elicited by the accumulation of activated IFN-γ-secreting T cells. Moreover, caspase 1, IL-18, IL-12, and IFN-γ are found to be highly expressed at the skin lesion, occurring simultaneously with elevation of eotaxin 2 and CCR3 expression. Therefore, the Th2-mediated immune response is not necessary for the development of AD-like skin disease in NC mice. The skin microenvironment that favored IFN-γ production tightly correlates with the skin disease in NC mice through the infiltration of eosinophils.

Received for publication August 31, 2001. Accepted for publication December 14, 2001.

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3 Abbreviations used in this paper: AD, atopic dermatitis; IP10, inducible protein 10; MDC, monocyte-derived chemotactic cytokine; Mfg, monokine induced by IFN-γ; SPF, specific pathogen free; TARC, thymus and activation-regulated chemokine.

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0022-1767/02/$02.00
affected skin regions, may be more important than Th2 cytokines for the development of AD in NC mice.

Materials and Methods

Mice

SFP NC/Nga mice were purchased from Clea (Tokyo, Japan). STAT6-deficient mice with a C57BL/6 background were a gift from Dr. S. Akira (Osaka University, Osaka, Japan) (18). The STAT6-deficient mice have been backcrossed to NC/Nga mice for more than six generations in the SPF condition. STAT6−/− NC mice were selected by genomic PCR using the primer specific for the downstream region of the targeted STAT6 gene and the targeted neo-resistant gene. STAT6+/− NC mice were mated, and STAT6−/− and STAT6−/+ NC mice were selected from their offspring.

Cytokines and Abs

The reagents for ELISA and intracellular cytokine staining, anti-IL-2 (JES6-1A12 and JES6-SH4 biotin), anti-IFN-γ (R-6-6A2 and XMGI2 biotin), anti-IL-4 (BVD4-1D11 and BVD6-2G42 biotin), anti-IFN-γ (XMGI2) FITC, and anti-IL-4 (11B1) PE, were purchased from BD Pharmingen (San Diego, CA). Anti-CD28 mAb (PV-1) was a gift from Dr. R. Abe (Science University of Tokyo, Tokyo, Japan).

Measurement of serum Ig concentration

Blood was drawn from the sinus cavernosus, and serum was separated by centrifugation at 1500 × g for 10 min. Serum levels of IgM, IgG1, IgG2a, IgG2b, and IgE were determined by ELISA using Ab pairs specific for different mouse Ig isotypes (BD Pharmingen).

Measurement of cytokine production in primary TCR activation and ELISA

Cells were incubated with anti-CD8 mAb (3.155) at 4°C and were placed on the plate-coated anti-mouse Ig (Cappel, Aurora, OH) to eliminate B and CD8+ T cells. This enrichment resulted in >80% CD4+ T cells. The CD4+ T cell populations (1 × 10⁶ cells/ml) were prepared from lymph nodes and stimulated with plate-bound anti-TCR plus anti-CD28 mAb for 48 h. The culture supernatants were harvested, and the cytokine concentration was analyzed by ELISA as described previously (22).

Measurement of cytokine production concentrated from effecter CD4+ T cells

The enriched splenic CD4+ T cells (1 × 10⁶ cells/ml) were stimulated with plate-bound anti-TCR (H57-597) mAb (30 µg/ml) plus the soluble form of anti-CD28 mAb (PV-1, 5 µg/ml). After 7 days, viable cells were collected and restimulated with plate-bound anti-TCR for 24 h, the culture supernatants were harvested, and the cytokine concentration was analyzed by ELISA.

Dermal histology

The dermal skin were resected, fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, stained with either H&E or Giemsa so-
cells skewed toward IFN-γ-secreting Th1 cells (Fig. 3B). The proportion of Th1 cells was significantly increased in mice developing the disease. Therefore, although the genetic background of NC mice has a propensity to facilitate the Th2 response, Th2-mediated effector functions, including Th2 cytokine production, and subsequent IgE responses may not be responsible for the development of AD-like skin disease in NC mice.

Next, we examined the possibility that the mechanisms regulating eczema formation in STAT6+/− NC mice may differ from those in STAT6+/+ mice. In a comparison of histological sections of dermal skin from the head and back of unaffected NC mice and NC mice that had developed lesions, it was found that the lesions showed a significant thickening of the dermis and epidermis and a cellular infiltrate in the dermis that contained eosinophils (Fig. 4B, H&E staining) and mast cells (Fig. 4C, Giemsa staining). The skin lesions elicited in STAT6+/− NC mice had histological features very similar to those of control NC mice (Fig. 4, D and E). Therefore, we concluded that there were no significant differences in the

FIGURE 1. Frequency and severity of the AD-like skin lesion in STAT+/+ (control) and STAT−/− NC mice. Aa, Control normal NC mice. Ab, The same litter of affected control NC mice. The severe lesions extended into the back, neck, and ear, and the ear was completely scratched out. B, The frequency of skin lesion was 10 of 19 for control littermate NC mice and 10 of 13 for STAT6−/− NC mice. The clinical severity of the AD-like skin lesion was assessed as the AD score with respect to four individual regions (ear, face, head, and back), and the total score was plotted. A score of zero represents normal skin, while a score of 4 represents the most severe skin lesion. ○, Control NC mice; ●, STAT6−/− NC mice. Mean values of the AD score are indicated by the horizontal bars.

FIGURE 2. Serum Ig concentration in AD-like skin lesion developing control and STAT−/− NC mice. Sera were obtained from control and STAT6−/− NC mice with (●) or without (○) AD-like skin lesion at the age of 8 mo, and serum levels of IgM, IgG1, IgG2a, IgG2b, and IgE were determined by ELISA. Each symbol indicates individual mice, and mean values are indicated by the horizontal bars.
pathogenesis of skin lesions between control and STAT6\textsuperscript{−/−} NC mice. These findings clearly indicate that the infiltration of eosinophils and mast cells tightly correlates with the development of disease, but increased IgE levels and Th2 cells were not necessary for these infiltration events.

The above observations in STAT6\textsuperscript{−/−} NC mice raised the question of whether the development of skin lesions is regulated by immunological responses via Th cells. It would be possible that nonimmunological stimuli under conventional conditions produce skin itching with the infiltration of eosinophils and mast cells. To investigate this possibility, we studied the pathogenesis of lymph nodes. The cervical, axillary, and brachial lymph nodes in the affected NC mice were markedly enlarged compared with those in unaffected littermates (Fig. 5Aa). The enlargements were specifically restricted to lymph nodes proximal to the skin lesions, while distal inguinal and popliteal lymph nodes in the affected NC mice remained normal (Fig. 5Aa). The enlarged cervical lymph node contained 10 times more cells compared with that of normal littermate NC mice (data not shown), suggesting that large numbers of T and B cells specifically migrate into the lymph nodes proximal to the skin lesion elicited in the ear and head. Indeed, histological and FACS analyses revealed that the cervical lymph node in the affected mice were replete with T and B cells (Fig. 5Ab). The proportion of B220\textsuperscript{−} B cells in the cervical lymph node was markedly higher than that in the distal inguinal lymph node, and more than half of the B cells showed low expression of surface IgM, indicating that many plasma cells migrate into the proximal lymph node (Fig. 5B). This was consistent with the histological features of the cervical lymph nodes in affected mice (Fig. 5Ab). Moreover, T cells in the cervical lymph nodes showed an enhancement of CD62 ligand\textsuperscript{low}, CD44\textsuperscript{high}-activated phenotype (Fig. 5B and data not shown). These results indicated that site-specific inflammation may contribute to the development of skin lesions.

Next, we studied the relevance of T cell-mediated immune responses at the site of the developing disease by assessing cytokine production. Purified CD4\textsuperscript{+} T cells from cervical lymph nodes were

FIGURE 3. Cytokine production and Th cell development in AD-like skin lesions in control and STAT\textsuperscript{6−/−} NC mice. A, Cytokine production of the activated effector T cells from control and STAT\textsuperscript{6−/−} NC spleens. The CD4\textsuperscript{+} T cells were separated from spleen cells of control and STAT\textsuperscript{6−/−} NC mice with (○) or without (□) AD-like skin lesions, and Th cell differentiation was introduced by stimulation with anti-TCR and CD28 mAbs for 7 days. The differentiated T cells were restimulated with anti-TCR mAb, and the concentrations of cytokines in the supernatant were measured by ELISA. Each symbol indicates individual mice, and mean values are indicated by the horizontal bars. B, Profile of Th cell development from spleens of control and STAT\textsuperscript{6−/−} NC mice. The preactivated CD4\textsuperscript{+} T cells were prepared as described in A and restimulated with anti-TCR mAb in the presence of monensin. After permeabilization, cells were stained with anti-IFN-\gamma-FITC and anti-IL-4-PE.
prepared and stimulated with anti-TCR and CD28 mAbs for 48 h. The levels of Th2 cytokines, IL-4 and IL-5, produced by lymph node T cells from the control affected mice were increased in some mice compared with those in unaffected controls, whereas IL-4 and IL-5 levels did not correlate with the development of AD in STAT6$^{-/-}$ NC mice (Fig. 5C). In contrast, IFN-γ production was 3- to 4-fold higher in most AD-developing mice, and an increase in IFN-γ production was consistently found in STAT6$^{-/-}$ NC mice (Fig. 5C). This indicates that accumulation of the IFN-γ-producing T cells is consistently observed at lymph nodes proximal to the dermal skin lesion.

To further examine whether the infiltration of cytokine-producing cells into the skin is associated with the development of disease, we analyzed the expression of cytokines, chemokines, and chemokine receptors in the skin lesions by RT-PCR. The expression of the Th2 cytokines, IL-4 and IL-5, was not detectable even in the AD-like skins from STAT6$^{+/+}$ NC mice. In contrast, IFN-γ expression was consistently found in the skin of AD-developing NC mice regardless of STAT6 (Fig. 6A). IL-18 is known to be a potent inducer of IFN-γ production in conjunction with IL-12. Consistent with the increased IFN-γ level, the expression of both IL-18 and IL-12 was clearly up-regulated in the skin lesions (Fig. 6A). Caspase 1, a cleaving enzyme that converts inactivated IL-18 to its active form, was prominently expressed in the skin, but the basal expression was not characteristic of NC mice, because skin from BALB/c and C57BL/6 mice exhibited the same level of caspase 1 expression (Fig. 6B). Consistent with a previous observation in caspase 1 transgenic mice that spontaneously developed AD-like skin disease with an elevation of serum IL-18 (26), caspase 1 expression became more prominent in the skin from AD developing NC mice (Fig. 6A). These results suggest that the cytokine environment that promotes the production of IFN-γ-producing cells.
was generated at the site of the developing AD-like skin disease, and that this process resulted in the infiltration of IFN-γ/H9253-producing T cells into dermal skin lesions from proximal lymph nodes. Therefore, it is reasonable to speculate that the infiltration of IFN-γ-producing T cells may be associated with eczema formation in NC mice.

The expression of inflammatory chemokines MDC in the skin of the head was not detectable even after the development of disease, while TARC and eotaxin 1 were prominently expressed. TARC expression was slightly increased with the development of disease, but eotaxin 1 was not affected. However, a dramatic enhancement of expression of eotaxin 2 and CCR, such as CCR3, -4, and -5, was found to coincide with the development of skin lesions (Fig. 6A). Eotaxin 2 has been identified as a CC chemokine with eosinophil-selective chemoattractant activity, and its expression is regulated by allergen challenge and IL-4 (21). However, the present study clearly demonstrated that the increased eotaxin 2 expression at the skin lesion-developing site in NC mice was STAT6 independent (Fig. 6A). The increased eotaxin 2 and CCR3 expression was found only when IFN-γ was constitutively expressed at the skin. Therefore, we speculate that the elevation of these chemokines and receptors may result from the IFN-γ-mediated responses.

Discussion
The NC mouse has been proposed as a model of human AD, which is described as a Th2-type disease at least in the initiating phase. Indeed, the disease-developing NC mice revealed predominant Th2 responses and elevated serum IgE levels. However, our finding in STAT6−−/− NC mice clearly demonstrated that the Th2-mediated immune response is not necessary for the development of AD-like skin disease in NC mice. The skin lesions were elicited in STAT6−−/− NC mice that had completely impaired Th2 differentiation and IL-4-mediated IgE class switching. Both control and STAT6−−/− NC mice revealed equivalent clinical severity in the AD skin lesions characterized by hypertrophy of the dermis and epidermis and massive infiltration of eosinophils and mast cells. Cytokine and chemokine expression analysis demonstrated that IFN-γ-producing T cells were accumulated at the local lymph nodes, and that IFN-γ, IL-18, and IL-12 were highly expressed at the skin site that developed AD-like disease, suggesting that the infiltration of IFN-γ-producing cells may account for eczema formation in NC mice.

Under conventional conditions, >50% of control NC mice spontaneously developed eczematous AD-like lesions around the age of 6 mo or later. The lesions appear to occur as a result of extensive scratching, based on the behavior of the mice. In this strain the level of serum IgE gradually increases and shows relatively high basal levels compared with age-matched mice of other strains. All the mice developing disease showed further elevation of serum IgE levels of about 10-fold (Fig. 2) and increased Th2 cytokines, such as IL-4, IL-5, and IL-10 (Fig. 3A). These observations in control NC mice were consistent with several previous
reports that the Th2-mediated immune response is probably responsible for the development of skin lesions in NC mice (11, 12, 14, 27). In many AD patients increased Th2 cytokines and serum IgE levels are regarded as a specific feature related with the development of disease (3–6, 28). These previous findings suggest that the NC strain would be a useful animal model for human AD (11).

However, STAT6−/− NC mice can elicit AD-like skin lesions to the same extent as control NC mice. The lesions completely fulfilled the pathologic criteria of the disease and were characterized by thickening of the outer layer and stratum spinosum of the epidermis and infiltration of eosinophils and mast cells in the dermis (Fig. 4). These results clearly demonstrated that the presence of IgE and Th2 cells is not a prerequisite for the development of AD-like lesions of NC mice at least in the initiating phase, although we cannot rule out the possibility that Th2 cytokines and IgE are responsible for the pathogenetic events in the chronicity and exacerbation of this disease. Current studies in humans have reported that the subgroup of AD patients with normal serum IgE levels and without specific IgE-mediated sensitization is characterized as nonallergic dermatitis (8–10, 30, 31). Therefore, the development of eczematous AD-like lesions in NC mice may contribute to the pathogenesis of nonallergic dermatitis. The elevated serum levels of IgE and the development of Th2 responses in normal affected NC mice may be consequences of the progression of the pathogenesis of AD-like disease.

The regional lymph nodes proximal to the skin developing the lesions exhibited massive enlargement elicited by the accumulation of activated IFN-γ-secreting T cells and plasma cells (Fig. 5). Inconsistent with previous observations (1), expression analysis of cytokines showed no expression of IL-4 and IL-5 at lesional skin (Fig. 6). The increased IFN-γ production tightly correlates with the eczema formation regardless of the STAT6-mediated signaling. Many potent inducers of IFN-γ production, such as caspase 1, IL-12, and IL-18, were significantly up-regulated at the skin lesion (Fig. 6). Caspase 1 converts IL-18 from an inactivated to an activated form by functioning as a cleaving enzyme, and the cleaved IL-18 acts as a potent inducer of IFN-γ production in conjunction with IL-12 (32, 33). It is well documented that IL-12 produced by Langerhans cells, eosinophils, and keratinocytes is a potential mediator for the induction of IFN-γ in T cells (34–36). Therefore, the cytokine microenvironment at pathologic sites leads to the accumulation of IFN-γ-secreting T cells during the development of skin lesions.

On the basis of these observations in STAT6−/− NC mice, the pathogenesis of the skin lesion was postulated to be regulated by IFN-γ-mediated responses. The expression profile of chemokines in the skin lesion showed increased expression of TARC, eotaxin 2, and CC chemokine receptors even in STAT6−/− NC mice. The TARC/MDC-CCR4 system has been shown to be involved in the Th2-mediated disease process (37, 38). However, consistent with the observation in NC mice that IFN-γ, but not IL-4, induces TARC expression in keratinocytes (15), our results indicate that the enhancement of TARC expression at the pathological site correlates with the increased IFN-γ expression. Eotaxin 2 is newly identified potent chemoattractant for eosinophils. Eotaxin 2 and its receptor, CCR3, were predominantly expressed in lesional skin. It was previously reported that IFN-γ plays a role as a potent inducer for eotaxin 2 and CCR3 (39). Therefore, we speculate that the elevation of these chemokines and receptors may result from IFN-γ-mediated responses. Recently, it has been demonstrated that CXCR3, predominantly expressed on human IL-2-stimulated T cells, is a receptor for IFN-γ-inducible protein 10 (IP10) and monokine induced by IFN-γ (Mig) (40). The induced IP10 and Mig activate eosinophils through CXCR3 that is also expressed on eosinophils (41). It would be possible that this IFN-γ-induced IP10/CXCR3 pathway plays a role in the disease site of NC mice, although we have not analyzed IP10, Mig, and CXCR3 expression in this study.

The pathological significance of IFN-γ proposed in this paper is consistent with previous reports using IFN-γ-deficient or transgenic mice indicating that IFN-γ-secreting cells play a significant role in the pathogenetic events of AD-like disease.

FIGURE 6. Total RNA was isolated from the dermal skin of normal and affected control NC mice. The expressions of cytokines, chemokines, and chemokine receptors were analyzed by RT-PCR using the set of primers described in Materials and Methods.
role in allergic eczema formation (29, 31). Moreover, recent re-
ports demonstrated that tranigenic mice expressing caspase 1 in keratinocytes spontaneously developed AD-like skin disease with an elevation of the active form of IL-18 in serum (37). Recent
linkage analysis demonstrated that the locus responsible for skin
disease in NC mice is on chromosome 9, and the IL-18 gene is near the locus (42). It is therefore reasonable to speculate that the in-
creased caspase 1, IL-18, and IL-12 expression in the skin micro-
environment may result in the increased IFN-γ production, leading
to the induction of eotaxin 2 expression that acts as a chemooat-
tractant for CCR3+ eosinophils. However, at this point the evi-
dence to support this idea remains correlative. We have attempted
to directly examine the role of IFN-γ by the administration of
neutralizing Abs, but it was difficult to control the timing of ad-
ministration, since the appearance of clinical signs varies among
individuals. Therefore, a nonimmune reaction may account for the
development of the pathogenesis in NC mice, and the IFN-γ-fa-
avored skin microenvironment may be a result of the pathogenesis
in NC mice. The establishment of Rag2−, caspase 1−, IL-18−, or
IFN-γ-deficient NC mice will directly answer these questions.

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