

Early up-regulation of Th2 cytokines and late surge of Th1 cytokines in an atopic dermatitis model

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SUMMARY

We investigated cytokine profiles in interleukin (IL)-4 transgenic (Tg) mice with a skin inflammatory disease resembling human atopic dermatitis. cDNA microarray revealed that the mRNAs encoding IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, tumour necrosis factor (TNF)- α , TNF- β and interferon (IFN)- γ were up-regulated in the skin of late lesion Tg mice and to a lesser degree in non-lesion Tg mice when compared to those of non-Tg mice. Real time reverse transcription–polymerase chain reaction (RT-PCR) analyses indicated that the cDNA copy numbers of IL-1 β , IL-4, IL-6, IL-10, TNF- α and IFN- γ from the skin of late, early and non-lesions increased significantly compared to non-Tg mice. IL-2 and IL-12p40 cDNA copy numbers were increased significantly in early, but not late, lesions. Interestingly, IL-1 β , IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, TNF- α , and IFN- γ cDNAs were increased significantly the skin of before-onset and/or non-lesion mice. Flow cytometry analyses demonstrated an increased percentage of keratinocytes producing IL-4 as the disease progressed. The percentage of IL-2, IL-4, IL-10 and IFN- γ -producing T cells and IL-12-producing antigen-presenting cells in skin-draining lymph nodes and inflammatory skin also increased, particularly in mice with late lesion. These results suggest that disease induction is primarily triggered by Th2 cytokines and that Th1, Th2 and non-Th proinflammatory cytokines are all involved in the disease process.

Keywords atopic dermatitis cytokine real time PCR Th1 Th2

INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease with increasing incidence in the developed nations [1–5]. The underlying immunological milieu of human AD is characterized by up-regulation of inflammatory cytokines, increased total serum IgE and inflammatory cell infiltration [1–5]. Previous studies suggest that an imbalance of Th2-predominating cytokine milieu may be responsible for the development of human AD [6–15]. We have generated an animal model of AD by expressing the critical Th2-type cytokine interleukin (IL)-4 in the basal epidermis of a transgenic (Tg) mouse line using a basal keratinocyte-specific keratin 14 (K14) promoter [16]. The Tg mice developed spontaneously a pruritic, chronic, inflammatory skin disease that resembles human AD clinically, histopathologically, serologically and bacteriologically and the mouse disease fulfills the diagnostic criteria for human AD and fulfills the majority of

the diagnostic criteria for dogs affected with AD [16–18]. Whether they were housed in special pathogen-free cages or conventional cages, the Tg mice developed identical clinical phenotypes, predominantly in relatively poorly haired areas such as ears, mouth and peri-orbital regions of the skin [16]. Because AD is an inflammatory disease, cytokines that are involved in the initiation and maintenance of inflammation are naturally essential components of investigation. Although many cytokine studies have been performed in human and animal AD [19–24], a quantitative analysis on the cytokine expression in before-onset, during early and late disease has not been performed previously. Reconstruction of the immunological sequence of events associated with AD could allow a better understanding of the step-by-step pathophysiology. In addition, previous studies on cytokine profiles of AD were carried out with only a few cytokines. We now know that *in vivo* inflammation is a complicated process involving a complex interaction of multiple cytokines [25]. Taking advantage of our mouse model, we performed quantitative analyses of the cytokine profiles in a large number of 12 cytokines in this K14 IL-4-Tg mouse line before the disease-onset, non-lesional skin at the early and late disease stages by cDNA

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microarray analyses, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and FACS analyses. To obtain a broader picture of the cytokine milieu, we tested not only the affected organ, the skin, but also the skin-draining lymph nodes (LNs), where the essential immunological interactions between T cells and antigen-presenting cells (APCs) take place. Our data suggest that Th2-type and non-Th-type proinflammatory cytokines are involved primarily in disease induction, while Th1-type, Th2-type and non-Th-type proinflammatory cytokines are involved in disease maintenance.

MATERIALS AND METHODS

Mice

Four- to 12-week-old IL-4 epidermal transgenic mice (IL-4 Tg) were used in the experiments. The non-Tg offspring served as age-matched, littermate controls. All mice were housed in the special pathogen-free room and fed with standard water and mouse chow.

Genotyping

Mice born from heterozygous IL-4-Tg mouse parents were genotyped for confirmation of their positive Tg status. The method we used was as follows: a small quantity of tail (about 0.5 cm long) was clipped from each mouse to extract DNA. The skin of the clipped tail was removed by scraping and the remaining tissue was digested in the presence of protease K (100 µg/ml) in an enzyme buffer consisting of 10 mM Tris (pH 8.0), 100 mM NaCl and 1 mM EDTA at 55°C overnight. After centrifugation for 5 min at 13 000/min the supernatant was collected and boiled at 100°C for 20 min; the resulting solution (5 µl per 25 µl reaction volume) was used for genotyping by the standard PCR method using mouse IL-4 primer pairs [16]. The PCR was performed in a Perkin-Elmer GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA) using the following cycling parameters: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min after preheating at 94°C for 4 min for 35 cycles, followed by a single 10-min extension at 72°C. A 357 base pairs (bp) DNA band corresponding to the mouse IL-4 nucleotide 70–427 [26] was visualized in a 1.5% agarose gel stained with ethidium bromide.

Disease phenotype classification

Using the above genotyping method, we observed all our mice for a minimum of 6 months in order to monitor skin disease development. We confirmed that all IL-4-Tg mice as determined by this genotyping method developed the characteristic inflammatory skin disease phenotype and none of the non-Tg mice developed the skin disease. This brings the disease induction rate in our IL-4-Tg mice to 100%. Skin lesions from IL-4 Tg mice that had developed over the course of 1 week or less are defined as early lesions. Skin lesions that had developed for 3 weeks or more are defined as late lesions.

Skin samples

Samples were obtained from normal skin of non-Tg mice (non-Tg), Tg mice before disease onset (Tg-BO), lesional skin from Tg mice with early lesion (Tg-EL), lesional skin from Tg mice with late lesion (Tg-LL) and non-lesional skin from Tg-LL mice (Tg-NL). All samples were collected from the ears except Tg-NL samples, taken from the normal-appearing skin on the mouse's back, which is distant from any lesion.

Histology

Skin samples from non-Tg, Tg-BO, Tg-EL, Tg-LL ($n = 3$ for each group) were fixed with formalin and processed in paraffin. Four-µm sections were stained for haematoxylin and eosin (H&E) and Giemsa. The number of purple-stained mast cells by Giemsa staining was counted per high-power field (HPF, 40× objective lens). Ten fields of each section were counted. Trachea, oesophagus, small intestine, bladder and oral mucosa were obtained similarly, processed, and were stained by H&E.

RT-PCR

To ensure that the Tg IL-4 is expressed exclusively in the skin and not in other epithelial tissues, trachea, oesophagus, small intestine, bladder and oral mucosa were collected from three non-Tg and three Tg-BO mice. The specimens were stored in RNAlater solution (Sigma, St Louis, MO, USA) at –20°C until RNA extraction. Total RNAs were extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) and treated with 1U DNase I (Invitrogen). One µg total RNA of each sample was reverse transcribed to cDNA by Retroscript Kit (Ambion, Austin, TX, USA). Primers used for amplifying IL-4 and GAPDH have been published previously [27,28]. PCR conditions for amplification of IL-4 and GAPDH were 3 min at 95°C, then 30 s at 95°C, 30 s at 55°C and 30 s at 72°C for 35 cycles followed by a final extension at 72°C for 5 min. The PCR products were resolved on a 1.5% agarose gel, stained with ethidium bromide and examined under UV light. The RNA extracted similarly from normal Tg mouse skin and non-Tg mouse skin was included as positive and negative control, respectively.

cDNA microarray

To screen for skin cytokine profiles, tissues were obtained from non-Tg mice ($n = 2$), lesional skin of Tg-LL ($n = 7$) mice and NL skin from Tg-LL mice ($n = 7$). The conditions for storage and total RNA extraction were the same as those described in the above RT-PCR procedure. For labelling cDNAs we used an Ampolabeling-LPR kit (Superarray, Frederick, MD, USA), which utilizes the linear polymerase replication method to amplify second-strand cDNA probes with gene-specific primers and biotin-labelled dUTP (Superarray); 0.5 µg total RNA was used in this procedure for each sample. The resultant biotinylated probes were then hybridized with the tetra-spotted cytokine-gene-specific cDNA on nylon membranes. Signal was developed after incubating the membranes sequentially with alkaline phosphatase-conjugated streptavidin and chemiluminescence substrate, according to the manufacturer's instructions. The images were scanned and recorded by using Scanalyse software developed by Michael Eisen at the Lawrence Berkeley National Laboratory and the University of California at Berkeley, and then analysed by using GEArrayAnalyser (Superarray). Results were recorded as fold increase between groups.

Quantitative real time RT-PCR

To determine quantitatively the initial quantities of cytokine mRNA in the skin of different stages of disease, real time RT-PCR was performed. First, total RNAs were extracted from non-Tg, Tg-BO, Tg-EL, Tg-LL and Tg-NL skin ($n = 10$ for each group). The total RNA of each sample was reverse transcribed as described in the above RT-PCR section. Primers for amplifying IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, tumour necrosis factor (TNF)-α, TNF-β, interferon (IFN)-γ and their

plasmid standards (except plasmids for IL-3, IL-5, IL-13 and TNF- β) have been published previously [27]; the GAPDH plasmid standard and its primers have also been published previously [28]. The amplicon lengths range from 95 to 236 bp. For real time PCR, we used SYBR green supermix (Bio-Rad, Hercules, CA, USA) with cytokine gene-specific primers (100 mM) and 25 ng cDNA templates of individual samples or 10-fold serial dilutions of the plasmid standards, with concentrations ranging from 5×10 to 5×10^6 copies. Total reaction volume was 50 μ l. For all cytokines, identical thermal cycling conditions were used: 15 s at 95°C and 1 min at 60°C with a total of 40 cycles followed by a melting curve data collection set up to identify the non-specific products or primer-dimers. The PCR was performed in an iCycler (Bio-Rad) in duplicate. The copy number of the gene present in the mRNA extract of the tissue was determined automatically by iCycler's software according to the standard curve of the plasmid DNA. In order to normalize for cDNA synthesis efficiencies and RNA input amounts, the highest copy number of GAPDH of all samples was divided by copy numbers of an individual sample to give a calculating factor. The normalized gene copy number was determined by multiplying the gene copy number obtained from the standard curve by this calculating factor. Because we did not have plasmids for IL-3, IL-5, IL-13 and TNF- α , quantification of these cytokines was calculated by the PCR values of the cytokines normalized to the values of the respective GAPDH, expressing in folds of increase over that of the non-Tg mouse samples.

FACS analyses of intracellular cytokines in LN inflammatory cells

For analysis of intracellular IL-2, IL-4, IL-10, IL-12 and IFN- γ expression, skin-draining LNs were collected from non-Tg and IL-4 Tg mice at different disease stages. Single LN cell suspensions were prepared and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma) for 4 h at 37°C with final concentrations at 20 ng/ml and 1 μ M, respectively, in RPMI-1640 with heat-inactivated 10% fetal calf serum (FCS). GolgiPlug (BD Biosciences, San Diego, CA, USA) containing brefeldin was also added into the culture to block the intracellular cytokine transport processes. After washing with phosphate-buffered saline (PBS), cells were incubated with antimouse CD16/32 (eBioscience, San Diego, CA, USA) in cold PBS/2% FCS/0.1% NaN₃ for 10 min on ice to block Fc receptor; the cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated hamster antimouse CD3 ϵ (IgG1) and Cy-Chrome conjugated rat antimouse CD8 (IgG2a, BD Biosciences) for 30 min on ice. For analysis of IL-12 intracellular expression, LN cells were stimulated with lipopolysaccharide (LPS) (Sigma) at a final concentration of 5 μ g/ml for 4 h and FITC-conjugated rat antimouse MHC class II (IA-IE) (IgG2b) (eBioscience) was used for cell surface marker staining. After the cell surface marker staining, the cells were fixed and permeabilized using Leucoperm (Serotec, Oxford, UK). During permeabilization, the cells were incubated with phycoerythrin (PE)-conjugated rat antibodies against mouse IL-2 (IgG2b), IL-4 (IgG1), IL-10 (IgG2b), IFN- γ (IgG1) or IL-12 (IgG2a) (eBioscience) for 30 min at room temperature. FACS was performed on a BD FACSCalibur Flow Cytometry System (BD Bioscience). Analysis of cell surface markers and cytokines was carried out using BD CellQuest software (BD Bioscience). In all experiments, samples prepared similarly with isotype controls were also included. Staining with control antibodies did not result in any shift in the fluorescence intensity. Because PMA/ionomycin

activation dramatically down-regulates CD4 expression, rendering CD4 staining ineffective, the CD4 cell population was gated in by CD3 ϵ positivity and CD8 negativity. All antibodies were used at a final concentration of 5 μ g/ml.

FACS analyses of intracellular cytokines in thymic epithelial cells and skin epidermal and inflammatory cells

For IL-4 intracellular expression in keratinocytes, ears obtained from non-Tg mice and IL-4 Tg mice at before-onset, early and late disease stages were washed in 70% ethanol and dried. Then, the ears were cut into small pieces in trypsin-GNK solution containing DNase (200 μ g/ml, Sigma) and stirred for 20 min at 37°C [29]. The single-cell suspension was filtered through a cell strainer and washed with RPMI-1640 medium. The cells were blocked with mouse CD16/32, and then stained with PE-conjugated rat antimouse CD11a (IgG2a, BD Bioscience) and Cy-Chrome-conjugated rat antimouse CD49f (IgG2a, BD Biosciences). After fixation, as described above, the cells were permeabilized and stained with FITC-conjugated rat antimouse IL-4 (IgG1, eBioscience). The keratinocytes were gated in by CD49f positivity and CD11a negativity. For analysis of IL-2, IL-4, IL-10, IL-12 and IFN- γ production in skin-infiltrating inflammatory cells, the single-cell suspensions from the skin of the early and late disease stages of Tg mice were stimulated *in vitro* and stained as described in cytokine analysis for LN cells. To examine IL-4 expression in thymic epithelial cells, thymus was obtained from three non-Tg and three Tg-BO mice and treated by trypsin-GNK solution, as described above. Single-cell suspensions (1×10^6 cells) were then fixed and permeabilized, and stained with PE antimouse IL-4 (eBioscience) and antikeratin AE3 (mouse antihuman cytokeratin IgG, which cross-reacts with mouse keratin; Chemicon, Temecula, CA, USA). After washing, the cells were incubated with FITC antimouse IgG. Flow cytometry was performed on a BD FACSCalibur Flow Cytometry System. Analysis of cell surface markers and cytokines was carried out using BD CellQuest software. In all experiments, samples prepared similarly with isotype controls were also included.

Statistical analyses

All experimental data were expressed as mean $\pm$ s.d. The significance of variation among different groups was determined by one-way ANOVA analysis and the difference between two groups was determined by the Tukey-Kramer multiple comparison test using GraphPad InStat Software. *P*-value ≤ 0.05 was considered to be significantly different.

RESULTS

Skin histology reveals increased infiltration of mononuclear cells and mast cells as the disease progresses

Figure 1 depicts the skin histology in H&E and Giemsa staining, showing the progressive increase of inflammatory cell infiltration in the dermis of Tg mice as the disease progresses. EL samples were characterized by moderate increases of epidermal thickness (acanthosis), dermal and epidermal leucocyte infiltration and spongiosis, resembling that of an acute inflammation in human AD [30]. LL samples are distinguished from EL samples by a substantial increase of acanthosis, leucocyte infiltrate and the presence of parakeratosis, a histological marker for chronic inflammation in human AD [30]. Interestingly, in the clinically normal skin of Tg-BO, increases of mononuclear cells (Fig. 1b) and mast cells

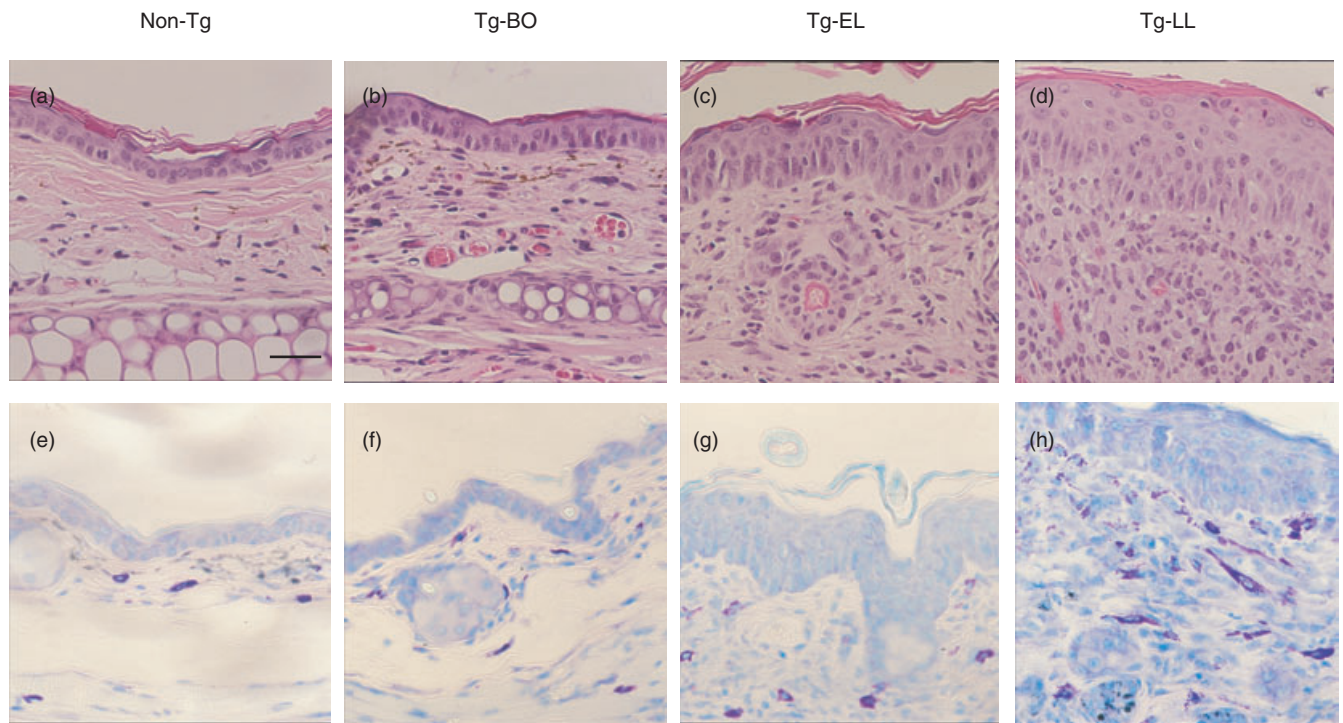


Fig. 1. Histology of skin samples obtained from non-Tg mouse (a,e), K14-IL-4-Tg mouse before disease onset (b,f), early lesions (c,g) and late lesions (d,h) were stained with H&E (a–d) and Giemsa (e–h). Bar, 34 μ m (a–h).

(Fig. 1f) are observed. The mast cell number increases in Tg-BO ($11.3 \pm 1.2/\text{HPF}$), Tg-EL (17.0 ± 2.90) and Tg-LL skin (32.4 ± 7.8) were statistically significant ($P < 0.01$; $P < 0.001$; $P < 0.001$, respectively) when compared to non-Tg mice skin (4.3 ± 0.9).

Tissue expression IL-4 mRNA is restricted to skin

RT-PCR analysis demonstrated that all non-skin epithelial tissues tested including trachea, oesophagus, small intestine, urinary bladder and oral mucosa did not express IL-4 mRNA, whereas IL-4 mRNA was expressed strongly in the skin of the Tg mice. The negative control non-Tg mouse skin also showed no IL-4 expression. Flow cytometric analysis of IL-4 expression in thymic epithelial cells revealed no positive staining (data not shown). This confirms that IL-4 is expressed exclusively in the skin of the K14-IL-4-Tg mice. The histology of trachea, oesophagus, small intestine, urinary bladder and oral mucosa revealed no inflammation, as seen in the skin of IL-4 Tg mice, supporting the exclusive expression of IL-4 in the skin, a keratinized squamous epithelium and the only epithelial type that expresses keratin 14.

cDNA microarray screening determines that both Th1 and Th2 cytokines are up-regulated in the skin of AD mice

Results from microgene array screening show that Tg-LL skin expresses more Th2 cytokines, including IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13, when compared to the Tg-NL skin and non-Tg mice skin, with average fold increase from 2.1 to 7.6 and 4.1–11.0, respectively. Th1 cytokines (IL-2, IL-12p40, IFN- γ , TNF- β) and other proinflammatory cytokines (IL-1 β , TNF- α) were also expressed highly in Tg-LL skin when compared to Tg-NL skin and non-Tg mice skin, with average fold increases from 1.1 to 11.1 and 3.2–23.1, respectively (Table 1). The highest fold increase of cytokine found in Tg-LL skin was IL-1 β , a non-Th-specific proin-

Table 1. Screening of cytokine expression by cDNA microgene array (fold increase)

Type	Cytokines	Tg-LL/Tg-NL	Tg-LL/non-Tg	Tg-NL/non-Tg
Th2	IL-3	7.6 ± 2.2	9.9 ± 2.5	1.5 ± 0.2
	IL-4	2.0 ± 1.0	6.8 ± 2.2	3.6 ± 1.7
	IL-5	5.6 ± 3.0	9.7 ± 4.1	4.3 ± 2.4
	IL-6	4.9 ± 1.7	11.0 ± 3.0	4.3 ± 2.4
	IL-10	4.5 ± 2.7	6.2 ± 2.3	2.2 ± 0.9
	IL-13	2.1 ± 1.4	4.1 ± 2.1	3.5 ± 2.0
Th2	IL-2	2.4 ± 1.0	3.2 ± 1.0	1.9 ± 0.9
	IL-12p40	4.2 ± 2.6	7.2 ± 3.1	2.8 ± 1.5
	IFN- γ	3.4 ± 1.2	8.8 ± 1.1	6.9 ± 2.3
	TNF- β	4.5 ± 2.4	5.7 ± 2.3	3.0 ± 1.0
Non-Th	IL-1 β	11.1 ± 2.0	23.1 ± 2.0	2.6 ± 0.6
	TNF- α	1.1 ± 0.5	4.9 ± 1.8	4.2 ± 1.3

Tg-LL: Tg mice, late lesional skin ($n = 7$); Tg-NL: Tg mice, non-lesional skin ($n = 7$); non-Tg: non-Tg mice, normal skin ($n = 2$).

flammatory cytokine. All the cytokines examined were also expressed in higher levels in Tg-NL skin when compared to skin of non-Tg mice (Table 1).

Quantitative analyses of skin cytokine mRNA show an earlier up-regulation of Th2 cytokines followed by a later surge of Th1 cytokine.

In order to verify the microarray data and to expand our study to include skin lesions at other stages, quantitative RT-real time PCR was then utilized on total RNA extracted from the mouse

skin of five different conditions: non-Tg, Tg-BO, Tg-NL, Tg-EL and Tg-LL. A bar graph presentation of the cytokine quantification was illustrated in terms of actual cDNA copy numbers (when cytokine plasmid DNA is available) or relative quantity (when cytokine plasmid DNA is not available) (Fig. 2a). One important finding from these quantitative analyses is that all Th2 cytokines (IL-3, IL-4, IL-5, IL-6, IL-10, IL-13) showed statistically significant increases in either Tg-BO, Tg-NL, or both conditions when compared to non-Tg mice (Fig. 2a). In contrast, none of the Th1 cytokines (IL-2, IL-12p40, TNF- β), except IFN- γ , showed a statistically significant increase in either the Tg-BO or Tg-NL condition (Fig. 2a). Consistent with the above data are the findings that all Th2 cytokine mRNA levels peaked earlier in the disease progression either at Tg-BO (IL-5), Tg-NL (IL-3) or Tg-EL (IL-4, IL-6, IL-10, IL-13) conditions, whereas all the Th1 cytokine mRNA levels peaked later in the disease progression either at the Tg-EL (IL-2, IL-12p40, IFN- γ) or the Tg-LL (TNF- β) condition (Fig. 2a,b). Not only were Th-type cytokines up-regulated in Tg mice, two essential non-Th proinflammatory cytokines (TNF- α and IL-1 β) were also up-regulated and increased in statistically significant levels in all conditions of Tg mice when compared to non-Tg mice: Tg-BO, Tg-NL, Tg-EL and Tg-LL. The trends of the cytokine mRNA level changes are depicted in line graphs of Fig. 2b.

Intracellular cytokine analysis demonstrates a combined Th1 and Th2 immune response in skin-draining LNs

Antigen presentation and T cell activation occur predominantly in the secondary lymph organs. In the case of AD, the activities occur in the lesional skin-draining LNs. In order to elucidate the cytokine production by T cells including CD4⁺ and CD8⁺ cells, we stimulated the LN cells from non-Tg mice and IL-4 Tg mice at different stages of disease with PMA and ionomycin *in vitro* for 4 h followed by FACS analysis. Representative dot-plots are illustrated in Fig. 3a. In general, our data showed that as the disease progressed, the percentage of cytokine-producing T cells increased, noted particularly in mice with late disease (Fig. 3b). Furthermore, there were higher percentages of Th1 cytokine-producing T cells than those producing Th2 cytokines based on the several cytokines we examined. Specifically, average FACS data showed that the average percentage of IFN- γ -producing CD4⁺ T cells increased steadily from only 1.7% in non-Tg mice to 3.8% in Tg-BO, to 4.6% in Tg-EL, and up to 14.7% in Tg-LL mice (Fig. 3b). A similar increase of IFN- γ -producing CD8⁺ T cells at a higher level than those of CD4⁺ T cells was documented: non-Tg mice, 14.5%; Tg-BO, 33.3%; Tg-EL, 28.0%; and Tg-LL, 37.9% (Fig. 3b). There was 31.4% of CD4⁺ T cells producing IL-2 in non-Tg mice, while 42.6%, 42.3% and 40.4% of CD4⁺ T cells producing this cytokine were detected in Tg-BO, Tg-EL and Tg-LL stages of disease, respectively. A slightly lesser percentage of CD8 cells produced IL-2 compared to CD4 cells: 25.5% in non-Tg mice, 29.3% in Tg-BO, 29.1% in Tg-EL and 32.2% in Tg-LL (Fig. 3b). The percentage of Th2 cytokines, including IL-4 and IL-10 in CD4⁺ T cells and CD8⁺ T cells, showed increases primarily at the Tg-LL stage. There were only about 2% of CD4 cells in non-Tg, Tg-BO and Tg-EL stages that produced IL-4 or IL-10, but at the Tg-LL stage about 6.0% of CD4 cells produced IL-4 and IL-10. In the case of CD8 cells, about 2.0% of these cells expressed IL-4 and IL-10 in the non-Tg, Tg-BO and Tg-EL stages. When the disease reached the Tg-LL stage, their expression increased slightly to 3.0% (Figs 3a,b).

We also investigated IL-12 production by MHC II⁺ (IA-IE⁺) cells after they were stimulated by LPS *in vitro*. Results showed that as the disease progressed more MHC II⁺ cells in the skin-draining lymph nodes produced IL-12 after *in vitro* LPS stimulation. As shown in Fig. 3a and b, 1.9 ± 0.57 , 1.8 ± 0.39 , $3.8 \pm 1.59\%$ and $20.7 \pm 1.52\%$ APC expressed IL-12 in non-Tg mice, Tg-BO, Tg-EL and Tg-LL stages, respectively.

As disease develops and progresses the percentage of epidermal keratinocytes expressing intracellular IL-4 in the Tg mice increases

In order to examine IL-4 expression in keratinocytes, we first obtained the single-cell suspension from skin. The cells were then stained with monoclonal antibodies against mouse CD11a and CD49f (integrin α chain). CD11a is expressed on all bone marrow-derived leucocytes and anti-CD11a was used for gating out leucocytes in the skin. CD49f is a cell surface marker for leucocytes and it is also expressed on keratinocytes [31]. Thus, we could identify keratinocytes by gating in CD11a⁻/CD49f⁺ cells with the combination of antibodies to CD11a and CD49f. By this gating method, the percentage of keratinocytes that contained intracellular IL-4 expression was identified in the IL-4 Tg-BO (1.7 ± 1.1) at the Tg-EL (2.7 ± 1.0) and Tg-LL (6.7 ± 1.0) stages, with no keratinocytes in the non-Tg mice expressing intracellular IL-4 (Fig. 4).

CD3⁺ T cells and MHC II⁺ APCs in the lesional skin produce both Th1 and Th2 cytokines

In our previous immunophenotyping and FACS analysis studies, we found that CD4⁺, CD8⁺ and MHC II⁺ cells infiltrated inflammatory skin [32,33]. In order to understand the type and the extent of cytokines produced by these inflammatory cells in the skin, skin cell suspension was cultured with PMA/ionomycin for IL-2, IL-4, IL-10 and IFN- γ examination or with LPS for IL-12 examination. FACS analysis showed that 36.7 ± 8.2 , 6.7 ± 1.0 , 10.5 ± 2.2 and $30.9 \pm 4.7\%$ of stimulated CD3⁺ cells in the late lesional skin produced IL-2, IL-4, IL-10 and IFN- γ respectively, while $26.9 \pm 8.0\%$, $2.4 \pm 1.7\%$, $3.7 \pm 2.1\%$, and $9.3 \pm 0.3\%$ of the cultured CD3⁺ cells in the Tg-EL stage produced these cytokines, respectively. In the skin APC population, $29.7 \pm 5.2\%$ of MHC II⁺ cells in the Tg-LL skin produced IL-12, slightly higher than that in Tg-EL ($21.1 \pm 6.9\%$) (Fig. 5). Due to the relatively scarce T cells and APCs in the skin of non-Tg mice and Tg-BO, we were not able to examine the corresponding cytokine protein expression by FACS analysis.

DISCUSSION

In this paper, we report the findings on our investigation of the cytokine milieu in the skin and LNs in the K14-IL-4-Tg mice at different disease stages, using non-Tg littermates as negative controls. In the skin, the mRNA expressions of 12 different cytokines, including six cytokines classified as Th2-type, four cytokines classified as Th1-type and two non-Th proinflammatory cytokines were screened by cDNA microarray followed by quantitative real time RT-PCR examination. In the LNs and the skin, the protein expressions of four major Th-type cytokines (two Th2-type, two Th1-type) in T cells and IL-12 in APCs were determined quantitatively by FACS analyses, as were IL-4 expression in the epidermal keratinocytes, the target of transgenesis. This extensive survey of cytokine expression occurred at different disease stages,

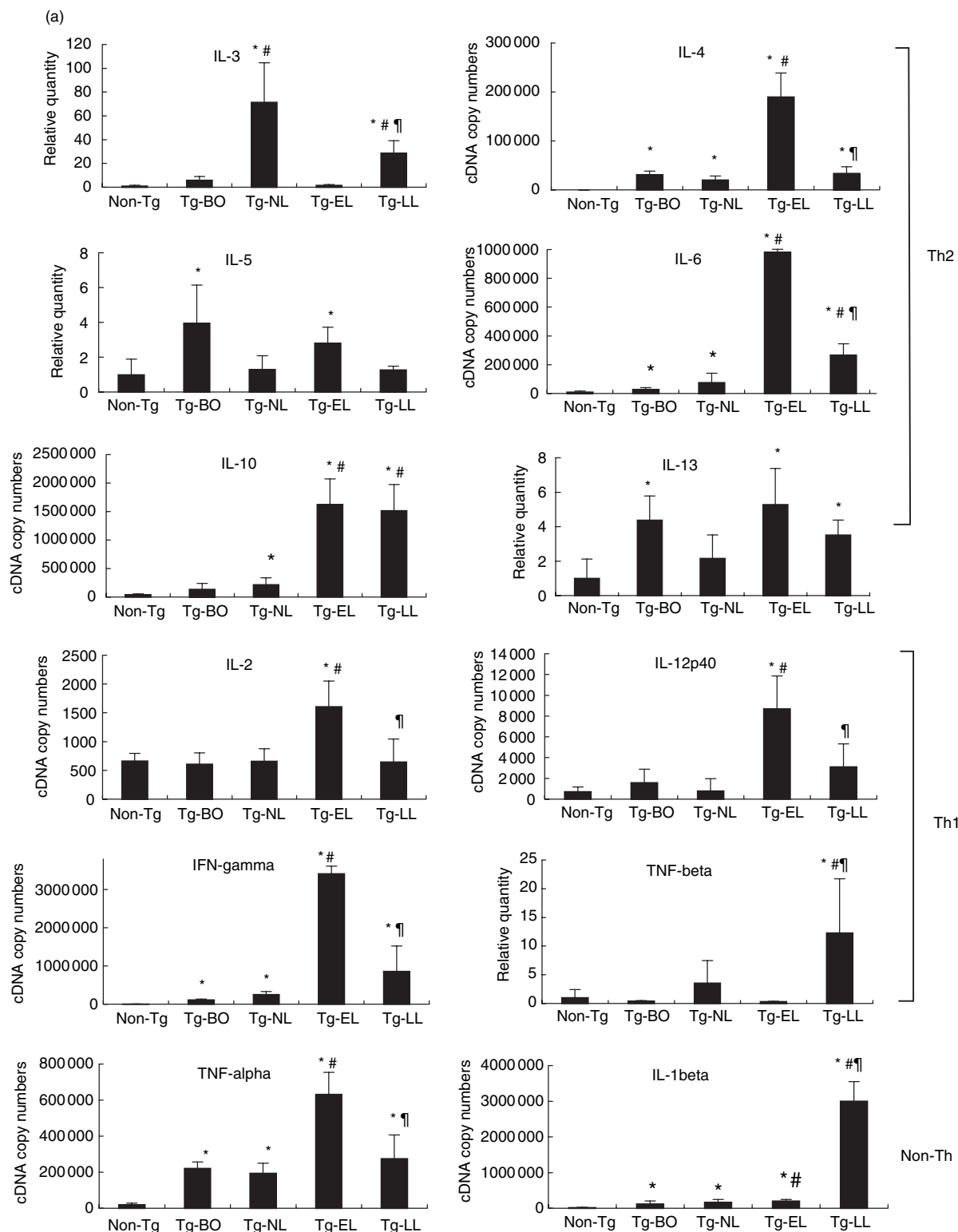


Fig. 2. RT-real time PCR quantitative analysis of cytokine mRNA expression in the skin. Total RNAs were first extracted from non-Tg, Tg-BO, Tg-NL, Tg-EL and Tg-LL mice ($n = 10$ for each group), and were then reverse transcribed. For real time PCR, we used SYBR green mix with cytokine gene-specific primers (IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN- γ and TNF- α) and cDNA template or 10-fold serial dilutions of the plasmid standards. For IL-3, IL-5, IL-13 and TNF- β , comparative quantification was performed and results were expressed in fold increase compared to non-Tg mice. (a) Copy numbers of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN- γ and TNF- α and fold increase of IL-3, IL-5, IL-13 and TNF- β . (b) Line graph of the cytokine expression at various stages of the disease. *Significant increase compared to non-Tg; #significant increase compared to Tg-BO; ¶significant difference between Tg-EL and Tg-LL.

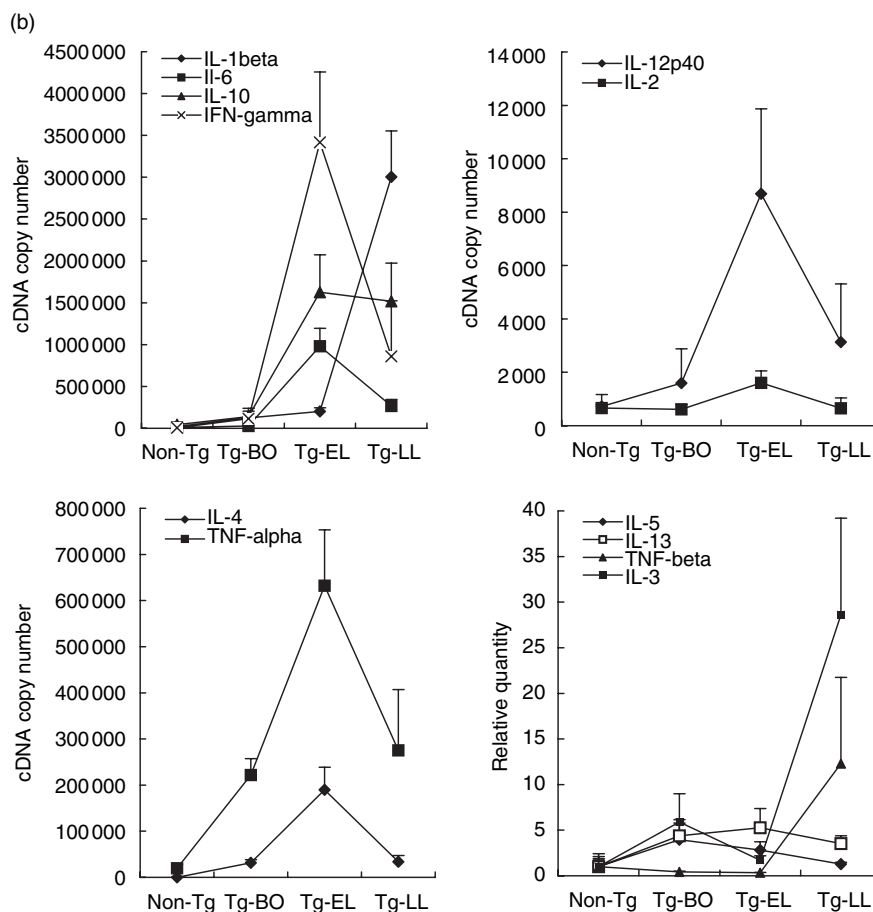


Fig. 2. Continued

aiming to establish a step-by-step immunological sequence of events that would assist us in understanding the pathogenesis of the inflammatory process in AD with regard to the role of cytokines.

Having confirmed the up-regulated cytokine expressions determined qualitatively by cDNA microarray study, our data from quantitative real time RT-PCR experiments delineate a clearer picture of cytokine involvement in the disease process (Fig. 2a). In all six of the Th2 cytokines we examined, a statistically significant increase of mRNA expression was detected in either the Tg-BO stage (two cytokines: IL-5 and IL-13), the Tg-EL stage (two cytokines: IL-3 and IL-10), or both (two cytokines: IL-4 and IL-6), in comparison with that of non-Tg mice. By contrast, only one (IFN- γ) of four Th1 cytokines we examined showed a statistically significant increase of mRNA expression in either the Tg-BO or Tg-EL stages. If cytokine profiles in skin of the Tg-BO and Tg-EL stages can be viewed as the underlying immunological milieu responsible for triggering the disease induction that leads to the subsequent Tg-LL clinical disease stage, our data suggest strongly that the clinical phenotype was induced primarily by Th2-type cytokines. The notion that disease induction involves primarily Th2-type cytokines is supported further, to some extent, by the findings that all the Th2 cytokine expressions we examined peaked either at the Tg-BO stage (one cytokine: IL-5), the Tg-EL stage (one cytokine: IL-3) or the Tg-LL stage (four cytokines: IL-4, IL-5, IL-10, IL-13) (Fig. 2a). In

contrast, all the Th1 cytokine expressions peaked either at the Tg-EL stage (three cytokines: IL-2, IL-12, IFN- γ) or the Tg-LL stage (one cytokine: TNF- β) (Fig. 2a). Our data also suggest that both Th1-type and Th2-type cytokines are involved in the maintenance of the disease; that is, to keep the disease going from the Tg-EL stage to the Tg-LL stage and beyond, as all six Th2 cytokines and all four Th1 cytokines we examined showed a statistically significant increase of expression either at the Tg-EL disease (three cytokines), the Tg-LL stage (two cytokines) or both (five cytokines), when compared to that of non-Tg mice.

Not only has the expression of Th-type cytokines been found to be up-regulated, the expression of two of the common non-Th proinflammatory cytokines we examined was also highly up-regulated in the skin of Tg mice (Fig. 2a). While the expression of TNF- α peaked at the Tg-EL stage, the expression of IL-1 β peaked at the Tg-LL stage. Regardless of when the peaked level occurred, the expression of both these cytokines was highly up-regulated at all four stages of the Tg mouse skin and all reached statistical significance when compared to non-Tg mice (Fig. 2a). Thus, our data suggest that not only Th-type cytokines, but also the non-Th proinflammatory cytokines, were involved in the induction and maintenance phases of this inflammatory process.

In order to confirm cytokine expression at the protein level and to examine the specific inflammatory cell types contributing to the skin cytokine milieu, FACS analyses were performed in T cells and APCs isolated from the skin and skin-draining LNs. In

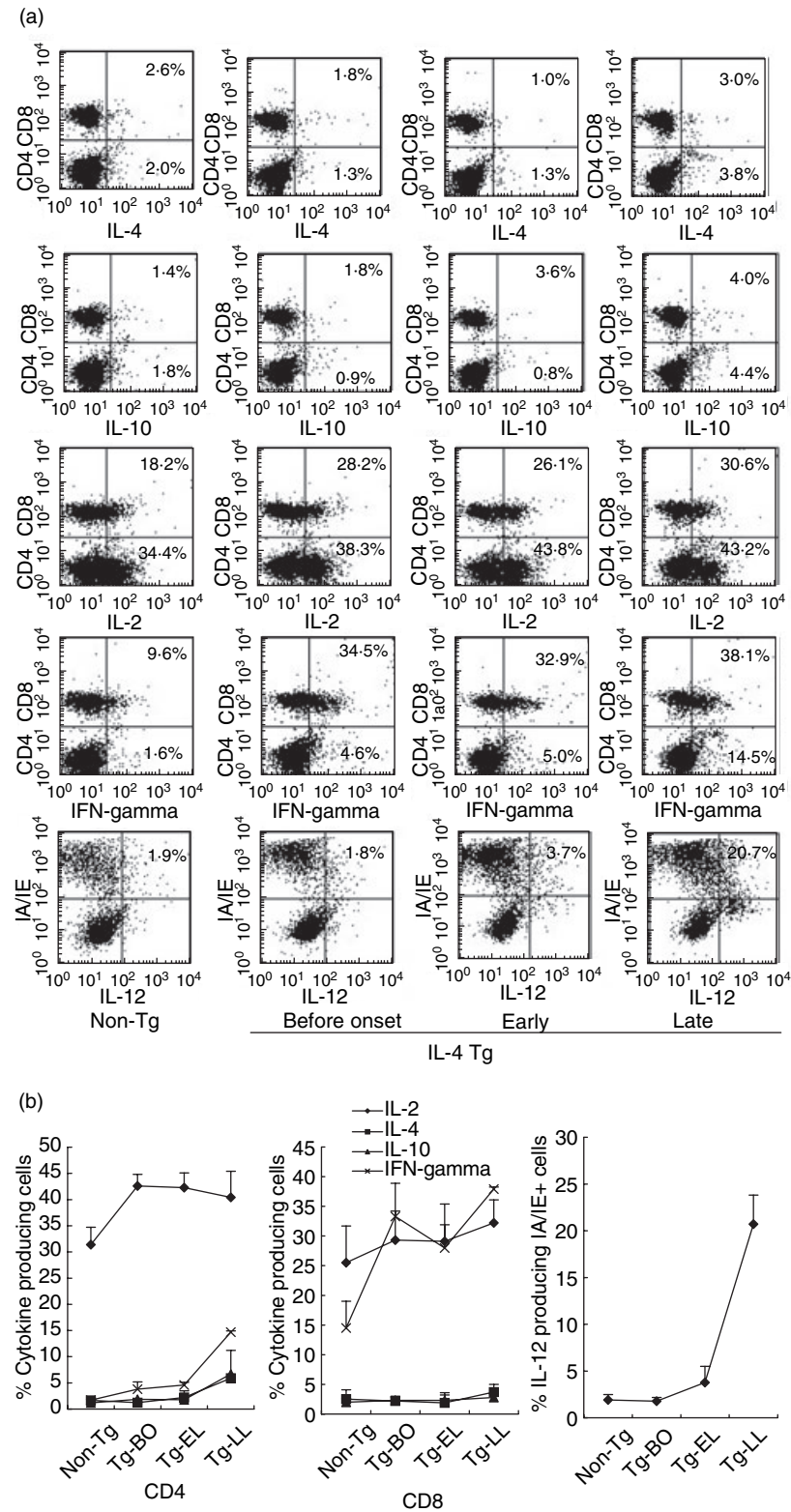


Fig. 3. Intracellular IL-2, IL-4, IL-10 and IFN- γ expression in CD4⁺ and CD8⁺ cells and IL-12 expression in IA-IE⁺ cells in LNs. Single-cell suspensions of LNs from non-Tg, IL-4 Tg mice at different stages of diseased mice were stimulated with PMA/ionomycin for IL-2, IL-4, IL-10 and IFN- γ analyses or stimulated by LPS for IL-12 analysis. Data shown are the summary of three similar experiments. (a) Representative dot-plot of intracellular cytokine staining from one of the three experiments. (b) Line graph of the cytokine expression in three experiments.

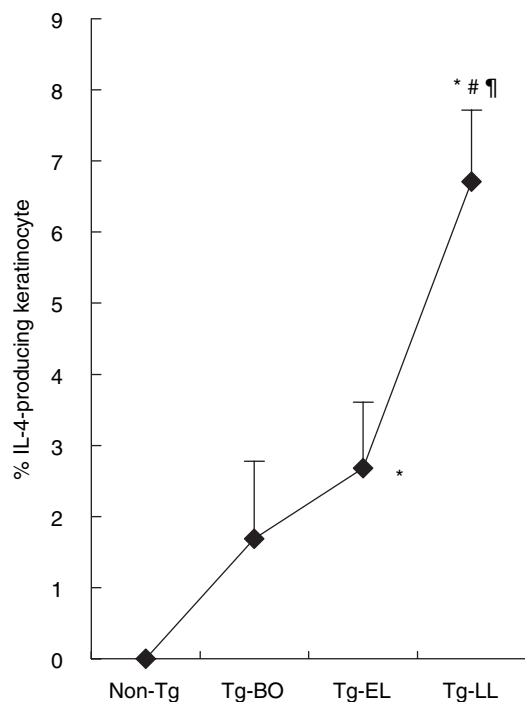


Fig. 4. Intracellular IL-4 expression in keratinocytes of epidermal IL-4 Tg mice. Single-cell suspensions of skin from non-Tg mice and IL-4 Tg mice at Tg-BO, Tg-EL and Tg-LL were stained with anti-CD11a, CD49f and anti-IL-4 followed FACS analysis as described in the Materials and methods section. CD11a⁺/CD49f⁺ cells were gated as keratinocytes. Data shown are the average frequencies of keratinocytes IL-4 intracellular expression in three experiments. *Significant increase compared to non-Tg; #significant increase compared to Tg-BO; ¶significant increase compared to Tg-EL.

all the cytokines we examined, both Th1 and Th2, the percentage of cytokine-producing inflammatory cells increased as the disease progressed (Figs 3 and 5). In almost all cytokines we examined, the highest percentage of cytokine-producing inflammatory cells were found in the Tg-LL stage, both in the LNs and in the skin (Figs 3 and 5). In previously performed experiments, we characterized this animal model by carrying out detailed phenotypical analyses of the inflammatory cells in the spleen, the LNs and skin. The results show that both T cells and dendritic cells are activated, T cells are expanded and there is a continued and progressive migration of inflammatory cells from the secondary lymphoid organs into the skin that may result in the pathology associated with AD [32]. Together with the findings in this paper, we would like to suggest that as the disease progresses, these inflammatory cells are activated continuously and remain productive in cytokine synthesis, and thus become part of a continuous source of cytokines for skin inflammation when they infiltrate the skin.

In order to examine to what degree the basal keratinocytes, the targets of the IL-4 transgenesis, contribute to the IL-4 cytokine milieu in the skin, FACS analyses were performed on skin keratinocytes by gating the CD49f⁺/CD11a⁺ cells. As the disease progresses, the percentage of IL-4-producing keratinocytes increases (Fig. 4). Thus, this epidermally derived IL-4 cytokine seems to serve as a continuous source of critical Th2 cytokine for the inflammatory process. The question that follows is whether a persistent supply of epidermal IL-4 is necessary to maintain the

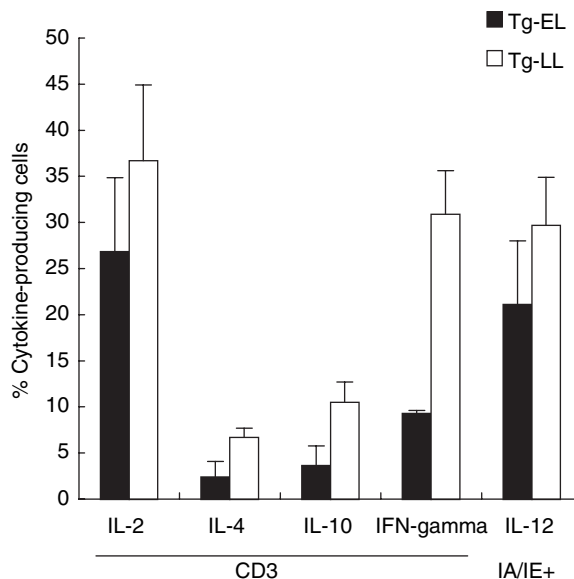


Fig. 5. Intracellular IL-2, IL-4, IL-10 and IFN- γ expression in CD3⁺ cells and IL-12 expression in IA-IE⁺ cells in the skin. Single-cell suspensions of skin from early and late stages of disease mice were stimulated with PMA/ionomycin for IL-2, IL-4, IL-10 and IFN- γ analyses or stimulated by LPS for IL-12 analysis as described in the Materials and methods section. Data shown are a summary of three similar experiments.

skin inflammation. We are currently back-crossing our IL-4-Tg mice with C57BL/6 mice to establish a syngeneic IL-4-Tg mouse line for the purpose of adoptive transfer. Adoptive transfer of T cells from diseased IL-4-Tg mice to non-Tg mice, which do not provide a continuous epidermal source of IL-4, will be performed in our future studies to clarify this point.

Although there is no parallel and quantitative study of cytokines in any human AD or other animal models of AD from which we can draw a direct comparison with our current study results, some degrees of comparative analyses could still be possible. In human AD studies some investigators, applying a semiquantitative *in-situ* hybridization method, have reported that at the early stage of the disease, only Th2 cytokine mRNA-expressing inflammatory cells, such as those carrying IL-4, IL-5 and IL-13, can be detected in significantly increased numbers in the skin lesions, compared to non-lesional skin [20,34]. However, at the later stage of the disease Th1 cytokines, such as IL-12, as well as IL-13, can be detected similarly in the skin lesions [20]. However other investigators, using the RT-PCR method, reported that the amplification signals for IFN- γ mRNA were increased in the late skin lesions of human patients compared to those of normal controls [19]. In canine spontaneous AD, one report using the method of semiquantitative RT-PCR identified a significant up-regulation of Th1 (IL-2, IFN- γ), Th2 (IL-4) and non-Th (TNF- α) cytokines in the skin lesions, although the stage of the skin lesions that were examined was not stated clearly [35]. In a spontaneous murine model of AD, NC/Nga mice skin showed RT-PCR-detectable mRNAs of IL-4 and IFN- γ only in lesional skin [22]. Cytokine studies were also performed by investigators who induced a murine model of AD by epicutaneous sensitization of allergen ovalbumin, revealing elevated levels of IL-4, IL-5 and IFN- γ mRNA in skin lesions using a semiquantitative RT-PCR method [36]. Another cytokine study was also conducted by investigators

who generated a murine model of AD by oral allergens, discovering significantly increased levels of IL-5 and IL-13 mRNA in the skin lesion also using semiquantitative RT-PCR [24]. Interestingly, in their model IFN- γ mRNA showed a non-significant increase and IL-4 mRNA showed no increase at all in the lesional skin [24]. Our data are in general agreement with these previous studies in the following aspects: (1) cytokines of the Th1-type and Th2-type were involved in the skin inflammatory process in varying degrees and at different stages; (2) the up-regulation of Th2-type cytokines preceded that of Th1-type cytokines; and (3) non-Th type cytokines could play a role in the inflammatory process.

In comparison with allergen contact dermatitis, extensive studies showed that the topical application of a contact allergen, dinitrochlorobenzene (DNCB), preferentially stimulated high levels of Th1 cytokines, IFN- γ [37–40] and IL-12 [38,39] over a 12–13-day period. Such results are different from those which we observed in our model during the early stage of disease development. Furthermore, as contact dermatitis is an acute rather than a chronic disease, studies on the cytokine profile of contact dermatitis are performed only for the acute stage and therefore cannot be compared in a parallel manner to our studies on cytokine profile changes at different stages of AD. Thus, the changes of these cytokines from BO to EL and to LL stages are specific to our animal model that are similar to human AD.

How, then, does this complex cytokine network observed in the skin participate in the inflammatory process of this animal model of AD? In accordance with the data on cytokine milieu, it seems likely that IL-4, the constitutively present transgene product in epidermis, may have induced T cells and other cells involved in the inflammatory response and triggered the up-regulation of other Th2 cytokines, IL-3, IL-5, IL-6, IL-10, IL-13 and non-Th cytokines, IL-1 β and TNF- α as shown by their statistically significant increase in the skin of Tg-BO and/or Tg-NL (Fig. 2a). IL-3 is produced by activated T cells, mast cells and eosinophils. It is a haematopoietic growth factor that stimulates colony formation of erythroid, megakaryocyte, neutrophil, eosinophil, basophil, mast cell and monocytic lineages. IL-3 was also found to regulate endothelial responses related to inflammation, immunity and haemopoiesis [41,42]. IL-5 is produced by T cells, eosinophils, mast cells and natural killer (NK) cells, and stimulates proliferation and differentiation of B cells and eosinophils and promotes IgE production [43]. IL-13 is produced by activated T cells and mast cells and shares biological activities with IL-4, including induction of IgE, although it is not as potent as IL-4 [44]. Many cell types in the skin could be responsible for the up-regulation of these cytokines. It is now known that mouse keratinocytes can produce IL-1 [45], IL-3 [46], IL-6 [47,48], TNF- α [49] and IL-10 [50]. Fibroblast, another major cell type in the

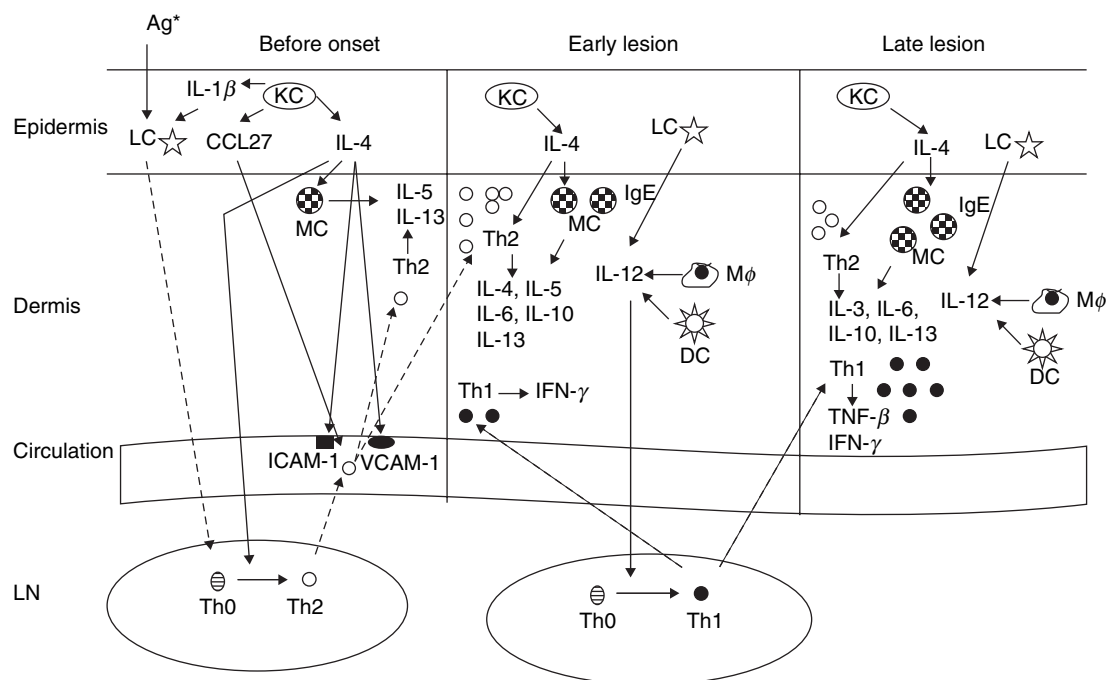


Fig. 6. Proposed immunological pathway in the IL-4 Tg AD mouse model. Before onset: unknown antigens or allergens activate LC. IL-1 β promotes LC to migrate to skin-draining LN. Activated LC and IL-4 convert Th0 to Th2 cells in the skin-draining LN. Up-regulated ICAM-1/VCAM-1 on blood vessels induced by IL-4 and skin-homing chemokine (CCL27) produced by keratinocytes promote Th2 cell to migrate into the skin. IL-4 stimulate mast cells to produce Th2 cytokines. Early lesion stage: as inflammatory cells build up in the skin, clinical inflammation is now observed. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which create a predominant Th2 environment. In addition, IL-4 induce B cell ϵ chain switching, inducing production of IgE, which upon binding mast cells causes further Th2 cytokine production. At the same time, IL-12 generated from activated DC/LC and M ϕ stimulates Th0 to Th1 cells, resulting in some Th1 cytokine secretion in the skin. Late lesion stage: as more Th1 lymphocytes infiltrate the skin, they produce larger amounts of Th1 cytokine IFN- γ and a new surge of TNF- β , both of which create a predominant Th1 milieu in the presence of Th2 cytokines. *Initiation step; $\rightarrow$: induction/production; $\dashrightarrow$: migration, LC: Langerhans cells; DC, dendritic cells; KC, keratinocytes; MC, mast cells; M ϕ , macrophages; $\bullet$: Th1 lymphocytes; $\circ$: Th2 lymphocytes; LN, skin-draining lymph node.

skin, is also known to produce several cytokines, including IL-6 and IL-1 β [51]. IL-10 is secreted by a wide number of cell types, including Th cells, APCs, mast cells, eosinophils and keratinocytes [52]. Human AD lesions had over-expressed IL-10 by large mononuclear cells in the dermis [53,54]. Dendritic cells in human AD also produced IL-10 [55]. In our study, the mRNA of IL-10 in the skin lesions was found to be increased markedly. The source was not clear, but could include APCs. Further experiment is needed to clarify this point in our animal model. Murine epidermal Langerhans cells (LC) produce IL-1, TNF- α and IL-6 [56]. The T cell is the main IFN- γ -producing cell type and the increase of CD3 $^{+}$ T cells in the dermis of non-lesional skin of Tg mice [33] may account for the increase of IFN- γ mRNA copy numbers in Tg-BO skin (Fig. 3). The relationship between IL-4 and the other cytokines up-regulated in the Tg mice skin before disease onset or non-lesional skin is not clear at this point. Together with IL-4, the up-regulation of these cytokines could be responsible for inducing the up-regulation of other non-Th cytokines IL-1 β and TNF- α (shown in Fig. 2a), as well as dermal microvascular endothelial adhesion molecule ICAM-1 [33]. It has also been reported that IL-4 increases the percentage of blood vessels expressing VCAM-1 and ICAM-1 on non-lesional skin obtained from human atopic dermatitis undergoing organ culture [57]. Another study identified that IL-4 increases expression of P-selectin and VCAM-1 on cultured human umbilical vein endothelial cells [58]. IL-4, IFN- γ or TNF- β alone increases endothelial cell adhesiveness for T cells [59]. These studies indicate that IL-4 is a critical cytokine for certain adhesion molecule expression on endothelial cells; thus it may initiate the inflammatory process by promoting leucocyte migration via its capability of induction of adhesion molecule expression. IL-1 β and TNF- α are known to be mandatory factors for the migration of LC and their early up-regulation in the skin BO implies their role in the initiation of the disease [60]. It is not clear why IFN- γ , one of the major Th1 cytokines, is also expressed in the skin of Tg-BO, although its mRNA copy number peaks at the Tg-EL stage. Its relative role in the disease initiation is probably not essential, because Th2 cytokines dominated the skin cytokine milieu of the early disease stages in our study.

The immunopathological pathways in the IL-4 Tg AD mouse model based on the findings of our present experiment and previous studies are proposed in Fig. 6. First, given our observation that inflammatory skin lesions occurred predominantly on hairless areas and that the mice do not develop skin lesions until few weeks after birth [16], we hypothesize that exposure to antigen is needed to initiate the skin inflammatory process. Therefore, as the initial event, unknown antigens or allergens activate LCs. IL-1 β , probably produced by keratinocytes [61], promotes the migration of activated LCs to the local LNs [60]. At the LNs, activated LCs present antigen to Th0 cells which under the influence of IL-4 are converted to the committed Th2 cells. In the skin dermal microvasculature, ICAM-1/VCAM-1, induced by IL-4 [57], and skin-homing chemokine (CCL27), produced by keratinocytes [62], facilitate Th2-cell migration into the skin from the circulation before disease onset. In addition, epidermal IL-4 exerts its effect on mast cells for their proliferation and for their Th2 cytokine production and secretion along with the cytokines released by infiltrating Th2 cells. The activities of these cellular and predominant Th2 cytokines (IL-4, IL-5, IL-13) result in early (acute) skin lesions; at this point, Th2 cells continue to produce substantial amounts of IL-4, IL-5, IL-6, IL-10 and IL-13, which maintain a strong Th2 immune response. Moreover IL-4, with its known

functional role in ϵ chain switching, activates B cells to produce large quantities of IgE [16]. By binding to the surface IgE receptor of mast cells, these abundant IgE activate them to degranulate [16] and secrete more Th2 cytokines and other proinflammatory molecules. At the same time, IL-12 generated from Th2 cell-activated DC/LC and/or M ϕ stimulates Th0 to Th1 cells. Th1 cells then produce large amounts of Th1 cytokines, including IL-2 and IFN- γ , which also contribute further to the inflammatory process and lead to late (chronic) skin lesions. At this point, IL-2, IL-12 and IFN- γ , together with a new surge of TNF- β , create a predominant Th1 milieu at the LL stage of the disease, whereas the reduced presence of Th2 cytokines IL-3, IL-4, IL-6, IL-10 and IL-13, along with non-Th proinflammatory cytokines (IL-1 β and TNF- α), provide additional fuel for the maintenance of the inflammation. In the absence of a strong inhibitory mechanism, this inflammatory process could go on indefinitely as observed in our Tg mice. The data from this current study will set up a framework for future detailed examination of the role of each of these cytokines in the pathogenesis of this inflammatory process.

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