VCAM-1 blockade delays disease onset, reduces disease severity and inflammatory cells in an atopic dermatitis model

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We investigated the functions of critical adhesion molecules ICAM-1 and VCAM-1 in a keratin-14 IL-4-transgenic (Tg) mouse model of atopic dermatitis, the skin lesions of which are characterized by prominent inflammatory cell infiltration, significantly increased mRNAs and proteins of ICAM-1, VCAM-1, E-selectin, P-selectin, L-selectin, and PSGL-1, and significantly increased numbers of dermal vessels expressing these adhesion molecules. We tested the hypotheses that deletion or blockade of these molecules may impede the inflammation by examining the disease progresses in the Tg mice crossed with ICAM-1-knockout mice and Tg mice received anti-VCAM-1-neutralizing antibody. Although the findings of the ICAM-1-knockout Tg mice (Tg/ICAM-11/−) developed skin lesions similar to wide-type ICAM-1 Tg mice (Tg/ICAM-11+/+) were surprising, a compensatory mechanism may account for it: the frequency of VCAM-1 ligand, CD49d, on CD3+ T cells in the lesional skin significantly increased in the Tg/ICAM-11/− mouse, compared with the Tg/ICAM-11+/+ mice. In contrast, anti-VCAM-1-treated Tg/ICAM-11/− or Tg/ICAM-11+/+ mice had significantly delayed onset of skin inflammation compared with isotype antibody-treated groups. Moreover, anti-VCAM-1 significantly reduced the skin inflammation severity in Tg/ICAM-11+/+ mice, accompanied with reduction of mast cell, eosinophil, and CD3+ T cell infiltration. VCAM-1 is more critical in developing skin inflammation in this model.

Keywords: atopic dermatitis; inflammation; ICAM-1; VCAM-1

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Received 6 August 2009; revised and accepted 1 December 2009; published online 12 January 2010

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ORIGINAL ARTICLE

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sions of these two molecules, we sought to provide direct evidence for their functions by either physical elimination through gene knockout or by functional blockade through antibody neutralization. Our results showed that VCAM-1, but not ICAM-1, had an important function in the development of skin inflammation in this model.

RESULTS
Significant upregulation of adhesion molecules in the skin of IL-4 Tg mice
To elucidate the function of vascular endothelial cell adhesion molecules in the development of skin inflammation in our model, we first sought to screen for the mRNA expressions of various molecules known to be important for skin inflammation. Using reverse transcription followed by quantitative real-time polymerase chain reaction (PCR), we found that the mRNA expressions of ICAM-1, VCAM-1, P-selectin, L-selectin, and PSGL-1 mRNAs in the skin of diseased IL-4 Tg mice in the early lesion (EL) stage significantly increased, in comparison to that of non-Tg mice. In addition, the ICAM-1, E-selectin P-selectin, and L-selectin expressions were also significantly increased in the Tg mice of the early lesion (EL) stage (Figure 1a). Western blot analysis further confirmed that protein level of ICAM-1 increased in the skin of EL and LL stages especially in the LL skin, paralleling the increase in mRNA levels (Figure 1b). We next sought to visualize the upregulation of these molecules at the dermal vascular endothelial cells. Using immunofluorescence microscopy, we determined the presence and the numbers of ICAM-1, VCAM-1, E-selectin, and P-selectin positively stained dermal vessels. Although there was no detection of expression of these four adhesion molecules in the vessels of non-Tg mice, small numbers of positively stained vessels were detected in the Tg mice at the disease onset (BO) stage (Figures 2a and b). As the disease progresses from BO stage to EL, and then to LL stage, there was a progressively larger number of positively stained vessels (Figures 2a and b). Parallel to the findings of increase vascular adhesion molecules in the skin, the skin infiltrating leukocytes bearing the L-selectin and PSGL-1 markers were also significantly increased in the diseased skin of Tg mice of the LL stage, compared with that of non-Tg mice (Figures 2c and d). The results provide indirect evidence that these adhesion molecules may have a function in the inflammation development of our model.

Generation of IL-4-Tg/ICAM-1-knockout mice
Having obtained indirect evidence, we next sought to provide direct evidence pointing to the function of these vascular adhesion molecules in the development of inflammation in our model. Toward that end, we first sought to examine the inflammation development in an ICAM-1 knockout mouse line in the context of IL-4 transgene. The scheme for generating the IL-4-Tg/ICAM-1-knockout (Tg/ICAM-1<sup>−/−</sup>) mice and control littermates are depicted in Figure 3a. Six groups of mice were generated from this method. The representative genotyping of these mice (with regard to the ICAM-1 gene) is depicted in Figure 3b.

ICAM-1 knockouts does not lessen the chronic disease severity of IL-4 mice
All the IL-4-Tg mice regardless of ICAM-1 genotype developed skin inflammation and the inflammation was mainly located on the ears. Seven weeks after the onset of the disease, the skin lesions were photographed and severity score recorded. To our surprise, the homozygous Tg/ICAM1<sup>−/−</sup> and Tg/ICAM1<sup>−/−</sup> had similar severity scores of skin inflammation (Figure 4). Similarly, there is no difference in disease severity between the heterozygous Tg/ICAM1<sup>+/−</sup> mice and the homozygous IL-4-Tg/ICAM1<sup>+/−</sup> mice (Figure 4). As expected, none of the non-Tg mice (non-Tg/ICAM1<sup>−/−</sup> and non-Tg/ICAM1<sup>+/−</sup>) had developed any skin lesions. The result indicates that ICAM-1 knockout alone does not block the skin inflammation development in our Tg mice and that there may be a compensatory inflammatory mechanism in the absence of ICAM-1.

Figure 1: Upregulations of adhesion molecules in the skin of IL-4-Tg mice parallel the disease progression. (a) mRNA expressions: total RNA from skin samples of non-Tg, Tg-BO, Tg-EL, and Tg-LL mice were extracted and reverse transcribed to cDNA. Copy numbers of ICAM-1, VCAM-1, E-selectin, P-selectin, PSGL-1, and L-selectin mRNA expression were determined by quantitative real-time PCR. (b) Protein expressions: skin protein extracts were obtained from the skins of non-Tg, Tg-BO, Tg-EL, and Tg-LL mice. After the SDS-PAGE and nitrocellulose membrane protein transfer, ICAM-1 was detected by an anti-ICAM-1 antibody followed by an Alexa Fluor 680-labeled secondary antibody. The membrane was then scanned by an Odyssey infrared scanner (Li-Cor). *P<0.05 compared with non-Tg mice.
Compensatory upregulation of expressions of ligands of VCAM-1 and P-selectin on T cells

In search for the possible compensatory mechanism that accounts for the undiminished inflammation in the absence of ICAM-1, we sought to examine the surface expression of CD49d [part of the VCAM-1 ligand, VLA-4 (CD49d/CD29)] on CD3+ cell in the skin lesions of the Tg/ICAM-1−/− mice, in comparison to that of the ICAM-1 Tg mice (Tg/ICAM-1+/+) mice. As shown in Figure 5a, 6.3% of CD3+ T cells in the lesional skin of Tg/ICAM-1−/− mice expressed CD49d, much higher than the 2.3% in Tg/ICAM-1+/+ mice (P<0.01). T cells express similar level of CD24 (P-selectin ligand) in Tg/ICAM-1−/− mice (15.8%) and Tg/ICAM-1+/+ mice (9.4%) (P>0.05; Figure 5a). In the skin-draining lymph nodes of Tg/ICAM-1−/− mice, CD49d expression on CD8+ T cells did have some, but not marked, increase compared with Tg/ICAM-1+/+ mice or non-Tg mice, but no increase was observed in CD4+ T cells (Figure 5b). CD24 expression on these two subsets of T cells in the lymph nodes of Tg/ICAM-1−/− mice, however, was significantly
upregulated compared with Tg/ICAM-1+/+ or non-Tg mice (Figure 5b). The results suggested that the upregulation of VCAM-1 and P-selectin may have compensatory functions for the skin inflammation in the absence of ICAM-1. Furthermore, the upregulation of CD49d seems to be a local event (in the skin), whereas the upregulation of CD24 may have occurred in the periphery.
Blockade of VCAM-1 delays disease onset and reduces disease severity

On the basis of the results above showing the upregulation of VCAM-1 ligand in the T cells of the Tg/ICAM-1−/− mice, we hypothesized that blocking the VCAM-1 in IL-4 Tg mice might impede the development of skin inflammation. Toward that end, Tg/ICAM-1−/− and Tg/ICAM-1+/+ mice were treated with monoclonal anti-VCAM-1 neutralizing antibody when the mice were 6 weeks old before the disease development and the treatment lasted continuously for 7 weeks. The results showed that Tg/ICAM-1−/− and Tg/ICAM-1−/− mice started to develop skin lesions at an average of 107 and 96 days of age, respectively, post-anti-VCAM-1 administration (Figure 6b), significantly delayed in comparison to the same groups of mice treated with isotype antibody control (61 and 64 days, P<0.01 and <0.05, respectively). There was no significant difference in the time of onset between anti-VCAM-1-treated Tg/ICAM-1−/− and Tg/ICAM-1−/− mice (Figure 6b). Interestingly, anti-VCAM-1 treatment significantly reduced the severity of the skin inflammation in Tg/ICAM-1−/− mice compared with isotype control-treated mice (Figure 6c). However, anti-VCAM-1 treatment did not reduce disease severity on the Tg/ICAM-1−/− mice (Figure 6c). Representative clinical phenotypes of these mouse groups were depicted in Figure 6d.

Histological document of VCAM-1 blockade of inflammatory cells

Having shown that VCAM-1 blockade reduced disease severity and delayed the disease onset in clinical phenotype, we sought to document the histological changes paralleling the reduction in clinical disease severity. By hematoxylin and eosin staining, the skin lesion in anti-VCAM-1-treated mice compared with that of isotype-treated mice has much less dense dermal inflammatory cell infiltration and thinner epidermis (Figure 6e). In particular, the numbers of mast cells (Figure 6f), eosinophils (Figure 6e), and CD3+ T cells (Figures 6g and h) present in the lesion of anti-VCAM-1-treated mice were significantly reduced with those of isotype-treated mice are substantially reduced (P<0.01). In addition, anti-VCAM-1 treatment also significantly downregulated mRNA expression of an inflammatory cytokine, IL-1β, and an eosinophil chemoattractant, CCL24 (Eotaxin 2) in the lesional skin (Figure 6f; P<0.01). mRNA expressions of IL-6 and IL-23 in anti-VCAM-1-treated group were also downregulated, though not significantly (Figure 6i). There were no changes of IL-4 between these two groups (data not shown). Furthermore, anti-VCAM-1 treatment significantly reduced the total serum IgE level (180.4 ng/ml−1) compared with that of isotype IgG-treated (400.3 ng/ml−1) Tg/ICAM-1−/− mice (Figure 6j, P<0.05).

DISCUSSION

In human beings with AD, the endothelial expressions of ICAM-1 and VCAM-1 were found to be significantly increased in the healthy-appearing skin (non-lesional skin) of these patients compared with the skin of normal individuals. The non-lesional skin showed a further increase of ICAM-1 and VCAM-1 expressions when cultured with medium alone, suggesting that these adhesion molecules are constitutively upregulated in the non-lesional skin of AD possibly because of the cytokines such as IL-4 released by cells in the skin. When the skin lesions developed, the endothelial expressions of ICAM-1 and VCAM-1 were found markedly increased in the lesional skin of AD patients compared with normal individuals. Moreover, some studies suggested a positive correlation of serum level of soluble ICAM-1 and VCAM-1 with the disease activity of AD. Furthermore, the soluble ICAM-1 level correlated significantly with the total numbers of leukocytes and lymphocytes in AD. A positive correlation was also found between the increase in soluble VCAM-1 level and both the clinical activities and the number of monocytes in AD. Therefore, it seems that ICAM-1 and VCAM-1 may be critical adhesion molecules in AD. To elucidate the direct function of ICAM-1 in our mouse model of AD, we first crossed this mouse line with ICAM-1−/− mice to generate Tg/ICAM-1−/− mice. However, the results showed that these Tg/ICAM-1−/− mice did not show any signs of reduction of skin inflammation, suggesting that ICAM-1 is not a pivotal adhesion molecule in this model with regard to the control of inflammatory cell migration. Our finding is in fact consistent with the other reports in human AD studies indicating that soluble ICAM-1 level has no correlation with the disease activity. Our result also suggests that a functional redundancy exists in the families of adhesion molecules. On the basis of the earlier reports that VCAM-1 is an important factor in human AD and the increase of CD49d, VCAM-1 ligand, on CD3+ T cells in the skin lesions of Tg/ICAM-1−/− mice in this study (Figure 5a), we reasoned that VCAM-1 may be the important redundancy of vascular adhesion and that VCAM-1 blockade may help elucidating its true function. Thus, we administered anti-VCAM-1-neutralizing antibody to the Tg/ICAM-1−/− and Tg/ICAM-1+/+ mice before the skin inflammation developed. The result showed that the treatment dramatically delayed the onset of skin inflammation in both Tg/ICAM-1−/− and Tg/ICAM-1+/+ mice, and alleviated the severity of skin lesions in Tg/ICAM-1+/+ mice, but not in Tg/ICAM-1−/− mice. Histologically, skin lesions of anti-VCAM-1-treated Tg/ICAM-1−/− mice had significantly less numbers of inflammatory cells infiltrate including T cells, mast cells, and eosinophils. Furthermore, this treatment reduced the mRNA expression of IL-1β, IL-6, and IL-23 in the skin lesions (Figure 6). IL-1β and IL-6 were two major pro-inflammatory cytokines that dramatically increased before disease onset skin or lesional skin of Tg mice (9). IL-23, a recently discovered IL-12-like T cell cytokine, was also slightly reduced after the treatment, correlating with the reduction of T cell skin infiltration (Figure 6). In addition, an eosinophil chemoattractant, CCL24, was significantly decreased after anti-VCAM-1 treatment, correlating with the reduction of eosinophil skin infiltration (Figure 6). Moreover, the anti-VCAM-1 treatment is associated with a reduction of total serum IgE as well as the numbers of mast cell infiltration in skin lesions (Figure 6). The reason for the decreased serum IgE level in the anti-VCAM-1-treated mice is not clear. The non-change of skin IL-4 between the anti-VCAM-1-treated and the isotype IgG-treated groups can possibly be explained by the constant presence of baseline IL-4 transgene in both groups. The reduction of these cytokines or chemokine explains the reduced skin inflammation after the treatment. Therefore, in our IL-4 Tg mice, VCAM-1 seems to have a more critical function in controlling the skin inflammation of this animal model. Our data are also in line with the findings of cutaneous delayed hypersensitive studies, showing that anti-VCAM-1 or VLA-4 antibody treatment diminished lymphocyte infiltrate in mice and monkeys. Further support for a greater importance of VCAM-1 over ICAM-1 included a study in which antibodies against VCAM-1 or VLA-4, but not ICAM-1 and lymphocyte function-associated antigen-1, prevented antigen-induced eosinophil infiltration of the mouse trachea, and the same treatments also inhibited CD4+ and CD8+ T cell infiltrates more potently than did ICAM-1 or lymphocyte function-associated antigen-1 blockade. Moreover, antibodies blocking alpha 4-integrin and VCAM-1 are capable of delaying onset of diabetes and decreased the incidence of the disease in mouse adoptive transfer studies accompanied by markedly reduced lymphocytic infiltration, whereas antibody specific for ICAM-1 had little effect on the onset or incidence of diabetes. The reason VCAM-1 has a greater function in these disease
Figure 6 VCAM-1-neutralizing antibody reduces skin inflammation in Tg ICAM-1+/- not in Tg ICAM-1-/- mice. (a) Timeline of anti-VCAM-1 treatment. (b) Neutralizing antibody to VCAM-1 delays the onset of skin inflammation and reduces the severity of skin disease (c) in comparison with those mice treated by non-specific isotype control (*P<0.01, **P<0.05 compared with their isotype control antibody-treated groups). (d) Representative clinical phenotypes of Tg/ICAM-1-/- and Tg/ICAM-1+/+ mice treated either with anti-VCAM-1 or isotype control. (e) Histopathology of Tg/ICAM-1+/+ mice treated either with anti-VCAM-1 or isotype control (hematoxylin/eosin stain, original magnification ×100). (f) Mast cells and eosinophils in the dermis after anti-VCAM-1 or isotype control treatment in Tg/ICAM-1+/+ mice. N=5 for each group (*P<0.01 compared with IgG control groups). (g) CD3+ T cell infiltrates in Tg/ICAM-1+/+ mice treated either with anti-VCAM-1 or isotype control. *P<0.01 compared with IgG control groups (g, original magnification ×250). (i) mRNA expression of IL-1β, IL-6, IL-23 and CCL24 in the lesional skin of anti-VCAM-1-treated Tg/ICAM-1+/- mice (*P<0.01 compared with IgG control groups). (j) Total serum IgE in anti-VCAM-1-treated Tg/ICAM-1+/- mice. n=10 in each group, *P<0.05 compared with IgG control groups.
models (delayed hypersensitivity, diabetes, asthma) may be due to the fact that key effector cells in these inflammatory disorders including T cells, monocytes, eosinophils, and basophils express alpha 4-integrin, a ligand for VCAM-1, whereas neutrophils, which are not important cell types in these disorders, express ICAM-1 ligand, but not VCAM-1 ligand.28 The same reason may be applicable to the findings in our model as T cells, eosinophils, and mast cells, but not neutrophils, are the key effector cells in AD.2–4 Although these studies provided encouraging evidence that inhibition of VCAM-1 and VLA-4 pathway-mediated adhesion could be a promising strategy in the treatment of autoimmune and allergic inflammatory diseases, these treatments targeting VCAM-1 and VLA-4 did not completely abolish the diseases. Thus, blockade of biologic redundancy involving other important pathways should be also considered, as we also found that there are upregulated expressions of E-selectin, P-selectin, L-selectin, and P-selectin ligand PSGL-1 in the skin lesions. Their direct functions in this animal model remain unclear and are needed to be further investigated.

It is interesting that Tg/ICAM-1+/− mice did not have any reduction in the skin inflammation, when compared with the Tg/ICAM-1+/+ mice. In fact the diseases in Tg/ICAM-1+/− mice are more severe than Tg/ICAM-1+/+ mice, although the difference was not statistically significant (Figure 4). Moreover, anti-VCAM-1 treatment reduced the severity of the skin inflammation in Tg/ICAM-1+/+ mice, not in Tg/ICAM-1+/− mice. One possible explanation is that the substantial increases of VCAM-1 ligand- and P-selectin ligand-bearing T cells as a result of ICAM-1 knockout may have, in a way similar to that of a competitive inhibition, rendered the blockage by a mixed quantity of anti-VCAM-1 less effective in the Tg/ICAM-1+/− mice than that in the Tg/ICAM-1+/+ mice. Alternatively, Knockout of ICAM-1 may have altered the immunological homeostasis in the Tg mice in such a yet-to-be-determined way that resulted in a more severe skin inflammation in Tg/ICAM-1+/− mice. The mechanism needs to be further elucidated.

In summary, we reported in this manuscript the findings that a blockade of VCAM-1 resulted in delay of skin disease onset, reduction of skin disease severity, and reduction of skin infiltration of mast cells, eosinophils, and T cells. Together with our earlier reported findings that blockade of T cell-selective chemokine CCL27 reduced the clinical disease phenotype and that the inflammatory disease progression correlated with the increasing inflammatory cell activation and migration toward the skin [10], the data from this Tg mouse AD model support a notion that the inflammation occurred in AD is an inflammatory cell-mediated process.

METHODS

Mice
Four to 12 weeks old IL-4 epidermal-Tg mice (IL-4 Tg) were used in the experiments.7–11,29 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. The study complied with the Animal Care Policies and Procedures of the University of Illinois at Chicago.

Tg/ICAM-1+/− mice were generated by crossing our IL-4-Tg mice (in CByB6 strain) with a commercially available homozygous ICAM-1−/− mouse line (Stock No. 002867, Jackson Lab, Bar Harbor, ME, USA). The homozygous ICAM-1−/− mice were first mated with IL-4-Tg mice to produce heterozygous Tg/ICAM-1−/− mice. The first five groups of mice did not have any reduction in skin inflammation when compared with the Tg/ICAM-1+/+ mice. Alternatively, Knockout of ICAM-1 may have altered the immunological homeostasis in the Tg mice in such a yet-to-be-determined way that resulted in a more severe skin inflammation in Tg/ICAM-1−/− mice. The mechanism needs to be further elucidated.

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Production of monoclonal antibody against mouse VCAM-1
Hybridoma producing anti-mouse VCAM-1-neutralizing monoclonal antibody (clone mK-2.7, rat IgG1) was purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI 1640 with 10% FCS and 2-mercaptoethanol. To produce an isotype control antibody, hybridoma AIIB2 (rat IgG1, anti-human integrin [β1-antibody, without cross-reaction with mouse tissue]) was purchased from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). Cells were cultured in Iscove’s Dulbecco’s modified Eagle’s medium as instructed. The proteins in culture supernatant were precipitated by ammonium sulfate, and then the IgG antibodies were purified using protein G Sepharose 4 column (Amersham Biosciences, Piscataway, NJ, USA). The purity of the IgGs was determined by SDS-PAGE to be >98% (data not shown).

Anti-VCAM-1 treatment and score of the disease severity
Tg/ICAM-1+/+ and Tg/ICAM-1−/− mice were treated with anti-VCAM-1-neutralizing antibody (0.5 mg per mouse, intra-peritoneal administration, every 5 days, n=10 for each group) started from 6 weeks of age before the skin lesions developed. This administration route and dose was chosen according to a protocol that successful blockaded the VCAM-1 function.29 The treatment was terminated 7 weeks after the disease onset (Figure 6a). For isotype control, two other groups of these mice (n=10 for each group) received the same amount of non-specific rat IgG1 treatment for the same treatment duration. The time of disease onset and severity scores were recorded as described.29 Briefly, it was determined by the number of location of skin affected by inflammatory skin lesions. The presence of inflammation, scale, or erythema, and severity on a scale of 0–4 and 0–3, respectively, was assessed as described.29

Quantitative real-time PCR
Total RNAs from skin tissues of non-Tg, Tg-BO, Tg-EL, and Tg-LL mice were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using Retro-script RT kit (Ambion, Austin, TX, USA) as described earlier.7 For real-time PCR, we used SYBR green PCR reaction mix (Bio-Rad, Hercules, CA, USA) with ICAM-1, VCAM-1, E-selectin, P-selectin, L-selectin, and PSGL-1 gene-specific primers (listed below) by method described earlier.9 cDNA templates of individual samples or 10-fold serial dilutions of the plasmid

Genotyping
For ICAM-1 genotyping, genomic DNA extracted from tail clipping was genotyped by PCR for homozygous knockout (ICAM-1−/−), heterozygous (ICAM-1−/+), or wild type (ICAM-1+/+) using a primer pair that detects neo gene in the ICAM-1 target mutation procedure: 5′-CTGAATGCAATGCGAGGCAAGA-3′; 5′-ATACCTTCTCGGGAGGAAA-GA-3′ and a primer pair that detects the wild type gene: 5′-CAGCTACATTACCCCCAGCTC-3′; 5′-TGAACGTGTTAAGGTCCTCT-CG-3′. The primer sequences and PCR conditions were obtained from Jackson Lab with the following parameters: preheating at 94°C for 1.5 min, followed by a 35-cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, then by an extension of 72°C for 2 min. The products were run on 1.8% agarose gel and then examinations and photos were conducted under UV light transillumination. The method for IL-4 genotyping was published earlier.9 As we have earlier determined that IL-4 transgene copy numbers do not correlate with disease severity,29 heterozygous IL-4-Tg mice were used in our experiment.

Disease phenotype classification
Skin lesions from IL-4 Tg mice that have been developed for 1 week or shorter in duration were defined as early lesion (EL). Skin lesions that have been developed for 3 weeks or longer in duration were defined as late lesion (LL). Tg mice before any skin lesion surfaces were defined as before onset of disease (BO), as described in our earlier publications.9–11,29
standards with concentrations range from 5×10^1 to 5×10^6 copies. For all adhesion molecules, 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 copy number of the gene present in the mRNA extract of the tissue was automatically determined by a software program in Mx3000P real-time PCR unit (Stratagene, La Jolla, CA, USA) according to the standard curve of the plasmid DNA. 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 GAPDH in an individual sample to give a calculating factor. The normalized adhesion molecule gene copy number was determined by multiplying this calculating factor with the gene copy number obtained from standard curve generated by PCR. Primers used included: E-selectin: forward: 5′-TTT-GAA-CTG-GGG-GTT-ATT-3′, reverse: 5′-GGA-CTT-CAA-GGC-TTC-ATC-TCC-3′; P-selectin: forward: 5′-GCC-AGT-CTA-TGG-GGC-ATG-AA-3′, reverse: 5′-GGC-AAA-GAT-TGC-TGG-ACA-CTT-3′; VCAM-1: forward: 5′-GTG-ATA-AGT-ATC-GGC-GTC-TCT-TT-3′, reverse: 5′-GGG-GAC-TGG-ATC-TGG-ACA-CT-3′; L-selectin, forward: 5′-ATC-TCA-CTG-GTG-GAG-AGG-3′, reverse: 5′-TTC-CGC-ACT-GGG-TAC-ATG-TG-3′; ICAM-1: forward: 5′-GCC-CAG-GTC-CAA-ATC-ACA-CT-3′, reverse: 5′-GCC-AGT-ATC-CAA-CGA-AA-3′, reverse: 5′-GCC-CTG-ATC-CCT-GAT-CAT-3′. Primers used for GAPDH were published earlier.\(^9\) In addition, relative quantities of cytokine mRNAs (IL-1β, IL-4, IL-6, IL-23) and chemokine mRNAs (CCL24/Eotaxin 2) in the skin lesions between Tg/ICAM-1+/− and Tg/ICAM-1−/− lesional skin 7 weeks after the onset of the skin inflammation were prepared as described earlier by Elbe-Burger et al.\(^35\) We also used the same method to analyze intercellular cytokine expression in CD3+ and MHC II cells from skin lesions.\(^9\) Cells were first incubated with Fc blocker (anti-CD16/32) (eBioscience, San Diego, CA, USA), then with FITC anti-CD3 (Clone 145–2C11), and PE anti-CD24 (Clone 30–F1) or CD49d (Clone R1–2) (eBioscience), and finally analyzed by Calibur FACs system (BD Biosciences). For analyses of CD42 and CD49d on CD3 cells in the skin lesions, single suspensions were stained with anti-ICAM-1+/− and anti-ICAM-1−/− mAb, and IF staining for the Tg/ICAM-1+/− and Tg/ICAM-1−/− groups were also performed by semi-quantitative real-time PCR as described in Chen et al.\(^9\) Primers used for IL-1β, IL-4, IL-6 were published earlier.\(^9\) Primers for CCL24 and IL-23 were earlier published in Maatta et al.\(^22\) and Broberg et al.\(^33\), respectively.

**Western blot analysis**

Mouse ears were collected from non-Tg, Tg-BO, Tg-EL, and Tg-LL mice and tissues were then homogenized in the presence of multiple protease inhibitors (Sigma, St Louis, MO, USA) containing lysing solution. After centrifugation, supernatants were collected and the amount of proteins was quantified by a mouse IgE enzyme-linked immunosorbent assay kit (Bethyl Laboratories, INC, Montgomery, TX, USA). The separated proteins were transferred to nitrocellulose membrane. Membrane was first blocked with an infrared scanner-specific blocking buffer (Li-Cor, Lincoln, NE, USA), then a rat anti-mouse ICAM-1 (Clone YN1/1.7.4, 0.25 μg ml⁻¹, Southern Biotech, Birmingham, AL, USA) was applied to the membrane at room temperature followed by incubation of an Alexa Fluor 680-labeled goat anti-rat IgG (Invitrogen). Image was acquired using Odyssey infrared scanner (Li-Cor).

**Histology**

Seven weeks after anti-VCAM-1 or isotype treatment in Tg/ICAM-1+/+ mice, lesional skin samples were collected, fixed in formalin, and processed for immunohistochemistry staining of formalin-fixed paraffinized skin samples were performed as earlier described with minor modifications.\(^34\) The primary antibody used was a monoclonal rat anti-mouse CD3 (Southern Biotech). Five different areas of each sample were counted per high power field and averaged (n=5).

**FACS analyses**

For analysis of CD24 and CD49d on CD3 cells in the skin lesions, single suspensions and immuno-staining for the Tg/ICAM-1+/− and Tg/ICAM-1−/− lesional skin 7 weeks after the onset of the skin inflammation were prepared as described earlier by Elbe-Burger et al.\(^35\) We also used the same method to analyze intercellular cytokine expression in CD3+ and MHC II cells from skin lesions.\(^9\) Cells were first incubated with Fc blocker (anti-CD16/32) (eBioscience, San Diego, CA, USA), then with FITC anti-CD3 (Clone 145–2C11), and PE anti-CD24 (Clone 30–F1) or CD49d (Clone R1–2) (eBioscience), and finally analyzed by Calibur FACs system (BD Biosciences). For analyses of CD24 and CD49d on CD4 and CD8T cells in the skin-draining lymph nodes of Tg/ICAM-1+/−, Tg/ICAM-1−/−, non-Tg/ICAM-1+/−, and non-Tg/ICAM-1−/− mice, single cells suspensions were stained with FITC anti-CD4 (Clone RM4-5)/CD8 (Clone 53–6.7) (eBioscience) and PE anti-CD24 or CD49d, then analyzed by FACS. In all experiments, samples with FITC or PE-conjugated isotype controls were also analyzed. All antibodies were used at a final concentration of 5 μg ml⁻¹. The reason for examining CD24 and CD49d on CD3+ cells instead of on CD4+ and CD8+ subsets in the skin lesions was that it was difficult to obtain enough CD4+ and CD8+ cells from the skin to analyze.

**Enzyme-linked immunosorbent assay**

Total serum IgE in anti-VCAM-1–treated Tg/ICAM-1−/− mice and IgG control mice (n=10 in each group) was determined using a commercially available mouse IgE enzyme-linked immunosorbent assay kit (Bethyl Laboratories, INC, Montgomery, TX, USA).

**Statistical analyses**

All experimental data were expressed as mean ± s.d. The significance of the variation among different groups was determined by one-way ANOVA analysis and the difference between two groups was determined by Tukey–Kramer Multiple Comparison Test using GraphPad Instat Software (San Diego, CA, USA).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We thank Mr Prakash Venkataramani and Dr Valeria I Barriuso for their technical help and helpful discussion. This work is supported in part by NIH grants (R01 AR47667, R03 AR47634, and R21 AR48438, L S Chan) and Albert H and Mary Jane Slepyn Fellowship Fund (L Chen).
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