

Immunopathology and Infectious Diseases

Stress-Induced Neurogenic Inflammation in Murine Skin Skews Dendritic Cells Towards Maturation and Migration

Key Role of Intercellular Adhesion Molecule-1/Leukocyte Function-Associated Antigen Interactions

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The skin continuously serves as a biosensor of multiple exogenous stressors and integrates the resulting responses with an individual's central and peripheral endogenous response systems to perceived stress; it also acts to protect against external challenges such as wounding and infection. We have previously shown in mice that stress induces nerve growth factor- and substance P-dependent neurogenic inflammation, which includes the prominent clustering of MHC class II⁺ cells. Because the contribution of dendritic cells (DCs) in response to stress is not well understood, we examined the role of DCs in neurogenic inflammation in murine skin using a well-established murine stress model. We show that sound stress increases the number of intradermal langerin⁺ and CD11c⁺ DCs and induces DC maturation, as indicated by the up-regulated expression of CD11c, MHC class II, and intercellular adhesion molecule-1 (ICAM-1). Blocking of ICAM-1/leukocyte function-associated antigen-1 interactions significantly abrogated the stress-induced numeric increase, maturation, and migration of dermal DCs *in vivo* and also reduced stress-induced keratinocyte apoptosis and endothelial cell expression of ICAM-1. In conclusion, stress exposure causes a state of immune alertness in the skin. Such adaptation processes may ensure protection from

possible infections on wounding by stressors, such as attack by predators. However, present-day stressors have changed and such adaptations appear redundant and may overrun skin homeostasis by inducing immune dermatoses. (Am J Pathol 2008, 173:1379–1388; DOI: 10.2353/ajpath.2008.080105)

Mammalian skin serves as a complex biological interface system that protects against external challenges such as wounding, infection, or UV radiation and even safeguards against viral and carcinogen-induced transformation. These complex functions only can be executed because skin represents far more than a simple mechanical barrier. It also constitutes a critical first barrier of immune defenses targeted against external stressors that crucially directs the linkage of innate and adaptive immunity.¹

Superimposed on this is the impact of psychological stress, which may challenge these complex interface functions of the skin.² In this context, we previously introduced the existence of the brain-skin connection³ by revealing that the skin and its appendages are not only vulnerable to key stress mediators such as Corticotropin-releasing hormone, substance P (SP), and nerve growth factor, but also are themselves potent sources of these stress response mediators.^{4–6} This—insufficiently defined—brain-skin connection provides the rationale for common skin responses to stress such as excessive

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sweating, flushing (facial erythema), pruritus, and neurogenic inflammation, and the triggering and/or exacerbation of chronic inflammatory dermatoses by stress.^{3,7}

Dependent on the dendritic cell (DC) phenotype, the cross talk between keratinocytes and DCs is now thought to either skew the adaptive immune response toward inflammation or to promote a state of nonresponsiveness (tolerance).^{1,8–10} Various phenotypes of cutaneous DCs have been described with different localization and function, eg, epidermal Langerhans cells (LCs), which express the surface marker langerin/CD207.^{11,12} Further, dermal DCs have been described, characterized by the expression of lectin DC-SIGN/CD209 in humans¹³ and the marker CD11c as well as lack of macrophage markers in mice.^{14–16}

To elicit a specific immune response, LCs migrate from the skin to the draining lymph nodes where antigen presentation to T cells takes place. Here, LCs change their phenotype and become mature DCs, expressing surface molecules such as MHC II and intercellular adhesion molecule-1 (ICAM-1) whereas other markers such as langerin are down-regulated.^{17–19} In contrast to LCs, which have to migrate to present antigens, dermal DCs might be able to present antigens *in situ* to T cells localized in the skin.²⁰ To facilitate such T-cell interaction with antigen-presenting cells (APCs), peripherally circulating leukocytes may be recruited to a specific site such as the skin.²¹ Molecules that play an upstream and critical role in controlling tethering (adhesion) and spreading of T cells are integrins such as leukocyte function-associated antigen-1 (LFA-1) on T cells (CD11a/CD18, $\alpha_1\beta_1$ integrin) and ICAM-1 on vascular endothelium.^{21,22}

Blocking of LFA-1/ICAM-1 in mice *in vivo* can abrogate the onset of inflammation, hereby preventing fetal rejection.²³ Moreover, LFA-1/ICAM-1 interactions also are important during graft-versus-host reactions,²⁴ the development of autoimmunity,²⁵ and neuropeptide SP-induced leukocyte migration.²⁶ The latter observation is particularly striking since we were recently able to show that stress leads to neurogenic inflammation in murine skin, comprised of sprouting nerve fibers, increased number and activation of MHC II⁺ APCs and mast cells, up-regulation of nerve growth factor, and the neuronal plasticity of dorsal root ganglia toward an increased presence of SP⁺ neurons.^{4,5,27} Remarkably, a wealth of published evidence indicates that the close anatomical relationship of the sensory nervous system and DCs is engaged in the modulation of antigen presentation in various examined tissues.^{28–31}

Established mouse models to investigate the impact of psychological stress on skin homeostasis are nowadays accessible.^{5,32,33} Here, our group and others use the exposure to sound stress, a stress model that has also become appreciated as a highly instructive tool for neuroimmunological stress research beyond the skin.^{34–37} We have now followed-up our previous observations on stress-induced neurogenic skin inflammation by focusing on DCs.

Specifically we asked whether sound stress in mice results in an increased number of CD11c⁺ DCs and/or langerin⁺ skin cells and/or changes key APC maturation

markers such as MHC II and ICAM-1. In subsequent experiments, we addressed the question whether blocking of LFA-1/ICAM-1 interactions in the context of stress-triggered skin inflammation affects the number and phenotype of skin DCs, alters stress-induced changes in endothelial ICAM-1 expression and/or in keratinocyte apoptosis. Finally, we wished to clarify by adoptive transfer experiments whether LFA-1⁺ cells are capable of migrating from the blood to dermal sites of inflammation.

Materials and Methods

Animals

Six- to eight-week-old female C57/BL6 mice were purchased from Charles River, Sulzfeld, Germany and maintained in an animal facility with a 12-hour light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to requirements of the authority for animal research conduct at the respective institutions. After depilation to induce a synchronization of the hair cycle stage in all mice (see below for method), we performed three experiments.

The first experiments were performed to investigate the effect of stress on cutaneous APC surface and maturation marker expression in stressed and nonstressed mice (for stress application see below). Subsequently, experiments were conducted to analyze the effect of blocking of ICAM-1/LFA-1-mediated intercellular adhesion events involved in DC maturation and migration after stress (for antibodies please see below). Mice were divided into different subgroups: one group served as a control, receiving 100 μ l of isotype control antibodies by intraperitoneal injection ($n = 10$) on days 11 to 15. Another group was exposed to stress on day 15 and received intraperitoneal injections of 100 μ l of isotype control antibodies on days 11 to 15 ($n = 10$). The third group was exposed to stress on day 15 and injected with monoclonal antibodies (mAbs) against ICAM-1 and LFA-1 on days 11 to 15 ($n = 10$). The last group was injected with mAbs against ICAM-1 and LFA-1 on days 11 to 15 without stress exposure ($n = 10$). All mice were sacrificed 16 days after depilation, since we previously observed the most profound stress-induced alterations of the skin immunity during this period. Lastly, experiments were performed to prove migration and local intercellular cross talk with the help of adoptive cell transfer of LFA-1⁺ cells injected into YFP mice (see below).

Synchronization of Hair Cycle by Depilation-Induced Anagen Induction

Anagen was experimentally induced by depilation, as previously published.^{38,39} Briefly, on day 0 mice were anesthetized with an intramuscular injection of ketamine hydrochloride (Ketanest, 10 mg/kg body weight; Parke-Davis, Freiburg, Germany) and xylazine hydrochloride (Rompun, 10 mg/kg; Bayer, Leverkusen, Germany). Then, a wax/rosin mixture was applied to the dorsal skin of mice with all hair follicles in telogen, as evidenced by

the pink back skin color. Peeling off the wax/rosin mixture removes all hair shafts and immediately induces homogeneous anagen development over the entire depilated back skin area of the mouse, thus inducing a highly synchronized anagen development. After full anagen development, the consecutive stages (catagen and telogen) then develop spontaneously in relatively homogeneous wave-like pattern, starting in the neck region.³⁹ Although depilation induces cutaneous changes that can influence the dermal immune responses, we decided to integrate this aspect in our experimental design because it will result in a synchronized hair cycle and thus, allow for direct comparison of mice.

Exposure to Stress

Mice were exposed to sound stress for the duration of 24 hours starting on day 14. The sound stress was emitted by a rodent repellent device (Conrad Electronics, Berlin, Germany) at a frequency of 300 Hz and an intensity of 75 dB at intervals of 15 seconds. The stress device was placed into the mouse cage so that the mice could not escape the sound perception. The paradigm of 24 hours of stress challenge and subsequent tissue analysis has been established by a wealth of previously performed experiments.^{4,5,23,27,32,34,36,40–43} It is based on pilot experiments using stress challenge ranging from 45 minutes, 2 hours, 4 hours, 12 hours, 24 hours, and 48 hours of sound, which led to appreciate 24 hours of sound challenge as a very moderate stressor. Other stressors, ie, restraint, have a more profound effect, ie, on the skin immune system, where 2 hours suffice to mirror the results seen on 24 hours of sound challenge.⁴⁰ Our previous work using the same stress paradigm in another context revealed that immune alterations occur within 6 hours and are best detectable between 24 to 48 hours. Using a single exposure, effects of stress challenge on the immune response will diminish after 48 hours.²³

Antibody Application

We used a mixture of anti-LFA-1, which reacts with the 180-kDa α 1-chain of LFA-1, a heterodimeric surface glycoprotein expressed on almost all leukocytes (clone M17/4, no. 553337; BD Pharmingen, San Diego, CA), and anti-ICAM-1 (clone 3E2, no. 553249; BD Pharmingen), and injected 25 μ g of each mAb/mouse/day in 100 μ l of sterile phosphate-buffered saline (PBS). Groups I and II received injections with the respective isotype controls for LFA-1 (hamster IgG1, no. 553968; BD Pharmingen) and ICAM-1 (rat IgG2a, no. 555840; BD Pharmingen).

Adoptive Transfer of LFA-1⁺ Cells into Transgenic CD11c Mice

To prove the migration of LFA-1⁺ cells from blood to tissue and intercellular cross talk between LFA-1⁺ and CD11c⁺ cells in the skin, we adoptively transferred LFA-1⁺ cells labeled with the cytoplasmic Cell Tracker™

Orange CMTMR {5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine}-mixed isomer (Invitrogen, Karlsruhe, Germany) into transgenic mice whose CD11c express enhanced yellow fluorescent protein (YFP).⁴⁴ The CD11c YFP mice were kindly provided by M. Nussenzweig (Rockefeller University, New York, NY). Based on the increased expression of LFA-1 on cells on stress challenge,²³ we first isolated LFA-1⁺ cells from spleens of stressed donor mice 24 hours after stress termination. Spleen cells were obtained by mincing the organs through a cell strainer, then cells were washed twice with sterile PBS and incubated (30 minutes, 4°C) with biotinylated rat anti-mouse LFA-1 (no. 557365, BD Pharmingen) diluted 1/200 in labeling buffer. After washing, cells were incubated (15 minutes, 4°C) with anti-biotin MicroBeads (Miltenyi Biotec, Gladbach, Germany) and processed using mini-MACS columns (Miltenyi Biotec) to collect LFA-1⁺ cells. Examination by flow cytometry revealed that >95% of selected cells expressed LFA-1. The phenotype of LFA-1⁺ cells used for subsequent adoptive transfer experiments was as follows: 65.5% CD3, 37.6% CD4⁺, 27.9% CD8⁺, 2.9% CD49b⁺, 18.7% Gr1⁺, 14.3% CD11c⁺. Isolated cells were labeled with cytoplasmic Cell Tracker Orange CMTMR (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Before LFA-1⁺ intravenous cell transfer, cell viability was checked and cell number adjusted to a concentration of 5×10^6 cells/100 μ l with sterile PBS. Then, 5×10^6 cells in 100 μ l of sterile PBS were injected intravenously into CD11c YFP recipient mice. Mice were sacrificed 24 hours after the adoptive LFA-1 cell transfer and skin was harvested as described in the following section.

Immunohistochemistry for MHC II, ICAM-1, Langerin, CD11c

On day 16 after depilation, at the time when control mice are just about to spontaneously enter the anagen/catagen transformation of their depilation-induced hair cycle,⁴⁵ all mice were sacrificed. Skin tissue from the neck region of murine back skin was harvested parallel to the vertebral line. Specimens were snap-frozen in liquid nitrogen and then covered with embedding medium, as described in detail in by Müller-Röver and colleagues.³⁹ Cryosections were then processed for immunohistochemistry. Cryostat sections (8 μ m) were incubated with peroxidase-, avidin-, and biotin-blocking solution (Vector Laboratories, Burlingame, CA), followed by another block using protein blocking agent (Immunotech, Marseille, France). The primary antibody (rat anti-mouse MHC II, no. T-2106 BMA; biotinylated hamster anti-mouse ICAM-1, no. 553251, BD Pharmingen; goat anti-mouse langerin, no. SC 22620, Santa Cruz Biotechnology, Santa Cruz, CA; biotinylated hamster anti-mouse CD11c, no. 553800, BD Pharmingen) was diluted 1/200 (langerin 1/100) in Tris-buffered saline containing 1% fetal calf serum and applied for 1 hour. After washing steps secondary antibody diluted 1/200 was applied (for MHC biotinylated goat anti-rat, no. 112-065-167 Jackson Im-

Table 1. Expression of ICAM-1 on Cutaneous Blood Vessels

	Control + IgG	Stress + IgG	Control + anti-LFA-1/ICAM-1	Stress + anti-LFA-1/ICAM-1
Blood vessels in dermis				
Total number of vessels	17.5 ± 4.1	20.1 ± 5.6	20.4 ± 4.3	17.5 ± 4.1
ICAM-1 (++)	14.9 ± 4.1	18.1 ± 5.0	14.3 ± 3.7	14.9 ± 4.1
ICAM-1 (+)	2.2 ± 1.5	1.8 ± 1.5	4.1 ± 2.8	5.0 ± 3.0
ICAM-1 (–)	0.4 ± 0.7	0.2 ± 0.4	2.0 ± 1.3	1.7 ± 1.5
Blood vessels in subcutis				
Total number of vessels	46.0 ± 9.5	42.7 ± 6.0	38.3 ± 4.4	39.8 ± 11.1
ICAM-1 (++)	35.8 ± 11.6	39.3 ± 7.5	20.1 ± 7.6	17.9 ± 14.4
ICAM-1 (+)	6.8 ± 3.7	2.8 ± 3.0	11.5 ± 3.0	15.5 ± 6.0
ICAM-1 (–)	3.4 ± 2.3	0.6 ± 0.8	6.6 ± 3.1	6.4 ± 3.8

ICAM-1 expression was scored as followed: (–) equals negative, (+) refers to weak, and (++) refers to high expression. The data presented here have been generated from an expression score, thus, no levels of significance have been tested on these arbitrary numbers. Percentages of highly ICAM-1-positive blood vessels were calculated and are presented in Figure 4. For representative photomicrographs see Figure 2, M–R.

munoResearch, West Grove, PA; for langerin biotinylated donkey anti-goat, no. 705-066-147, Jackson ImmunoResearch) where necessary. As an amplification and revealing system, we used avidin-biotin alkaline phosphatase 1:100 in Tris-buffered saline for 30 minutes. The signal was detected by incubating sections with 0.2 mg/ml of diaminobenzidine (Sigma-Aldrich, St. Louis, MO) and 0.05% hydrogen peroxide, followed by light counterstaining with 0.1% Mayer's hematoxylin. The signal intensity of ICAM-1 on blood vessels was scored as follows: –, negative; +, weak; ++, high (Table 1). On each section an area of 1.25 mm² (dermis and subcutis) was evaluated. ICAM-1/PECAM double staining was performed to identify blood vessels [rat anti-mouse CD 31 (PECAM), no. 550274; BD Pharmingen]. Total number of blood vessels and numbers of blood vessels scored highly positive were used to calculate percentages indicated in figures.

Immunofluorescence histochemistry was performed adapting established protocols.^{5,46} Briefly, cryostat sections (10 mm) were incubated overnight at room temperature in a humidity chamber with the primary antisera to MHC II (rat anti-mouse, no. T-2106, BMA, dilution 1/50) or CD11c (hamster anti-mouse, no. 550283, dilution 1/200; BD Pharmingen). This was followed by an incubation of 1 hour at 37°C with tetramethylrhodamine-isothiocyanate or carbocyanine (Cy) 2-conjugated F(ab)2 fragments of goat anti-rat or hamster anti-rat IgG (Dianova, Hamburg, Germany) at a dilution of 1:200. Washing steps with Tris-buffered saline were interspersed between all staining steps. All antibodies were diluted in Tris-buffered saline at pH 7.4 containing 2% normal goat serum. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole.⁴⁷ For double-immunofluorescence detection of MHC II- and CD11c-positive cells application of protocols was combined. Slides were examined using a Zeiss Axioscope light microscope and a Zeiss Axioscope fluorescence microscope (Zeiss, Oberkochen, Germany). Photo documentation was performed using digital image analysis systems (Zeiss KS400 and VisiTron Systems).

Terminal dUTP Nick-End Labeling (TUNEL) Staining

To evaluate apoptotic cells in murine hair follicles, we used the TUNEL staining method as described before.⁴⁸

Ten-μm cryostat sections of murine back skin were freshly prepared and fixed in formalin, postfixed in ethanol/acetic acid, and incubated with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT), using a commercially available kit (Intergen, Oxford, UK). TUNEL-positive cells were visualized by anti-digoxigenin fluorescein isothiocyanate-conjugated F(ab)2 fragments, then counterstaining was performed using 4,6-diamidino-2-phenylindole dye (1 μg/ml methanol; Roche, Mannheim, Germany) in a subsequent incubation step. Finally, sections were mounted using VectaShield (Vector Laboratories). Negative controls for the TUNEL staining were made by omitting TdT, according to the manufacturer's protocol.

Statistics

Differences were judged as significant if the *P* value was ≤0.05 as determined by the Mann-Whitney *U*-test. The α level was adjusted by Bonferroni correction where necessary (α = 0.0125 for four comparisons). Results are presented as mean values ± SEM.

Results

Stress Exposure Results in Increased Number of Langerin⁺ and CD11c⁺ DCs and Induces DC Maturation in Murine Skin

To characterize and phenotype dermal MHC II⁺ cell clusters we described earlier^{4,5} and to identify additional populations with the potential to present antigens in the skin in response to stress, we analyzed the expression of CD11c, langerin, and ICAM-1 besides MHC II in the skin of stressed mice compared to nonstressed control mice. With the present data, we were able to confirm our previous observations with regard to an increased number of cell clusters positive for MHC II after stress (Figure 1A). These cell clusters are localized in the dermis as shown in Figure 2A. Further, we observed an increased number of cell clusters positive for the DC maturation marker ICAM-1 (Figure 1B), CD11c (Figure 1C), as well as langerin (Figure 1D) in the skin of stressed mice compared to nonstressed controls. Double immunofluorescence

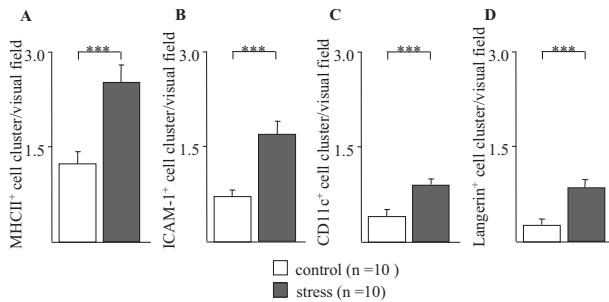


Figure 1. Stress increases the number of cell clusters positive for MHC II (A), ICAM-1 (B), CD11c (C), langerin (D). *** $P < 0.001$.

confirmed that the majority of the MHC II⁺ cell clusters were also positive for CD11c. Representative photomicrographs of the data presented in Figure 1, A–D, are shown in Figure 2, A–H, examples for the population of CD11c⁺ MHC II⁺ cells, shown by immunofluorescence, is provided in Figure 2, J–L.

Blocking of LFA-1/ICAM-1 Cross Talk Abrogates Stress-Induced Increase of Cutaneous DC Cell Clusters

To elucidate whether ICAM-1/LFA-1-dependent pathways are involved in the increased migration of APCs to the site of inflammation, we blocked ICAM-1/LFA-1-mediated intercellular adhesion events. As shown in Figure 3, A and B, blocking ICAM-1/LFA-1 interaction in stressed mice significantly decreased the number of langerin⁺ and CD11c⁺ cell clusters to an expression present in nonstressed control mice, indicating that stress-induced migration of DCs could be successfully blocked by anti ICAM-1/LFA-1. In the epidermis the number of langerin⁺ cells did not change with stress application or blocking of ICAM-1/LFA-1 (Figure 3E).

Blocking of LFA-1/ICAM-1 Cross Talk Affects the Phenotype of Skin APCs

Because the cross talk between co-stimulatory molecules on APCs, such as ICAM-1, and the respective ligand LFA-1 on leukocytes is crucial in inflammatory settings,^{21,23–26,49} we analyzed the phenotype of APC clusters in the skin in response to LFA-1/ICAM-1 blockade. As depicted in Figure 3, C and D, such blockade of ICAM-1/LFA-1 cross talk significantly abolished the stress-induced increase of cell clusters expressing the DC maturation markers MHC II and ICAM-1.

Blocking of LFA-1/ICAM-1 Cross Talk Abrogates Stress-Induced Increase of Endothelial ICAM-1 Expression in Murine Skin

To prove the influence of stress on adhesion molecule expression, we scored the endothelial expression of ICAM-1 in blood vessels of dermis and subcutis (Table 1). Stress increased the percentage of high positive blood vessels (Figure 4, A and B), and levels of signifi-

cance were reached in the subcutis (Figure 4B). Application of anti LFA-1/ICAM-1 dramatically down-regulated the percentage of highly ICAM-1-positive blood vessels in both dermis and subcutis (Figure 4, A and B). Representative photomicrographs are shown in Figure 2, M–R.

Blocking of LFA-1/ICAM-1 Cross Talk Abrogates Stress-Induced Increase of Keratinocyte Apoptosis

In the present study we were able to confirm earlier results that showed that stress-induced inflammation in the skin is associated with increased keratinocyte apoptosis, as revealed by an increased number of TUNEL⁺ cells in the hair follicle bulge region.^{4,32} A representative photomicrograph is shown in Figure 2S. Further, we were able to show that the increase in keratinocyte apoptosis could be abrogated by LFA-1/ICAM-1 blockade (Figure 5). Very few TUNEL⁺ cells could be detected in the epidermis (in average less than 0.1 cell per visual field at $\times 200$ magnification, (representative photomicrograph Figure 2T, $\times 400$ magnification) and no significant differences were observed between the groups.

LFA-1⁺ Cells in Blood Migrate to Dermal Sites of Inflammation

To prove the migration of LFA-1⁺ cells from blood to the skin and the LFA-1/ICAM-1 cross talk in the skin *in vivo*, we isolated LFA-1⁺ cells from stressed donor mice and adoptively transferred them. Here, we used CD11c YFP-transgenic mice as hosts because these mice provide a tool to visualize and evaluate DC networks *in vivo*.⁴⁴ Immunohistochemical analysis revealed that adoptively transferred LFA-1⁺ cells indeed migrate to the skin within 24 hours (Figure 2, U and V). Interestingly, these LFA-1⁺ cells could be identified in close proximity to DC11c⁺ skin cells, strongly suggesting a direct cross talk between DC and LFA-1⁺ cells.

Discussion

With the present work, we provide experimental evidence that mature APCs accumulate in the skin in response to stress. Further, we substantiate that stress exposure induces cell migration and up-regulates expression of adhesion molecule on vascular epithelium in the skin. These effects were highly dependent on the co-stimulatory molecule ICAM-1 and the respective ligand LFA-1, which are engaged in cell migration as well as in the cross talk between T cells and APCs in an array of inflammatory settings. The relevance of our findings was further corroborated by showing that blocking of LFA-1/ICAM-1 also significantly reduced stress-associated keratinocyte apoptosis. Further, we were able to confirm that peripheral LFA-1⁺ cells indeed migrate into the skin and establish contact to skin DC.

During tissue residence, DCs might receive a wealth of signals from the tissue environment, which may influence

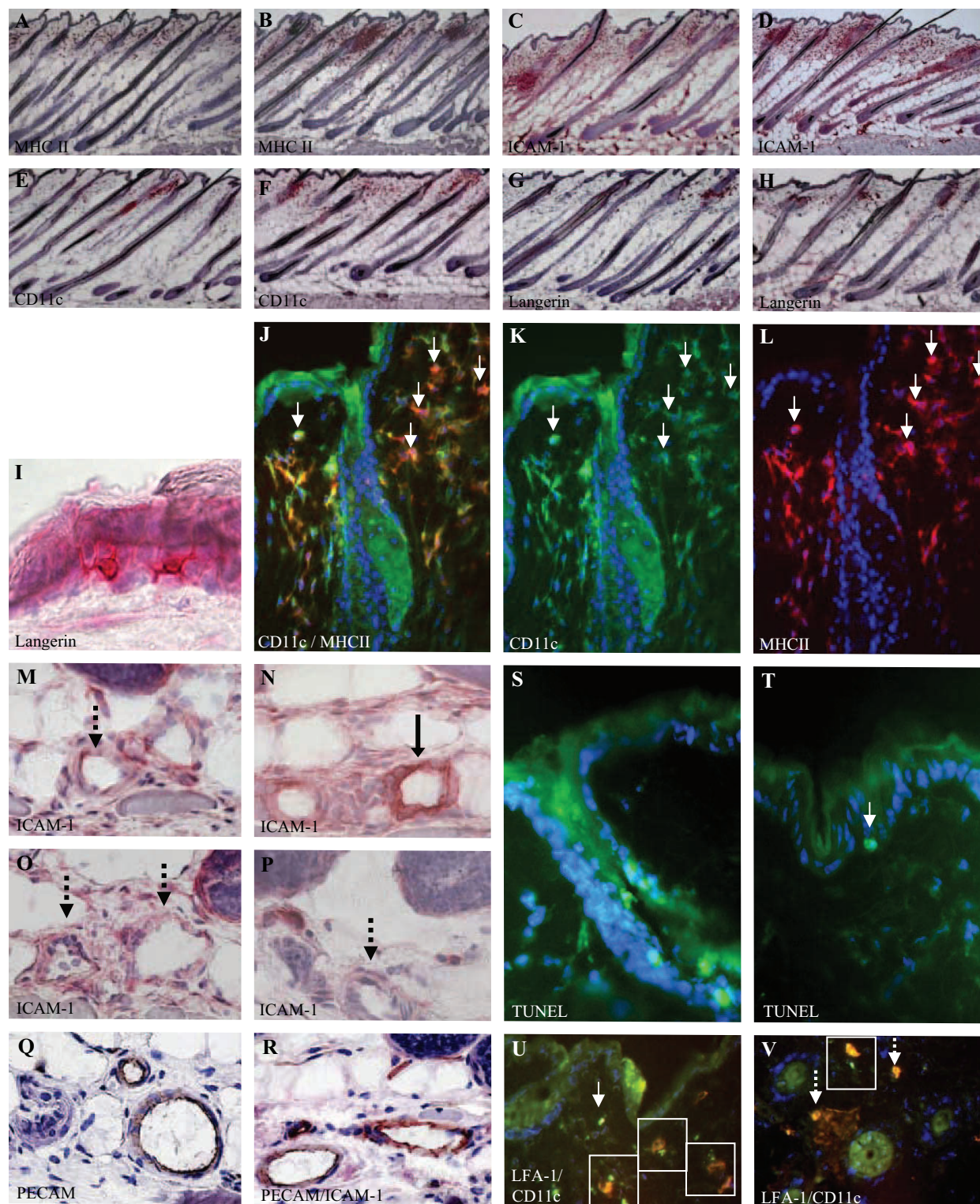


Figure 2. Representative photomicrographs of immunohistochemistry (IH) of skin sections from nonstressed (A, C, E, G) versus stressed (B, D, F, H) mice. MHC II (A, B), ICAM-1 (C, D), CD11c (E, F), langerin (G, H), langerin in epidermis (I). Double immunofluorescence for MHC II and CD11c (J; merged; K: CD11c; L: MHC II). ICAM-1 detection (IH) in subcutis of nonstressed (M, O) and stressed mice (N, P), without (M, N) and with blockade of LFA-1/ICAM-1 (O, P) (dotted arrows: weak positive expression; full arrows: strong positive expression). PECAM (blood vessel-specific antigen) single staining (Q) and PECAM/ICAM-1 double staining (R). Immunofluorescence showing apoptosis (TUNEL) in hair follicle (S) and epidermis (T). Immunofluorescence showing skin sections from transgenic mice whose CD11c express enhanced yellow fluorescent protein (YFP) after adoptive transfer of orange CMTMR-labeled LFA-1⁺ cells isolated from stressed mice (U, V). Original magnifications: $\times 100$ (A–H); $\times 500$ (I); $\times 400$ (J–V).

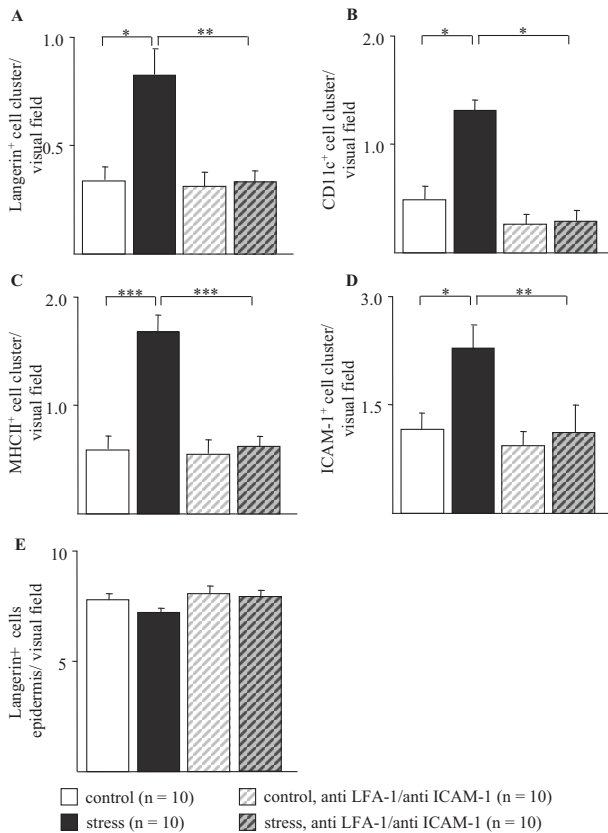


Figure 3. Blocking of LFA-1/ICAM-1 cross talk abrogates stress-induced increase of cutaneous DC cell clusters and affects APC phenotype. Numbers of cell clusters positive for langerin (A), CD11c (B), MHC II (C), ICAM-1 (D). Number of langerin⁺ cells in epidermis (E). $\alpha = 0.0125$ for four comparisons, $^*P < 0.0125$, $^{**}P < 0.0025$, $^{***}P < 0.00025$.

the maturation process of DC and hence, determine their phenotype and function.⁵⁰ In our murine model, exposure to stress resulted in an increase of mature DCs in the skin, based on markers indicating antigen-presenting potential, such as MHC II or ICAM-1. We did not observe significant differences with regard to the number of langerin⁺ cells in the epidermis and it remains to be elucidated whether the accumulation of langerin cells in the dermis in response to stress results from increased mi-

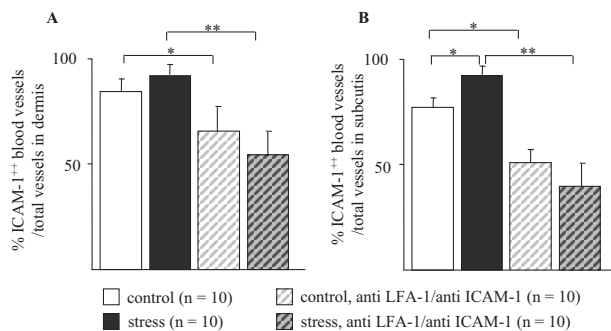


Figure 4. Percentage of highly ICAM-1⁺ blood vessels is increased after stress in subcutis (B) but not in dermis (A). Application of anti-LFA-1/ICAM-1 significantly reduces percentage of highly ICAM-1⁺ in subcutis and dermis in nonstressed and stressed animals. $\alpha = 0.0125$ for four comparisons, $^*P < 0.0125$, $^{**}P < 0.0025$.

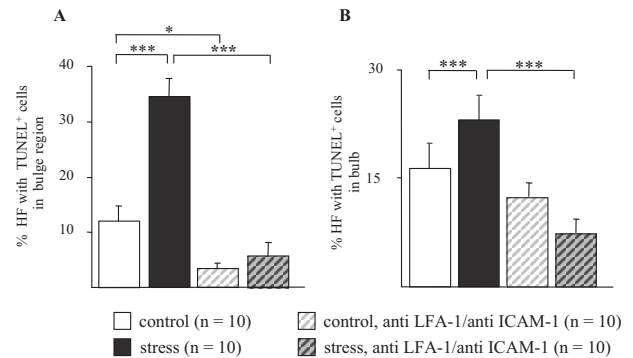


Figure 5. Stress induces increased percentages of TUNEL⁺ cells in bulge region of hair follicles (A) and bulb (B). This stress effect can be blocked by application of anti-LFA-1/ICAM-1. $\alpha = 0.0125$ for four comparisons, $^*P < 0.0125$, $^{***}P < 0.00025$.

gration from peripheral blood or is a consequence of trafficking from epidermis.

Surprisingly, the endogenous regulation of DC function is still poorly understood. Based on experiments performed *in vitro*, Dunzendorfer and colleagues⁵¹ proposed that peripheral neuropeptides might guide immature DCs to peripheral nerve fibers, whereby one function of sensory nerves may be to fasten DCs at sites of inflammation. Further, SP has been shown to evoke Ca²⁺ transients in DCs, which strongly promotes the notion that sensory nerves modulate DC function.⁵² Our previous evidence accumulated within the context of the brain-skin connection strongly supports the concept of DC phenotype modulation via neurogenic pathways because we were able to abrogate the expression of the DC maturation marker MHC II when antagonizing the neuropeptide SP high-affinity receptor NK1.^{4,32}

An increased number of skin APCs, which we could identify by increased expression of maturation markers and co-stimulatory molecules such as MHC II and ICAM-1 in response to stress in mice unaltered by skin diseases, is a prominent feature of inflammatory skin diseases in humans. For example, in patients suffering from atopic dermatitis, or other inflammatory skin conditions such as psoriasis, increased numbers of LCs⁵³ and/or increased numbers of DCs have been reported.⁵⁴ Because epidemiological trials corroborate an aggravation of such skin diseases in response to stress, we now propose that the stress-triggered migration and maturation of skin DCs might initiate and/or perpetuate such skin diseases.

Additional pathways of DC activation might result from an overall inflammatory environment, eg, as present in murine skin in response to stress. Here, skin mast cell activation could be identified as cardinal switchboard of neurogenic skin inflammation and deficiency of mast cells, as present in WW^v mice, protected from stress-induced up-regulation of MHC II cells in the skin.⁴⁰ Because DCs and mast cells are located in close proximity in various tissues, a regulatory function of mast cell products in the DC maturation process and subsequent skew of the adaptive immune response has been proposed.^{55,56} Such mast cell products, synthesized or released, comprise a plethora of pro-inflammatory media-

tors and cytokines including tumor necrosis factor- α . Interestingly, a synergy of tumor necrosis factor- α along with anti-inflammatory cytokines such as interleukin-4 or interleukin-13 has been proven to induce the production of thymic stromal lymphopoietin in human skin explants.⁵⁷ Emerging evidence indicates that thymic stromal lymphopoietin, an epithelial cell-derived cytokine that strongly activates DCs, is involved in allergic inflammation such as atopic dermatitis.^{58,59} Hence, it is appealing to speculate that allergic inflammation is aggravated via tumor necrosis factor- α /thymic stromal lymphopoietin-dependent DC activation in response to mast cell activation.

Cutaneous endothelial cells (ECs) form a continuous lining that normally preclude blood-borne T lymphocytes from entering the skin. However, within the response to stress, we observed a significant increase of leukocyte adhesion molecule ICAM-1 in the subcutis but not in dermis. As shown earlier, during the induced hair cycle vascular dermal ICAM-1 expression is high in the late anagen/early catagen.⁶⁰ Here, we observed that expression level of ICAM-1 after stress is comparable in both compartments, dermis and subcutis, whereby dermal ICAM-1 expression in control animals is already high because of this respective phase of the hair cycle. Meanwhile, the expression of ICAM-1 in subcutis is considerably lower.

On the other hand, activated T cells may activate dermal ECs themselves via cytokine secretion, thereby increasing the capacity to recruit memory and effector T-cell populations in an antigen-independent manner. We were able to confirm the migration of LFA-1⁺ cells into the skin in the present study. This finding also supports the hypothesis that stress may aggravate inflammatory dermatoses, such as chronic urticaria, via enhanced homing of T-cell populations into the skin. In patients diagnosed with chronic urticaria a significantly higher ICAM-1 expression could be observed at vascular and perivascular dermal sites, compared to healthy controls.⁶¹ Further, an increased ICAM-1 expression has been observed in non-lesional skin of patients with atopic dermatitis.⁶²

Clearly, a variety of functions is dependent on ICAM-1/LFA-1 interaction. ICAM-1 expression on ECs participates in trafficking of inflammatory cells,^{21,22} whereas ICAM-1 expression on tissue-residing APCs is engaged in cell-cell interactions subsequently skewing the adaptive immune response.⁶³ Based on the experimental design of the present work, the individual role of LFA-1 or ICAM-1 could not be dissected because the cross talk between both was inhibited. Inhibition of ICAM-1 or LFA-1 alone may have provided additional clues of the impact of either mediator, however, because we observed an increased expression of both LFA-1 as well as ICAM-1 in response to stress,²³ we primarily focused on the complete inhibition of their cross talk. Interestingly, LFA-1 cell migration into the skin also occurred when LFA-1⁺ splenocytes from stressed mice were adoptively transferred into nonstressed mice. Considering the profound effect of complete inhibition of LFA-1/ICAM-1 cross talk on skin immune hemostasis, future studies should address to dissect the kinetics of up-regulation of such a migratory marker, which may then allow a highly targeted

therapeutic approach. It would be interesting to prove our findings in a knockout model. However, ICAM-1 knockout mice show increased numbers of circulating monocytes⁶⁴ that would delimit comparability of the data accumulated with respect to the CD11c expression in wild-type mice. Because we were able to show an up-regulation of ICAM-1 on ECs as well as in the skin in response to stress, exposure to stress interferes with cell trafficking into the skin as well as with dermal cell-cell interaction.

In conclusion, as drawn in the hypothetical scenario (Figure 6) we propose that the cross talk between co-stimulatory molecules such as ICAM-1 on ECs and DCs

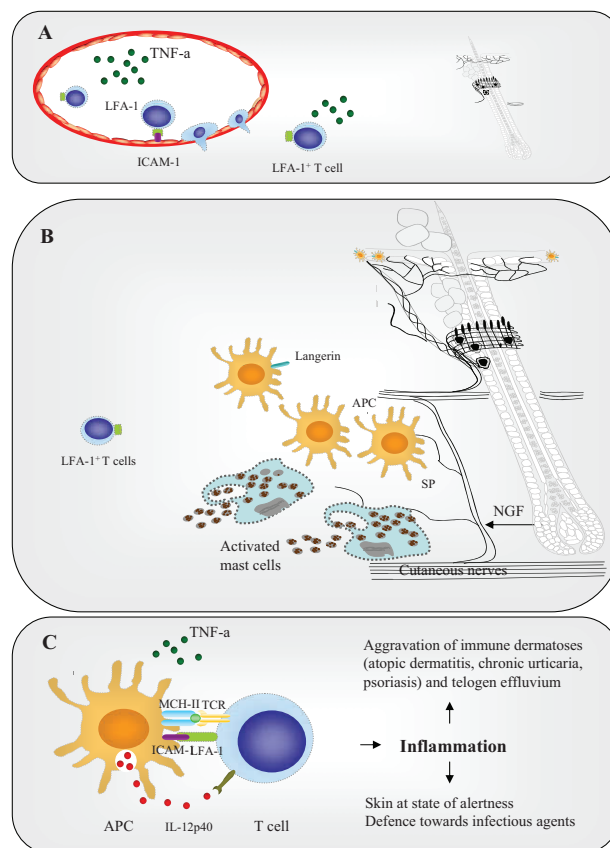


Figure 6. Hypothetical scenario on the role of ICAM-1/LFA-1 cross talk in the context of signaling events in response to stress along the brain-skin connection. **A:** Dermal ECs, which generally form a continuous lining that normally preclude blood-borne T lymphocytes from entering the skin, up-regulate surface protein expression of ICAM-1 in response to stress, thereby promoting homing of circulating LFA-1⁺ T cells. Also, activated T cells in blood may activate dermal ECs via cytokine secretion. Subsequently, an increased migration of LFA-1⁺ (inflammatory) cells into the skin occurs. **B:** In the skin, the number of mature APCs increases in response to stress. Mediators involved in such increase of maturation to APCs, migration, and expression of stimulatory and co-stimulatory molecules may comprise peripheral neuropeptides such as SP and/or activated mast cells, both of which have been shown to increase in the skin in response to stress via nerve growth factor-dependent pathways. **C:** Up-regulation of co-stimulatory molecules like ICAM-1 on APCs and the presence of T cells carrying the respective ligand for DC-T cell interaction (LFA-1) in the skin may initiate and/or perpetuate skin inflammation in response to stress by polarizing the adaptive immune response. Here, it still remains elusive whether such inflammatory reaction to stress is a physiological response, which may promote a state of alertness, allowing the individual to deal with infectious agents entering the body via skin lesions. On the other hand, such overshooting of the skin immune response may be involved in triggering or aggravating immune dermatoses such as atopic dermatitis, chronic urticaria, or psoriasis.

and its respective ligand LFA-1 on T cells play a pivotal role in initiating and perpetuating skin inflammation in response to stress. Inhibiting the ICAM-1/LFA-1-dependent interaction between ECs/DCs and T cells might not only prevent the activation and homing of T cells, but also arrest DCs in a state of immaturity. However, it still remains elusive whether such adaptation processes to stress are physiological responses, which may promote the generation of DC-dependent pathogen-specific immune responses at antigenic portals such as the skin. Here, cardinal cell subsets such as skin DCs clearly have the potential to initiate or perpetuate autoimmunity or chronic inflammation, as present in immune dermatoses such as atopic dermatitis, chronic urticaria, or psoriasis.

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