

ORIGINAL ARTICLE

OX40 ligand and OX40 are increased in atopic dermatitis lesions but do not correlate with clinical severity

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Abstract

Background The interaction between the OX40 ligand (OX40L) and OX40 has been suggested to have pathogenetic significance in atopic dermatitis (AD).**Objective** The purpose of this study was to investigate the expression and relevance of OX40L and OX40 in AD skin.**Methods** OX40L and OX40 were stained immunohistochemically on the cryosections of the lesional and non-lesional skin of 17 subjects with moderate-to-severe AD and of 10 patients with psoriasis vulgaris. Phorbol myristate acetate (PMA) stimulated keratinocytes and cell membrane preparations from PMA-stimulated keratinocytes or LAD-2 mast cells were incubated with peripheral blood mononuclear cells (PBMC) in the presence or absence of blocking monoclonal antibodies to OX40L, CD30L or ICAM-1.**Results** We show for the first time that the staining intensity of OX40L and the number of OX40⁺ cells are significantly greater in the lesional dermis than in the healthy-looking dermis in AD ($P < 0.001$ in both comparisons) and also in psoriasis ($P = 0.01$ and $P < 0.001$ respectively), but neither molecule correlate significantly with the clinical severity of AD. Living keratinocytes and cell membranes from LAD-2 mast cells and keratinocytes increased the PBMC proliferation response. Anti-OX40L antibody inhibited, in a similar fashion as anti-ICAM-1 and anti-CD30L, PBMC proliferation induced by LAD-2 membranes, but stimulated that induced by keratinocytes.**Conclusion** Our findings provide evidence for the involvement of OX40 and OX40L in the pathogenesis of AD though they are not specific to AD and *in vitro* results suggest complex interaction.

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Conflicts of interest

The authors state no conflict of interest.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease where numerous proinflammatory cells interact and participate in the inflammation, such as different T cell subsets, mast cells (MC), antigen presenting cells (APC), endothelial cells and sensory nerves. In AD, an early Th2 response with IL-4 and IL-5 expression is induced during the development of an acute lesion, but chronic lesions seem to contain a heterogeneous population of Th1, Th2 and Th0 cells.¹ The aetiopathogenesis of AD is, however, largely obscure.

The OX40 ligand (OX40L) has been suggested to play an essential role in the pathogenesis of AD.² OX40L and its receptor OX40 are members of the TNF and TNF receptor superfamilies, respec-

tively.³ OX40L is expressed by APCs, endothelial cells and T cells,^{4–6} whereas OX40 is found mainly on activated T cells.^{7,8} The expression of OX40L on dendritic cells (DC) is induced by IgE-stimulated mast cells (MC)⁹ and by thymic stromal lymphopoietin (TSLP).¹⁰ TSLP is a cytokine produced by epithelial cells after bacterial, viral and non-specific inflammatory stimuli (IL-1 and TNF- α) or after physical trauma such as scratching.¹¹ TSLP is highly expressed in the keratinocytes of AD lesions.¹² TSLP-activated DCs migrate into lymph nodes where OX40/OX40L interaction triggers the differentiation of inflammatory Th2 cells that produce IL-4, IL-5, IL-13 and TNF- α , but no or only little IL-10.^{2,13} Additionally, human MCs have been shown to activate T cells *in vitro* by direct cross-talk between OX40L on MCs and

OX40 on T cells.¹⁴ These results indicate the crucial role of OX40 and OX40L in the pathogenesis of allergic inflammation.

However, the role of OX40L and OX40 in the clinical course of AD is still unclear. We have investigated the expression of OX40L and OX40 in the skin of AD patients by using immunohistochemistry, and associated the results with the clinical severity of the disease. To clarify disease specificity, the results were compared with findings obtained from the skin biopsies of psoriasis patients. In order to clarify the functional significance of OX40L, we examined in cell cultures whether LAD-2 mast cell line and keratinocytes express OX40L, and whether this cell membrane-associated ligand can stimulate peripheral blood mononuclear cells (PBMC) isolated from patients with AD.

Patients, material and methods

Study subjects and skin biopsies

Seventeen subjects with moderate-to-severe AD (11 women and 6 men, mean age 35 years, range 19–69) volunteered for the study. The patients were recruited from the outpatient clinic of the Department of Dermatology, Kuopio University Hospital. The general entry criteria were: age 18 years or over, diagnosis of AD using standard criteria, moderate-to-severe AD according to the Rajka-Langeland Severity Index (score range 3–9; score 3–4 meaning mild, 4.5–7.5 moderate and 8–9 severe disease),¹⁵ willing to comply with the protocol, and written informed consent obtained prior to entry. The subjects had not received any potent systemic therapy (such as corticosteroids, cyclosporine, cytostatic drugs and biologics), any UV light treatment, or any potent local treatment (such as potent topical corticosteroids and calcineurin inhibitors) during the preceding two months. For the evaluation of the current clinical severity status, the Eczema Area and Severity Index (EASI, score range 0–72) and the Dermatology Life Quality Index (DLQI, score range 0–30)¹⁶ were used. The mean level of itching over the past week was measured on a Visual Analogue Scale (VAS, 0–10 cm, where 0 = no itching and 10 = worst itching ever experienced). In addition, the mean total serum IgE level was measured.

To study the specificity of the findings on OX40L and OX40 in AD, another study group consisting of 10 patients with psoriasis vulgaris (six men and four women, mean age 63 years, range 41–85) was included. The patients had not received any systemic therapy, phototherapy or potent local therapy during the preceding two months. Psoriasis was chosen because it is a chronic T-cell-mediated disease but its response is rather Th1-Th17-mediated, whereas primary response in AD is Th2-mediated.

After local anaesthesia (1% lidocaine with adrenalin), 4-mm punch biopsies were collected from the lesional and healthy-looking skin of study participants. The non-lesional skin biopsy was taken at least 2 cm from lesions. The specimens were immediately embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and frozen for preparing 5 µm thick cryosections.

The protocol was approved by the Ethics Committee of the Kuopio University Hospital, Kuopio, Finland.

Immunohistochemical staining and analyses of skin biopsies

All the histochemical techniques used were developed by the research group.^{17,18} Cryosections were fixed in cold acetone for 10 min. OX40 and OX40L were identified using commercially available mouse monoclonal antibodies (sc-20073, 1 µg/mL and sc-80271, 40 µg/mL, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The bound monoclonal anti-OX40 antibodies were demonstrated by using a secondary antibody and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) with 0.05% 3,3'-diaminobenzidine, 0.04% nickel chloride, and 0.03% hydrogen peroxide.¹⁹ For OX40L visualization, Vectastain ABC-AP Mouse IgG kit was used. Unrelated mouse IgG was used as control at the same concentration as the specific antibodies.

OX40L and OX40 expression levels in different cell types were analysed microscopically. The number of OX40-positive cells in upper dermis (depth 0.6 mm, width at least 1 mm) was counted on 2–3 sections per skin sample. The results are expressed as cells/mm². OX40L immunostaining intensity was analysed separately in dermis and epidermis, and owing to the large number of cells it was graded semiquantitatively as weak, medium and strong. All the analyses were performed in a blinded fashion.

Cultivation of LAD-2 mast cells and keratinocytes and preparation of plasma membranes

A mast cell line LAD-2 was cultured using StemPro-34 SFM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 ng/mL rh-SCF, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.²⁰ Keratinocytes from human foreskin specimens were cultured in Keratinocyte-SFM medium (Invitrogen) supplemented with epidermal growth factor, bovine pituitary extract, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

After large numbers of LAD-2 cells and keratinocytes had been obtained, the cells ($1\text{--}2 \times 10^6/\text{mL}$ in 175 cm² flasks) were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MA, USA) or diluent control using incomplete (without rh-SCF) StemPro-34 medium for LAD-2 cells or incomplete (without epidermal growth factor and bovine pituitary extract) Keratinocyte-SFM medium for keratinocytes, at 37°C and 5% CO₂. After 1-day stimulation, the non-adhered cells were collected and combined with adhered cells (in the case of keratinocytes) detached from the plastic surface with a cell scraper. The cells were washed once in PBS, and after centrifugation suspended in 15 mL of lysis buffer containing 30 mM NaCl, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (Merck, Whitehouse Station, NJ, USA), Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany), and 10 mM sodium phosphate buffer, pH 7.4. Thereafter, the cells were broken by freeze-thawing five times. Cell

membranes were isolated by layering the lysed cells on 8 mL of 41% sucrose containing 30 mM NaCl, 1 mM MgCl₂ and 10 mM sodium phosphate buffer, pH 7.4. The lysed cells were centrifuged in 50.2 Ti ultrafuge polycarbonate tubes using a Beckman L 90-K ultrafuge and chilled 50.2 Ti rotor at 100 000 × g for 60 min, as described previously.²¹ Finally, cell membranes were washed in PBS and stored in aliquots in a freezer.

Cultivation of peripheral blood mononuclear cells with plasma membranes from LAD-2 cells and keratinocytes

A heparinized peripheral blood sample was taken from the antecubital vein of three AD study subjects and PBMCs were separated by Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) at 1000 × g for 10 min as described previously.²²

PBMCs (1×10^6 /mL) were incubated in RPMI-1640 for 48 h with plasma membranes from PMA-stimulated and non-stimulated keratinocytes (corresponding to 0.5×10^6 cells/mL), or with those from LAD-2 mast cells (0.5×10^6 /mL). The experiments were performed in the presence of a range of different concentrations (5, 15 or 30 µg/mL) of blocking mouse monoclonal antibodies to OX40L (clone RR19; Santa Cruz), CD30L (clone 116614; R&D Systems, Minneapolis, MN, USA) and ICAM-1 (clone BBIG-II; R&D Systems). Mouse IgG₁ was used as the control (R&D Systems). To measure the proliferation response of PBMCs, 5 µCi/mL ³H-thymidine in RPMI-1640 was added to wells. After incubation for 24 h, the cells were collected and washed using a Combi Cell Harvester (Skatron Instruments AS, Lier, Norway). ³H-thymidine incorporation was analysed with a liquid scintillation counter using ULTIMA Gold™ fluid. In addition, culture supernatants were collected after incubation, and cytokine release was measured using commercial Human Th1/Th2/Th17 Cytokines Multi-Analyte Profiler ELISArray Kit (SABiosciences, Frederick, MD, USA).

In additional studies, living keratinocytes were used in a co-culture with PBMCs from three other AD patients. In this experiment, keratinocytes (30 000 cells were seeded in each well) were first cultured in the wells of a 24-well plate in complete Keratinocyte-SFM medium overnight. Thereafter, the medium was replaced by fresh incomplete medium. About six hours later the cells were stimulated with 100 ng/mL PMA or diluent control overnight, followed by washing twice with PBS. Then 150 µL RPMI-1640 was added to the wells. PBMCs (1×10^6 /mL) were incubated with PMA-stimulated and non-stimulated keratinocytes for 48 h in the presence of 30 µg/mL blocking anti-OX40L or anti-ICAM1 monoclonal antibodies, or mouse IgG₁ as the control. Non-adherent cells were collected with the cell harvester, and proliferation response was measured using ³H-thymidine incorporation as described above.

Immunostaining of LAD-2 mast cells and keratinocytes

Some non-stimulated and PMA-stimulated MCs were processed for preparing cytopins, followed by fixation in methanol and

immunocytochemistry of OX40L. Visualization was made using 0.05% 3,3'-diaminobenzidine, 0.04% nickel chloride, and 0.03% hydrogen peroxide. OX40L expression was analysed microscopically, and the percentage of OX40L-positive cells among LAD-2 cells was calculated.

Keratinocytes were cultured in 4-well chamber slides (Nunc Lab-Tek™, Nunc, Roskilde, Denmark) for 48 h in incomplete Keratinocyte-SFM medium in the presence of TNFα, PMA or diluent control. After fixation with methanol, OX40L was immunostained as described above. For OX40L visualization, the Vector Vectastain ABC-AP Mouse IgG kit was used. OX40L expression was analysed using a microscope.

Statistical analysis

All statistical analyses were performed using SPSS for Macintosh (SPSS, Chicago, IL, USA). Cell culture experiments and the differences in OX40L staining intensity between lesional and non-lesional skin were evaluated with the paired Student's t-test. The Wilcoxon and Mann-Whitney tests were used to compare the differences in OX40-positive cells between lesional and healthy-looking skin or in AD and psoriasis, respectively. Linear associations between variables were tested using the Spearman correlation coefficients.

Results

OX40L staining intensity is greater in lesional than in healthy-looking dermis

The data for OX40L immunostaining are shown in Figs 1 and 2. OX40L expression varied greatly between subjects, but was apparent on the surface of keratinocytes in whole epidermis and on the surface of infiltrating cells in upper dermis. OX40L immunostaining was more intense in the lesional than in non-lesional dermis of both AD and psoriasis patients ($P < 0.001$ and $P = 0.01$ respectively). Neither disease showed OX40L immunostaining of the strongest intensity in healthy-looking dermis. No significant difference in OX40L staining intensity was found between the lesional and non-lesional epidermis in either disease.

Although the groups are not directly comparable, the staining intensity appeared to be somewhat more intense in the lesional skin in AD than in psoriasis. However, the difference was not statistically significant.

The number of OX40-positive cells is greater in lesional than in non-lesional dermis

The mean number of OX40-positive cells was $133.7 \pm 116.2/\text{mm}^2$ and $13.8 \pm 17.4/\text{mm}^2$ in lesional and healthy-looking AD dermis, respectively. A 10-fold increase in cell numbers ($P < 0.001$) was observed in lesional AD skin (Fig. 3a,b). In psoriasis, the mean number of OX40-positive cells was $128 \pm 70/\text{mm}^2$ and $7 \pm 9/\text{mm}^2$ in lesional and non-lesional dermis, respectively (an 18-fold increase $P < 0.001$, see Fig. 3c,d). The number of positive

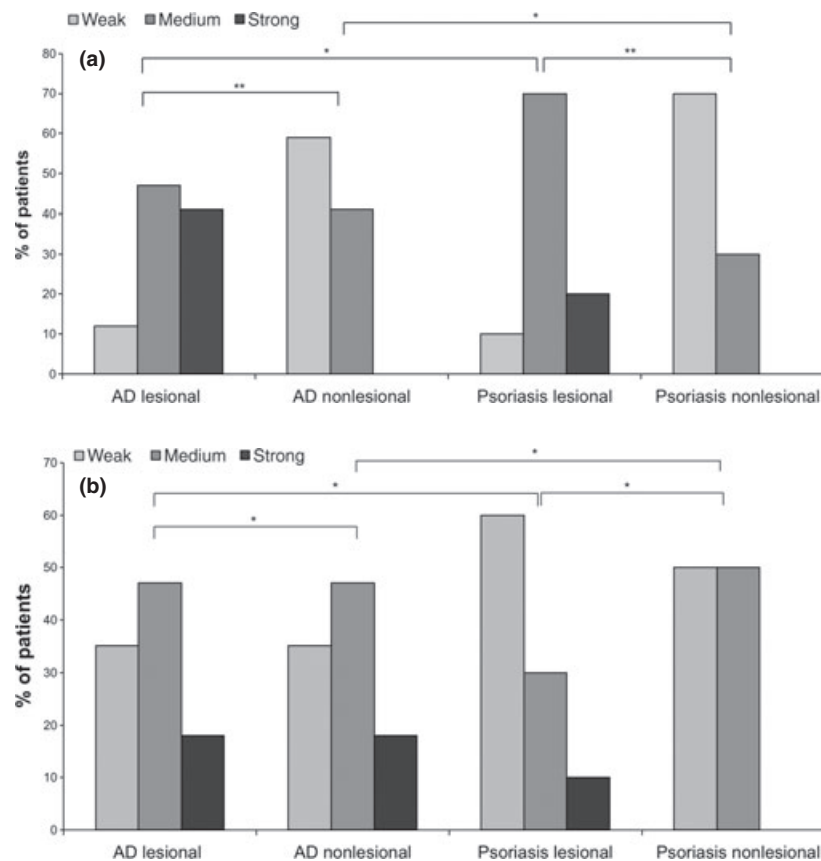


Figure 1 OX40L immunostaining is increased in the lesional dermis of atopic dermatitis and psoriasis patients but not in the lesional epidermis of either disease. The intensity of the staining in the (a) dermis and (b) epidermis was graded visually as weak, medium and strong. * $P > 0.05$, ** $P < 0.05$.

cells did not differ between AD and psoriasis. No immunostaining was detected when unrelated mouse IgG was used.

The number of OX40-positive cells did not correlate with OX40L staining intensity neither in lesional nor in healthy-looking skin in AD or psoriasis. In both diseases, OX40L and OX40 expressing cells were confined mainly to upper dermis. However, OX40L-positive cells were also found in epidermis, where only a few OX40-positive cells were detected.

No correlations between clinical severity of AD and OX40L or OX40

We further clarified associations between OX40L or OX40 expression and the clinical severity of AD. The mean score of the Rajka-Langeland Severity Index was 7 ± 1 (range 5–9); 6 of 17 patients had severe AD. The mean EASI score was 12.3 ± 6.6 (range 6–28.9), the mean DLQI was 12.7 ± 6.8 (range 4–27), and the mean level of itching according to the VAS was 5.4 ± 2 (range 2–10). The mean total serum IgE was 1862.3 ± 1873.6 kU/L. These clinical parameters did not correlate with the number of OX40-positive cells in dermis or the staining intensity of dermal

or epidermal OX40L. A tendency to stronger OX40L staining intensity along with decreased IgE levels was noted both in the lesional and non-lesional epidermis but no statistical significance was reached according to the Spearman correlation test.

OX40L blocking inhibits PBMC proliferation induced by LAD-2 mast cell membranes

Both non-stimulated and PMA-stimulated LAD-2 human MCs expressed OX40L as determined by immunocytochemistry. Unexpectedly, as much as 81% of non-stimulated and only 66% of PMA-stimulated LAD-2 cells expressed OX40L. To test the functional activity of OX40L on MCs, we performed culture experiments with PBMCs from three AD patients and cell membrane preparations from non-stimulated and PMA-stimulated LAD-2 cells (Table 1). The PBMC proliferation response was strongly increased by cell membranes from PMA-activated LAD-2 MCs in all experiments. The proliferation response of PBMCs was inhibited by up to 30 $\mu\text{g/mL}$ anti-OX40L mAb (11–58% of the controls). The inhibition by up to 30 $\mu\text{g/mL}$ anti-CD30L and anti-ICAM-1 mAb was similar to that by anti-OX40L (12–71%

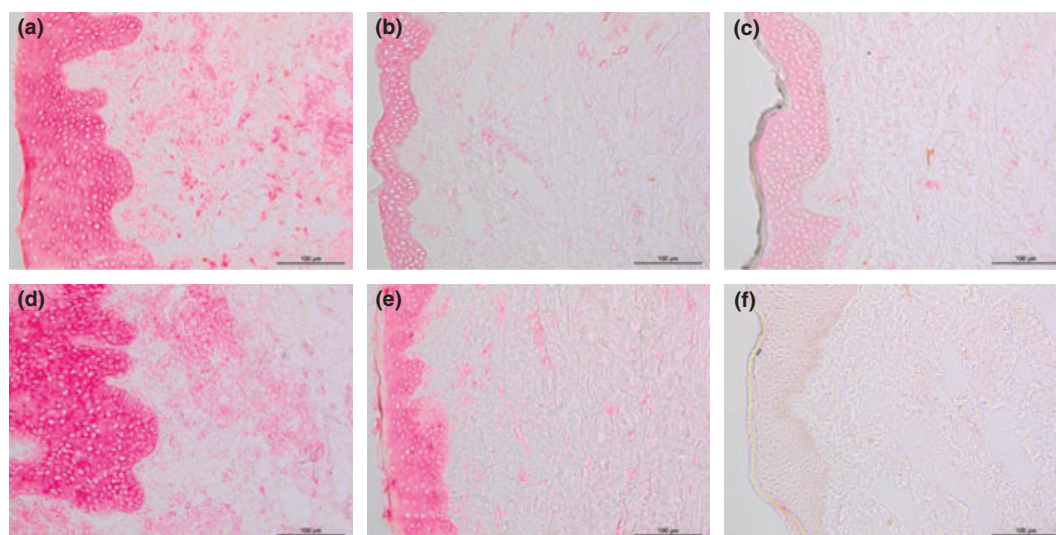


Figure 2 OX40L immunostaining in the skin of atopic dermatitis (AD) and psoriasis patients. Representative cryosections from (a) the lesional AD skin showing strong staining in dermis and epidermis; (b) non-lesional AD skin showing moderate staining in dermis and epidermis; (c) non-lesional AD skin showing weak staining in dermis and epidermis; (d) lesional psoriatic skin; (e) non-lesional psoriatic skin. (f) Unrelated mouse IgG isotype control for AD.

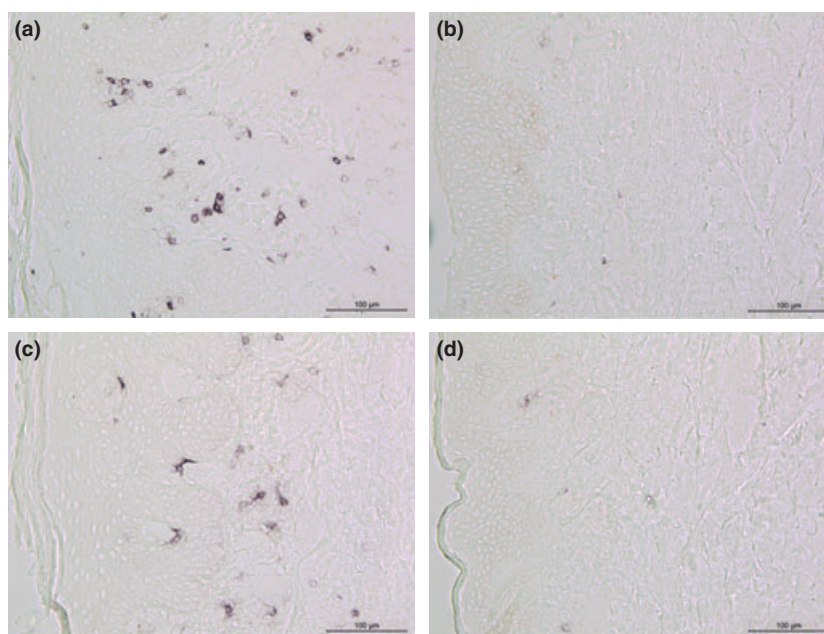


Figure 3 Increased number of OX40-positive cells in lesional dermis. Representative cryosections from (a) the lesional skin and (b) non-lesional skin of a patient with atopic dermatitis and from (c) the lesional skin and (d) non-lesional skin of a patient with psoriasis are shown.

and 22–79%, respectively). However, there was variation between the study subjects.

Cytokine secretion by PBMCs was screened from supernatants of two experiments using Th1, Th2 and Th17 ELISArray. In both

experiments, non-stimulated PBMCs and PBMCs stimulated by membranes from non-activated LAD2 cells secreted IL-2 and TGF β 1 (data not shown). IL-12 and TNF α secretion was detected in only one of the two experiments. PBMC stimulation by

Table 1 Proliferation response of peripheral blood mononuclear cells (PBMC) from atopic dermatitis patients in co-culture experiments with PBMC and a cell-membrane preparation of non-activated or PMA-activated LAD-2 mast cells in the presence of monoclonal antibodies

Co-culture system	PBMC proliferation (%)			
	Patients			Mean
	I	II	III	
Diluent control + PBMC + diluent control	1,5	4,5	4,6	3,5
Non-act. LAD-2 + PBMC + IgG1 30 µg/mL	1	7,2	5,9	4,7
Act. LAD-2 + PBMC + IgG1 30 µg/mL	100	100	100	100 ^{*,†}
Act. LAD-2 + PBMC + anti-OX40L 5 µg/mL	119	78	96	98
Act. LAD-2 + PBMC + anti-OX40L 15 µg/mL	43	58	78	60
Act. LAD-2 + PBMC + anti-OX40L 30 µg/mL	11	58	46	38 ^{*,‡}
Act. LAD-2 + PBMC + anti-ICAM1 5 µg/mL	57	81	82	73
Act. LAD-2 + PBMC + anti-ICAM1 15 µg/mL	51	72	70	64
Act. LAD-2 + PBMC + anti-ICAM1 30 µg/mL	22	79	65	55 ^{***,‡}
Act. LAD-2 + PBMC + anti-CD30L 5 µg/mL	76	85	98	86
Act. LAD-2 + PBMC + anti-CD30L 15 µg/mL	49	77	110	79
Act. LAD-2 + PBMC + anti-CD30L 30 µg/mL	12	68	71	50 ^{***,‡}

* $P < 0.001$; ** $P = 0.01$; *** $P = 0.06$.

P values of the differences between PBMC responses were determined using a paired 2-tailed Student's t test: †non-activated vs. activated LAD-2 cells, ‡ activated LAD-2 cells vs. activated LAD-2 cells and monoclonal antibodies.

PMA-activated LAD-2 membranes resulted in slight increases in TNF α and TGF β 1 secretion in both experiments, and in IL-12 and IL-13 secretion in one experiment. In the same experiment, IL-13 secretion returned to the baseline level after inhibition by anti-OX40L mAb.

OX40L blocking stimulates, whereas ICAM-1 blocking inhibits, PBMC proliferation induced by keratinocytes

Since OX40L staining was detected in epidermis, immunostaining of stimulated keratinocytes was performed. Both TNF α - and PMA-stimulated keratinocytes weakly expressed OX40L. Additionally, the functional activity of OX40L on keratinocytes was tested. In three culture experiments, a cell-membrane preparation from PMA-stimulated keratinocytes increased the PBMC proliferation response significantly more than that from non-stimulated keratinocytes (Fig. 4a). Surprisingly, the proliferation response of PBMCs was stimulated by blocking anti-OX40L mAb in a dose-dependent manner in every experiment. The stimulation was comparable to the effect of phytohaemagglutinin on PBMC proliferation. Instead, anti-ICAM-1 mAb at 30 µg/mL inhibited the proliferation of PBMCs induced by PMA-activated keratinocyte membranes.

As a result of the unexpected stimulation of the PBMC proliferation by anti-OX40L mAb, additional co-cultures of living keratinocytes and PBMCs were performed (Fig. 4b). PMA-activated keratinocytes stimulated a fourfold greater PBMC proliferation response than that induced by non-activated keratinocytes. A further rise (10–25%) in the PBMC proliferation rate was detected in the presence of 30 µg/mL anti-OX40L mAb in all three indepen-

dent experiments. In contrast, blocking anti-ICAM-1 mAb at 30 µg/mL inhibited the PBMC proliferation.

Discussion

Surprisingly, there have been no previous studies on the expression and relevance of OX40L and OX40 in AD skin, even though this ligand-receptor interaction has been shown to have pathogenetic and clinical significance.² In this study, we show that OX40 and OX40L are expressed in AD skin. The expression of both the receptor and the ligand was significantly higher in lesional than in healthy-looking dermis. Both molecules were found in the same compartment of the dermis (mainly in upper dermis) making receptor-ligand interactions possible. However, OX40/OX40L interactions are not specific to AD pathogenesis, since a similar expression was found in the biopsies of psoriatic skin. Moreover, no clear difference in OX40L staining intensity or in the number of OX40-positive cells was seen between the two diseases. The expression and location of OX40 and its ligand in different skin diseases has been investigated in two previous studies.^{23,24} In the cryosections obtained from lesional skin of two eczema patients, OX40L was detected only in dilated capillary vessel walls in the papillary dermis, and no OX40 was seen in lymphocytes. Since the biopsies were taken from areas of beginning eczema, the authors explained the results with the possibility of transient expression of OX40. The differences between these results and those of the present study can also be explained by the difference in the number of studied biopsies and the absence of AD patients in the previous study. In formalin-fixed and paraffin-embedded biopsies from psoriatic lesional skin, OX40L and OX40 were expressed in and around the

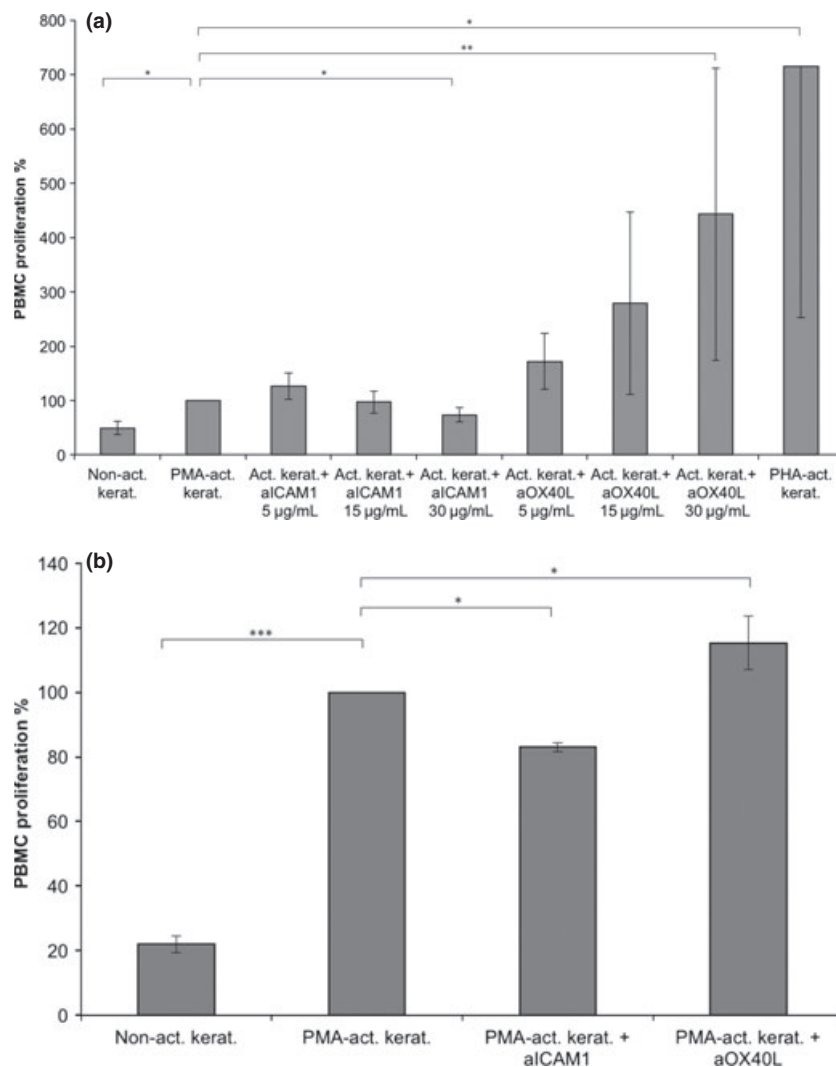


Figure 4 The PBMC proliferation is stimulated by a) a cell-membrane preparation from PMA-stimulated keratinocytes and b) PMA-activated keratinocytes significantly more than by non-stimulated keratinocytes. Blocking anti-OX40L mAb further stimulated the proliferative response of PBMC. PHA, phytohaemagglutinin; PMA, phorbol myristate acetate. * $P > 0.05$, ** $P = 0.05$, *** $P < 0.05$.

vessels of papillary dermis, respectively, and at lower levels in sub-papillary dermis. In contrast to present study, no OX40 or OX40L were expressed in non-lesional skin. However, the embedding of biopsies in paraffin may negatively affect antigen-antibody reaction and decrease the intensity of immunostaining.

Despite differences in OX40L staining intensity or in the number of OX40-positive cells between lesional and non-lesional AD skin, no correlations were found between the expression of OX40/OX40L and the clinical severity of the disease. This is not surprising, as inflammatory processes in AD are complicated and involve numerous factors.

OX40L has previously been detected in lung and tonsillar MCs, and upregulation of OX40L expression has been shown after FcεRI aggregation.¹⁴ In the current study, the immunocytochemical

staining of OX40L was positive in LAD-2 mast cell line. Membranes from PMA-stimulated LAD-2 MCs induced PBMC proliferation significantly more (mean 95%) than those from non-stimulated cells, and anti-OX40L antibodies inhibited almost two thirds of the obtained proliferation in co-culture experiments. This result is consistent with that in a previous report where anti-OX40L blocking antibodies significantly reduced T cell proliferation and IFN and IL-17 secretion in co-cultures with IgE- and antigen-stimulated MCs,²⁵ although no changes in IFN and IL-17 secretion were seen in our work. In another study, human MCs stimulated through FcεRI induced T cell proliferation by OX40-OX40L interaction.¹⁴ The OX40/OX40L pathway seems to play an important role both in initial and later phases of allergic inflammation.^{13,26} In the absence of IL-12, OX40/OX40L interaction

stimulates Th2 differentiation into inflammatory Th2 cells that produce proinflammatory cytokines (e.g. TNF α) but not IL-10. In the presence of IL-12, in contrast, the interaction induces Th polarization into TNF-producing Th1 cells.¹⁰ Additionally, it has been suggested that activated MCs expressing OX40L and TSLP may attract T cells to inflammation sites and support their survival.²⁶ In addition to MCs, OX40L on DCs and on endothelial cells stimulates T cell proliferation.^{9,27,28}

In the LAD-2 MC membrane and PBMC co-culture experiments, CD30L and ICAM-1 were targeted as control molecules. Expression of both molecules is increased in AD skin.^{29,30} CD30L has been found predominantly on MCs in AD lesions, in the same area as its receptor CD30.³¹ Another study demonstrated CD30 on CD4+ T cells, and a positive correlation between the number of CD30 positive cells and the severity of AD.³² Activation of CD30 on T cells *in vitro* has been found to lead to IFN- γ secretion in Th1 cells and IL-4 and IL-5 secretion in Th2 cells.³³ The adhesion molecule ICAM-1 on MCs can interact with LFA-1 on T cells, leading to T cell activation.³⁴ This interaction is thought to be of pathogenetic importance for the development of atopic dermatitis lesions.³⁵ Here we show that the inhibition level of the PBMC proliferation by anti-CD30L and anti-ICAM-1 antibodies was similar to that by anti-OX40L.

OX40L positive cells were detected in epidermis, where very few OX40 positive cells were seen. In additional stainings, we also found that keratinocytes express OX40L. However, no significant differences were found in OX40L staining intensity between lesional and non-lesional epidermis. In our co-cultures, both PMA-stimulated keratinocytes and the cell-membrane preparation from PMA-stimulated keratinocytes increased PBMC proliferation more than non-stimulated ones. This finding corroborates earlier results where PMA-stimulated keratinocytes induced proliferation of both allogeneic and autologous PBMCs.³⁶ In the same work, the proliferation was also induced by paraformaldehyde-fixed PMA-stimulated keratinocytes but not by supernatants, suggesting the critical role of membrane-associated factors. One important factor in such direct keratinocyte-lymphocyte interaction seems to be ICAM-1, since monoclonal antibodies against ICAM-1 were found to reduce partially the PBMC proliferation response.³⁶ Similar result was observed in our study. The role of OX40/OX40L interaction is more controversial. Unexpectedly, blocking anti-OX40L antibodies did not decrease the proliferation response of PBMCs but seemed to further increase it. A possible explanation for this result may be an interplay between costimulatory pairs ICAM-1/LFA-1 and OX40/OX40L.³⁷ Moreover, OX40 is expressed both on regulatory and effector T cells.³⁸ In our study, MCs, keratinocytes and PBMCs from different donors were used, causing the possibility for allogeneic cell interactions.³⁹ The net effect of these mechanisms can be unpredictable. Nevertheless, the absence of OX40 in epidermis suggests that the OX40L-OX40 interaction between epidermal keratinocytes and T cells has no marked pathogenetic significance.

This is the first study evaluating the cutaneous expression of OX40L and OX40 and their association with the clinical severity of AD patients. We have shown that OX40 and OX40L are strongly expressed in lesional AD skin and their interaction leads to PBMC proliferation *in vitro*. We suggest that the OX40/OX40L costimulatory pair may play an important role in the pathogenesis of AD, and blocking their interaction may provide opportunities for drug development. However, the finding that PBMC proliferation was not decreased by anti-OX40L and keratinocytes suggests that the interactions between OX40L and OX40 are more complex than in the case of ICAM-1/LFA-1, and therefore the results warrant further mechanistic studies.

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