

CD44, but not L-selectin, is critically involved in leucocyte migration into the skin in a murine model of allergic dermatitis

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Abstract: CD44 and L-selectin (CD62L) are major adhesion receptors that mediate leucocyte recruitment at inflammatory sites and lymph nodes, by supporting cell rolling under blood flow. Both CD44 and CD62L have been implicated in inflammatory skin disorders, but their specific involvement in an immediate-type allergic reaction remains uncertain. We used mice deficient in CD44 or CD62L or both in order to determine whether one or both of these molecules were required for leucocyte extravasation in an atopic dermatitis-like allergic response. Wild-type (WT) mice and mice deficient in CD44, CD62L or both were immunized with ovalbumin (OVA). Inflammatory reaction in the ear was elicited once by means of intradermal injection of OVA. Effective sensitization of CD62L knockout (KO) mice required intraperitoneal antigen injection; however, OVA-specific T helper 2 (Th2)-type immune responses and IgE production in mice lacking CD44, CD62L or both were comparable to those in WT mice following intraperitoneal immunization. We employed intravital videomicroscopy to monitor the recruitment of fluorescence-labelled leucocytes to the ear tissue following challenge with OVA. The number of adherent leucocytes was significantly reduced in CD44 KO and CD44/CD62L double KO mice, indicating that CD44 was involved in firm adhesion, the committed step of leucocyte extravasation. Histology of the OVA-challenged ears showed a diminished leucocyte infiltration in the ears of CD44 KO and double KO mice. The results of our study demonstrate that CD44, but not CD62L, is required for leucocyte extravasation during a Th2-type inflammatory response.

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Introduction

Leucocyte migration to inflammatory sites or lymphoid tissue is a multistep process governed by specific interactions between adhesion molecules expressed in endothelial cells and leucocytes (1). The selectin family (L-selectin, E-selectin and P-selectin) of adhesion receptors (2,3) and CD44 (4) are involved in mediating the initial capture and rolling of leucocytes along the endothelium of postcapillary venules under blood flow. Rolling leucocytes can be arrested via chemokine-induced interactions between cell surface integrins and

integrin ligands (5). Arrested (adherent) cells that resist detachment from the endothelium under the shear force of blood flow can then migrate across the vessel wall into the tissue, using a distinct set of adhesion receptors (6).

L-Selectin (CD62L) is expressed on most leucocytes, and binds sulphated carbohydrate ligands expressed in vascular beds within lymphoid organs and inflamed tissues (7). Although first identified as a lymph node-specific homing receptor (8), CD62L was also shown to be required for leucocyte migration to non-lymphoid tissue in certain models of inflammation (9–12). It has

been reported that CD62L-deficient mice do not respond well to repeated epicutaneous sensitization with allergens (13). However, other studies have found that the failure of CD62L-null mice to mount a proper allergic reaction lies in the inability of T lymphocytes to home to skin-draining lymph nodes where they could meet antigen-presenting cells and undergo activation (14,15). Unlike CD62L, whose expression is restricted to leucocytes, CD44 is expressed on the surface of both endothelial cells and leucocytes (16). As the principal receptor of hyaluronan (HA) (17), CD44 expressed in endothelial cells anchors extracellular HA to the surface of vascular endothelium on which activated leucocytes can roll in a CD44-dependent manner (16). A number of studies implicated CD62L (9–12) and CD44 (18–22) in leucocyte migration to inflamed tissues in a variety of inflammatory conditions, but the specific contribution of these adhesion receptors to leucocyte recruitment in a T helper 2 (Th2) lymphocyte-mediated skin disease remains uncertain.

Atopic dermatitis (AD) is a common skin disorder, characterized by recurrent inflammatory episodes in human patients (23–25). AD patients demonstrate evidence of Th2-type lymphocyte activation in the skin lesions, and most have elevated serum levels of IgE antibodies against a variety of allergens (23,24,26). Experimental animals, sensitized with a protein antigen, such as ovalbumin (OVA), and challenged locally with the same antigen, also exhibit a Th2-type response (27). As in humans, the inflammatory reaction(s) in animal models of AD begins with an immediate-phase response (IPR), characterized by rapid influx of mast cells and tissue oedema (28). The IPR is followed several hours later by a late-phase response (LPR), i.e. influx of a large number of leucocytes, and a further increase in tissue swelling (29). The Th2-polarized, biphasic response of actively (intraperitoneally) sensitized animals to local challenge (28,30), together with an increased IgE production (13), shows similarities to the immunological and clinical manifestations of AD in human patients (24–26,31).

Using mice lacking CD44, CD62L or both, we, in this study, investigated the requirement for these adhesion molecules for the development of a Th2-type response, as well as for inflammatory leucocyte recruitment and extravasation in an AD-like reaction, induced by intradermal injection of OVA following systemic sensitization. We found that CD44, but not CD62L, was required for effective leucocyte recruitment in the post-capillary venules of the skin, whereas neither was necessary for immune activation. Showing

an impaired leucocyte adhesion in the absence of CD44, intravital videomicroscopy (IVM) provided a key explanation for the reduced skin inflammation observed in CD44-deficient mice.

Materials and methods

Mice and immunization

Mice deficient in CD44 were generated by targeted gene disruption as described (20), and CD62L-null mice (32) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Using the marker-assisted speed congenic breeding method (33), both mutant strains were backcrossed into BALB/c (National Cancer Institute, Frederick, MD, USA) as described earlier (12). In order to generate CD44/CD62L double knockout (KO) mice, CD44- and CD62L-null BALB/c mice were intercrossed, and homozygous double KO offspring was selected (12). The experimental procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Rush University Medical Center. Allergic dermatitis was induced by systemic immunization, followed by intradermal challenge with OVA, as described by others (28,34,35). In brief, WT, CD44 KO, CD62L KO and CD44/CD62L double KO mice at 8–10 weeks of age were injected intraperitoneally with 10 µg of chicken egg OVA in 0.5 ml of aluminium hydroxide gel (alum) containing 6.5 mg of Al(OH)₃ (both from Sigma, St Louis, MO, USA), and boosted 3 weeks later with the same amount of OVA and alum. Mice injected with PBS in alum served as non-sensitized controls. Two weeks after the second intraperitoneal injection, the local reaction was elicited by intradermal administration of 5 µg of OVA in 10 µl of sterile PBS into the ventral side of one of the ears. The other ear, injected with PBS only, was regarded as a non-challenged control. Only those mice whose ear retained all the injected material were used.

Determination of serum concentrations of OVA-specific IgE, IgG1 and IgG2a

Serum concentrations of OVA-specific antibodies were determined in blood samples taken from the mice 24 h after local challenge with the antigen. OVA-specific IgE levels were measured employing a purified anti-IgE antibody and biotin-conjugated OVA in an enzyme-linked immunosorbent assay (ELISA) system (30). In brief, the wells of Maxisorp microtiter plates (Nunc, Rochester, NY, USA) were coated with rat anti-mouse IgE monoclonal antibody (clone R35-72; BD Biosciences-Pharmingen, San Diego, CA, USA) in carbonate coating buffer (pH 9.6) at 0.15 µg/100 µl/well. Non-specific binding sites were blocked with 3% bovine serum albumin (BSA; Sigma) in PBS. Serially diluted (1:50–1:12 500) serum samples were incubated in the anti-IgE-coated plates overnight at 4°C. Unbound material was removed by repeated washing with PBS containing 0.5% Tween. OVA-specific IgE, bound to immobilized anti-mouse IgE antibody, was detected by incubation with biotinylated OVA for 2 h, and the colour reaction was developed employing horseradish peroxidase (HRPO)-conjugated streptavidin (Zymed, San Francisco, CA, USA), *o*-phenylenediamine and hydrogen peroxide (both from Sigma). Optical density was read at 490 nm using an ELISA reader. Purified mouse IgE (BD Pharmingen), directly coated onto wells at increasing concentrations and detected with biotinylated anti-mouse IgE (clone R35-92; BD Pharmingen), was used as a standard for estimation of the concentrations of OVA-specific IgE in the serum samples.

OVA-specific IgG1 and IgG2a antibodies were measured using a standard ELISA protocol. In brief, Maxisorp plates

(Nunc) were coated with OVA (0.5 µg/well), washed and blocked with 3% BSA in PBS. Serially diluted serum samples were incubated in the OVA-coated wells overnight at 4°C. Following washing, biotin-labelled rat anti-mouse IgG1 or IgG2a (BD Pharmingen) was added at 0.15 µg/well and incubated for 2 h. The colour reaction was developed, and the serum antibody levels were calculated using purified mouse IgG1 and IgG2a standards, as described for the IgE ELISA.

Determination of OVA-specific T-cell responses

Lymphocytes were harvested from the spleens of OVA-sensitized mice 24 h after the injection of OVA into the ears. T-cell stimulation assays were performed as described previously (20,36). In brief, freshly isolated spleen cells were suspended in Dulbecco's Modified Eagle medium (DMEM, Sigma) containing 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) and cultured in 96-well plates (3×10^5 cells/well in triplicate wells) for 72 h in the absence or presence of OVA (50 µg/well). The concentrations of IFN- γ and IL-4 in 72-h culture supernatants of spleen cells were determined using ELISA kits (BD Pharmingen), according to the instructions of the manufacturer.

IVM

Separate groups of OVA-sensitized mice were used for IVM, performed on control (PBS-injected) and OVA-injected ears. In order to assess the time course of leucocyte-endothelial interactions during the IPR and LPR phases of the first episode of the AD-like inflammation, intravital experiments were performed before (0 h) and 1, 2, 6, 12 and 24 h after intradermal OVA injection. Before microscopy, mice were anaesthetized by means of intramuscular injections of a ketamine-xylazine cocktail. A drop of blood was drawn from the retroorbital venous plexus of each mouse for peripheral blood leucocyte (PBL) counting. The mouse was wrapped in gauze sheets to maintain constant body temperature, and was placed on a glass plate under the objective lens (magnification: 10 $\times$) of the intravital microscope (model E600FN, Nikon, Garden City, NY, USA). Using a Q-tip, the ear was gently flattened between two layers of immersion oil on the glass plate (to increase the resolution of fluorescence), and a glass coverslip was placed over the oil on the dorsal surface of the ear. The mouse was then injected intravenously with the fluorescent cell-permeable DNA-binding dye, rhodamine 6G (Molecular Probes, Eugene, OR, USA), which stains the nucleated blood cells (leucocytes) in the circulation without having any effect on the *in vivo* adhesion behaviour of the cells (12,37,38). The movement of leucocytes in the postcapillary venules of the ear was recorded for 1 min using streamline acquisition of serial images by a digital camera (RS Photometrics, Trenton, NJ, USA) (12). All video recordings were performed over the pinna of the ear, at a distance of 2.5–3 mm from the site where the needle was inserted during the injection of OVA or PBS. Microvessel diameters were measured using an on-screen caliper, and postcapillary venules with similar diameters ($d = 35 \pm 5$ µm) were selected for video recording. Upon playback of the video records, the following parameters were determined: (i) the frequency of rolling interactions (number of cells rolling along the endothelium under blood flow per minute), (ii) the velocity of rolling (µm/second) and (iii) the number of firm adherent leucocytes (cells immobile for at least 30 s) within a 100 µm long segment of the venule. These parameters were analysed off-line using a digital image processing and analysis system (MetaVue, Universal Imaging, West Chester, PA, USA) (12).

Measurement of ear swelling, histology and immunohistochemistry

Ear thickness was measured with a constant-tension microcaliper (Wilha Werkzeuge GmbH, Schonach, Germany) before (0 h) and 1, 6, 12 and 24 h after OVA challenge. Following the 1-h and 24-h ear thickness measurements, some of the mice were killed, and the ears were harvested; 1–2 mm-wide strips were cut from the central region of the ear, and processed for histology. The tissue slices were fixed in 10% buffered formalin and embedded in paraffin, or were embedded in OCT compound, frozen and sectioned in a cryostat. Sections of 6 µm thick were cut from the paraffin-embedded tissue and stained with hematoxylin and eosin (for conventional histology) or with safranin O (for identification of mast cells). The number of leucocytes and mast cells infiltrating the tissue was counted in 10 high-power fields (HPF; magnification: 100 $\times$) per section (six ears per animal group) using light microscopy (Nikon Microphot-FXA microscope, Nikon, Garden City, NY, USA).

Immunohistochemistry was performed as described previously (20). In brief, frozen sections of the ears were fixed with acetone and non-specific binding sites were blocked with 5% normal goat serum. Granulocytes and T helper cells were identified on adjacent tissue sections by immunostaining with biotinylated rat anti-mouse Gr-1 (BD Pharmingen) and anti-CD4 monoclonal antibody (R&D Systems, Minneapolis, MN, USA), respectively, and the colour reaction was developed using HRPO-conjugated streptavidin, hydrogen peroxide substrate and diaminobenzidine chromogen (DAB tablets; Sigma). The sections were postfixed with 10% buffered formalin, and counterstained with hematoxylin. Gr-1⁺ or CD4⁺ cells, as well as infiltrating cells negative for these markers (designated 'unstained' or 'other'), were counted on each pair of adjacent sections.

Statistical analysis

Data are expressed as the mean $\pm$ SEM. Statistical analysis of data was performed using SigmaStat software (SPSS, Chicago, IL, USA). The one-way analysis of variance with the Student's *t*-test was used in order to determine significant differences between the WT group and any of the gene-deficient groups. The threshold for statistical significance was set at $P < 0.05$.

Results

OVA-specific immune responses in WT, CD44 KO, CD62L KO and CD44/CD62L double KO mice

Intraperitoneal sensitization and intradermal challenge of WT, CD44-deficient, CD62L-deficient and CD44/CD62L double KO mice with OVA resulted in the production of substantial amounts of antigen-specific IgE (Fig. 1a), thus confirming the allergic character of the immune response (30,34). The concentrations of OVA-specific IgG1 in serum were several orders of magnitude higher than those of IgG2a (Fig. 1b,c), indicating the dominance of Th2-type immune responses in all four groups of OVA-immunized animals. As in the case of IgE, neither IgG1 nor IgG2a production was significantly different between the WT and the gene-deficient groups of mice. Control animals injected intraperitoneally with PBS in alum showed

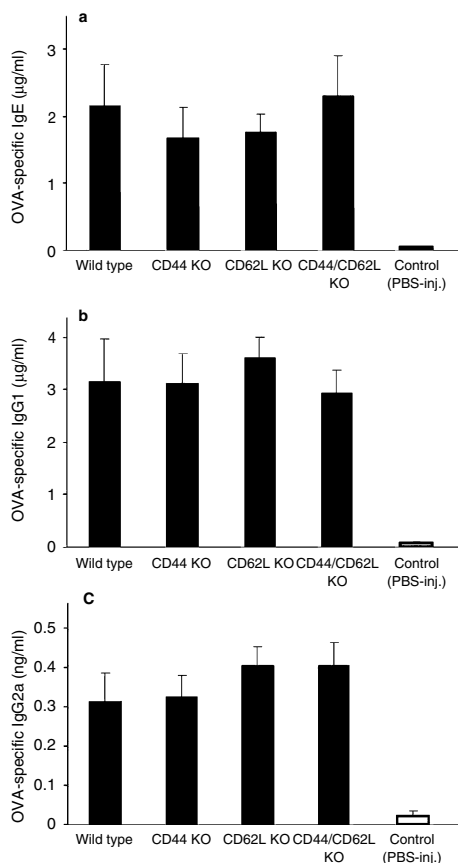


Figure 1. Comparison of the serum levels of ovalbumin (OVA)-specific IgE and IgG among WT, CD44 KO, CD62L KO and double (CD44/CD62L) KO mice after intraperitoneal sensitization and intradermal challenge with OVA. Serum levels of OVA-specific IgE (a), IgG1 (b) and IgG2a (c) are shown. Control mice (control, PBS-injected) were treated identically, but they received PBS instead of OVA. Data are the mean $\pm$ SEM (6–8 mice per group). The antibody profiles were consistent with an allergic, Th2-polarized immune response, and were similar in all genotypes of mice.

no evidence of OVA-specific antibody responses (Fig. 1a–c).

Predominant production of OVA-specific antibodies of the IgG1 isotype suggested a Th2-polarized immune response. This was further confirmed by the cytokine profile of antigen-stimulated spleen cells (Fig. 2). Spleen T cells from mice immunized with OVA in aluminium hydroxide (as an adjuvant) responded to *in vitro* OVA stimulation with the production of modest amounts of the Th1-type cytokine IFN- γ (Fig. 2a). This concentration of IFN- γ , however, was very low, as spleen cells from the same strain of mice (BALB/c) were reported to produce 1000-fold higher amounts of IFN- γ when the animals were immunized with an antigen in Th1-polarizing adjuvants (39). Spleen cells from OVA/alum sensitized mice also produced detectable amounts of the Th2-type cytokine IL-4

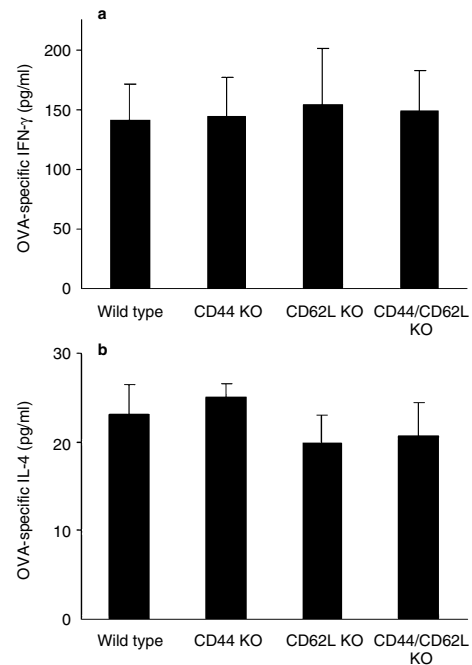


Figure 2. Production of IFN- γ and IL-4 by splenic T cells of ovalbumin (OVA)-sensitized mice. Spleen cells were harvested from WT, CD44 KO, CD62L KO and CD44/CD62L double KO mice following sensitization and intradermal challenge with OVA, and assayed for cytokine production as described in Materials and methods. The data showed are the mean $\pm$ SEM (six mice per group; triplicate samples from each). Comparison of IFN- γ levels (a) in the supernatants of OVA-stimulated cells revealed no statistically significant differences between WT and KO mice. IL-4 concentrations (b), measured in the same media samples, were also similar in all genotypes of mice. Note: IFN- γ concentrations were $<50 \text{ pg/ml}$, and IL-4 was not detectable in the supernatants of spleen cells in the absence of OVA.

(Fig. 2b). The concentrations of IL-4 were slightly lower in the supernatants of CD62L and double KO cells than in the WT cultures, but the differences were not significant statistically (Fig. 2b).

Kinetics of ear swelling

Consistent with an immediate-type allergic reaction (28), ear thickness increased slightly by 1 h after OVA injection in all genotypes of mice (Fig. 3). As the PBS-injected control ears were also slightly thicker 1 h after than before injection, this early swelling could be due, in part, to the extra volume ($10 \mu\text{l}$) injected into both OVA-challenged and control ears, or to an instant reaction to the minor tissue injury caused by the injection. Ear swelling gradually increased between 6 and 24 h in OVA-challenged WT and CD62L-deficient mice; these ears were

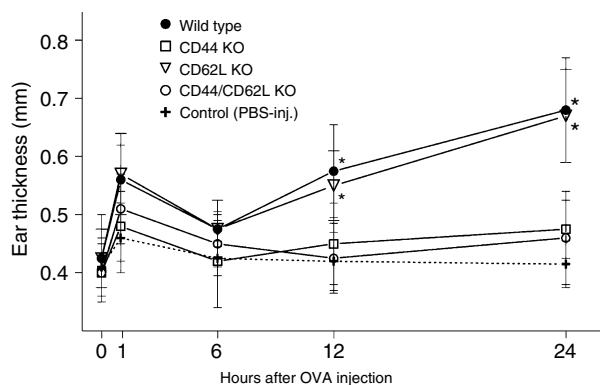


Figure 3. Time course of ear swelling in ovalbumin (OVA)-sensitized WT (solid circles), CD44 KO (open squares), CD62L KO (open triangles) and double KO (open circles) mice in response to intradermal injection of OVA or PBS. Ear thickness was measured with a microcaliper (at 0.05 mm precision) before (0 h) and after the injection of OVA at the indicated time points in six mice per group. Data are expressed as the mean $\pm$ SEM. Control (PBS-injected ears; crosses) represents the mean $\pm$ SEM (four PBS-injected ears from each genotype). Asterisks indicate significant differences between control and OVA-injected ears ($P < 0.01$).

significantly thicker than the PBS-injected controls 24 h after antigen challenge. In contrast to WT and CD62L-null animals, only minimal swelling was detected in the ears of CD44 KO and double KO mice (Fig. 3).

Leucocyte–endothelial cell interactions in the ear vessels

As both CD62L and CD44 can mediate leucocyte rolling, it was of interest to determine the effects of the lack of one or both of these receptors on leucocyte behaviour in the ear microvessels following OVA challenge. The rolling and firm adhesion interactions of leucocytes with the endothelium of postcapillary venules in the OVA-challenged ears of the four genotypes of mice were recorded in real time using IVM. Intravital experiments were performed immediately before (0 h) and 1, 6, 12 and 24 h after intradermal injection of OVA (or PBS) into the inner side of the ears. Snapshots of video records illustrate that fluorescent leucocytes are rarely seen in the vessels of a PBS-injected ear (Fig. 4a), but they are present and interact avidly with the vascular endothelium in the OVA-injected ears of WT mice 1 h (Fig. 4b) and 12 h (Fig. 4c) after antigen challenge. Only a few fast-rolling leucocytes were observed in non-challenged (PBS-injected) ears, whereas both rolling and firm adherent cells were seen 1 h after OVA injection in the ears of all four genotypes of mice (Figs 4

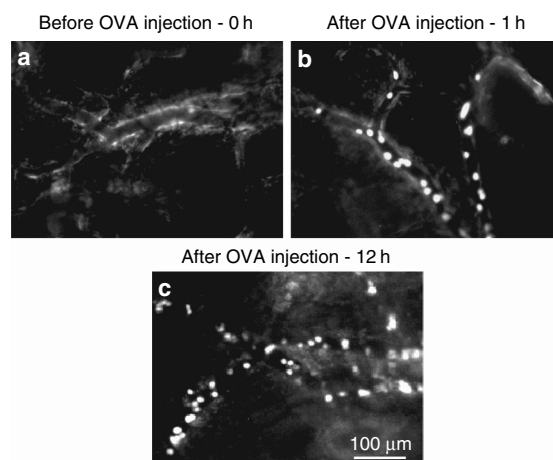


Figure 4. Snapshots of intravital videomicroscopy (IVM) records of leucocyte–endothelial cell interactions in the postcapillary venules of non-challenged (PBS-injected; a) and OVA-challenged (b and c) ears of WT mice. Fluorescence IVM was performed on the ears of mice following intravenous injection of rhodamine 6G. The snapshots illustrate leucocyte traffic in non-challenged (PBS-injected) and OVA-challenged ears of WT mice only. The venules of the control ear (a) appear 'empty', due to the very low number of leucocytes interacting with the vascular endothelium, most of which are fast-rolling cells that leave no visible mark of fluorescence on a single image. One hour after the OVA challenge, there is an increase in leucocyte–endothelial cell interactions, as illustrated by the numerous fluorescent cells (white spots) within the postcapillary venules of the ear (b). Leucocyte traffic becomes even more intensive in the WT ear 12 h after intradermal OVA injection (c).

and Fig. 5). This finding, together with the early onset of ear swelling (Fig. 3) confirmed the immediate component (IPR) of the inflammatory response. Detailed analysis of the IVM records on OVA-injected ears indicated a continuous increase in the number of rolling cells by 12 h, relative to 1 h, which was followed by a decline in the rolling interactions by 24 h in WT mice (Fig. 5a). In the venules of CD44 KO ears, the number of rolling leucocytes was high throughout the entire postchallenge period, whereas the rolling interactions gradually decreased between 1 and 24 h in the ears of CD62L KO mice, and increased between 6 and 24 h in the double KO ears (Fig. 5a). The velocity of rolling decreased over time in all OVA-injected ears, with the smallest decrease in double KO mice (Fig. 5b). The rolling speed at 1 h after OVA injection was the highest in mice lacking CD44 or CD62L, but velocity dropped more quickly in CD62L KO than in CD44 KO mice between 1 and 6 h post-injection (Fig. 5b). The number of firm adherent (immobile) cells increased until 12 h postchallenge, and decreased afterwards in the ears of WT and L-selectin KO mice (Fig. 5c). Only a few adherent leucocytes were observed at each time point in

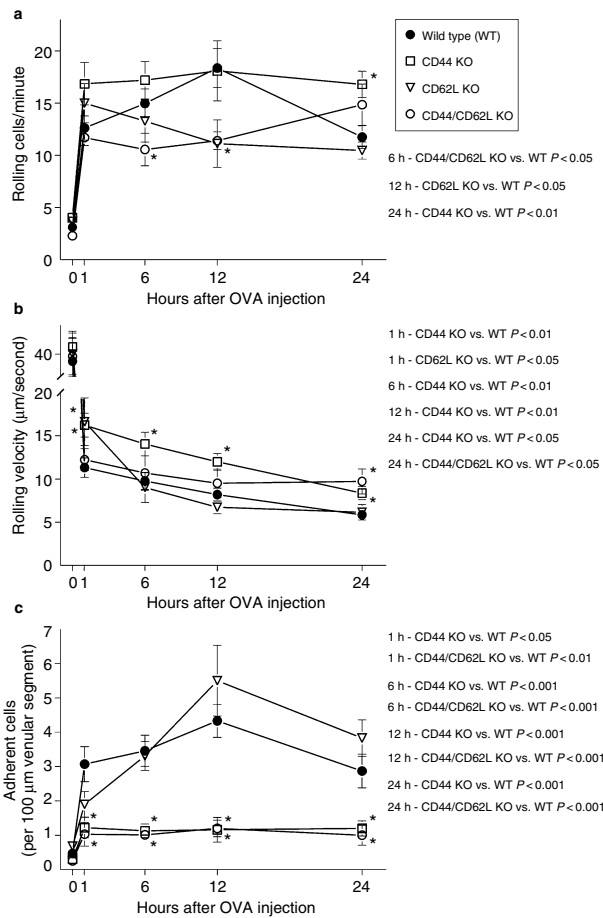


Figure 5. Kinetics and distinctive characteristics of leucocyte-endothelial cell interactions in the ovalbumin (OVA)-challenged ears of WT, CD44 KO, CD62L KO and CD44/CD62L double KO mice. The frequency of rolling leucocytes (number of rolling cells/minute) (a), the velocity of rolling ($\mu\text{m}/\text{second}$) (b) and the number of firm adherent (immobile) cells (c) in the OVA-challenged ears of WT (solid circles), CD44 KO (open squares), CD62L KO (open triangles) and CD44/CD62L double KO (open circles) mice were plotted as a function of time. Results shown are the mean $\pm$ SEM (8–11 mice per group at each indicated time point). Statistically significant differences between WT and any group of KO mice at specific time points are indicated at the right side of each graph.

CD44 and double KO ears, i.e. the number of cells that adhered tightly to the endothelium was significantly less in CD44 KO and double KO than in WT ears (Fig. 5c). Leucocyte counts in the blood samples taken before videomicroscopy were similar in each genotype of mice.

Histopathology and infiltrating leucocyte populations of OVA-injected ears

Histopathology of the OVA-injected ears, dissected 1 h after the challenge, demonstrated a modest thickening of all of the ears examined. Whereas infiltrating leucocytes and mast cells were rarely seen in the ears before OVA challenge

(on average, 0.3 leucocytes and 0.1 mast cells/HPF), a 10-fold increase in the number of these cells was observed 1 h after antigen injection (2.6–3.1 leucocytes/HPF and 0.8–1.2 mast cells/HPF) in the ears of all genotypes of mice. In the ears dissected 24 h after the challenge, massive leucocyte infiltrates were seen in WT and CD62L KO mice, but much fewer extravasated inflammatory cells were found in CD44 KO and double KO ears (Fig. 6a). Semiquantitative comparison of tissue-infiltrating leucocytes in hematoxylin-stained ear sections showed a significant reduction in the overall number of infiltrating cells, relative to WT, in CD44 KO and double KO, but not CD62L KO, mice (Fig. 6b). The cellular infiltrates contained a large number of polymorphonuclear and mononuclear cells, as well as a few (1.1–1.4 cells/HPF) degranulated mast cells. Counting of positive cells in cryosections after immunostaining with leucocyte subset-specific antibodies showed that the predominant infiltrating cells were granulocytes and CD4⁺ T cells in the ears of WT and CD62L KO mice, and that these cell types were significantly less abundant in CD44 KO and double KO, than in WT, ears (Fig. 6b). The rest of infiltrating cells (indicated 'other' in Fig. 6b) were not significantly different in number among the different genotypes of mice.

Discussion

In the present study, mice deficient in CD44, CD62L or both were used in order to examine the requirement for expression of these adhesion molecules for the development of OVA-induced immediate-type allergic skin reaction that resembles the first episode of AD. CD62L-deficient mice respond poorly to epicutaneous sensitization ((13) and our unpublished observations), primarily due to the inability of CD62L-null lymphocytes to home to, and undergo activation in, skin-draining lymph nodes (14,15). Presuming that systemic immunization could provide antigen delivery to the spleen, a secondary lymphoid organ to which lymphocytes home in a CD62L-independent manner (40), we chose the peritoneal cavity (28,34,35) for the introduction of the antigen in all genotypes of mice. Following intraperitoneal sensitization with OVA in alum, mice deficient in CD44, CD62L or both were able to mount an IgE-mediated, Th2-polarized immune response (34), which was similar in magnitude to that in WT animals. This finding reinforced the observation that activation of naïve T cells proceeds normally in the mutant mice if the antigen is delivered to a lymphoid organ (spleen) that lymphocytes can

enter in a CD62L- or CD44-independent fashion (14,40). Despite the unaltered activation state of the adaptive immune system in the absence of CD44 (or both CD44 and CD62L) expression, CD44 KO and double KO mice demonstrated significant reductions in leucocyte influx and ear swelling after local challenge with OVA, whereas inflammation in mice lacking CD62L only was comparable to WT. These results suggested that CD44, but not CD62L, was required for the entry of effector cells into the inflamed skin. Furthermore, immunohistochemistry showed a clear negative impact of CD44 deficiency on the ability of both T helper cells and polymorphonuclear granulocytes (including eosinophils) to infiltrate the ear.

IVM on the microcirculation of the ears provided new information about the time course of leucocyte–endothelium interactions in the skin vessels during the IPR and LPR phases of the AD-like hypersensitivity reaction, as well as a clue to the mechanistic basis of the defective leucocyte extravasation in CD44 KO and double KO mice. Unlike the ear swelling that showed a

biphasic pattern, IVM recording of leucocyte traffic showed a single peak at 12 h after OVA challenge even in WT and CD62L KO mice that both developed full-blown inflammation. In other words, at least in these two genotypes of mice, we found no evidence of biphasic cell migration. A likely explanation for this apparent discrepancy between the clinical signs of inflammation and the kinetics of leucocyte traffic is that during the IPR, the rapid influx of mast cells and release of mast cell products could cause vascular leak and subsequent tissue swelling, whereas during the LPR, increased vascular permeability and oedema could result from the continuous intravascular accumulation and extravasation of activated leucocytes (25,28).

Using IVM for the first time in an animal model of allergic dermatitis, we found that the number of leucocytes that rolled on the endothelium in the OVA-challenged ears was reduced at 6 and 12 h, but not at 24 h, in mice lacking CD62L (both single and double KO), whereas the frequency of rolling interactions was significantly increased at 24 h in CD44 KO mice as compared with WT. As

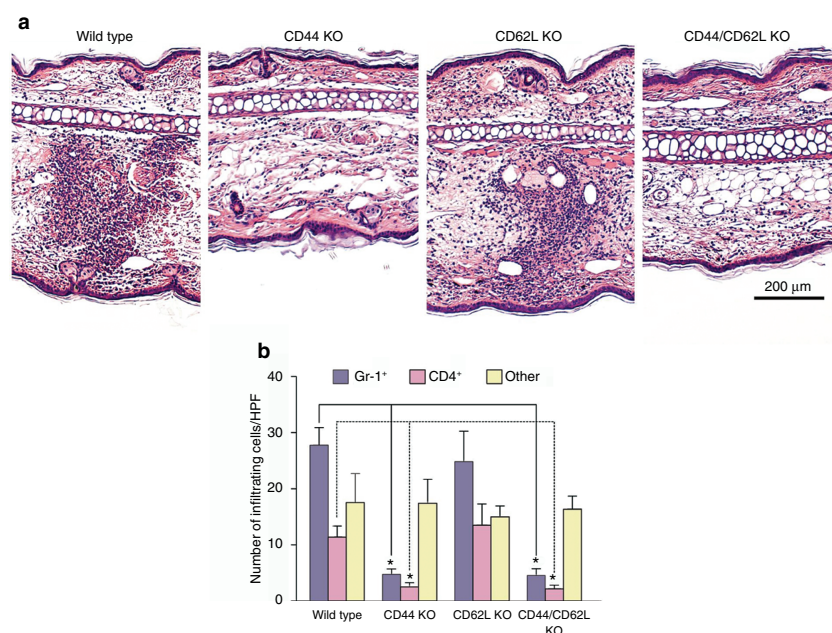


Figure 6. Histopathology and immunohistochemistry of the ears of ovalbumin (OVA)-sensitized mice 24 h after intradermal OVA injection. (a) Representative sections of OVA-injected ears from WT, CD44 KO, CD62L KO and double KO mice, respectively, are shown. Hematoxylin and eosin staining; the scale bar (indicated in the right bottom corner) is the same for all sections. Dense leucocyte infiltrates were typically seen in the ears of WT and CD62L KO mice, whereas only a few inflammatory cells were found in the ears of mice lacking CD44 or both CD44 and CD62L. Consistent with the results shown in Fig. 3, thickening of the ears, due to swelling, is greater in WT and L-selectin KO than in CD44 KO or double KO mice. As the ears were injected with OVA only once, they did not show evidence of epidermal thickening (hyperkeratosis) that develops after repeated allergen exposure. (b) The number of different inflammatory leucocytes, as assessed by counting the tissue-infiltrating cells on serial sections of the ears, dissected 24 h after OVA challenge. Adjacent sections were immunostained for granulocytes (Gr-1⁺ blue bars) and CD4⁺ T cells (pink bars), respectively, followed by counterstaining with hematoxylin. Yellow bars ('other') represent infiltrating cells that stained with hematoxylin, but not with Gr-1-specific or CD4-specific monoclonal antibody. The results shown are the mean + SEM (six ears per group) of average leucocyte numbers per high-power field (HPF; magnification: 100 $\times$); * $P < 0.05$.

CD44 has been showed to mediate leucocyte rolling on HA-rich endothelium (4), it is somewhat surprising that rolling was not compromised by the absence of CD44 expression. Although the reason for this leucocyte behaviour is unclear, previous studies on other inflammation models also reported unaltered leucocyte rolling in CD44-deficient mice (12,41). The velocity of rolling is expected to decrease, as the activation state of the vascular endothelial cells and leucocytes increases (2,42). Indeed, the rolling speed decreased gradually, at least during the first 12 h of the 24-h observation period in the OVA-challenged ears of all four genotypes of mice. Compared to WT animals, however, mice lacking CD44 (or both CD44 and CD62L) demonstrated an elevated rolling velocity. In addition, firm adhesion was clearly compromised in CD44 KO and double KO mice. The velocity of rolling and the propensity of leucocytes to exhibit firm adhesion are not independent aspects of cell behaviour, as slowly rolling leucocytes have longer exposure to locally produced chemokines that promote firm adhesion by activating integrins (1,6). It was not surprising, therefore, that CD44 KO and CD44/CD62L KO leucocytes, which rolled faster than WT cells or those lacking CD62L only, showed a significant reduction in firm adhesion. Adherence to the endothelium is the committed step of leucocyte emigration from the circulation into the inflamed tissue (1,42). Consequently, leucocytes that failed to adhere firmly could not extravasate, as indicated by the reduced ear swelling and cell infiltration in CD44 KO and double KO mice. Our observations are similar to those in other studies that reported normal rolling, but impaired adhesion and leucocyte extravasation at inflammatory sites in CD44 KO mice (12,41). Although CD44 was thought to mediate cell rolling, it could be involved, possibly via cooperation with integrins, in the stabilization of leucocyte adhesion. Indeed, a previous study reported that a molecular interaction between CD44 and a leucocyte integrin was essential for rolling lymphocytes to exhibit firm adherence (43).

In summary, employing single and double gene-deficient mice and IVM to elucidate the role of CD44 and CD62L in an immediate-type allergic reaction, we found that the expression of CD44, but not CD62L, was critical for leucocyte emigration into the skin. In agreement with other investigators reporting therapeutic benefits from anti-CD44 antibody treatment in allergic conditions in mice (18,22), in this study we have identified CD44 as a potential target for intervention in an AD-like skin disease.

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