Ph.D. Thesis

THE ROLE OF CD44 AND L-SELECTIN IN LEUKOCYTE-ENDOTHEL INTERACTIONS IN DIFFERENT MURINE MODELS OF INFLAMMATION

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Introduction

Leukocyte influx into tissues is one of the hallmarks of physiological reactions to inflammatory stimuli. Leukocyte emigration into the tissues after an inflammatory stimulus is a process consisting of several distinct steps. These have been described as (1) leukocyte rolling along activated endothelium, (2) leukocyte activation, (3) firm adhesion to the endothelium and (4) transendothelial migration.

Leukocyte capture and rolling are mediated via receptor-ligand interactions between the members of the selectin family and carbohydrate moieties on glycoproteins. L(leukocyte)-selectin (CD62L), which is constitutively expressed on the surface of leukocytes, supports lymphocyte rolling in peripheral lymph nodes (PLNs) and neutrophil rolling in inflamed extralymphoid tissue. E-and P-selectins are expressed by endothelial cells under inflammatory stimuli. P-selectin glycoprotein ligand-1 (PSGL-1), the major leukocyte ligand for P-selectin, can mediate rolling on inflamed endothelium. Exposure of rolling cells to proinflammatory or "homing" chemokines, via interaction with chemokine receptors and subsequent signaling, can result in the activation of β_1 (e.g. very late activation Ag-4 (VLA-4)) or β_2 (Mac-1 or LFA-1) integrins on leukocytes. Binding of leukocyte integrins to counterreceptors on endothelium (e.g., VCAM-1 or ICAM-1) leads to deceleration, arrest, and firm adhesion. Integrins are unable to establish an adhesion interaction between the leukocyte and endothelium under flow unless the cells have rolled first, with the exception of VLA-4, which is capable of initiating a rolling interaction.

The other molecule, which also plays a role in the leukocyte extravasation under inflammatory conditions is CD44, a transmembrane glycoprotein, the principal cell surface receptor for hyaluronan (HA). In a manner similar to the interaction between selectins and their carbohydrate ligands, binding on leukocyte CD44 to vascular HA serves to tether

leukocytes to the vessel wall, and may also facilitate the transendothelial migration of these cells to the site of inflammation. Both the cell surface density and the ligand (hyaluronan) binding capacity of CD44 are enhanced upon exposure of cells to proinflammatory stimuli.

The requirement for L-selectin or CD44 for inflammatory responses has been investigated intensively with antibodies or gene knockout (KO) mice. Both molecules have been shown to play crucial roles in different models of inflammations, although the results are sometimes conflicting, indicating that the process of leukocyte-endothel interactions depends on different kind of other factors than the adhesion molecules (e.g. type of inflammation, cytokines, chemokines specific for the tissue microenvironment etc.) and still not fully understood.

Atopic dermatitis (AD) is a common inflammatory skin condition characterized by severe pruritus, chronic relapses with frequent periods of exacerbation, and distinctive clinical morphology and distribution of skin lesions. Most patients have increased serum levels of IgE Ab against many kinds of allergens. After the challenge with relevant antigen, sensitized animals exhibit immediate phase responses (IPR), which is caracterized by oedema.. When a high concentration of allergen is used, oedema and erythema usually persist at the challenged site for a 6-24h period (LPR: late phase response) , histologically with infiltration of mononuclear cells, neutrophils, basophils and eosinophils.

Examinations of affected skin lesions in atopic dermatitis suggests, that T cells play an important role in the pathogenesis of the disease. In acute lesions of of AD, there is a significant increase in the number of cells expressing IL-4 and IL-5mRNA and protein, suggesting preferential accumulation of Th2 cells. In the chronic skin lesions of AD, cells containing IFN-γ mRNA and protein predominate over those containing IL-4 and IL-5.

It has been previously reported that the Ig-E mediated biphasic skin reaction in passively sensitized mice with monoclonal anti –DNP Ig-E antibody could serve as an animal model for

atopic dermatitis. Later it was showed that active sensitization with ovalbumin could produce the same biphasic skin reaction after the challenge with the relevant antigen, and the skin inflammation resembles atopic dermatitis as well.

Ag-induced arthritis (AIA) can be induced in mice by s.c. or intradermal immunization with methylated BSA (mBSA) followed by intra-articular (i.a.) injection of the same Ag into the knee joint. AIA shows similarities with rheumatoid arthritis (RA), including the involvement of the adaptive immune system in the initiation of the disease, infiltration of the synovium by inflammatory leukocytes, synovial hyperplasia, and cartilage erosion. Unlike the systemic autoimmune forms of murine arthritis, AIA has a well-defined onset, i.e., it starts after i.a. injection of the Ag, so the time-course changes of the inflammatory parameters can be investigated easier.

Objectives

1, To determine wether in the absence of L-selectin molecule local inflammatory reaction could be induced or not after intraperitoneal administration of the relevant antigen.

2, To investigate the contribution of L-selectin and CD44 adhesion molecules to the antigen specific immune responses and morphology of inflammations in the mouse model of allergic dermatitis and AIA.

3, To determine the effect of CD44-L-selectin double deficiency in different kind of inflammations.

4, To apply intravital videomicroscopy and analyse the inflammatory leukocyte-endothel interactions in the absence of CD44, L-selectin, or both.

Materials and methods

Mice and immunization

Mice deficient in CD44 were generated by targeted gene disruption, and CD62L-null mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Both mutant strains were backcrossed into BALB/c (National Cancer Institute, Frederick, MD, USA) for 6-8 generations using a marker-assisted speed congenic breeding method. Mice lacking both CD44 and CD62L were obtained through a two-step breeding process.

In the model allergic dermatitis wild type (WT), CD44 knockout (KO), L-selectin KO, and CD44/L-selectin double KO mice were injected intraperitoneally with chicken ovalbumin (OVA) in aluminum hydroxide gel, and boosted 3 weeks later with the same amount of OVA and adjuvant. Two weeks after the second intraperitoneal injection, the local reaction was elicited by intradermal administration of OVA in sterile PBS into the inner side of one of the ears.

Mice with the same gene disruption as above were injected 2 times with methylated BSA in adjuvant subcutan/intradermally and intraperitoneally. Two to three weeks after the boost, mice were injected with the same antigen into the right knee to induce AIA.

Detremination of antigen specific immune responses

Serum concentrations of OVA- and mBSA specific antibodies were determined in blood samples with ELISA.

OVA-specific IgE, bound to immobilized anti-mouse IgE antibody, was detected by incubation with biotinylated OVA for 2 hours at room temperature, and the color reaction was developed employing horseradish peroxidase (HRPO)-conjugated streptavidin, *o*-phenylenediamine, and hydrogen peroxide .

Antigen specific IgG antibodies were measured using biotin-labeled rat anti-mouse IgG1or IgG2a and peroxidase-conjugated rabbit anti-mouse IgG .

For lymphocyte stimulation assays, spleens (and in the model of allergic dermatitis the submandibular lymphnodes) were harvested from the ear-injected animals 24 hours, in AIA 5 days after the antigen challenge. The isolated cells were then incubated in the presence or absence of the relevant antigens. Proliferation was determined by incorporation of (3H) thymidine.

The concentrations of IFNγ and IL-4 in 72-hour culture supernatants of spleen cells were determined using ELISA kits.

Flow cytometry

To determine the expression of CD44 and L-selectin , spleen cells were harvested 1 day after induction of AIA, were incubated with biotinylated anti mouse CD62L (clone MEL-14) or anti CD44 (clone IRAWB14), followed by staining with PE-conjugated streptavidin. Expression of early activation marker CD69 in lymphocytes was examined using two-colour fluorescence labeling (anti-CD3-PE with anti-CD69-FITC for T cells and biotinylated anti CD19 and streptavidin-PE with anti-CD69-FITC for B cells.

Inflammatory cells were harvested from the knees of mice at different time points after i.a. injection of mBSA. In addition to immunostaining with MEL-14 and IRAWB14, the collected cells were also stained with mAb against the granulocyte marker Gr-1 and anti-CD3.

Granulocyte-specific expression of adhesion molecules, including L-selectin, Mac-1, LFA-1, VLA-4 and PSGL-1, in peripheral blood was determined by two-color fluorescence labeling, during which the cells were coincubated with biotinylated or PE-conjugated anti-Gr-1 mAb and either a biotinylated or PE-conjugated adhesion molecule specific mAb.

Intravital videomicroscopy

Separate groups of OVA-sensitized mice were used for intravital videomicroscopy, performed on control (PBS-injected or non-injected) and OVA-injected ears. To assess the time course of leukocyte-endothelial interactions during the IPR and LPR phases of the inflammatory reaction, intravital experiments were conducted before (0 hour) and 1, 2, 6, 12, and 24 hours after intradermal OVA injection. The anesthetized mice were wrapped in gauze sheets to maintain constant body temperature, and was placed on a glass plate under the objective lens of the intravital microscope. Mice were then injected intravenously with the fluorescent cell-permeable DNA-binding dye, rhodamine 6G which stains the nucleated blood cells (leukocytes) in the circulation without having any effect on the in vivo adhesion behavior of the cells. The movement of leukocytes in the post-capillary venules of the ear was recorded for 1 minute using streamline acquisition of serial images by a digital camera.. Microvessel diameters were measured using an on-screen caliper, and post-capillary 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: (a) the frequency of rolling interactions (number of rolling cells/minute), (b) the number of firm adherent leukocytes (cells immobile for at least 30 seconds) within a 100-μm long segment of the venule; and (c) rolling velocity (μm/second), which was calculated from the distance traversed by a fluorescent cell during the 1-minute recording time. The numbers of rolling and adherent leukocytes and the velocity of rolling were analyzed off-line using a digital image processing and analysis system. The actual counts of circulating PBL were determined in the blood samples taken immediately before intravital microscopy.

In the model of AIA the synovium of the mouse knee joint was accessed throug an anterior opening. After excision of the shaved skin above the joint, the patellar ligament was transected below the patella. Then the ligament was lifted to expose the fatty synovial tissue.

The synovium was continuously superfused with sterile warm PBS. The real-time recording and analysis of leukocyte-endothelial interactions of the synovial postcapillary venules were the same as above.

Clinical and histopathological assesment of inflammations

Ear thickness was measured with a constant-tension microcaliper before (0 hour), and 1, 6, 12, and 24 hours after OVA challenge. Following the 1-hour and 24-hour ear thickness measurements, some of the mice were killed, and the ears harvested. One-to-two 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. Six μm-thick sections 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). Granulocytes and T helper cells were identified on adjacent tissue sections by immunostaining with biotinylated rat anti-mouse Gr-1 and anti-CD4 mAb .

In the model of AIA animals were monitored for joint swelling at defined time points between 4h and 5 days postinjection by measuring the knee joint diameter with a constant-tension microcaliper. For histopatholgy, the knees were dissected, fixed in formalin, decalcified, embedded in parafin, sectioned, and stained with H&E.

Statistical analysis

Data are expressed as the mean ± SEM. The one-way analysis of variance with the Student's *t*-test was used 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

Cell surface expression of CD44 and L-selectin in mBSA imunized WT, CD44 KO, L-selectin KO, and CD44/L-selectin double ko mice

Expression of L-selectin in the spleen cells of mBSA-immunized CD44KO mice was significantly lower than in the cells of WT littermates. In contrast, CD44 expression in L-selectin KO and WT lymphocytes was comparable.

OVA and mBSA specific immune responses int he WT and gene knockout groups

L-selectin deficient mice that exhibit impaired homing of naive T cells to PLNs are not able to mount a full response to Ag delivered from the skin to the lymph nodes. Indeed, immunization with mBSA via the s.c./intradermal route resulted in significantly lower cell stimulation in mice lacking L-selectin than in WT or CD44-deficient animals, although the BSA-specific IgG response did not seem to be affected by the absence of L-selectin. Intraperitoneal administration of mBSA, where the spleen is the major Ag-draining lymphoid organ, resulted in normal T cell response, as well as normal production of BSA-specific IgG, in both L-selectin deficient and double KO mice.

Intraperitoneal sensitization and intradermal challenge of WT, CD44-deficient, L-selectin-deficient and CD44/L-selectin double KO mice with OVA resulted in the production of substantial amounts of antigen-specific IgE, thus confirming the allergic character of the immune response . The concentrations of OVA-specific IgG1 in serum were several orders of magnitude higher than those of IgG2a, 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. The Th2-polarized immune response was further confirmed by the cytokine profile of antigen-stimulated spleen cells. Spleen cells from mice immunized with OVA

responded to in vitro OVA stimulation with production of modest amounts of the Th1-type cytokine IFNγ, and detectable amounts of the Th2-type cytokine IL-4.

The OVA-induced T cell proliferation of spleen cells were comparable in all four genotypes, lymph node cells harvested from L-selectin-null, but not from CD44/L-selectin-deficient, mice demonstrated significantly reduced proliferation in the presence of OVA.

Clinical features of OVA induced allergic dermatitis and mBSA induced AIA in mice lacking CD44, L-selectin or both

Consistent with an immediate-type allergic reaction, ear thickness increased slightly by 1 hour after OVA injection in all genotypes of mice. Ear swelling gradually increased between 6 and 24 hours in OVA-challenged WT and L-selectin deficient mice; these ears were significantly thicker than the PBS-injected controls 24 hours after antigen challenge. In contrast to WT and L-selectin animals, only minimal swelling was detected in the ears of CD44 KO and double KO mice.

After the i.a. injection of mBSA, mice were then monitored for the developement of arthritis at defined intervals between 4h and 5 days. Joints injected with mBSA showed swelling in all four experimental groups, with a peak response at 24h.Knee swelling was significantly less in L-selectin KO and CD44/L-selectin double KO than in WT mice at all time points between 12 h and 5 days.

Histology and immunohystochemistry of the ears and knee joints of mice

Histopathology of the OVA-injected ears, dissected 1 hour after the challenge, demonstrated a modest thickening of all of the ears examined, with minimal leukocyte infiltration. In the ears dissected 24 h after the challenge, massive leukocyte infiltrates were seen in WT and L-selectin KO mice, but much fewer extravasated inflammatory cells were found in CD44 KO and double KO ears. Semi-quantitative comparison of tissue-infiltrating leukocytes in hematoxylin & eosin-stained ear sections revealed a significant reduction in the overall

number of infiltrating cells, relative to WT, in CD44 KO and double KO, but not L-selectin KO mice. The cellular infiltrates contained polymorphonuclear and mononuclear cells, as well as a few degranulated mast cells. Counting of positive cells in cryosections after immunostaining with leukocyte subset-specific antibodies revealed that the predominant infiltrating cells were granulocytes and $CD4^+$ T cells in the ears of WT and L-selectin KO mice, and that these cell types were significantly less abundant in CD44 KO and double KO, than in WT ears.

On histological sections, infiltration of the i.a. soft tissue by inflammtory cells was seen as early a 4 h after mBSA injection in WT and CD44-deficient mice, whereas the knee joints of L-selectin-deficient and double KO animlas showed little evidence of inflammtion at this time point. Synovitis progressed rapidly in WT mice. 24 hours after teh i.a. Ag injection, massive cellular infiltrates were observed at the synovium-cartilae interface and underneath the patellar ligament, comprising predominantly neutrophil leukocytes. At 24 hours the degree of inflammatory cell infiltration appeared to be somewhat lower in the synovium of CD44-deficient animals than in the WT controls. In L-selectin KO and double KO mice, only mild synovial inflammtion could be seen at 24h, with the participation of both mononuclear cells and neutrophils. Whereas the histological appearance of the joints was clearly different on day 1, the inflammatory features of AIA became similar in all four genotypes of mice by day 5 after mBSA injection, with heavy infiltration of the soft tissue by granulocytes and synovial hyperplasia.

Leukocyte-endothelial cell interactions in the postcapillary venules of the ears and joints of mice following the challenge with the relevant antigens

The rolling and firm adhesion interactions of leukocytes with the endothelium of post-capillary venules in the OVA-challenged ears of the four genotypes of mice were recorded in real time using intravital videomicroscopy. Intravital experiments were performed

immediately before (0 hour), and 1, 6, 12, and 24 hours after intradermal injection of OVA (or PBS) into the inner side of the ears. Snapshots of video records illustrate the virtual absence of fluorescent leukocytes in the vessels of a normal ear before (0 hour) OVA challenge, and the high abundance of cells interacting with the vascular endothelium in the OVA-injected ears of WT mice 1 hour and 12 hours after antigen challenge. Only a few fast-rolling leukocytes were observed in non-challenged (or PBS-injected) ears, while both rolling and firm adherent cells were seen 1 hour after OVA injection in the ears of all four genotypes of mice. Detailed analysis of the real-time videomicroscopy records on OVA-injected ears indicated a continuous increase in the number of rolling cells by 12 hours, relative to 1 hour, which was followed by a decline in the rolling interactions by 24 hours in WT mice. In the venules of CD44 KO ears, the number of rolling leukocytes was high throughout the entire post-challenge period, whereas the rolling interactions gradually decreased between 1 hour and 24 hours in the ears of L-selectin KO mice, and increased between 6 and 24 hours in the double KO ears. The velocity of rolling decreased over time in all OVA-injected ears, with the least decrease seen in double KO mice. The rolling speed at 1 hour after OVA injection was the highest in mice lacking CD44 or L-selectin, but velocity dropped more quickly in L-selectin KO than in CD44 KO mice between 1 and 6 hours post-injection. The number of firm adherent cells increased until 12 hours post challenge, and decreased afterwards in the ears of WT and L-selectin KO mice. Only a few adherent leukocytes were observed at each time point in CD44 and double KO ears.

To gain insight into the kinetics of leukocyte recruitment throughout early and later phases of the inflammtory process, we performed intravital microscopy on the knee joints 4h, 12 h, 1 day, and 5 days after i.a. injection of mBSA. A few rolling leukocytes, but essentially no firmly adherent cells, were observed in the postcapillary venules of control joints.

Numerous leukocytes interacted with the endothelium in the joints of WT mice, and the vast majority of these cells adhered tightly to the vessel walls 4 h after Ag injection.At this early phase of AIA, in the synovial venules of CD44 KO mice, significantly more cells rolled, but many fewer cells exhibited firm adhesion than int he vessels of WT joints. The firm adhesion interactions were also reduced in the joints of L-selectin-deficient and double KO mice. At 12 h, the frequency of rolling cells increased slightly in the WT and decreased in the CD44 KO joints, whereas the number of adherent cells did not changed. Differences between WT and KO animals in firm adhesion events could be still detected 1 day after the initiation of AIA. The overall rate of leukocyte recruitment (frequency of rolling and adherent cells), however decreased by day 1 and was also low at day 5. Leukocytes in all gene-targeted mice showed a tendency to roll faster than WT cells, and this was most obvious at 4h after i.a. mBSA injection. The average rolling velocity was higher in the KO groups than in WT mice, but the differences did not reach statistical significance in any KO group at any of the time points.

Leukocytes in the arthritic joints and peripheral blood of WT, CD44-deficient, L-selectin deficient and double KO mice.

The cellular composition of infiltrates in the joints of mBSA-injected mice was quantitatively determined using flow cytometry. Four hours after i.a. Ag injection, 88-92% of the infiltrating cells expressed Gr-1 in the knee joints of WT and CD44-deficiet mice, but this ratio was only 29-36% in the absence of L-selectin. At 24h, >98% of joint-infiltrating cells were granulocytes in WT and CD44 Ko animals, and 48-76% in L-selectin-deficient and double KO mice. 5 days after mBSA injection, granulocytes represented >90% of the infiltarting cells in all arthritic joints. The percentage of CD3-positive T cells was very low in each group at 4 h postinjection.This ratio increased in the joints of all KO mice, and was the highest in the double KO group at 24h, but declined by day 5 postinjection.

The CD11b, CD18, VLA-4 and PSGL-1 were expressed in similar proportions of circulating neutrophils and at comaparable cell surface densities in WT and all three groups of gene-targeted mice.

Discussion

CD44 and L-selectin have been shown to be involved in leukocyte extravasation in different models of inflammation. They are involved in mediating the initial capture and rolling of leukocytes along the endothelium of postcapillary venules under blood flow. Rolling leukocytes can be arrested via chemokine-induced interactions between cell surface integrins and integrin ligands. Adherent cells that resist detachment from the endothelium under the shear force of blood flow can than migrate across the vessel wall into the tissue, using a distinct set of adhesion receptors.

The requirement for L-selectin or CD44 for inflammatory responses has been investigated intensively with antibodies or gene knockout (KO) mice. Both molecules have been shown to play crucial roles in different models of inflammations, although the results are sometimes conflicting, indicating that the process of leukocyte-endothel interactions depends on different kind of other factors than the adhesion molecules (e.g. type of inflammation, cytokines, chemokines specific for the tissue microenvironment etc.) and still not fully understood.

Intravital microscopy allows observing interactions of virtually any blood cell with the endothelial wall in vivo. Principally this technique can be applied to any organ or tissue, which can be surgically prepared to be placed under a microscope. The skin is a unique organ in as much as no surgical procedure needs to be applied to access its microvasculature.

Using mice lacking CD44, L-selectin or both, we, in this study, investigated the requirement for these adhesion molecules for the development of an OVA induced Th2 type

cells mediated allergic dermatitis (resembling the acute phase of atopic dermatitis) and an mBSA induced antigen induced arthritis.

In the model allergic dermatitis wild type (WT), CD44 knockout (KO), L-selectin KO, and CD44/L-selectin double KO mice were injected intraperitoneally with chicken ovalbumin (OVA, and boosted 3 weeks later with the same amount of OVA. Two weeks after the second intraperitoneal injection, the local reaction was elicited by intradermal administration of OVA into the inner side of one of the ears.

Mice with the same gene disruption as above were injected 2 times with methylated BSA in adjuvant subcutan/intradermally and intraperitoneally. Two to three weeks after the boost, mice were injected with the same antigen into the right knee to induce AIA.

It has been reported that L-selectin-deficient mice do not respond well to subcutan/epicutaneous sensitization. This hyporesponsiveness is primarily due to the inability of L-selectin-deficient lymphocytes to home to, and undergo activation in, the regional (skin-draining) lymph nodes. Indeed, mBSA specific immune responses were significantly reduced in mice lacking L-selectin after s.c./intradermal Ag administration. However T cells were adequately activated, and the magnitude of their responses to Ag was normal in L-selectin Ko mice after i.p. immunization. The most likely explanation for the emergence of adequate T cell memory in L-selectin KO mice in this case is that the antigen from the peritoneal cavity is delivered to splenic lymphocytes that could enter the white pulp of the spleen in an L-selectin independent manner.

Despite comparable immune responses to OVA and mBSA, the morphological features of allergic dermatitis and AIA were different in WT, CD44-deficient, L-selectin-deficient, and CD44/L-selectin double KO mice.

In the absence of CD44 (or both CD44 and L-selectin) expression, CD44 and double KO mice demonstrated significant reductions in leukocyte influx and ear swelling after local challenge

with OVA, whereas inflammation in mice lacking L-selectin only was comparable to WT. These results suggested that CD44, but not L-selectin, 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.

Joint swelling was diminished in CD44 KO animals for 2 days after i.a. mBSA injection and was strongly reduced throughout the 5-day observation period in mice lacking L-selectin or both L-selectin and CD44. Histopathology of the knees and flow cytometric analysis of the joint infiltrating cells revealed a rapidly increasing influx of granulocytes (neutrophils) into the joints of WT and CD44-deficient mice at the early phase of AIA.In comparison, L-selectin KO and double KO mice exhibited a delay in leukocyte extravasation, with a slower accumulation of granulocytes in the joints. By day 5 of AIA, all Ag-challenged knee joints became heavily infiltrated predominantly by neutrophil granulocytes.

One of the possible explanations for the predominance of joint-homing neutrophils in this otherwise T-cell dependent model of arthritis is the presence of immune complexes (ICs), that form between the locally injected mBSA and BSA-specific serum Abs. ICs, besides activating the complement system, can stimulate the local production of chemokinesthat preferentially attract neutrophils.

We found that extravasation of granulocytes, but not the recruitment of T cells, was diminished in the joints of mice lacking L-selectin at the early phase of AIA. These data indicate, that L-selectin is necessary for the early influx of neutrophils into the joints, whereas most T cells can enter these sites ina n L-selectin-independent manner.

We showed in the present study that in comparison with WT mice, CD44-deficient mice exhibited only a moderate reductions in joint swelling and neutrophil infiltration during the first day of AIA.

Intravital microscopy (IVM) on the microcirculation of the ears provided new information about the time course of leukocyte-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 leukocyte extravasation in CD44 KO and double KO mice. Unlike the ear swelling that showed a biphasic pattern, IVM recording of leukocyte traffic showed a single peak at 12h after OVA challenge even in WT and L-selectin KO mice that both developed full-blown inflammtion. A likely explanation for this apparent discrepancy between the clinical signs of inflammation and the kinetics of leukocyte 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 leukocytes. Using IVM for the first time in an animal model of allergic dermatitis, we found that the number of leukocytes 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 L-selectin (both single and doule KO), whereas the frequency of rolling interactions was significantly increased at 24 h in CD44 KO mice as compared with WT.

The velocity of rolling is expected to decrease, as the activation state of the vascular endothelial cells and leukocytes increase. 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 of all four genotypes of mice. Compared to WT animals, however, mice lacking CD44 (or both CD44 and L-selectin) demonstrated an elevated rolling velocity. In addition, firm adhesion was clearly compromised in CD44 KO and double KO animals. Slowly rolling leukocytes have longer exposure to locally produced chemokines that promote firm adhesion by activating integrins. It was not surprising, therefoer, that CD44 Ko and double KO leukocytes showed a significant reduction in firm adhesion.

Using IVM, inflammatory cell recruitment into the joints in the model of AIA was the most intense at the earliest time point (4h) of video recording after i.a. Ag injection. In WT mice, numerous leukocytes engaged in firm adhesion interactions with the endothelium, and only a few cells rolled. The frequency of rolling leukocytes was significantly higher and the number of firmly adherent cells was significantly lower in the vessels of CD44-deficient animals than in those of WT mice. In L-selectin-deficient and double KO mice rolling leukocytes were still more frequent than in WT mice. The function of L-selectin was not compensated for by CD44, because leukocytes in L-selectin-deficient and double KO animals exhibited almost identical rolling and adhesion behavior. The lack of any discernible effect of CD44 deficiency on the rolling of L-selectin-deficient cells and exaggerated down-regulation of L-selectin in CD44 KO, compared with WT cells, together suggested that the moderate reduction in leukocyte influx into the joints of CD44-deficient mice could be the result of diminished L-selectin expression rather than the consequence of a missing CD44 function.

In comparison with WT mice, the frequency of rolling cells was elevated in all KO groups at the initial phase of AIA. Because L-selectin was either absent or reduced and CD44 was not appreciably involved in the rolling interactions, the majority of these cells must have rolled in a PSGL-1 or VLA-4-dependent manner. This rolling was less „productive" than rolling via L-selectin. Our results suggest that L-selectin in neutrophils must be more effective than PSGL-1 or VLA-4 in preparing these cells for integrin-mediated firm adhesion in vivo. In addition, the absence of secundary leukocyte-leukocyte interactions via L-selectin-PSGL-1 binding could contribute to the reduced/delayed recruitment of mutant cells in the inflamed joints.

The absence of both L-selectin and CD44 was expected to have either additive or synergistic negative effects on the inflammatory reactions. However, the combination of L-selectin and CD44 deficiency resulted in an inflammatory response indistinguishable from

that exhibited by mice deficient in CD44 in the model of allergic dermatitis and L-selectin only in the mBSA-induced AIA.

In conclusion, the reason for the different kinetics of the leukocyte-endothel interactions in the two models of inflammations could be the followings:

a, The allergic dermatitis induced by OVA is a Th2-polarized reaction, while in the model of AIA the effector cells were predominantly neutrophil leukocytes.

b, The different kind of inflammatory cells, the elevated IgE level in the AD-like dermatitis resulted in different chemokine profile, and cell movement.

c, CD44 and L-selectin molecules could be involved, possibly via cooperation with other adhesion molecules, in the stabilization of leukocyte adhesion. Previous studies reported, that a molecular interaction between CD44 and a leukocyte integrin (VLA-4) was essential for rolling lymphocytes to exhibit firm adherence. The absence of this mechanism could play a role in the reduction of adherence of CD44 KO leukocytes in the lymphocyte mediated model of allergic dermatitis. On the other hand, in AIA the absence of L-selectin contributed to the reduced interactions between L-selectin and PSGL-1 and that resulted in reduced/delayed recruitment of mutant cells in the inflamed joints.

The frequency of rolling interactions, as well as the velocity of rolling, are regulated by multiple factors, including the density of leukocyte and endothelial adhesion receptors and ligands/counterreceptors, the avidity and membrane distribution of adhesion molecules, and the specificity and concentration of locally released chemokines. The density and distribution of endothelial ligands and the avidity of receptor-ligand interactions however could not be determined in vivo. Additional factors (e.g, the presence of ICs in the tissue and expression of adhesion molecules, cytokines, and chemokines specific for the tissue microenvironment),

could also influence the rolling behavior of leukocytes, whose adhesion interactions with endothelium have already been destabilized by the lack of CD44, L-selectin, or both adhesion receptors.

Summary

Using mice deficient in CD44, L-selectin, or both we developed two different inflammatory models.

In the model allergic dermatitis, that shows similarities to the acute phase of atopic dermatitis (elevated Ag-specific IgE level, Th2 type cell activation), the mice were injected intraperitoneally with chicken ovalbumin (OVA), and boosted 3 weeks later with the same amount of OVA. Two weeks after the second intraperitoneal injection, the local reaction, was elicited by intradermal administration of OVA in sterile PBS into the inner side of one of the ears.

Mice with the same gene disruption as above were immunized 2 times with methylated BSA in adjuvant subcutan/intradermally and intraperitoneally. Two to three weeks after the boost, mice were injected with the same antigen into the right knee to induce antigen induced arthritis (AIA).

Despite comparable immune responses to OVA and mBSA, the morphological features of allergic dermatitis and AIA were different in WT, CD44-deficient, L-selectin-deficient, and CD44/L-selectin double KO mice.

In the absence of CD44 (or both CD44 and L-selectin) expression, CD44 and double KO mice demonstrated significant reductions in leukocyte influx and ear swelling after local challenge with OVA, whereas inflammation in mice lacking L-selectin only was comparable to WT.

These results suggested that CD44, but not L-selectin, was required for the entry of effector cells into the inflamed skin.

Joint swelling was strongly reduced throughout the 5-day observation period in mice lacking L-selectin or both L-selectin and CD44. Histopathology of the knees and flow cytometric analysis of the joint infiltrating cells revealed a delay in leukocyte extravasation, with a slower accumulation of granulocytes in the joints in the absence of L-selectin.

Extravasation of granulocytes, but not the recruitment of T cells, was diminished in the joints of mice lacking L-selectin at the early phase of AIA.

Using intravital microscopy (IVM), in CD44 KO animals the rolling velocity and frequency was higher, the adherence of leukocytes was diminished as compared with the WT mice.

In the model of AIA, L-selectin-deficient and double KO mice rolling leukocytes were more frequent than in WT mice, while the ability of these cells to adhere to the vessel wall was reduced. The moderate reduction in leukocyte influx into the joints of CD44-deficient mice could be the result of diminished L-selectin expression rather than the consequence of a missing CD44 function.

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