Mitigation of Delayed-Type Hypersensitivity Reactions by a CD44 Variant Isoform v3-Specific Antibody: Blockade of Leukocyte Egress

Simone Seiter,*† Peter Engel,* Nicole Föhr,* and Margot Zöller*‡

*Department of Tumor Progression and Immune Defense, German Cancer Research Center, Heidelberg, Germany; †Department of Dermatology, University of the Saarland, Germany; ‡Department of Applied Genetics, University of Karlsruhe, Karlsruhe, Germany

In allergic alterations of human skin the majority of infiltrated leukocytes express CD44v3, but no other CD44 variant isoform. Vessel endothelium, too, is brightly stained with a CD44v3-specific antibody. Being concerned about therapeutic intervention, it became of importance to define whether expression of CD44v3 on the endothelial cells or on the leukocytes or on both is of functional importance. As expression of CD44v3 in the mouse on activated endothelium and on subpopulations of activated $CD4^+$ cells, B cells and monocytes was similar to the expression in the human, we answered the question in a mouse delayed-type hypersensitivity model. The effect of anti-CD44v3 was compared with the effect of anti-CD44s and anti-CD44v10, both known to suppress delayed-type hypersensitivity reactions. Anti-CD44v3 mitigated the delayed-type hypersensitivity reaction in dinitrofluorobenzene sensitized and challenged mice comparable with anti-CD44s and anti-CD44v10. The seemingly similar effects of CD44 isoform-specific antibodies, however, resulted from a distinct modulation of response. Anti-CD44s mainly suppressed T cell activation and interleukin-2 as well as interferon-γ expression. Anti-CD44v10 inhibited the activation of monocytes in the draining lymph nodes and in the infiltrate, which led to a strong reduction in the proinflammatory cytokines tumor necrosis factor-α and interleukin-12 and in edema formation. Anti-CD44v3 had only a weak effect on cytokine expression by isolated subpopulations of leukocytes, but suppressed cytokine production by helper T cells when cocultured with antigen-presenting cells, i.e., blocked an interaction between antigen-presenting cells and helper T cells. The dominating effect of anti-CD44v3, however, relied on a blockade of leukocyte extravasation. As leukocytes transferred into dinitrofluorobenzene sensitized, anti-CD44v3-treated and lethally irradiated mice did not infiltrate the sensitized skin, anti-CD44v3 most likely prevented leukocyte extravasation by blocking CD44v3 on endothelial cells. ***Key words: CD44 isoforms/cytokines/delayed-type hypersensitivity/leukocyte migration. J Invest Dermatol 113:11–21, 1999***

CD44 comprises a set of transmembrane glycoproteins, whose members differ by glycosylation (Lesley *et al*, 1993) and by insertion of up to 10 variant exons between exon 5 and exon 6 of the CD44 standard isoform (CD44s) (Screaton *et al*, 1993). Considering functions of CD44 on hematopoietic cells, CD44s has been described originally as a lymphocyte homing receptor (Jalkanen *et al*, 1986). Meanwhile, it is known also to be involved in lymphocyte maturation (Kincade *et al*, 1993; Siczkowski *et al*, 1993; Sugimoto *et al*, 1994; Toyama-Sorimachi *et al*, 1995), traffic (Camp *et al*, 1993; Wu *et al*, 1993; Mikecz *et al*, 1995; DeGrendele *et al*, 1997, 1993), and activation (Funaro *et al*, 1994; Shimizu *et al*, 1989; Pierres *et al*, 1992; Galandrini *et al*, 1993; Naujokas *et al*, 1993). Knowledge of functional activity of CD44 variant isoforms is still scarce: CD44v3 seems to be involved in hematopoiesis, particularly myelopoiesis (Bennett *et al*, 1995; Jackson *et al*, 1995). Lymphocyte activation has been shown to be accompanied by upregulation of CD44v6 (Arch *et al*, 1992; Koopman *et al*, 1993; Galluzzo *et al*, 1995; Levesque and Haynes, 1996). CD44v10 is involved in the activation of virgin B cells, in a process which requires an interaction between conventional antigen-presenting cells (APC) and the virgin B cell (Rösel *et al*, 1998). Considering the relevance of CD44v expression in pathology it has been demonstrated in animal models that anti-CD44s and anti-CD44v6 interfere with collagen-induced arthritis (Verdrengh *et al*, 1995; Brennan *et al*, 1997). Anti-CD44v7, but neither anti-CD44s nor anti-CD44v6 nor anti-CD44v10, cures a lethal T_H1 (T helper cell)-mediated experimental colitis (Wittig *et al*, 1998). Delayed-type hypersensitivity (DTH) reactions can be mitigated by anti-CD44s, anti-CD44v6, and anti-CD44v10 (Camp *et al*, 1993; Rösel *et al*, 1997; Weiss *et al*, 1997).

The mode by which CD44 communicates with the intracellular signal transduction pathway has not yet been unraveled, but there is evidence that the tyrosine kinase $p56^{lck}$ may play a central part

Manuscript received September 16, 1998; revised February 9, 1999; accepted for publication April 5, 1999.

Reprint requests to: Dr. Margot Zöller, Department of Tumor Progression and Immune Defense, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

Abbreviations: APC, antigen-presenting cells; CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; OVA, ovalbumin; PBL, peripheral blood leukocytes; PE, phycoerythrin; PEC, peritoneal exudate cells; T_H, helper T cells.

0022-202X/99/$14.00 · Copyright © 1999 by The Society for Investigative Dermatology, Inc.

(Taher *et al*, 1996). Cross-linking of CD44 has also been described to initiate intracellular Ca^{2+} mobilization (Galluzzo *et al*, 1995). Furthermore, CD44 has been assumed to stimulate production of the cytokines interleukin (IL)-1, IL-2, monocyte colony-stimulating factor and of the insulin-like growth factor (Webb *et al*, 1990; Gruber *et al*, 1992; Noble *et al*, 1993). Alternatively, CD44 could anchor cytokines and chemokines via proteoglycan side chains and by this means influence lymphocyte attraction and motility (Tanaka *et al*, 1993; Bennett *et al*, 1995; Weber *et al*, 1996). Finally, CD44 has been described to function as the ligand of a costimulatory molecule (Guo *et al*, 1996).

As mentioned above, T_H1-mediated DTH reaction can be mitigated by application of CD44v10 (Rösel *et al*, 1997). As leukocyte infiltrates as well as endothelial cells in patients with allergic skin reactions express CD44v3, the question arose whether anti-CD44v3 also would interfere with the DTH reaction. Furthermore, it became of interest (i) whether both CD44v isoforms function via the same mechanism, and (ii) whether the pathologic over-reactivity could be most efficiently blocked, e.g., by an antibody, at the level of the leukocytes or the endothelial cells. We approached the question by an *in vivo* model of a 2,4-dinitro-1-fluorobenzene (DNFB) induced DTH reaction. Anti-CD44v3, like anti-CD44s and anti-CD44v10, efficiently mitigated the inflammatory response. CD44s, CD44v3, and CD44v10, however, display distinct modes of action. CD44s is involved directly in T and B cell activation as well as in leukocyte traffic. CD44v10 supports monocyte activation. CD44v3 takes part in a T_H–monocyte interaction, but mainly is required for the extravasation of activated leukocytes.

MATERIALS AND METHODS

Blood samples Heparinized blood (10 ml) was collected from 24 healthy donors (10 female, 14 male; mean age, 32 ± 8 y; range, 24–54 y), from 10 patients with viral and bacterial infections of the skin (four female, six male; mean age, 48 ± 17 y; range, 20–77 y) and from 15 patients (eight female, seven male; mean age, 50 ± 14 y; range, 25–74 y) with an allergic reaction of the skin (e.g., urticaria, contact eczema, atopic dermatitis). The diagnosis had been verified for all patients by conventional clinical criteria. peripheral blood leukocytes (PBL) from patients were collected before therapeutic treatment. PBL were separated by Ficoll Hypaque centrifugation. Where indicated, PBL were stimulated for 48 h with phytohemagglutinin A (PHA) (2 μg per ml) in RPMI 1640, supplemented with antibiotics, L-glutamine, 10^{-5} M 2-mercaptoethanol, 10^{-3} M HEPES buffer, 10% fetal bovine serum.

Mice and treatment BALB/C mice were obtained from WIGA (Sulzfeld, Germany). They were kept under specific pathogen-free conditions and were fed sterilized tap food and water ad libitum. Animals were used for experiments at the age of 8 wk. Contact allergy was induced by painting 20 μl of 0.5% DNFB in 4:1 acetone/olive oil on the rear footpads of mice for a successive 2 d. Four days later, mice were challenged by painting 10 μl of 0.2% DNFB to each side of both ears (Tang *et al*, 1996). In most experiments mice received concomitantly with the first painting and 4 d thereafter either rat immunoglobulin (Ig)G, anti-CD44s, anti-CD44v3, or anti-CD44v10 (200 μg per 100 μl phosphate-buffered saline), intravenously. Where indicated, antibody was applied only at the first contact with DNFB or at the time of challenge. Ear thickness was measured near the root and the tip of the ears 48 h after challenge.

Monoclonal antibodies The following monoclonal antibodies were used: Anti-CD44s (IM-7, rIgG2b), anti-CD44v10 (K926, rIgG2a) (34), anti-CD44v3 (PTS33, rIgG2b),[1] anti-interferon (IFN)-γ (R4–6A2, anti-rIgG), anti-μ (131.12, rIgG2b), anti-CD4 (YTA3.2.1, rIgG2b), anti-CD8 (YTS169.4.1, rIgG2b), anti-Mac-1 (YBM6.1.1, rIgG2a), anti-IL-2R (7D4, rIgG2b), and anti-ICAM-1 (YN1/1.7.4, rIgG2b) (ECACC). The 25–32 hybridoma (anti-hCD44s, mIgG2b) was obtained from the ECACC. The human CD44 variant specific antibodies vFF327 (anti-CD44v3, mIgG1), vFF8 (anti-CD44v5, mIgG1), vFF7 and vFF18 (anti-CD44v6, mIgG1), vFF9 (anti-CD44v7, mIgG1), vFF17 (anti-CD44v7-v8, mIgG2b), and vFF16 (anti-CD44v10, mIgG1) were kindly provided by Bender & Co. GMBH (Vienna, Austria), and have been described in detail elsewhere (Heider *et al*, 1993). Culture supernatants were purified by passage over Protein G-Sepharose and monoclonal antibodies were used *in vitro* at a concentration of 10 μg per ml. Anti-CD40, anti-CD40L, anti-CD80, anti-CD86, anti-CD28, anti-CTLA4 and pairs of anti-IL-2, anti-IL-12, anti-IFN-γ and anti-tumor necrosis factor (TNF)-α were obtained commercially (Pharmingen Hamburg, Germany). For flow cytometry, 3–5 × 10^5 cells were stained according to routine procedures using either fluorescein isothiocyanate-labeled and phycoerythrin (PE)-labeled isotype-specific secondary antibodies or biotinylated first antibodies and streptavidin–PE for detection. When assaying cytokine producing cells, lymphocytes were fixed and permeabilized in advance. As far as they had been stimulated *in vitro*, 5 μM monensin was added to the culture medium during the last 8 h of culture. Fluorescence was determined with an EPICS XL (Coulter, Hialeah, FL).

Lymphocyte separation and *in vitro* stimulation Leukocytes were isolated after sensitization or after sensitization and challenge with DNFB from draining lymph node cells, the infiltrate of the ear and the peritoneal cavity (PEC). Single cell suspensions of infiltrating leukocytes were obtained by incubating the ear, inner side down, for three times 30 min at 37°C in 0.5% trypsin, collecting the cells in the supernatant. Lymph nodes were meshed through fine gauze. The cell suspensions were washed twice with phosphate-buffered saline. Where indicated, lymphocyte populations were depleted of adherent cells (monocytes) by 2 × 1 h plastic adherence. B cells were enriched by panning on anti-μ coated plates (Wysocki and Sato, 1978) collecting the adherent fraction. Lymph node cell preparations were enriched for $CD4^+$ and $CD8^+$ cells by panning on anti-μ-coated plates and by depletion of plastic adherent cells collecting in both instances the nonadherent fraction. These nonadherent cell populations were then incubated with either anti-CD4 (rIgG2b) or anti-CD8 (rIgG2b) and after washing were plated on anti-rat IgG-coated plates, from where the adherent population was collected. According to analysis by flow cytometry the individual populations were enriched by 90%–95%. For flow cytometry of cytokine expressing subpopulations of leukocytes, cells were cultured for 48 h in medium containing DNP-OVA (dinitrophenyl-ovalbumin) (100 μg per ml) and 10 μg per ml of either rat IgG or anti-CD44v3 or anti-CD44v10. The culture medium contained in addition 10% supernatant of concanavalin A (Con A) activated rat spleen cells as a source of T cell growth factor after Con A has been blocked by α-methylmannoside. In experiments where cytokine expression by $CD4^+$ cells was determined after coculture with B cells, the B cells had been irradiated (3000 R); when $CD4^+$ cells were cocultured with monocytes care was taken to collect only the nonadherent cells. For the determination of cytokine secretion, cells were cultured in flat bottom 96 well plates, which had been coated with anti-interleukins. Cytokine secretion was determined after 24 h of culture in the medium described above.

Enzyme-linked immunosorbent assay and enzyme-linked immunosorbent spot assay Plates were coated with the CD44v3 peptide EDFTDYPSFSG (10 μg per ml). After washing and blocking with bovine serum albumin, 50 μl of biotinylated anti-CD44v3 (2 μg per ml) was added. Plates were washed again, alkaline phosphatase-labeled streptavidin and finally the substrate were added. The optical density of the enzyme reaction was measured after 20 min at 450 nm. For the enzyme-linked immunosorbent spot assay plates were coated with anti-IL-2, anti-IL-12, anti-IFN-γ, or anti-TNF-α in bicarbonate buffer, pH 8.9. Plates were washed and blocked with bovine serum albumin (100 μg per ml). Cells were added to the coated plates and were incubated for 24 h at 37°C in RPMI 1640, supplemented with antibiotics, L-glutamine, 10^{-5} M 2-mercaptoethanol, 10^{-3} M HEPES buffer, 10% fetal bovine serum and 100 μg DNP-OVA per ml. Thereafter, cells were lysed, plates were washed three times and 50 μl biotinylated antibody was added. Plates were incubated overnight at 4°C. After four washings, the enzyme (streptavidin–alkaline phosphatase) was added in phosphate-buffered saline, gelatin, and Tween20. Plates were incubated for 90 min at 37°C, they were washed five times before adding the substrate in 1% agar. Spots were counted after incubation at 37°C for 4 h.

Normal human tissue and biopsies of patients with alteration of the skin Normal human skin was derived from surgical specimens (discarded tissue). Tissues from patients with allergic alterations of the skin were derived from biopsies undertaken as routine diagnostic procedure, i.e., the tissue has been left after routine immunohistochemical staining and was not part of any study. All biopsies were taken at first diagnosis and before systemic treatment. Biopsies were obtained from patients

[1]Engel P, Zöller M: Functional activity of CD44v3 in hematopoietic progenitor maturation. In preparation.

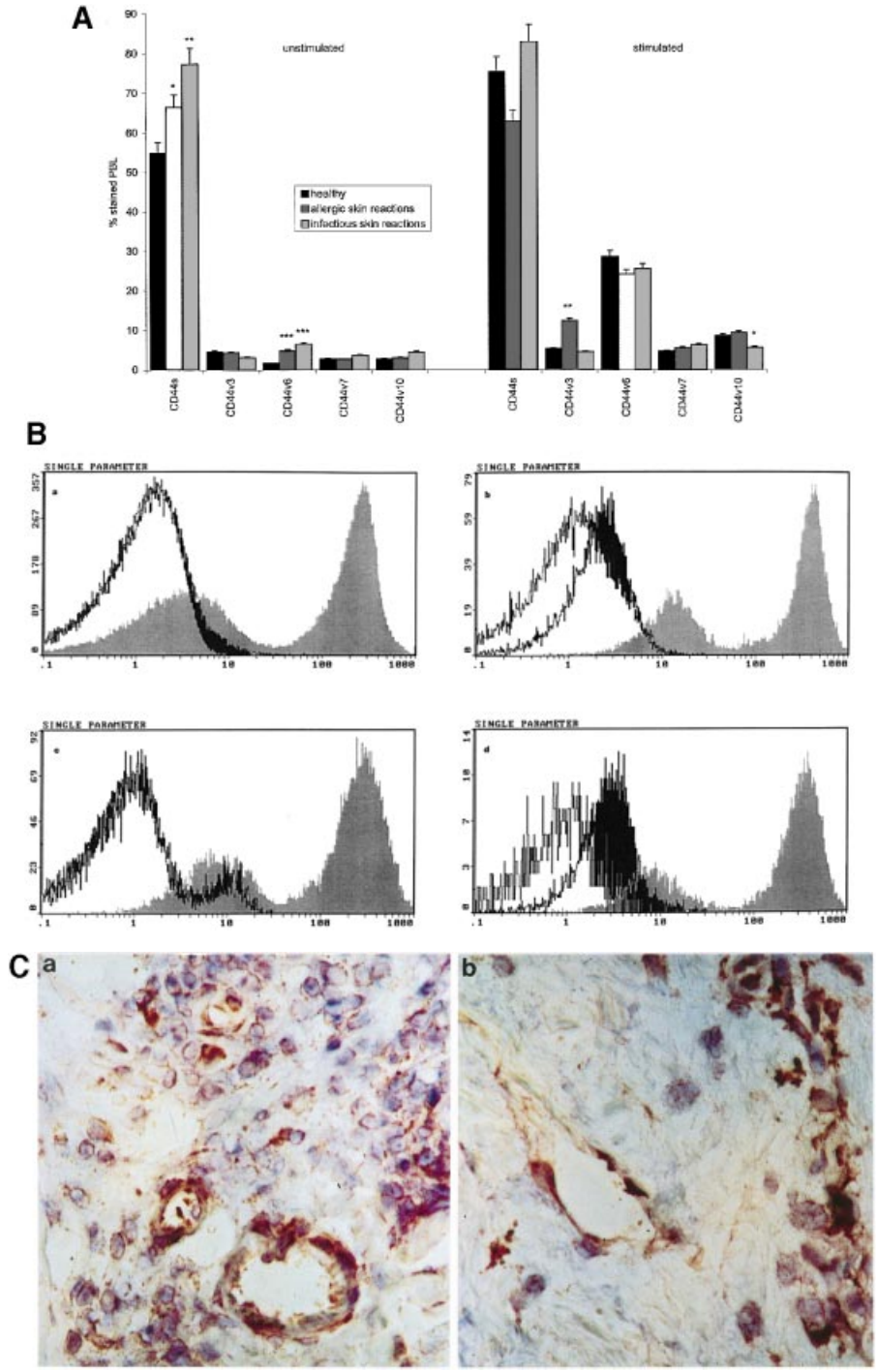

Figure 1. Expression of CD44 variant isoforms in allergic skin reactions. (*A*) PBL of 15 healthy donors, of 15 patients with allergic and of 10 patients with infectious alterations of the skin were stained with anti-CD44s, anti-CD44v3, anti-CD44v6, anti-CD44v7, and anti-CD44v10. The mean percentage (+SD) of freshly harvested PBL and of PBL stimulated for 48 h with PHA (2 μg per ml) are shown. Significance of differences are indicated by asterisks (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$). (*B*) Freshly harvested (*a*, *b*) and activated (2 μg per ml PHA, 48 h) (*c*, *d*) PBL of a healthy donor (*a*, *c*) and a patient with disseminated contact eczema (*b*, *d*) were stained with anti-CD44s and anti-CD44v3. Single parameter overlays are shown. *White area*: negative control. *Black area*: anti-CD44v3-stained cells. *Gray area*: anti-CD44s stained cells. (*C*) A skin biopsy of a patient with a contact eczema has been stained with anti-CD44v3 (*a*) and of a patient with a maculopapular cutaneous drug eruption has been stained with anti-CD44v10 (*b*). The majority of the few infiltrated cells were stained by anti-CD44v3, some of the infiltrated cells were stained by anti-CD44v10. Vessel endothelia were clearly stained by both antibodies.

with urticaria (three), atopic dermatitis (two), contact eczema (five), and maculopapular cutaneous drug eruption (one). The mean age of the patients was 43 ± 19 y (range, 21–74 y). Biopsies were from six female and five male patients. Diagnosis of all patients was verified by clinical and pathologic criteria.

Immunohistology Human biopsies and sensitized skin of mice were excised and either fixed in formaldehyde for paraffin sections or snap frozen in liquid nitrogen for immunohistology (Cattoretti *et al*, 1988). Sections of about 0.4 cm^2 were cut in 4–6 μm thick sections, mounted on gelatin coated slides and air-dried overnight. They were fixed in acetone for 10 min at –20°C and stored at –80°C. Fixed and dried sections were incubated for 1 h at room temperature with the first antibody. Slides were washed intensively and incubated at room temperature for 30 min with a second, biotinylated anti-rat IgG antibody. After washing, sections were incubated with a horseradish-peroxidase-conjugated streptavidin complex. The substrate for the enzyme was either 3-amino-9-ethylcarbazole or Texas Red (Sigma, St. Louis, MI). The peroxidase reaction resulted in an intense red precipitate. The sections were faintly counterstained with Mayer's hematoxylin, air-dried and mounted. For negative controls staining of each sample was performed without applying the primary antibody or by using an antibody of irrelevant specificity. No staining was observed in these samples.

Statistics Significance of differences were calculated by the two-tailed Student's t test.

RESULTS

Expression of CD44 variant isoforms in PBL and infiltrates of patients with allergic skin reactions Freshly harvested as well as PHA-stimulated PBL from healthy donors and patients

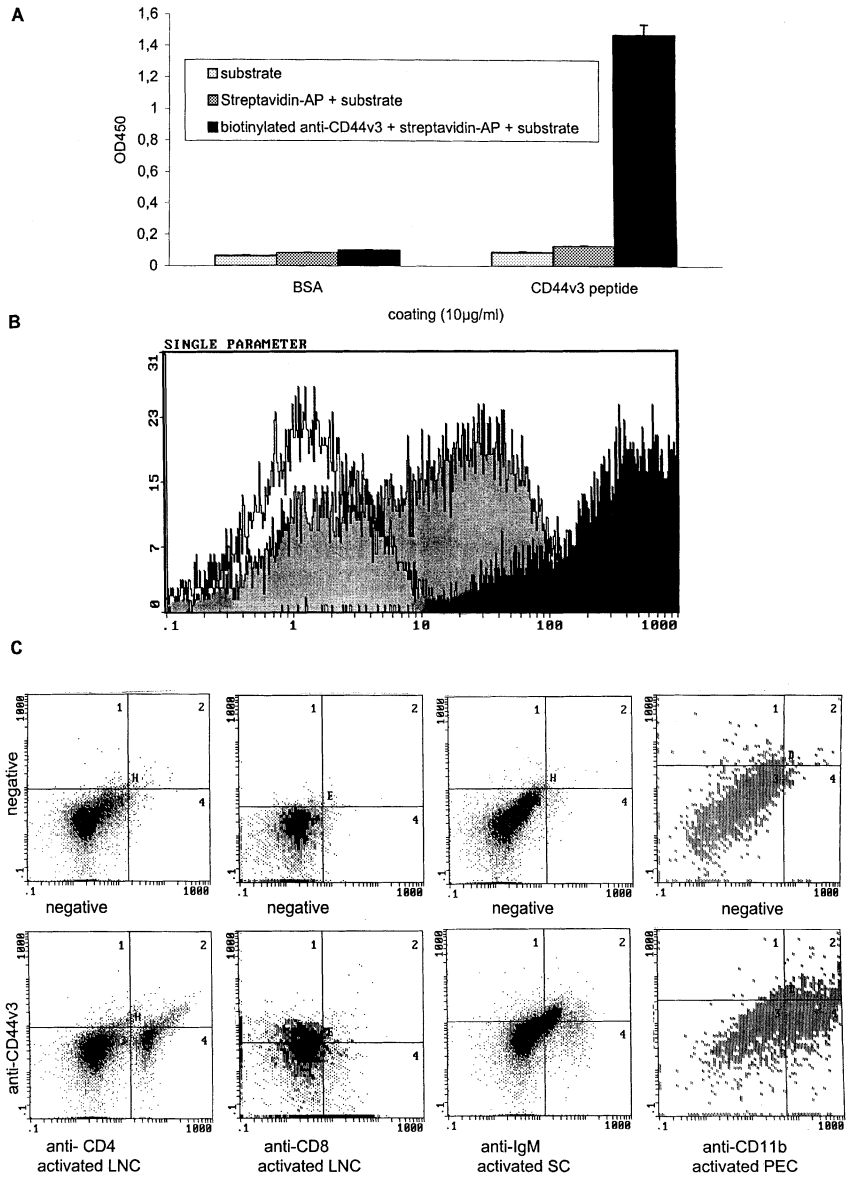

Figure 2. Expression of CD44v3 on murine leukocytes. (*A*) The newly generated anti-CD44v3 antibody PTS33 specifically binds to CD44v3 peptide-coated plates. (*B*) Staining of *in vitro* stimulated lymph node cells (DNP-OVA plus T cell growth factor) with anti-CD44s and anti-CD44v3. Single parameter overlays are shown. *White area*: negative control. *Gray area*: anti-CD44v3-stained cells. *Black area*: anti-CD44s-stained cells. (*C*) Double fluorescence analysis of Con A activated lymph node cells (anti-CD4, anti-CD8), lipopolysaccharide (LPS) activated spleen cells (SC) (anti-sIgM) and LPS activated PEC (anti-CD11b) with anti-CD44v3. Activated lymph node cells, SC and PEC have been stained with anti-CD4, anti-CD8, anti-µ and anti-CD11b (first antibody) and counterstained with fluorescein isothiocyanate-labeled anti-rat IgG (fluorescence 1, X-axis) as well as with biotinylated anti-CD44v3 (first antibody) and with streptavidin–PE for detection (fluorescence 2, Y-axis). The negative control has been incubated with rat IgG, fluorescein isothiocyanate-labeled anti-rat IgG and streptavidin–PE. The population of large cells (lymph node cells, SC) and of large and more granulated cells (PEC) has been gated.

with allergic skin reactions were stained with CD44 isoform specific antibodies and were analyzed by flow cytometry (**Fig 1*A***). Expression of CD44s and CD44v6 was elevated in freshly harvested PBL of patients with allergic skin reactions as compared with PBL of healthy controls. After *in vitro* stimulation, expression of CD44s and CD44v6 was augmented on PBL of healthy donors and the difference to PBL of patients with allergic skin reactions had disappeared. Instead, a small, but significantly increased population of PBL from patients expressed CD44v3. As demonstrated in **Fig 1(*B*)**, a low percentage of $CD44v3^+$ cells was detected even in activated PBL of a healthy donor. Yet, a significantly higher percentage of $CD44v3^+$ cells was detected in the PBL of a patient with a contact eczema. As compared with the intensity of CD44s expression, CD44v3 expression was more than a decade lower. Distinct to freshly harvested PBL, the intensity of expression of CD44s on activated PBL was comparable in healthy donors and in patients with allergic skin reactions.

It has been described before that leukocytes infiltrating the skin contain few, but strongly $CD44v3^+$ cells. Infiltrates of patients with allergic and other alterations of the skin rarely stain with anti-CD44v10 and do not stain with anti-CD44v6 and anti-CD44v7 (Seiter *et al*, 1998). Notably, endothelial cells in unaltered (data not shown) and allergen-reactive skin are $CD44v3^+$ as well as $CD44v10^+$ (**Fig 1*C***).

The rather selective expression of CD44v3 on PBL, dermal infiltrates and activated endothelial cells of patients with allergic skin reactions prompted us to evaluate in a mouse model of a DNFB-induced DTH reaction the possible functions of this CD44 variant isoform. Since anti-CD44s as well as anti-CD44v10 are known to interfere with T_H1 DTH reactions, evaluation of these isoforms has been included in the study.

Expression of CD44v3 and CD44v10 on subpopulations of activated murine leukocytes The newly generated CD44v3-specific antibody PTS33 has been derived from a fusion of Ag8 cells with rat spleen cells after immunization with a synthetic peptide covering the amino acids EDFTDYPSFSG of the CD44v3 exon product. As shown in **Fig 2(*A*)**, the antibody specifically binds to CD44v3 peptide coated plates. It also recognizes Ag8 cells transfected with CD44v3, but not nontransfected Ag8 cells (data not shown).

In a pilot experiment it was tested whether murine lymphocytes display a comparable pattern of CD44v3 expression as observed on human PBL. Expression of CD44v10 on resting and activated murine leukocytes has already been described, i.e., CD44v10 is not expressed on resting murine lymph node cells, but 10%–15% of activated lymph node cells express CD44v10. CD44v10 was predominantly expressed on monocytes and B cells (Rösel *et al*,

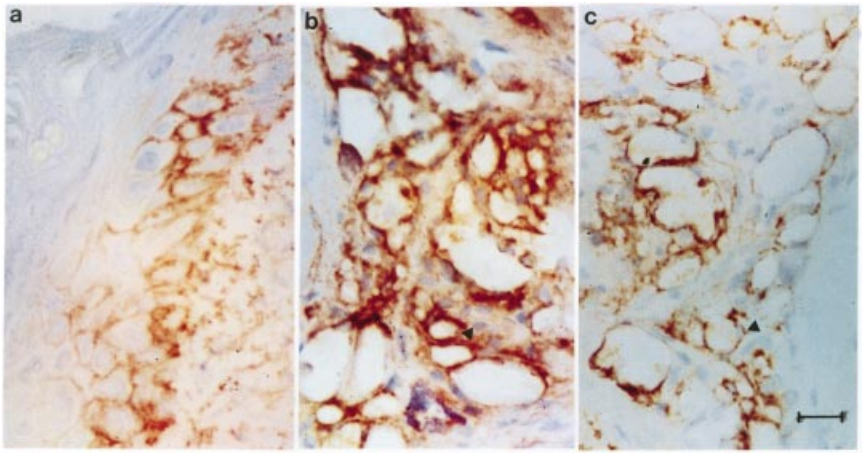

Figure 3. Expression of CD44v3 and CD44v10 in dermal infiltrates of DNFB sensitized mice. Mice were sensitized with DNFB, the skin was excised 48 h after sensitization and was shock frozen. Sections of 5 μm were stained with anti-CD44v3 and anti-CD44v10. Basal layers of the epidermis were stained by anti-CD44v3 (*a*). Part of the infiltrated leukocytes were stained by anti-CD44v3 (*b*) and, distinct to the human skin, also by anti-CD44v10 (*c*). Notably, endothelial cells (*arrowhead*), too, expressed CD44v3 and CD44v10. *Scale bar*: 2.5 μm.

1997, 1998). The CD44v3-specific antibody also did not stain nonactivated lymph node cells (data not shown), but stained 20%–30% of blasts, although the intensity of expression was weak as compared with CD44s (**Fig 2*B***, **Table I**). Staining of leukocyte subpopulations (**Table II**) and double fluorescence analysis (**Fig 2*C***) revealed that CD44v3 is expressed on a part of $CD4^+$ cells, B cells and monocytes, but hardly on $CD8^+$ cells. A similar distribution has been observed on activated human PBL.[2]

Considering expression of CD44 variant isoforms in epidermis and dermis of the mouse (**Fig 3**), expression of CD44v10 is restricted to the basal layers of the epidermis (Rösel *et al*, 1997). CD44v3, too, is expressed in the epidermis. Both, CD44v3 and CD44v10 are moderately expressed on vessel endothelia and infiltrating leukocytes. As there is no distinct marker for Langerhans cells in the mouse, CD44 variant isoform expression on these cells could not be defined. In the human, however, some but not all Langerhans cells express CD44v3 (Seiter *et al*, 1998).

CD44v3 and CD44v10 specific antibodies influence a T_H1-mediated DTH reaction A DTH reaction was induced by painting with DNFB, which after challenge of the ears provides a strong inflammatory stimulus with erythema, edema, and massive leukocyte infiltration. The peak reaction is observed after 2 d, at which time-point the draining lymph nodes and the infiltrate were analyzed. All three CD44-specific antibodies mitigated the DTH reaction. Considering edema formation, anti-CD44v10 exerted the strongest effect, but the differences in the efficiency to anti-CD44v3 and anti-CD44s were minor (**Table III**). The number of infiltrated cells, however, was more strongly reduced in anti-CD44v3 and anti-CD44v10 than in anti-CD44s-treated mice (**Fig 4**). Anti-CD44 antibodies obviously also influenced expansion of lymphocytes in the draining lymph node. Interestingly, a reduction in the number of lymphocytes in the draining lymph node was only seen with anti-CD44s and anti-CD44v10. Instead, mice receiving anti-CD44v3 showed a significantly increased number of cells in the draining lymph node.

The uniform influence of the anti-CD44 antibodies on leukocyte infiltrates in combination with the differential expression pattern of CD44 isoforms on leukocyte subsets and the opposing influence on the number of cells in the draining lymph nodes indicated that CD44v3 and CD44v10 may exert distinct modes of action despite the seemingly similar end result. To obtain hints as to the possible mechanisms, we next evaluated the composition of leukocytes and expression of costimulatory molecules in draining lymph node cells and the infiltrate.

[2]Wittig B, Seiter S, Schmidt DS, Zuber M, Neurath M, Zöller M: Selective upregulation of CD44 variant isoforms on peripheral blood leukocytes of patients with chronic inflammatory bowel disease. *Lab Invest*, in press.

Table I. Expression of CD44v3 and CD44v10 on activated murine lymphocytes

Antibody	% Stained cells[ab]	Intensity of staining (peak)[b]
Negative control	2.3 ± 0.2	0.1 ± 0.03
Anti-CD44s	96.5 ± 7.4	242.8 ± 23.1
Anti-CD44v3	19.4 ± 2.2	5.9 ± 0.71
Anti-CD44v10	12.3 ± 1.9	8.2 ± 1.14

[a] Lymph node cells were stimulated *in vitro* by Con A (7.5 μg per ml) for 48 h.
[b] The mean ± SD of five experiments are shown.

Table II. Expression of CD44v3 and CD44v10 on subpopulations of activated leukocytes

	% CD44 variant isoform expressing cells[b]	
Leukocyte subset[a]	CD44v3	CD44v10
$CD4^+$	29.6 ± 3.6	4.8 ± 0.9
$CD8^+$	6.1 ± 1.0	7.2 ± 1.2
$CD11b^+$	28.8 ± 2.6	27.3 ± 2.8
$sIgM^+$	22.6 ± 2.7	11.9 ± 2.1

[a] Draining lymph node cells and PEC (for $CD11b^+$ cells) were collected 5 d after sensitization with DNFB and were separated by panning according to the description in *Materials and Methods*. The purity of the separated populations was in the range of 90%– 95%.
[b] The mean ± SD of five experiments are shown.

Table III. Edema after DNFB sensitization and challenge: influence of anti-CD44

	Ears: mean thickness[b] (mm) (p values)	
Antibody[a]	Root	Tip
Control IgG	3.11 ± 0.32	0.25 ± 0.027
Anti-CD44s	1.18 ± 0.09 (<0.001)	0.12 ± 0.016 (<0.001)
Anti-CD44v3	1.16 ± 0.11 (<0.001)	0.12 ± 0.014 (<0.001)
Anti-CD44v10	1.00 ± 0.14 (<0.001)	0.10 ± 0.011 (<0.001)

[a]Mice were sensitized and challenged with DNFB and received concomitantly with the first sensitization and 3 d thereafter 200 μg antibody, i.v.
[b]Edema formation of the ears was determined by measuring the thickness towards the root and the tip of the ears at 48 h after challenge, mean ± SD of 10 mice/group are shown.

Leukocyte subset composition in the draining lymph node and in the infiltrate in DTH reactions under the influence of anti-CD44v3 and anti-CD44v10 The distribution of $CD4^+$ cells, $CD8^+$ cells, and monocytes in the draining lymph nodes of DNFB sensitized and challenged mice showed only minor differ-

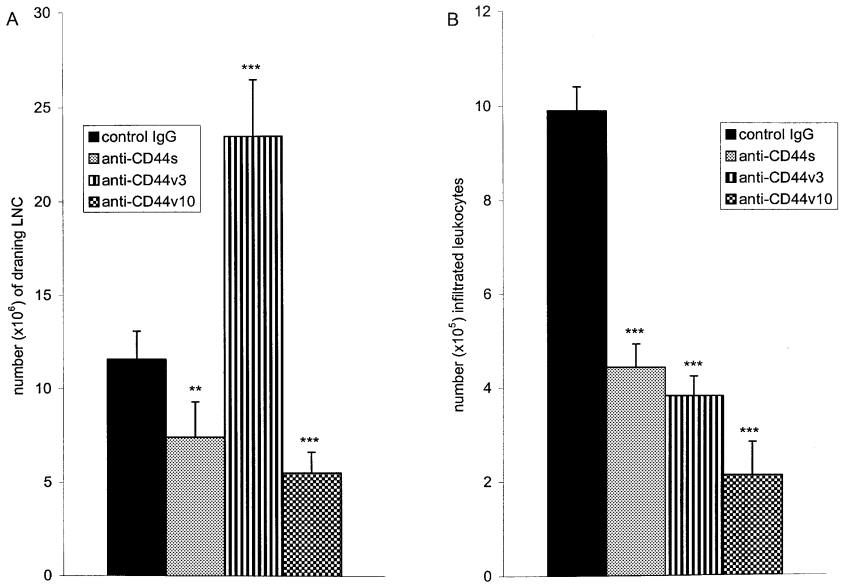

Figure 4. Influence of anti-CD44v3 and anti-CD44v10 on leukocyte expansion and infiltration in DNFB-induced DTH reactions. Mice were sensitized and challenged with DNFB and received concomitantly with the first sensitization and 3 d thereafter an intravenous injection of 200 μg antibody. Mice were killed 2 d after the challenge, the draining lymph nodes were excised and leukocytes infiltrating the ear were isolated as described in *Materials and Methods*. The mean (+SD) number of leukocytes in the draining lymph node (*A*) and the infiltrate (*B*) are shown. Values were derived from 10 mice per group. Significance of differences are indicated by asterisks (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$).

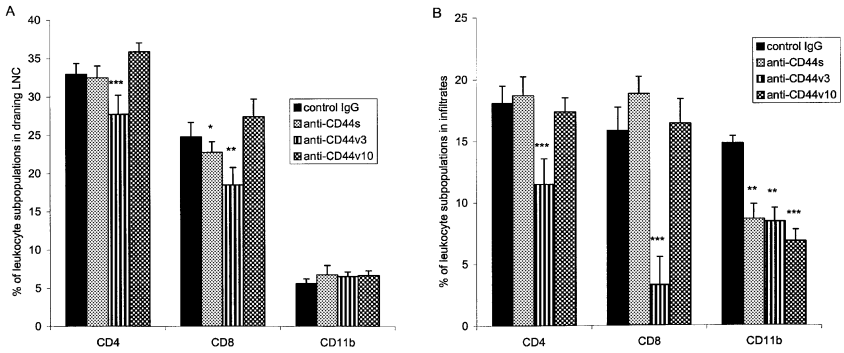

Figure 5. Distribution of leukocyte subpopulations in the draining lymph node and the infiltrate of DNFB sensitized and challenged mice. DTH was induced as described above and leukocytes from the draining lymph node and the infiltrate were isolated 48 h after challenge. Cells were stained with anti-CD4, anti-CD8, and anti-CD11b. The mean (+SD) percentage of stained leukocytes in the draining lymph node (*A*) and the infiltrate (*B*) are shown. Values were derived from 10 mice per group. Significance of differences are indicated by asterisks (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$).

ences in dependence on the application of anti-CD44 antibodies (**Fig 5**). This was different in the infiltrate, where the number of $CD4^+$ and $CD8^+$ cells was strongly reduced in anti-CD44v3-treated mice. In anti-CD44s and anti-CD44v10-treated mice only the relative percentage of monocytes was significantly reduced. None of the three antibodies exerted a major effect on the expression of T cell costimulatory molecules. Yet, expression of the ligands for costimulatory molecules on APC, like CD40, CD80 and CD86, was slightly but consistently reduced in anti-CD44v3-treated mice (data not shown).

In view of the distinct influence of anti-CD44s, anti-CD44v3, and anti-CD44v10 on leukocytes infiltrating the injured organ, it became tempting to speculate that anti-CD44s and anti-CD44v10 may interfere with leukocyte activation and migration/extravasation, whereas anti-CD44v3 seemed to influence particularly leukocyte migration/extravasation. The following experiments were particularly concerned with an elucidation of the latter phenomenon.

Influence of CD44 isoform-specific antibodies on leukocyte activation and leukocyte migration To differentiate between an influence of anti-CD44s, anti-CD44v3, and anti-CD44v10 on leukocyte activation *versus* migration and extravasation, the antibodies were applied either during the sensitization or during the elicitation period and leukocyte expansion. Edema formation and leukocyte infiltration into the injured organ were monitored (**Table IV**). When the antibodies were given only during the sensitization period, expansion of lymphocytes in the draining lymph node was still strongly impaired in anti-CD44s-treated and anti-CD44v10-treated mice. The number of draining lymph node cells recovered from anti-CD44v3-treated mice was increased, although to a minor degree. Edema formation was reduced, but the reduction was less pronounced, particularly in anti-CD44s-treated animals. The number of infiltrated cells also had been reduced, the reduction being less pronounced in anti-CD44v10-treated mice. When the antibodies were applied only at the time of challenge, expansion of lymphocytes in the draining lymph node was hardly affected. In anti-CD44v3-treated and anti-CD44v10-treated mice the reduction in edema formation was as strong as in mice receiving antibody treatment throughout the test period; only anti-CD44s exerted a weaker effect. Considering the number of infiltrated cells, only anti-CD44v10 was less effective when given only during the elucidation phase; the reduction by anti-CD44v3 and anti-CD44s was as strong as when given repeatedly.

These data confirmed our working hypothesis that anti-CD44s and anti-CD44v10 influence both lymphocyte expansion and leukocyte migration/extravasation, anti-CD44s exerting a stronger effect on lymphocyte expansion, anti-CD44v10 interfering more efficiently with leukocyte recruitment/extravasation. Instead, anti-CD44v3 strongly inhibits leukocyte migration/extravasation, only.

Antibody blockade of CD44v3 on endothelial cells hampers leukocyte extravasation We have mentioned above that endothelial cells are stained by anti-CD44v3 and anti-CD44v10. To evaluate whether expression of CD44v3 and CD44v10 on endothelial cells actively contributes to leukocyte extravasation, mice were sensitized with DNFB, lethally irradiated and treated with anti-CD44v3 or anti-CD44v10. Mice were rested for 48 h to allow for excretion/degradation of the bulk of unbound antibody. Thereafter, i.e., at a time the majority of unbound antibody will have left the circulation, the abdominal skin was painted with DNFB and mice received an intravenous injection of ^{51}Cr-labeled draining lymph node cells from DNFB sensitized mice. Circulation and homing of the injected lymphocytes was followed for 48 h.

The majority of lymphocytes had left the circulation after 48 h (**Fig 6*A***). Yet, retention in the circulation was slightly increased

Table IV. Anti-CD44 isoform specific antibodies differentially interfere with leukocyte sensitization and effector functions

Antibody	Sensitization + challenge	Sensitization	Challenge
Influence of anti-CD44 on leukocyte expansion in the draining lymph node No. ($\times 10^6$) of draining lymph node cells[a] (p values) in dependence on antibody application time[b]			
Control IgG	14.0 ± 2.1	14.2 ± 1.3	13.9 ± 1.1
Anti-CD44s	8.1 ± 0.7 (0.014)	9.4 ± 0.9 (<0.001)	13.5 ± 1.2 (ns)
Anti-CD44v3	26.9 ± 2.8 (<0.001)	18.9 ± 1.5 (0.001)	14.3 ± 0.9 (ns)
Anti-CD44v10	7.5 ± 0.9 (<0.001)	8.8 ± 1.5 (<0.001)	11.3 ± 1.1 (0.011)
Influence of anti-CD44 on edema formation ears: mean diameter towards the root[a] (mm) (p values) in dependence on antibody application time[b]			
Control IgG	3.00 ± 0.28	3.00 ± 0.25	3.05 ± 0.27
Anti-CD44s	1.20 ± 0.11 (<0.001)	1.55 ± 0.12 (<0.001)	1.50 ± 0.18 (< 0.001)
Anti-CD44v3	1.15 ± 0.13 (<0.001)	1.22 ± 0.11 (<0.001)	1.15 ± 0.16 (< 0.001)
Anti-CD44v10	1.02 ± 0.12 (<0.001)	1.19 ± 0.12 (<0.001)	1.02 ± 0.18 (< 0.001)
Influence of anti-CD44 on leukocyte extravasation No. ($\times 10^5$) of infiltrated cells[a] (p values) in dependence on antibody application time[b]			
Control IgG	9.9 ± 0.71	10.2 ± 0.77	10.4 ± 0.68
Anti-CD44s	5.5 ± 0.49 (<0.001)	5.9 ± 0.54 (<0.001)	5.0 ± 0.60 (< 0.001)
Anti-CD44v3	4.0 ± 0.46 (<0.001)	4.1 ± 0.46 (<0.001)	4.0 ± 0.42 (< 0.001)
Anti-CD44v10	2.5 ± 0.49 (<0.001)	7.0 ± 0.63 (<0.001)	6.0 ± 0.76 (< 0.001)

[a]Expansion of draining lymph node cells, edema formation and the numbers of infiltrated cells were determined 48 h after challenge, mean ± SD of five mice per group are shown; ns, not significant.

[b]Mice were sensitized and challenged with DNFB and received either concomitantly with the first sensitization or at the time of challenge or at both times an intravenous injection of 200 μg antibody.

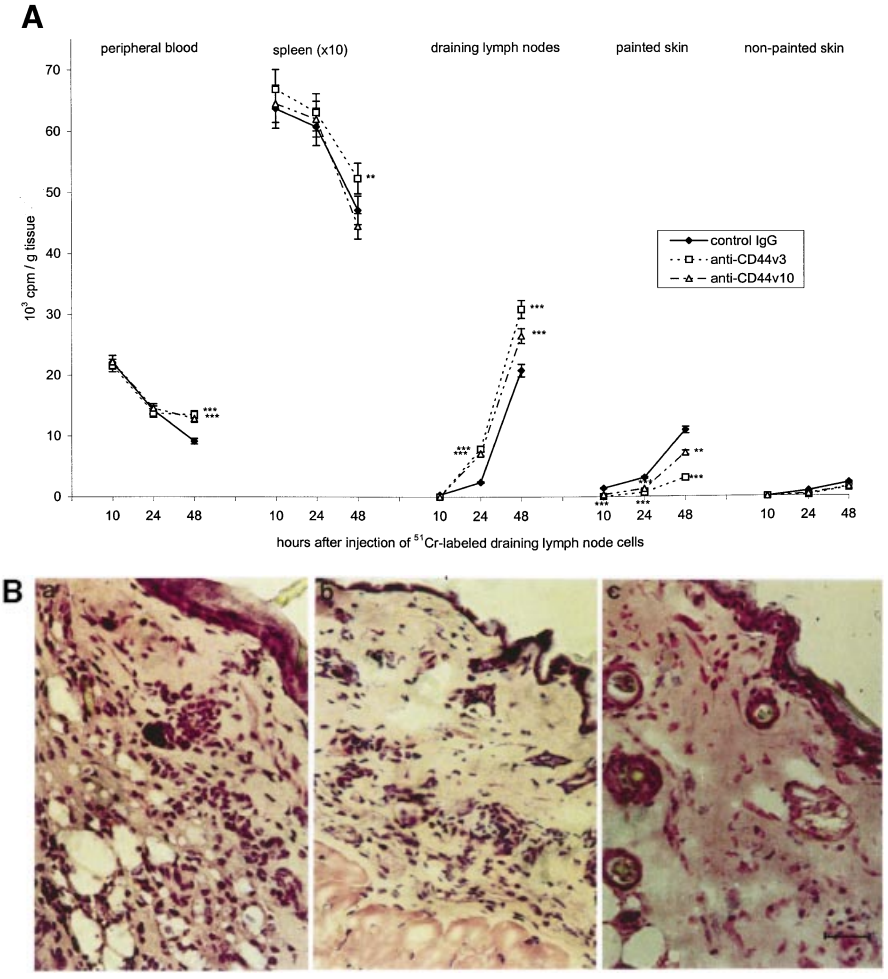

Figure 6. Blockade of CD44v3 on endothelial cells interferes with leukocyte extravasation. DNFB sensitized BALB/C mice were lethally irradiated and received an intravenous injection of control IgG or anti-CD44v3 or anti-CD44v10. Forty-eight hours later they were reconstituted with ^{51}Cr-labeled draining lymph node cells from DNFB-sensitized mice and the abdominal skin was painted with DNFB. Distribution of the injected lymphocytes was followed for 48 h. (*A*) The recovery of lymphocytes in the peripheral blood, bone marrow, spleen, and skin has been evaluated by excision of the organs, weighing and counting in a γ counter. The mean number of counts per g tissue (± SD) are shown. Significance of differences are indicated by asterisks (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$). Values were derived from five mice per group. (*B*) Hematoxylin–eosin-stained frozen sections of the painted skin of sensitized, lethally irradiated, and reconstituted mice (48 h after reconstitution), which received after irradiation and 48 h before reconstitution either a control IgG (*a*), anti-CD44v10 (*b*), or anti-CD44v3 (*c*). The number of infiltrated cells is strongly reduced in anti-CD44v10-treated mice. In anti-CD44v3-treated mice hardly any infiltrating leukocytes could be detected. The experiment was repeated two times (and three times with antibody application 24 h before reconstitution). All repetitions showed comparable results.

in anti-CD44v3-treated and anti-CD44v10-treated animals. Degradation of lymphocytes as revealed by recovery of ^{51}Cr from the liver, the lung, and the kidney was independent of the antibody treatment (data not shown). Notably, in control IgG-treated mice and, although with delay, in anti-CD44v10-treated mice, transferred cells were recovered in the painted area of the skin. In

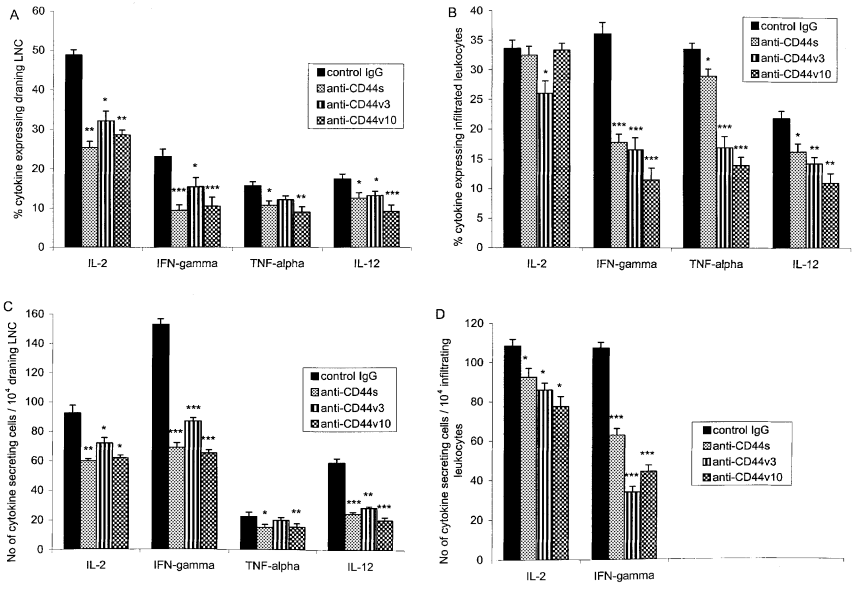

Figure 7. Influence of anti-CD44v3 and anti-CD44v10 on cytokine expression and secretion in a DNFB-induced DTH reaction. DTH was induced as described above and leukocytes from the draining lymph node (*A*, *C*) and the infiltrate (*B*, *D*) were isolated 48 h after challenge. Cells were stained with anti-IL-2, anti-IFN-γ, anti-TNF-α, and anti-IL-12 (*A*, *B*). (*C*, *D*) Cells were pooled from five mice and were seeded (1×10^4 cells per well) in triplicates on plates coated with anti-IL-2, anti-IFN-γ, anti-TNF-α, and anti-IL-12. Cells were cultured for 24 h in the presence of DNP-OVA to allow for cytokine secretion. After lysis of the cells, cytokine secreting cells were visualized by staining with detector antibodies. After 4 h at 37°C for development of the substrate, individual spots were counted. The mean (+SD) percentage of stained leukocytes (*A*, *B*) and the mean number of stained cells (*C*, *D*) are shown. Values were derived from 10 individually tested mice per group (*A*, *B*) or from three individually tested pools (each consisting of five mice) per group (*C*, *D*). Significance of differences are indicated by asterisks (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$).

anti-CD44v3-treated animals, hardly any leukocytes immigrated into the painted skin (**Fig 6*A*, *B***). Yet, a significantly increased proportion of cells was retained in the draining lymph node (**Fig 6*A***).

We have shown above that anti-CD44v3 inhibits most strongly the extravasation of $CD8^+$ cells, which by themselves do not express CD44v3. Thus, the strongly reduced egress of leukocytes in anti-CD44v3-treated mice cannot rely only on an alteration of leukocyte migration, but is quite impressively influenced by the occupancy of CD44v3 on endothelial cells of the dermis.

Influence of anti-CD44v3 and anti-CD44v10 on cytokine production and secretion in a DTH reaction As stated above the distinct influence of anti-CD44s, anti-CD44v3, and anti-CD44v10 on leukocyte expansion has been suggestive for an involvement of the anti-CD44 antibodies not only in leukocyte migration, but also in leukocyte activation. As DNFB induces a T_H1 reaction, the hypothesis was tested by characterizing IL-2 and IFN-γ as well as IL-12 and TNF-α expression, the latter two as representatives of proinflammatory cytokines in T_H1 reactions. Cytokine expression was evaluated by flow cytometry, cytokine secretion by the enzyme-linked immunosorbent spot assay.

All three anti-CD44 antibodies led to a small reduction of IL-2 and a strong reduction of IFN-γ expression in the draining lymph node, anti-CD44v3 being the least effective. A strong reduction in the expression of the proinflammatory cytokines TNF-α and IL-12 was seen only in anti-CD44v10-treated mice (**Fig 7*A***). Cytokine secretion (**Fig 7*C***) roughly corresponded to the pattern of cytokine expression.

A comparison of cytokine expression on cells in the infiltrate *versus* cells in the draining lymph node should provide an additional hint as to the influence of anti-CD44 on lymphocyte activation *versus* migration. Anti-CD44v10 inhibited cytokine expression and secretion in the infiltrate in a similar way as in the draining lymph node. Instead, in anti-CD44s-treated mice IL-2 expression and secretion by infiltrated cells was hardly reduced. Whether this phenomenon relates to the preferential blockade of monocyte extravasation by anti-CD44s will be discussed. In anti-CD44v3-treated mice, on the other hand, the reduction in the number of cytokine expressing and secreting cells was much stronger in the infiltrate than in the draining lymph node (**Fig 7*B***, ***D***). The latter observation is in line with the above-mentioned interpretation that anti-CD44v3 hampers the egress of activated leukocytes.

In summary, anti-CD44s exerted the strongest effect on cells expressing the inflammatory cytokines IL-2 and IFN-γ, anti-CD44v10 more strongly suppressed expression of the proinflammatory cytokines, whereas anti-CD44v3 efficiently inhibited IL-12 and IFN-γ expression. Taking additionally into account that CD44v10 is preferentially expressed on B cells and monocytes, whereas CD44v3 is expressed on $CD4^+$ cells, B cells, and monocytes, it was tempting to speculate that anti-CD44v3 and anti-CD44v10 may interfere with the activation of APC rather than of T cells.

Influence of anti-CD44v3 and anti-CD44v10 on cytokine expression by leukocyte subsets To answer the question whether anti-CD44v3 and anti-CD44v10 indeed block the activation of APC, their influence on cytokine expression by enriched subpopulations of leukocytes was tested. Mice were sensitized with DNFB. Draining lymph node cells were collected and were enriched for leukocyte subsets as described in *Materials and Methods*. Cells were cultured for 48 h in medium containing DNP-OVA and T cell growth factor, the latter being used as an unspecific growth stimulus plus either control IgG or anti-CD44v3 or anti-CD44v10. Cytokine expression (IL-2, IL-12, IFN-γ, and TNF-α) was evaluated by flow cytometry (**Fig 8**).

Anti-CD44v10, indeed, interfered strongly with IL-12, IFN-γ, and TNF-α expression by monocytes, exerting only marginal effects on $CD4^+$ and $CD8^+$ cells as well as B cells. Anti-CD44v3 hardly affected cytokine expression by isolated leukocyte subpopulations, solely IFN-γ expression by $CD8^+$ cells and monocytes and IL-12 expression by $CD4^+$ cells and B cells has been slightly reduced. As the effect of anti-CD44v3 appeared much weaker in the isolated subpopulations than in unseparated leukocytes recovered after *in vivo* sensitization, a second experiment was performed where T cells were cocultured with irradiated B cells or with monocytes. In the latter case only the nonadherent cells were collected for flow cytometry (**Fig 8*B***). The effect of anti-CD44v10 on $CD4^+$ cells cocultured with irradiated B cells or with monocytes roughly resembled the one on the separated $CD4^+$ cells. Anti-CD44v3, however, strongly suppressed cytokine expression by $CD4^+$ cells when cocultured with irradiated B cells or with monocytes.

These data confirm that the major target of anti-CD44v10 are monocytes. The effect of anti-CD44v3 on T_H cells cultured in the presence of APC indicates that CD44v3 is not only required for leukocyte extravasation but also for a T_H–APC interaction, which initiates cytokine production by T_H cells. In line with the finding was the observation of a striking downregulation of CD40, CD80, and CD86 on monocytes cultured in the presence of anti-CD44v3 (data not shown).

DISCUSSION

CD44 isoforms like many adhesion molecules are not only involved in leukocyte adhesion and homing, but take actively part in

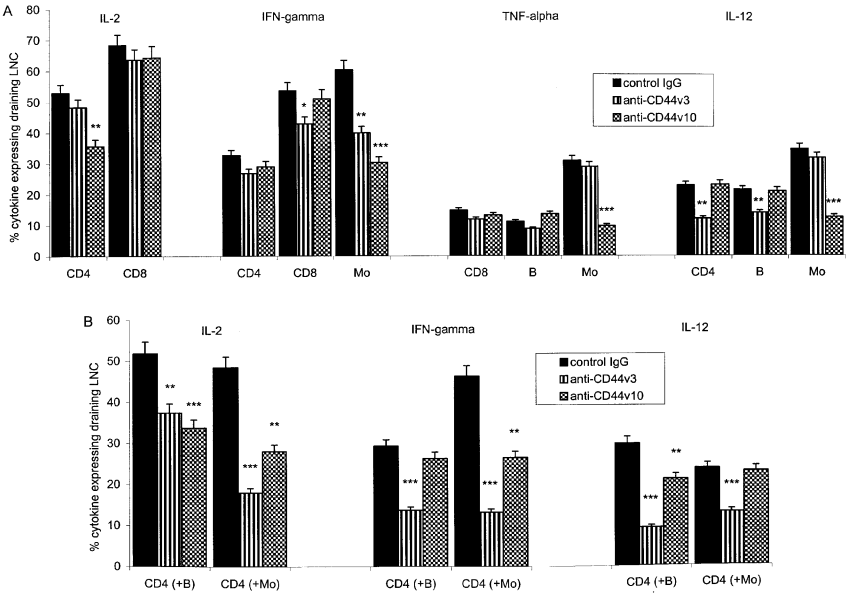

Figure 8. CD44v3 and CD44v10 specific antibodies influence cytokine production by distinct subpopulations of leukocytes. Mice were sensitized with DNFB and were killed after 6 d. PEC were collected and draining lymph node cells were excised. Subpopulations of leukocytes were separated as described in *Materials and Methods* and were cultured in the presence of DNP-OVA, T cell growth factor as an unspecific growth stimulus and control IgG or anti-CD44. As far as $CD4^+$ cells have been cocultured with B cells, the latter have been irradiated; in cocultures of $CD4^+$ cells with monocytes, only the nonadherent cells were collected to assess solely cytokine expression by $CD4^+$ cells. Cells were collected after 48 h and were stained with anti-IL-2, anti-IFN-γ, anti-TNF-α, and anti-IL-12. The mean percentage (+SD) of stained cells in three independently performed experiments is shown. Significance of differences are indicated by asterisks (*$p < 0.1$, **$p < 0.01$, ***$p < 0.001$).

leukocyte activation and regulation of response. This has been known for several years for CD44s (Shimizu *et al*, 1989; Pierres *et al*, 1992; Galandrini *et al*, 1993; Naujokas *et al*, 1993; Funaro *et al*, 1994) and has been suggested for some of the variant isoforms, particularly CD44v6, but also for CD44v3 and recently for CD44v7 and CD44v10 (Arch *et al*, 1992; Koopman *et al*, 1993; Bennett *et al*, 1995; Galluzzo *et al*, 1995; Jackson *et al*, 1995; Levesque *et al*, 1996; Weiss *et al*, 1997; Rösel *et al*, 1998; Wittig *et al*, 1998). Here we demonstrate in a model system the involvement of distinct CD44 isoforms in a pathologic DTH reaction. Furthermore, we provide evidence that CD44v3 and CD44v10 exert distinct functional activities. CD44v10 functions predominantly as a receptor molecule on monocytes, whereas CD44v3 functions as a ligand on APC for an as yet undefined costimulatory molecule on $CD4^+$ cells. In addition, expression of CD44v3 on endothelial cells is of particular importance for leukocyte extravasation.

Ear swelling, but most pronounced, infiltration of lymphocytes in DNFB-induced DTH reactions was impaired when mice were treated with anti-CD44s, anti-CD44v3, or anti-CD44v10, but a state of tolerance could not be induced, i.e., upon re-challenge an unimpaired DTH reaction was observed (data not shown). Interestingly, the inhibitory effect of the three antibodies was comparably strong. This finding was puzzling, because CD44 variant isoforms are expressed at a very low level and on small subpopulations of cells, whereas CD44s is strongly expressed on the majority of lymphocytes and expression is further upregulated during the activation process. As a possible explanation we took into consideration that the antibody blockade may not have functioned exclusively on the level of leukocytes. To support our hypothesis, we first evaluated the influence of anti-CD44 antibodies on leukocyte activation, which included the analysis of expression and secretion of IL-2 and IFN-γ as prototype T_H1 cytokines (Shanley *et al*, 1995) and of IL-12 and TNF-α as prototype T_H1 proinflammatory cytokines (Trinchieri, 1995), the latter being known to be of critical importance for provoking clinical manifestations after exposure to contact allergens (Grabbe and Schwarz, 1996). Second, we examined whether CD44 isoform-specific antibodies interfere with leukocyte extravasation and whether such an interference is due to a blockade of activated leukocytes or activated endothelial cells.

In the described model of a DNFB-induced DTH reaction, treatment with anti-CD44s affected both leukocyte activation and the elucidation phase with edema formation and leukocyte infiltration. Accordingly, the DTH reaction was mitigated, although to a lesser degree, when the antibody was given either during the sensitization or the exacerbation period. With respect to lymphocyte activation, anti-CD44s most strongly affected expression of IL-2 and IFN-γ. The finding could well be explained by a blockade of T_H1 activation. Yet, it remains to be explored whether this results from a blockade of CD44s functioning as a ligand or as a costimulatory molecule, both features have been ascribed to CD44s: Cross-linking of CD44s on T cells provides a costimulatory signal (Gruber *et al*, 1992; Pierres *et al*, 1992). A CD40–CD40L interaction initiates a rapid upregulation of CD44 on APC, which by binding to a so far unknown ligand on T cells provides a strong stimulus for proliferation (Guo *et al*, 1996).

DeGrendele *et al* (1997) have described that blockade of the CD44s isoform interferes not only with lymphocyte activation but also with leukocyte emigration, particularly of monocytes. Our study and reports by other groups (Camp *et al*, 1993; Weiss *et al*, 1997) confirm this inasmuch as anti-CD44s inhibited edema formation and leukocyte infiltration even when applied only during the effector phase.

If anti-CD44s functions during sensitization and elucidation, how do we explain that anti-CD44s had less effect on IL-2 expression in infiltrated cells than in draining lymph node cells? In line with the study by deGrendele *et al* (1997), we noted that in anti-CD44s-treated mice the percentage of monocytes in the infiltrate was significantly reduced. Therefore we suggest that, although fewer T cells will become activated in the presence of anti-CD44s, the egress of T cells may not be influenced significantly and, as a consequence, the percentage of activated, IL-2 expressing T cells in the infiltrate will not be significantly reduced in anti-CD44s-treated mice.

Thus, in the T_H1-mediated DTH model anti-CD44s blocked T cell activation and, preferentially, monocyte extravasation. Notably, there has been no evidence for a linkage of the two processes.

Anti-CD44v10, too, has been shown to interfere strongly with T_H1-mediated DTH reactions (Rösel *et al*, 1997). The presented data extend this earlier study inasmuch as they demonstrate that anti-CD44v10 suppressed most strongly TNF-α, IL-12, and IFN-γ expression and secretion. The analysis of cytokine expression by subpopulations of leukocytes revealed that the blockade of cytokine production by anti-CD44v10 was clearly restricted to APC. Furthermore, in anti-CD44v10-treated, like in anti-CD44s-treated mice, the infiltrate contained a significantly reduced percentage of monocytes. Different to anti-CD44s, cytokine expression was blocked in the draining lymph node as well as in the infiltrate of anti-CD44v10-treated animals. We interpret these findings in the sense that anti-CD44v10 directly inhibits the activation of monocytes in the draining lymph node, where they may function as APC as well as in the infiltrate during the elucidation phase of

the response. Considering the latter aspect, we want to mention that anti-CD44 has been described to interfere with TNF release (Zembala *et al*, 1994) and to block nitric oxide release by activated monocytes (DelPozo *et al*, 1995). Although these studies were not concerned about distinct CD44 isoforms, according to our study it is tempting to speculate, although not yet proved, that CD44v10 has been involved. Irrespective of this open question, we clearly could demonstrate that mitigation of a DTH reaction by anti-CD44v10 proceeds quite exclusively via a blockade of the monocytes. It may be added that sensitization with DNFB induces a rather severe DTH reaction with fever and weight loss. As compared with anti-CD44s and anti-CD44v3, anti-CD44v10 had the strongest effect on improving the overall physical conditions of the DNFB-treated mice.

Anti-CD44s and anti-CD44v10 are known to mitigate a DTH reaction and have been included in the study as "positive controls", but also to explore whether they block alike or distinct activities of elements of the immune system. Yet, the prime object of our study was the definition of possible functions of CD44v3. The interest in CD44v3 derived from the observation that dermal infiltrates of patients with autoimmune and allergic skin reactions contain $CD44v3^+$ cells, but neither $CD44v6^+$ nor $CD44v7^+$, and rarely $CD44v10^+$ cells (Seiter *et al*, 1998). Furthermore, in humans and mice, vessel endothelium of the dermis can be $CD44v3^+$.

The most striking effects of anti-CD44v3 were (i) a strongly reduced number of infiltrated cells, and (ii) an overshooting expansion of draining lymph node cells. We have no explanation for the latter phenomenon. Yet, it should be mentioned that the molecule has been described to function as receptor for basic fibroblast growth factor and for heparin-binding growth factor (Bennett *et al*, 1995; Jackson *et al*, 1995) and possibly for MIP-1β (Tanaka *et al*, 1993). Furthermore, although the influence of anti-CD44v3 on cytokine production by leukocyte subpopulations was minor, the antibody strongly interfered with cytokine production by $CD4^+$ cells cultured together with irradiated B cells or monocytes. Thus, CD44v3 does not only function as a receptor for cytokines, but CD44v3–CD44v3 ligand binding plays an essential part in triggering cytokine production by $CD4^+$ cells. Whether this in turn supports clonal expansion of T cells remains to be explored. We are currently establishing a CD44v3 receptor globulin to identify the ligand for CD44v3 and to elucidate by which mechanism CD44v3–CD44v3 ligand interaction initiates cytokine production.

Considering the strongly reduced number of cells in the infiltrate of anti-CD44v3-treated mice, the fact that activated vessel endothelium strongly expressed CD44v3 has to be taken into account. Thus, and despite the fact that anti-CD44v3 exerted some effect on leukocyte activation/cytokine expression, we presume that anti-CD44v3 interfered with the DTH reaction predominantly by a blockade of leukocyte extravasation. In line with this interpretation are the findings that (i) application of anti-CD44v3 during the effector phase sufficed for a strongly impaired extravasation of leukocytes, and that (ii) extravasation of leukocytes could not be detected in the anti-CD44v3 pretreated, lethally irradiated and reconstituted host. According to the latter aspect it could well be that mitigation of a DTH reaction by anti-CD44v3 is, at least partly, due to the blockade of CD44v3 rather on endothelial cells than on leukocytes. The finding that anti-CD44v3 inhibits most strongly the egress of $CD8^+$ cells, which rarely are $CD44v3^+$, strengthens our interpretation.

In conclusion, CD44s, CD44v3, and CD44v10 isoform-specific antibodies interfere with T_H1-mediated DTH reactions. The seemingly similar effect is based on different functional activities. Anti-CD44s interferes with T cell activation and with the egress preferentially of monocytes. Anti-CD44v10 predominantly prevents the activation of monocytes. Anti-CD44v3 hampers an interaction between APC and $CD4^+$ T cells with the consequence of an impaired T_H cell activation. Yet, the dominating effect of anti-CD44v3 is the strong inhibition of the extravasation of activated leukocytes, which likely includes a blockade of CD44v3 on the vessel endothelium. Knowledge of these distinct activities will be important for any consideration of therapeutic interference.

This work was supported by the Deutsche Forschungsgemeinschaft, grant no. Zo40-5/3. We thank Drs. G.Adolf and E.Patzelt, Bender and Co. GMBH, Vienna, for the kind provision of the human CD44 variant isoform-specific antibodies.

REFERENCES

Arch R, Wirth K, Hofmann M, *et al*: Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science* 257:682–685, 1992

Bennett KL, Jackson DG, Simon JC, *et al*: CD44 isoforms containing exon v3 are responsible for the presentation of heparin-binding growth factor. *J Cell Biol* 128:687–698, 1995

Brennan FR, Mikecz K, Glant TT, *et al*: CD44 expression by leukocytes in rheumatoid arthritis and modulation by specific antibody: implications for lymphocyte adhesion to endothelial cells and synoviocytes in vitro. *Scand J Immunol* 45:213–220, 1997

Camp RL, Scheynius A, Johansson C, Pure E: CD44 is necessary for optimal contact allergic responses but is not required for normal leukocyte extravasation. *J Exp Med* 178:497–507, 1993

Cattoretti G, Berti E, Schirren R, D'Amato L, Valeggio C, Rilke F: Improved avidin-biotin-peroxidase complex (ABC) staining. *Histochem J* 20:5–80, 1988

DeGrendele HC, Estess P, Siegelman MH: Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 278:672–675, 1997

DelPozo MA, Sanchez-Mateos P, Nieto M, Sanchez-Madrid F: Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. *J Cell Biol* 131:495–508, 1995

Funaro A, Spagnoli GC, Momo M, Knapp W, Malavasi F: Stimulation of T cells via CD44 requires LFA–1 interactions and IL-2 production. *Hum Immunol* 40:267–278, 1994

Galandrini R, Albi N, Tripodi G, *et al*: Antibodies to CD44 trigger effector functions of human T cell clones. *J Immunol* 150:4225–4231, 1993

Galluzzo E, Albi N, Fiorruci S, *et al*: Involvement of CD44 variant isoforms in hyaluronate adhesion by human activated T cells. *Eur J Immunol* 25:2932–2938, 1995

Grabbe S, Schwarz T: Immunoregulatory mechanisms involved in elucidation of allergic contact hypersensitivity. *Am J Contact Dermat* 7:238–246, 1996

Gruber MF, Webb DS, Gerrard TL: Stimulation of human monocytes via CD45ev, CD44 and LFA–3 triggers macrophage–colony–stimulating factor production. Synergism with lipopolysaccharide and IL–1 beta. *J Immunol* 15:1113–1118, 1992

Guo Y, Wu Y, Shinde S, Sy MS, Aruffo A, Liu Y: Identification of a costimulatory molecule rapidly induced by CD40L as CD44H. *Exp Med* 184:955–961, 1996

Heider KH, Hofmann M, Horst E, van den Berg F, Ponta H, Herrlich P, Pals ST: A human homologue of the rat metastasis-associated variant of CD44 is expressed in colo-rectal carcinomas and adenomatous polyps. *J Cell Biol* 120:227–233, 1993

Jackson DG, Bell JI, Dickinson R, Timans J, Shields J, Whittle N: Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. *J Cell Biol* 128:673–685, 1995

Jalkanen S, Bargatze RF, de los Toyos J, Butcher EC. A lymphoid cell surface protein involved in endothelial cell recognition and lymphocyte homing in man. *Eur J Immunol* 16:1195–1202, 1986

Kincade PW, He Q, Ishihara K, Miyake K, Lesley J, Hyman R: CD44 and other cell interaction molecules contributing to B lymphopoiesis. *Curr Top Microbiol Immunol* 184:215–222, 1993

Koopman G, Heider KH, Horst E, *et al*: Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J Exp Med* 177:897–904, 1993

Lesley J, Hyman R, Kincade PW: CD44 and its interaction with extracellular matrix. *Adv Immunol* 54:271–335, 1993

Levesque MC, Haynes BF: In vitro culture of human peripheral blood monocytes induces hyaluronan binding and up-regulates monocyte variant CD44 isoform expression. *J Immunol* 156:1557–1565, 1996

Mikecz K, Brennan FR, Kim JH, Glant TT: The role of adhesion molecules in the development of autoimmune arthritis. *Scand J Rheumatol Suppl* 101:99–106, 1995

Naujokas MF, Morin M, Anderson MS, Peterson M, Miller J: The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell* 74:257–268, 1993

Noble PW, Lake FR, Henson PM, Riches DW: Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor-alpha-dependent mechanism in murine macrophages. *J Clin Invest* 81:2368–2377, 1993

Pierres A, Lipcey C, Mawas C, Olive D: A unique CD44 monoclonal antibody identifies a new T cell activation pathway. *Eur J Immunol* 22:413–417, 1992

Rösel M, Seiter S, Zöller M: CD44v10 expression in the mouse and functional activity in delayed type hypersensitivity. *J Cell Physiol* 171:305–317, 1997

Rösel M, Föger N, Zöller M: Involvement of CD44 exon v10 in B cell activation. *Tissue Antigens* 52:99–113, 1998

Screaton GR, Bell MV, Bell JI, Jackson DG: The identification of a new alternative exon with highly restricted tissue expression in transcripts encoding the mouse

Pgp-1 (CD44) homing receptor. Comparison of all 10 variable exons between mouse, human, and rat. *J Biol Chem* 268:12235–12238, 1993

Seiter S, Schadendorf D, Tilgen W, Zöller M: CD44 variant isoform expression in a variety of skin-associated autoimmune diseases. *Clin Immunol Immunopathol* 89:79–93, 1998

Shanley TP, Warner RL, Ward PA: The role of cytokines and adhesion molecules in the development of inflammatory injury. *Mol Med Today* 1:40–45, 1995

Shimizu Y, van Seventer GA, Siraganian R, Wahl L, Shaw S: Dual role of the CD44 molecule in T-cell adhesion and activation. *J Immunol* 143:2457–2463, 1989

Siczkowski M, Andrew T, Amos S, Gordon MY: Hyaluronic acid regulates the function and distribution of sulfated glycosaminoglycans in bone marrow stromal cultures. *Exp Hematol* 21:126–130, 1993

Sugimoto K, Tsurumaki Y, Hoshi H, *et al*: Effects of anti-CD44 monoclonal antibody on adhesion of erythroid leukemic cells (ELM-l-1) to hematopoietic supportive cells (MS-5): CD44, but not hyaluronate-mediated cell-cell adhesion. *Exp Hematol* 22:488–494, 1994

Taher EI, Taher LS, Griffioen AW, Schilder-Tol EJM, Borst J, Pals ST: Signaling through CD44 is mediated by tyrosine kinases. *J Biol Chem* 271:2863–2867, 1996

Tanaka AS, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S: T cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. *Nature* 361:79–82, 1993

Tang A, Judge TA, Nickoloff BJ, Turka LA: Suppression of murine allergic contact dermatitis by CTLA4Ig. Tolerance induction of Th2 responses requires additional blockade of CD40-ligand. *J Immunol* 157:117–125, 1996

Toyama-Sorimachi N, Sorimachi H, Tobita Y, *et al*: A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. *J Biol Chem* 270:7437–7444, 1995

Trinchieri G: Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 13:251–276, 1995

Verdrengh M, Holmdahl R, Tarkowski A: Administration of antibodies to hyaluronan receptor (CD44) delays the start and ameliorates the severity of collagen arthritis. *Scand J Immunol* 42:353–358, 1995

Webb DS, Shimizu Y, van Seventer GA, Shaw S, Gerrard TL: CD44 and CD45: physiological triggers of human monocyte TNF and IL-1 release. *Science* 14:1295–1297, 1990

Weber GF, Ashkar S, Glimcher MJ, Cantor H: Receptor–ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271:509–512, 1996

Weiss JM, Sleeman J, Renkl AC, *et al*: An essential role for CD44 variant isoforms in epidermal Langerhans cell and blood dendritic cell function. *J Cell Biol* 137:1137–1147, 1997

Wittig B, Schwärzler C, Föhr N, Günthert U, Zöller M: Curative treatment of an experimentally induced colitis by a CD44 variant exon v7 specific antibody. *J Immunol* 161:1069–1073, 1998

Wu L, Kincade PW, Shortman K: The CD44 expressed on the earliest intrathymic precursor population functions as a thymus homing molecule but does not bind to hyaluronate. *Immunol Lett* 38:69–75, 1993

Wysocki LJ, Sato VL: Panning for lymphocytes: a method for cell selection. *Proc Natl Acad Sci USA* 75:2844–2848, 1978

Zembala M, Siedlar M, Ruggiero I, Wieckiewicz J, Mytar B, Mattei M, Colizzi V: The MHC class II and CD44 molecules are involved in the induction of tumor necrosis factor (TNF) gene expression by human monocytes stimulate with tumor cells. *Int J Cancer* 56:269–274, 1994