CD44 Is Necessary for Optimal Contact Allergic Responses but Is Not Required for Normal Leukocyte Extravasation

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Summary

The in vivo administration of certain monoclonal antibodies (mAbs) against the adhesion receptor, CD44, into normal mice induces both a modulation of CD44 from the surface of peripheral lymphocytes, and a concomitant increase in the amount of soluble CD44 in the serum. CD44-negative lymphocytes isolated from anti-CD44-treated mice exhibit normal homing patterns upon adoptive transfer, and are capable of reexpressing CD44 upon activation. The treatment of hapten-sensitized mice with anti-CD44 mAb inhibits their ability to mount a cutaneous delayed-type hypersensitivity (DTH) response within the first 24 h after hapten challenge. This inhibition reflects a block in both the edema and leukocyte infiltration of the cutaneous site of DTH, whereas the extravasation and accumulation of leukocytes in the draining lymph nodes progress normally. After 72 h, the leukocytes that extravasate into the site of antigen challenge express CD44. These results indicate that CD44 is not necessary for normal leukocyte circulation but is required for leukocyte extravasation into an inflammatory site involving nonlymphoid tissue.

CD44 (pgp-1) is a type I transmembrane glycoprotein found on the surface of most leukocytes, fibroblasts, keratinocytes, and epithelial cells. The principal, known ligand for CD44 is the glycosaminoglycan, hyaluronic acid (HA),[1] a major constituent of extracellular matrices (1–4). CD44 is thought to play a role in several processes critical to normal immune system development and function, including leukocyte extravasation, myelo- and leukopoiesis, and leukocyte activation (5–7). In addition, recent evidence has demonstrated that CD44 and its alternatively spliced isoforms (CD44R) endow some tumor cells with enhanced metastatic proclivity (8–15). Many of these functions may be mediated by interactions between CD44 and HA (1, 16); however, other counter-receptors for CD44 may exist, for example on endothelial cells (17, 18).

Several lines of in vitro evidence have suggested that CD44 promotes the binding of lymphocytes to specialized or high endothelial venules (HEVs), which support the extravasation of circulating lymphocytes from the blood into lymphoid organs (5, 9, 16). However, efforts to confirm the importance of CD44 in lymphocyte extravasation in experimental animals, or to define novel roles for CD44, have been hampered by the lack of anti-CD44 mAbs that block specific functions. Miyake et al. (1) have described an anti-murine CD44 mAb that inhibits the interaction between CD44 and HA; however, this antibody fails to block the binding of lymphocytes to HEVs (2). CD44-deficient mice have yet to be developed through genetic manipulation.

To study the role of CD44 in the periphery of mature animals, we injected hapten-sensitized mice with various CD44-specific mAbs, and analyzed their capacity to mount a delayed-type hypersensitivity (DTH) reaction. During the course of these experiments we discovered that the in vivo administration of certain mAbs to CD44 results in the complete modulation of CD44 from the surface of lymphocytes in the peripheral lymph nodes (PLN) and blood, and a substantial decrease in CD44 expression from lymphocytes in the spleen and mesenteric lymph nodes. In contrast to existing evidence from in vitro experiments, these CD44-negative cells are equally as adept at entering both peripheral and mucosal lymphoid organs upon adoptive transfer as CD44-positive cells from control mice. However, CD44-deficient mice exhibit a marked reduction in the inflammation associated with the early stages of a cutaneous DTH response. In parallel experiments, mAbs specific for LFA-1 inhibited normal lymphocyte homing and ablated both the early and late phases of the cutaneous DTH response (19). Unlike anti-LFA-1 treat-

[1] *Abbreviations used in this paper:* DNFB, 2,4-dinitro-1-fluorobenzene; DTH, delayed-type hypersensitivity; ECM, extracellular matrix; HA, hyaluronic acid; HEV, high endothelial venule; PLN, peripheral lymph node.

ment, anti-CD44 treatment does not reduce the number of cells found in the lymph nodes draining DTH sites. These results clearly distinguish the functions of CD44 and LFA-1 in leukocyte trafficking, and suggest that CD44 may be important in mediating the extravasation of leukocytes into inflamed nonlymphoid tissue, but that it is not critical for normal leukocyte migration into lymphoid organs.

Materials and Methods

Production and Purification of mAbs. The following anti-mouse mAbs were used in this study: 2C11 (CD3, hamster) (20), obtained from American Type Culture Collection (ATCC; Bethesda, MD); IM7 (CD44, rat IgG2b) (21), a generous gift of I. Trowbridge (Salk Institute, La Jolla, CA); KM201 (CD44, rat IgG1) (7), a generous gift of P. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK); IRAWB14.4 (CD44, rat IgG2a) (22), a generous gift of J. Lesley (Salk Institute); 23G2 ($CD45R_B$, rat IgG2a) (23), produced by M. Birkeland and E. Puré (The Rockefeller University, New York, NY); YN-1/1.7.4 (ICAM-I [CD54], rat IgG2b) (24), obtained from ATCC; Mel-14 (L-selectin, rat IgG2a) (25); FD441.8 (LFA-1 [CD11a/CD18], rat IgG2b) (26), obtained from ATCC; M1/70 (MAC-1 [CD11b/CD18], rat IgG2b) (27), obtained from ATCC.

Hybridoma cells were expanded in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Hazelton Biologics, Lenexa, KS), antibiotics, antimycotic, and L-glutamine. Cells in log-phase growth were washed free of FCS and grown to maximal density in serum-free medium containing Nutridoma-SP® (Boehringer Mannheim Biochemicals, Indianapolis, IN). Antibody was purified by precipitation with ammonium sulfate (50% saturation), dialyzed against PBS, and filter sterilized.

Animals and mAb Administration Protocols. 8-20-wk-old female CD_2F_1 (BALB/c × DBA/2)F_1 mice were purchased from The Trudeau Institute (Saranac Lake, NY). Mice were injected intraperitoneally with various concentrations of mAb or normal rat IgG (Jackson ImmunoResearch, West Grove, PA) diluted in isotonic saline to 1 mg/ml.

In Vivo Cell Migration Assay. Peripheral lymph node cells were removed from mice treated (intraperitoneally) for 12–24 h with the anti-CD44 mAb IRAWB14 (300 μg), the anti-LFA-1 mAb FD441.8 (200 μg), or normal rat IgG (300 μg). 10^7 cells were suspended in 1 ml HBSS containing 5 μg ^{111}indium oxyquinoline (Amersham Clinical Products, Arlington Heights, IL) for 30 min at ~25°C. Cells were washed twice with complete medium containing 20% FCS, twice with medium with 10% FCS, and once with isotonic saline. Cells (0.1–0.3 cpm/cell) were resuspended at 10^7/ml, and 300 μl (3 × 10^6 cells) was injected into the tail veins of normal mice. 1–18 h later, mice were anesthetized by exposure to methoxyflurane (Metofane®; Pitman-Moore, Mundelein, IL), and peripheral blood was recovered by retro-orbital bleeding before death. Organs were removed, weighed, and assayed for their incorporation of radiolabeled cells using an auto-gamma scintillation spectrometer (5220; Packard Instrument Co., Inc., Downers Grove, IL).

Generation and Evaluation of DTH Responses. 20 μl of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) (Sigma Chemical Co., St. Louis, MO) in 4:1 acetone:olive oil (Sigma Chemical Co.) was painted on the rear footpads of mice for a successive 2 d. 4 d later, mice were challenged by applying 10 μl of 0.2% DNFB (in the same vehicle) to each side of each ear. In each experiment, unsensitized mice, painted only with the vehicle on their footpads at the time of sensitization, and later challenged with DNFB on the ears, were included as controls. Ear thickness was measured before challenge and at 24, 48, and 72 h postchallenge, using an engineer's micrometer (Mitutoyo Co. Mfg. Ltd., Tokyo, Japan). Each ear was measured twice, for a total of four measurements per animal.

Isolation of Leukocytes. Cells were isolated from lymph nodes and spleens by teasing, and the resulting cell suspensions were washed, counted, and analyzed as described. Splenocytes were depleted of RBC by incubating them for 2 min in a solution of 10 mM Tris/HCl, pH 7.5, 0.17 M NH_4Cl at ~25°C. Peripheral blood leukocytes were partially purified from heparinized blood by adding one-half volume 2% dextran T500 (Pharmacia Fine Chemicals, Piscataway, NJ) in PBS, and allowing RBC to agglutinate and settle for 30 min. The RBC-depleted cell suspension was centrifuged and the resulting pellet treated with RBC lysis buffer as above.

In Vitro Stimulation of Lymphocytes. Partially purified cell suspensions from PLN were incubated in complete medium for 12 h at a density of 4 × 10^6 cells/ml, in the presence or absence of 100 ng/ml phorbol dibutyrate and the anti-CD3 mAb, 2C11 (25% [vol/vol] culture supernatant). Where indicated, cells were additionally treated with 0.1 μg/ml actinomycin-D (Sigma Chemical Co.) or 2.5 μg/ml cycloheximide (Sigma Chemical Co.).

Fluorescence Flow Cytometry. For unconjugated antibodies, cells were incubated for 30 min with 5–10 μg/ml purified antibody or culture supernatant (30% [vol/vol]) diluted in PBS containing 1 mg/ml BSA and 0.02% sodium azide. Cells were then stained with $F(ab')_2$ FITC-conjugated mouse anti-rat IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA), washed, fixed with 3.7% formaldehyde, and analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). For double labeling, cells were incubated with PE-conjugated antibodies against CD4 or IgD, in combination with FITC-conjugated antibodies against CD44 (IM7) or CD8. Cells were fixed and analyzed as above. To distinguish viable from nonviable cells, unfixed cells were treated with 5 μg/ml ethidium bromide immediately before analysis.

Radioimmunoassay. Flexible microtiter plates were coated with the anti-CD44 mAb, KM201 (20 μg/ml) for 3 h at room temperature (RT). Plates were washed with H_2O and quenched with PBS containing 0.2% BSA (PBS/BSA) for 1 h at RT. Serum was prepared from the clotted, nonheparinized blood of mice previously treated for 12 h with normal rat IgG, an anti-LFA-1 mAb (FD441.8), or anti-CD44 mAbs (IRAWB14 and IM7). Serum samples were clarified by centrifugation, serially diluted in triplicate in PBS/BSA, and added to the plates for 2 h at RT. Plates were washed with H_2O, and 10^6 cpm/ml of ^{125}I-labeled IM7 (anti-CD44) (sp act, ~1.1 × 10^7 cpm/μg) was added for 1.5 h at RT. Plates were washed, dried, and individual wells were cut and counted separately using an auto-gamma scintillation spectrometer (5220; Packard Instrument Co., Inc.).

Immunohisto- and Immunocytochemical Staining. Tissue specimens were kept in RPMI or Histocon® (HistoLab, Gothenburg, Sweden) at 4°C (for <8 h) until they were snap-frozen in chilled isopentane and stored at −80°C. Acetone-fixed, 6-μm-thick cryostat sections or cytospin preparations of lymph node cells were incubated in 0.3% H_2O_2 in PBS for 15 min at RT to block endogenous peroxidase, and then reacted with normal rabbit serum (diluted 1:10) for 10 min to reduce nonspecific staining. Binding of rat mAbs was visualized with biotinylated rabbit anti-rat IgG (2.5 μg/ml) and avidin-biotin-peroxidase complexes (Vectastain ABC® kit; Vector Laboratories, Inc., Burlingame, CA). The peroxidase reaction was developed with 3',3' diaminobenzidine tetrahydrochloride (Polysciences, Inc., Warrington, PA). The sections and cells were counterstained

with Mayer's hematoxylin and mounted in Kaiser's glycerin-gelatin (Merck, Darmstadt, Germany). The optimal dilutions of the antibodies were determined by using sections from normal spleen, lymph nodes, and skin. Each ear specimen was also processed for hematoxylin and eosin staining.

Results

Anti-CD44 mAbs Induce the Modulation of CD44 from Lymphocytes In Vivo. The intraperitoneal injection of the anti-CD44 mAbs IRAWB14 or IM7 induces a striking reduction in the amount of cell surface CD44 expressed on lymphocytes in the periphery as determined by fluorescence-activated flow cytometry and immunohistochemistry. This reduction is obvious at 5 h postinjection, and maximal at 12–24 h (Fig. 1). Cells isolated from PLN bear no detectable CD44 on their surfaces by 12 h postinjection. Mesenteric lymph node cells exhibit nearly complete modulation, whereas splenocytes retain ~50% of their CD44 (Fig. 1). The overall number of cells as well as the percentage of IgD^+ B cells, and $CD4^+$ and $CD8^+$ T cells, in peripheral lymph nodes are not affected by anti-CD44 mAb treatment (Table 1, and data not shown). Peripheral blood B and T cells are also CD44 negative, and the number of circulating leukocytes was similar in antibody-treated and control animals (Fig. 2, and data not shown). The varying extent of CD44 modulation in different lymphoid organs may reflect distinctions in the subpopulations of lymphocytes residing in each. Alternatively, there may be a difference in the efficiency of mAb delivery to different lymphoid organs after intraperitoneal administration. In view of this distinction, all adoptive transfer experiments in this study were carried out with PLN cells. In mice treated with isotype-matched mAbs to two other adhesion molecules, LFA-1 and ICAM-1, the antibodies remain associated with the surface of LFA-1-positive leukocytes and ICAM-1-positive leukocytes and endothelial cells, respectively (19), suggesting that the downregulation of CD44 expression occurs by a specific mechanism.

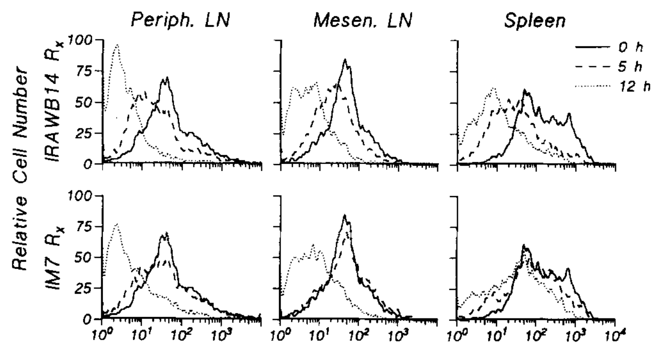

Figure 1. The in vivo administration of anti-CD44 mAbs modulates CD44 from the surface of lymphocytes. Cells were isolated from the peripheral and mesenteric lymph nodes and spleens of normal mice (0 h) or normal mice injected (300 μg i.p.) with the anti-CD44 mAbs IRAWB14 or IM7 (5 and 12 h). Cells were then stained with the anti-CD44 mAb KM201, visualized with a FITC-conjugated secondary antibody, and analyzed by fluorescence flow cytometry.

We analyzed the cell surface phenotype of CD44-negative lymphocytes from anti-CD44-treated mice using a panel of mAbs against a variety of surface antigens, including adhesion receptors and activation markers (Fig. 3). The removal of CD44 from the surface of lymphocytes does not result in the comodulation of the other cell surface receptors analyzed (LFA-1, ICAM-1, L-selectin, CD4, CD8, and IgD), nor does the activation state of these lymphocytes appear to

Table 1. *Anti-CD44 mAb Treatment Does Not Reduce the Number of Cells in Peripheral Lymph Nodes*

Exp.	Treatment	Antibody	Dose	Cells/PLN
			μg	× 10^6
A	Normal, unsensitized	Normal rat IgG	300*	2.1
		IRAWB14	300	2.0
		FD441.8	200	1.3
B	DNFB unsensitized, challenged	–	–	2.2 ± 0.7
	DNFB sensitized and challenged	Normal rat IgG	400‡	6.7 ± 0.6
		IRAWB14	400	6.2 ± 0.8
		IM7	400	6.8 ± 0.4

Leukocytes were removed from the PLN of normal mice, or the draining lymph nodes of DNFB-challenged mice (unprimed or DNFB sensitized). In exp. A, lymph nodes were removed 24 h after antibody injection, whereas in exp. B they were harvested 24 h after DNFB challenge on the ears (see Fig. 8). Lymph nodes were placed in culture medium on ice and gently teased. The resulting cells were counted using a hemocytometer and checked for viability by trypan blue exclusion. In all cases viability was >75%. Results shown are from typical experiments and are expressed as the number of cells per lymph node based either on cell counts from a combined pool of 30–36 lymph nodes from five to six mice in each group (exp. A), or cell counts from four individual draining lymph nodes isolated from two mice in each group (exp. B).

* Administered 12 h before death.

‡ Administered 20 h (300 μg) and 1 h (100 μg) before challenge (see Fig. 8).

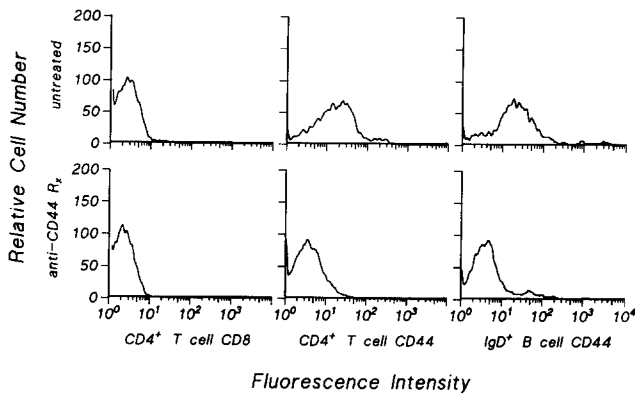

Figure 2. Treatment with anti-CD44 mAb modulates CD44 from the surface of peripheral blood B and T cells. Blood was removed from saline-treated (*untreated*) mice or mice treated for 12 h with 300 μg anti-CD44 mAb IRAWB14 (*anti-CD44 R_x*). Enriched suspensions of leukocytes were prepared according to Materials and Methods. Cells were double labeled with a combination of PE-conjugated anti-CD4 or anti-IgD and FITC-conjugated anti-CD8 or anti-CD44 (IM7). Cells were gated for CD4+ T cells or IgD+ B cells, and analyzed for their expression of CD44 or CD8 (negative control).

be different based of the expression of $CD45R_B$ (Fig. 3, and data not shown) (23, 28).

Anti-CD44 mAb Treatment Induces the Shedding of CD44 into the Serum. To gain insight into whether internalization or shedding accounted for the modulation of CD44 from the surface of lymphocytes, we analyzed acetone-fixed and permeabilized tissue sections of PLN from mAb-treated animals, for cell-associated CD44 (Fig. 4). When probed for the presence of the injected anti-CD44 mAb, using a polyclonal anti–rat Ig antibody, most lymphocytes in the PLN are devoid of CD44; however, a subpopulation of cells that do not appear in single-cell suspensions prepared from lymph nodes (Fig. 3) are intensely stained (Fig. 4). These cells may consist of dendritic cells, fibroblasts, macrophages, and/or interdigitating cells. To detect all CD44, including that not bound by the in vivo administered mAb, we stained the sections with an anti-CD44 mAb followed by secondary antibody. This analysis fails to reveal any additional CD44 (Fig. 4). Analysis of acetone-fixed cytospin preparations of isolated lymphocytes reveals similar results (data not shown). However, internalized, significantly degraded CD44 or anti-CD44 could presumably escape detection using these techniques.

Given the lack of evidence for internalized CD44 or anti-CD44 immune complexes in lymphocytes, we analyzed the levels of soluble CD44 found in the serum of anti-CD44-treated and control mice, using a sandwich RIA (Fig. 5). Serum from control animals and humans contains significant levels of soluble CD44 (Fig. 5) (29). IRAWB14-treated mice exhibit a 1.5–2-fold increase in serum CD44 levels, consistent with mAb-induced shedding of CD44 (Fig. 5). We could not detect high levels of soluble CD44 in the serum of IM7-treated animals using ^{125}I-labeled IM7, suggesting that the injected antibody remains complexed with shed CD44 (data not shown). We have not yet determined the mechanism of shedding of CD44 induced by anti-CD44 mAb in vivo; however, our data are consistent with results from a recent study that demonstrated a partial shedding of CD44 from cells induced by in vitro exposure to immobilized anti-CD44 (30).

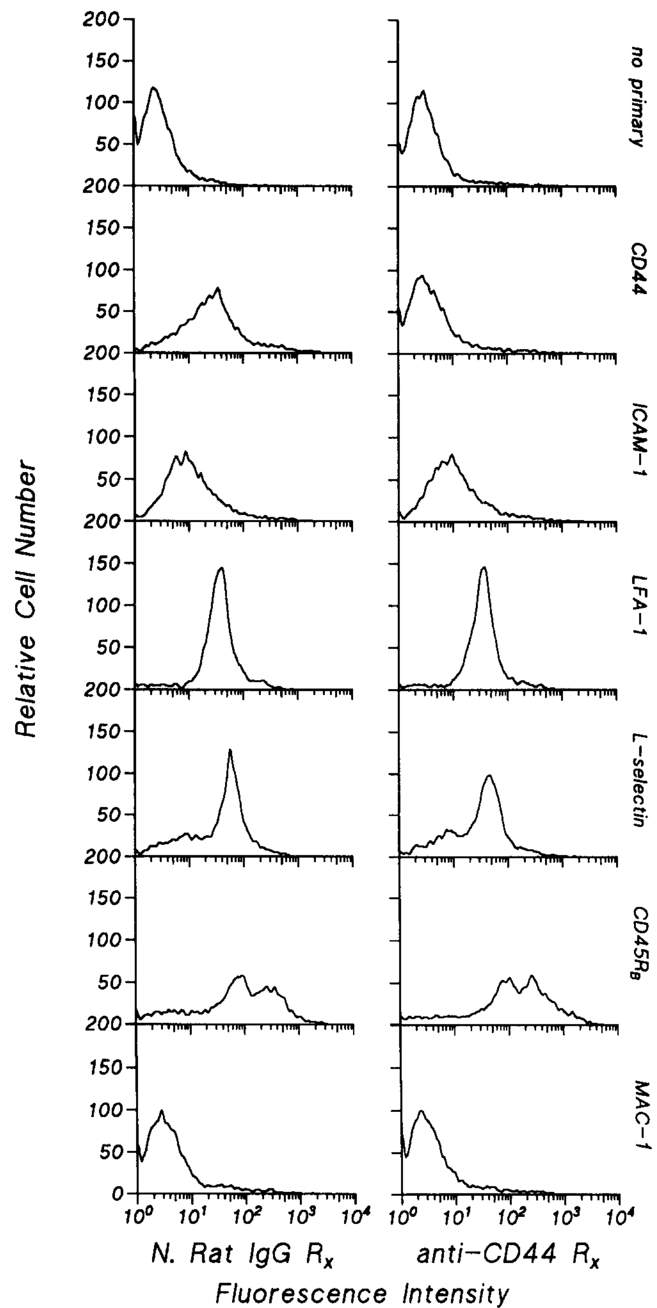

Figure 3. The cell surface phenotype of CD44-positive and CD44-negative lymphocytes. Lymphocytes were isolated from the PLN of mice treated for 12 h with 300 μg normal rat IgG (*N. Rat IgG R_x*) or IM7 (*anti-CD44 R_x*), and stained with mAbs to the indicated surface antigens. mAb binding was visualized with a FITC-conjugated secondary antibody, and analyzed by fluorescence flow cytometry.

The Reexpression of CD44 Requires Lymphocyte Activation. CD44-negative lymphocytes explanted from the PLN of mice 12 h post–mAb injection fail to reexpress CD44 on their surface after a 12–20-h culture in vitro in the absence of stimu-

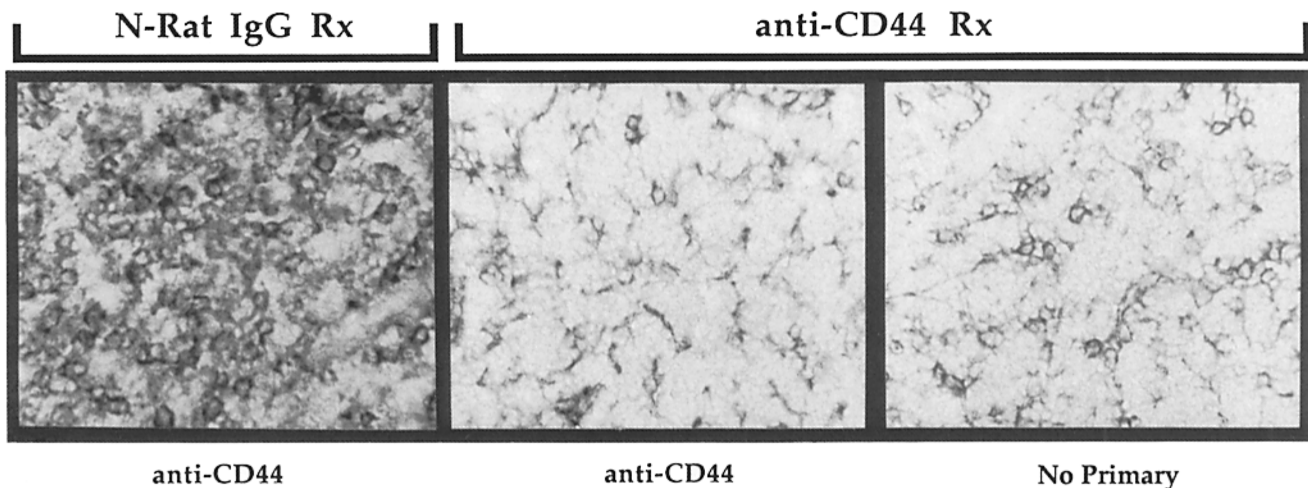

Figure 4. In situ analysis of CD44 expression in PLN of mice treated with anti-CD44 mAb. DNFB-sensitized mice were injected with 300 μg i.p. of IRAWB14 (*anti-CD44 R_x*) or a control (*N-Rat IgG R_x*) and challenged with DNFB 2 h later. After 24 h, draining lymph nodes were removed and acetone-fixed cryostat sections prepared. Sections were stained with secondary antibody alone (*No Primary*), or IRAWB14 followed by secondary antibody (*anti-CD44*).

lant (Fig. 6). However, stimulation with a combination of phorbol esters and a mitogenic anti-CD3 mAb enhances the expression of CD44 on CD44-positive cells (from control mice) and induces CD44 expression on CD44-negative cells (from mAb-treated mice), resulting in similar levels of expression on the two populations. The enhanced expression on cells from control mice and the reexpression of CD44 on cells from anti-CD44-treated animals are completely blocked by cycloheximide, which inhibits protein synthesis. PCR amplification of the mRNA from CD44-negative cells demonstrates that these cells retain CD44-specific mRNA (data not shown). Resting T cells express little, if any, CD44R (12, 13), and the elevated expression of CD44 after T cell activation, as detected by the mAbs used in this study, is primarily due to the enhanced expression of CD44, not CD44R (Camp, R. L., and E. Puré, unpublished observations). Below, we also describe evidence for activation-induced reexpression of CD44 in vivo.

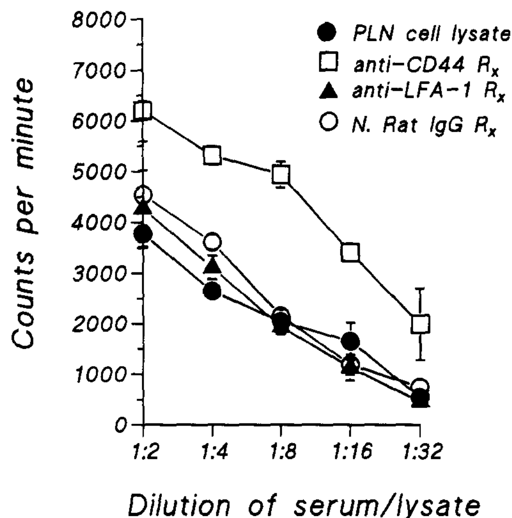

Figure 5. The administration of anti-CD44 mAbs in vivo results in increases in the level of CD44 in the serum of mice. Serum from normal mice treated for 12 h with 300 μg control antibodies (*N. rat IgG R_x*) or 200 μg FD441.8 (*anti-LFA-1 R_x*), or 300 μg anti-CD44 mAbs (IRAWB14), was analyzed in a sandwich RIA using plate-bound KM201 and ^{125}I-labeled IM7. Lysate from normal PLN cells was used as a positive control. Results expressed are the average of serum samples from three to four mice ± SEM, subtracted from a background mean of 62 cpm.

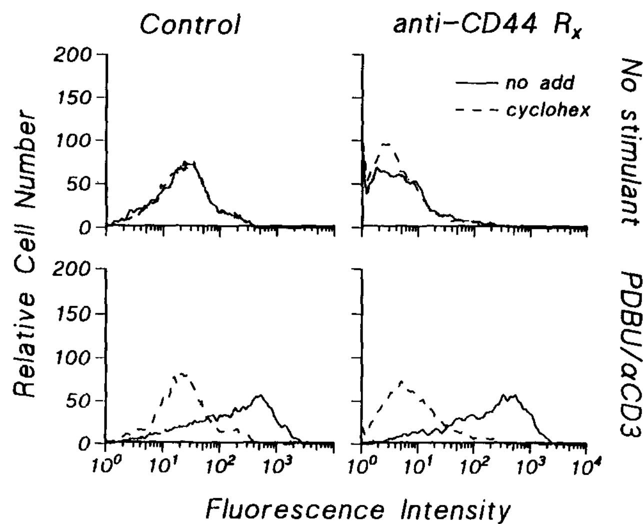

Figure 6. The stimulation of CD44-negative T cells in vitro induces the expression of CD44. Lymphocytes were isolated from the PLN of normal mice treated for 12 h with 300 μg N. rat IgG (*Control*) or 300 μg IRAWB14 (*anti-CD44 R_x*). Cells were cultured for 12 h in the absence (*No stimulant*) or presence of a combination of phorbol ester and an activating anti-CD3 mAb, 2C11 (*PDBU/αCD3*). Cells were coincubated in the absence (*no add*) or presence of cycloheximide (*cyclohex*). Viable cells were selected according to their ability to exclude ethidium bromide, and their expression of CD44 was analyzed with a FITC-conjugated anti-CD44 mAb (IM7).

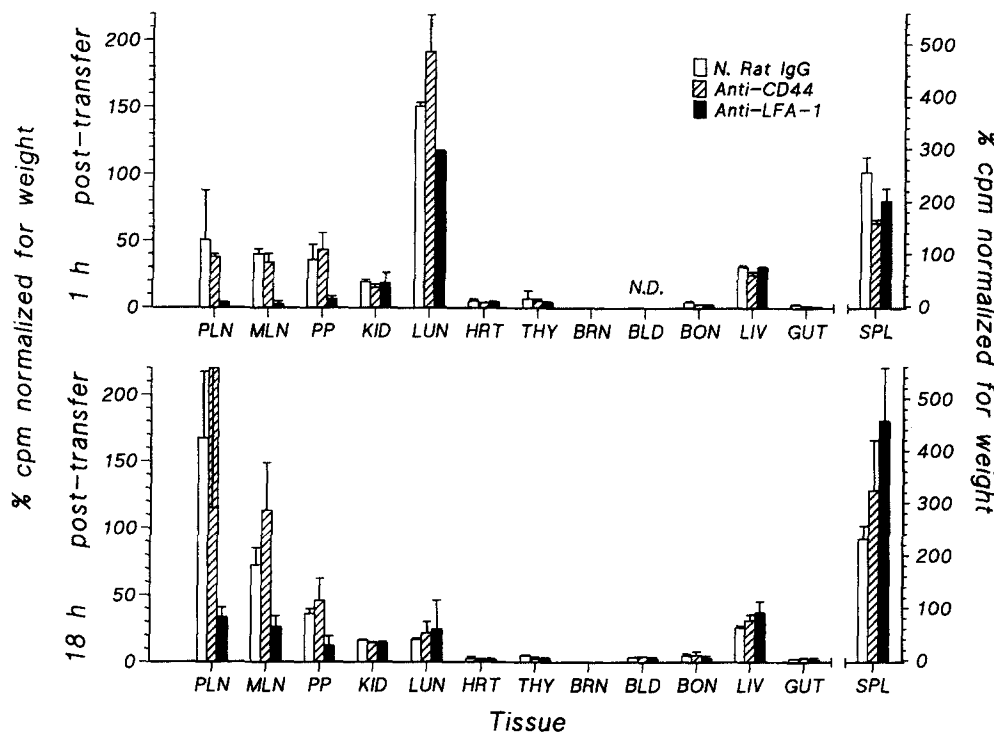

Figure 7. The migratory patterns of CD44-negative and CD44-positive lymphocytes in normal mice. Lymphocytes were isolated from the PLN of normal mice injected with 300 μg normal rat IgG, 200 μg FD441.8 (*anti-LFA-1*), or 300 μg IRAWB14 (*anti-CD44*) 12 h before death. Cells were labeled in vitro with ^{111}In, washed, and injected into the tail veins of normal mice (3 × 10^6 cells/mouse). 1-18 h posttransfer, injected mice were killed, their organs weighed, and the amount of incorporated radioactivity was measured. Each data point represents an average of results from two mice ± SEM. To normalize for variability between mice, results are expressed as the percentage of total radioactivity recovered per organ, divided by the organ's weight in grams. The absence of error bars for some data points indicates error values that are too small to be visualized graphically. MLN, mesenteric lymph node; PP, Peyer's patch; SPL, spleen.

CD44-negative Lymphocytes Home to Peripheral and Mucosal Lymphoid Organs. To determine the homing potential of CD44-negative lymphocytes, we isolated PLN cells from normal mice injected with mAbs against CD44 or LFA-1, or normal rat IgG. These cells were analyzed by flow cytometry and determined to be CD44 negative, saturated with LFA-1, or CD44 positive, respectively (Fig. 3, and data not shown). The cells were subsequently labeled with ^{111}indium, washed and injected intravenously into normal mice. Mice were killed 1 or 18 h later and various organs were removed, weighed, and assayed for the presence of labeled cells. Lymphocytes from untreated or control mice, injected in this manner, are initially trapped in the lungs, and subsequently disseminate to the peripheral and mesenteric lymphoid organs and the spleen (Fig. 7). After 18 h, the vast majority of labeled cells has marginated from the blood into other tissues (Fig. 7). The specificity of this extravasation is demonstrated by the lack of cell accumulation in highly vascularized tissues (i.e., lung, liver, and kidney).

We and others have previously determined that cells coated with an anti-LFA-1 mAb migrate normally to nonlymphoid organs and the spleen, but fail to migrate into the peripheral and mesenteric lymph nodes or the Peyer's patches (Fig. 7) (19, 31). In contrast, CD44-negative, unactivated lymphocytes exhibit migratory patterns identical to those of lymphocytes from control mice (treated with normal rat IgG), in terms of their ability to extravasate into peripheral and mesenteric lymphoid organs and spleen (Fig. 7). To eliminate the possibility that CD44-negative cells reexpress their CD44 in vivo after adoptive transfer, we performed similar homing experiments using recipient mice that were injected with anti-CD44 mAb 12 and 3 h before adoptive transfer. These experiments failed to reveal any significant difference between the migratory patterns of normal and CD44-negative lymphocytes (data not shown).

Consistent with the data above, the number of cells recovered from the PLN of anti-CD44-treated mice is identical to that obtained from control mice, whereas the number of cells isolated from the lymph nodes of mice treated with anti-LFA-1 is 62% of controls at 24 h post-mAb administration (Table 1, exp. A).

Treatment with Anti-CD44 mAbs Inhibits the Early Phase of the DTH Response. To investigate the immune competence of hapten-sensitized mice injected with anti-CD44 mAbs, we analyzed their ability to exhibit DTH in response to hapten challenge presented by skin painting. The administration of mAbs against CD44 before and during antigenic challenge delays the onset of DTH by at least 24 h (Fig. 8). Ultimately, the peak inflammatory response is similar to that observed in animals injected with control antibody. A histological analysis of the DTH sites at 24 h demonstrated that anti-CD44 treatment inhibits both the edema and the cellular infiltrate associated with early inflammation (Fig. 9). The normal cellular infiltrate in a murine DTH reaction at 24 h is predominately composed of T lymphocytes (32). Analogous treatment of mice with mAb to LFA-1 also inhibits the DTH response, but this suppression is protracted and inhibits at both early (<24 h) and late (>48 h) time points (19). Anti-LFA-1 mAbs also inhibit the influx of cells into lymph nodes draining DTH sites, whereas anti-CD44 mAbs have no effect on the number of cells recovered (19, and Table 1, exp. B).

The DTH response in sensitized mice given a single dose

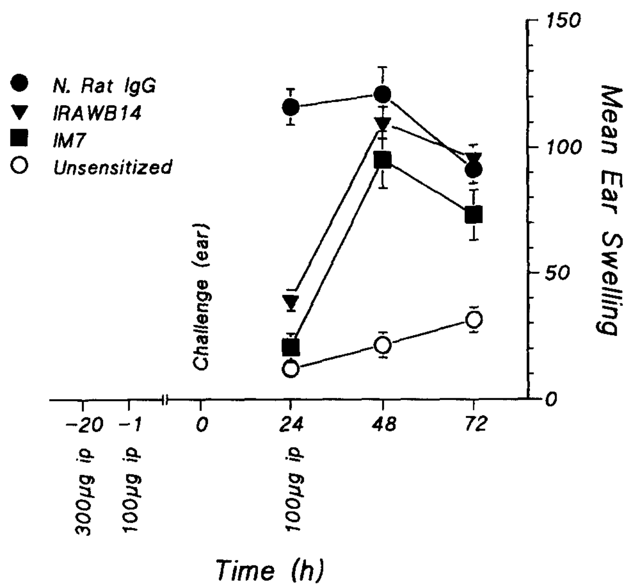

Figure 8. The administration of anti-CD44 mAbs in vivo inhibits the early phase of DTH responses. DNFB-sensitized mice (six per group) were injected with 300 μg control antibody (*N. rat IgG*) or 300 μg of anti-CD44 mAb (*IM7* or *IRAWB14*) at the indicated times. Mice were challenged on the ears with DNFB, and mean ear swelling was determined 24–72 h later. DNFB-challenged, unsensitized mice were used as a negative control (*Unsensitized*). Two animals in each group were killed at 24 and 72 h, and organs were taken for further analysis. Results are expressed as the increase in ear thickness (mean value of both ears measured at the indicated times postchallenge minus mean value before challenge) ± SEM. The anti-CD44-treated animals showed a reduced ear swelling that was statistically significant at 24 h postchallenge ($p < 0.0005$, student's t test) compared with N. rat IgG injected animals.

(300 μg) of anti-CD44 mAb 12 h before hapten challenge is similarly suppressed (data not shown). In contrast, the administration of mAbs against CD44 (including KM201, which blocks CD44/HA interactions) 1–3 h before challenge has little effect on the resulting DTH response (data not shown).

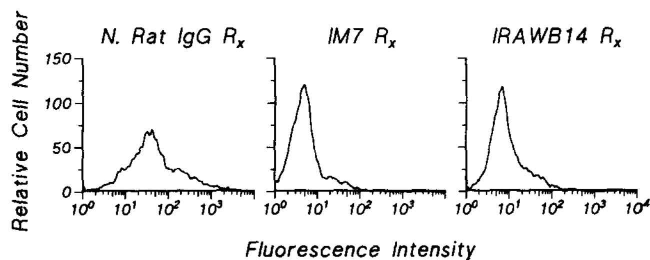

Figure 10. Lymphocytes in the lymph nodes draining DTH sites remain CD44 negative. Leukocytes were isolated from the draining lymph nodes of the mice analyzed in Fig. 8, at the 72-h time-point. Cells were stained with a FITC-conjugated anti-CD44 mAb (KM201).

Given that we have established that the modulation of CD44 from the surface of lymphocytes in vivo takes at least 12 h (Fig. 1), this result suggests that CD44 modulation must occur before challenge to achieve an inhibition of the DTH response.

The delay in the presentation of a full-scale DTH response in anti-CD44-injected animals might imply that CD44 expression is required only during the early phase of DTH, and that CD44-negative cells could extravasate during the later phase of the response. Alternatively, the delay may represent the time required for cells to become activated and subsequently reexpress CD44 on their surface, after which the DTH response would proceed normally. To investigate these possibilities, we looked at the expression of CD44 on lymphocytes in both DTH-inflamed skin and the adjacent draining lymph nodes at 72 h. The vast majority of lymphocytes isolated from the draining lymph nodes at 72 h remain CD44 negative, although a small number of cells is CD44 positive (Fig. 10). In contrast, those leukocytes that eventually gain entrance to the DTH site (by 72 h postchallenge) are CD44 positive (Fig. 11). These cells may represent CD44-positive cells that have escaped CD44 modulation and migrated into

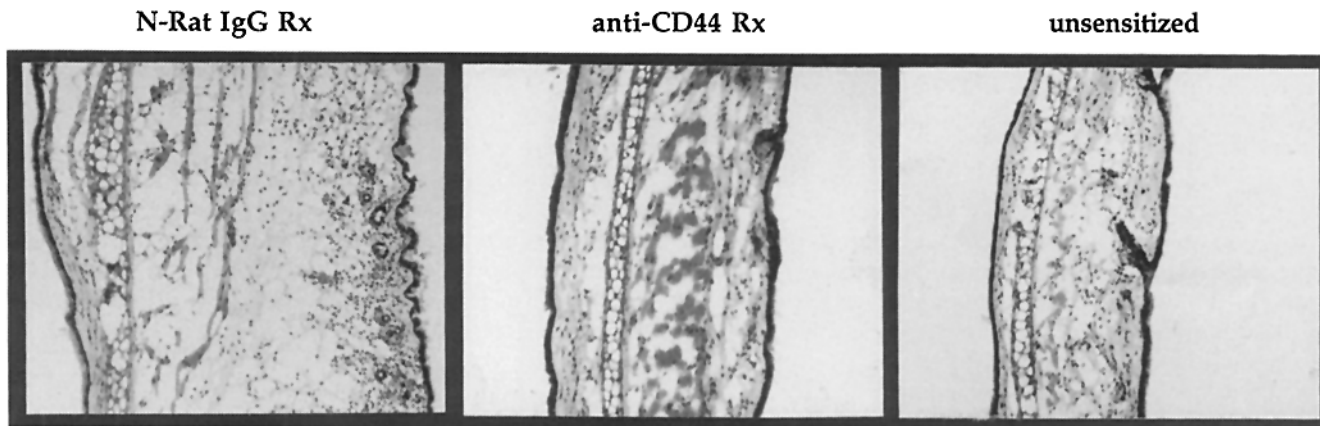

Figure 9. Anti-CD44 mAb treatment reduces both the edema and cell infiltration at DTH sites. Hematoxylin- and eosin-stained cryostat sections of ears 24 h after DNFB challenge of an unsensitized mouse, a sensitized mouse given control antibody (*N. Rat IgG R_x*), or a sensitized mouse injected with IM7 (*anti-CD44 R_x*) before challenge (see Fig. 8 for doses and times of injections). Shown are cross-sections of comparable areas of ears at identical magnifications.

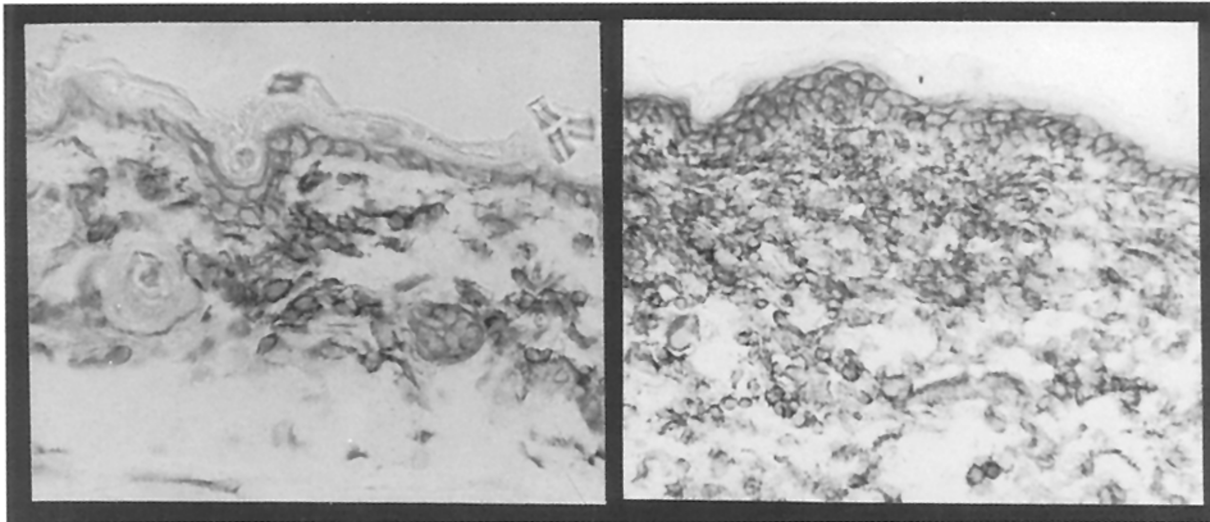

Figure 11. Lymphocytes in the site of DTH are CD44 positive in the late phase of the response. Cryostat sections of ears from mice 72 h after DNFB challenge were stained with an anti-CD44 mAb (IM7). Shown are ears from an unsensitized mouse and a sensitized mouse injected with IM7 (see Fig. 8 for doses and times of injections). The sections are counterstained with Mayer's hematoxylin. In addition to infiltrating lymphocytes, keratinocytes are also CD44 positive in both sections.

the DTH site, or lymphocytes that have been activated distally or locally and reexpress CD44. The presence of CD44R on the surface of keratinocytes (8) may explain the resistance of these cells to anti-CD44-induced CD44 modulation (Fig. 11).

Discussion

In vitro studies have defined several potential functions for CD44; however, the extent to which these activities are important in vivo is unknown. There is mounting evidence to suggest that CD44 and its associated isoforms (CD44R) play a central role in the cell adhesion and migration of primary leukocytes and the metastatic proclivity of certain tumors (8–13). HA, a glycosaminoglycan found ubiquitously as an extracellular matrix (ECM) component, is the principal ligand defined for CD44. Of particular interest in view of our findings, is the presence of HA as a component of the ECM of the vascular intima and the accumulation of HA in inflammatory lesions (33–35).

Several studies have suggested that CD44 may play a role in the extravasation of lymphocytes into lymphoid organs, in part through a putative glycoprotein ligand ("addressin") expressed on HEVs (17). In humans, an anti-CD44 mAb, Hermes-III, blocks the binding of lymphocytes to the HEVs on tissue sections of mucosal lymphoid organs. Hermes-III also partially blocks the binding of activated T cells to IL-1-stimulated human umbilical vein endothelial cells (36). In addition, a polyclonal serum against CD44 blocks the binding of lymphocytes to PLN HEVs and inflamed synovia (5).

The initial binding of lymphocytes to HEVs appears to be independent of CD44/HA interactions (2). However, other studies suggest that CD44 can bind endothelial cells in an HA-dependent manner (16, 22). Consequently, the relationship between HA and the endothelial addressin is unclear (2, 16, 22). HA may be a component of the HEV addressin, and/or act as a separate ECM-derived ligand, particularly in nonlymphoid tissue. The HA and addressin binding sites on CD44 appear to be distinct (2). The close proximity of the HA-rich vascular ECM to the vascular cell surface makes it difficult to determine the site of lymphocyte–endothelial interactions (i.e., cell–cell vs. cell–ECM) in in vitro binding assays.

Our data are inconsistent with the deduction that CD44 is a lymphoid organ–specific homing receptor (5, 17, 18, 36, 37). We find no evidence in vivo that CD44 is critical for the extravasation of unactivated murine lymphocytes into any lymphoid organ during normal trafficking. Since the cells used for the adoptive transfer experiments were stripped of CD44, the lack of an effect on lymphocyte migration as described herein is conclusive evidence that the receptor is dispensable with regard to this function. Resting T cells do not express detectable levels of CD44R (12, 13). The lack of an effect on lymphocyte homing cannot be attributed to the reexpression of CD44 on adoptively transferred cells because CD44 reexpression is not detectable for at least 24 h in vivo, and requires lymphocyte activation in vitro. In addition, CD44-negative cells adoptively transferred into host mice treated with anti-CD44 mAb before transfer migrate normally.

The ability to generate lymphocytes devoid of CD44 precludes the need for anti-CD44 mAbs specific for functional epitopes, which is particularly advantageous considering that CD44 appears to contain multiple functional epitopes (1, 5, 22). In addition, the modulation of CD44/mAb complexes from the cell surface also overcomes the potential for mAb-coated cells to be bound and opsonized by FcR-bearing cells.

An alternative approach would be to develop CD44-negative primary cells through genetic manipulation (i.e., "knock-out" mice). However, this has not been achieved, to our knowledge. Furthermore, since CD44 may be critical for development, CD44 knock-out mice may not be amenable to investigating the role of CD44 in mature mice. We are presently investigating whether CD44 is important for long-term leukocyte recirculation, and whether it is involved in the migration of lymphocytes after extravasation (i.e., the movement of different lymphocyte types to their appropriate lymphoid compartments).

Although CD44 is not necessary for the extravasation of lymphocytes into lymphoid organs, we demonstrate that CD44 plays a role in the generation of DTH responses. As with many other sites of increased cellular migration, such as the developing embryonic limb bud (38), inflammatory sites (33), and around metastatic tumor deposits (33), the deposition of HA within the DTH site is markedly enhanced (34, 35). Given that CD44 binds HA, each of these sites is a potential area in which to look for CD44 receptor function. DTH responses are particularly interesting because they involve a cascade of events that may be influenced by CD44. DTH responses are dependent upon the prior generation of antigen-primed T cells during the sensitization phase (39, 40). After challenge, antigen-primed T cells are restimulated and release lymphokines (i.e., IFN-γ and IL-2) that promote, both directly and indirectly, increased vascular permeability and leukocyte activation and extravasation at both the DTH site and in the draining lymph nodes (41–43). HA-rich edema precedes the cellular infiltrate, and is maximal by 24 h postchallenge (44). The subsequent migration of cells into DTH sites in mice is characterized by the early influx of mononuclear cells (1–12 h postchallenge), followed by the enhanced extravasation of lymphocytes, neutrophils, and eosinophils (12–24 h). By 24 h, lymphocytes predominate (32, 44, 45).

Anti-CD44 treatment could potentially block any of these steps, resulting in temporary immunosuppression. However, anti-CD44 does not inhibit the facilitated migration of lymphocytes into the draining lymph nodes after antigenic challenge. This observation demonstrates that the DTH response is initiated even in the presence of anti-CD44 mAb, and implies that the T cell memory component of DTH remains intact, and antigen presentation, T cell activation, and at least some cytokine release are not inhibited. In contrast, events occurring at the DTH site at 24 h, including the development of edema and the extravasation of leukocytes, are inhibited. This may either reflect a direct blockade of leukocyte extravasation, or a defect in the activation or cytokine production of T cells, PMN, monocytes, and/or NK cells. There is in vitro evidence to suggest that CD44 plays a role in each of these processes (46–49). The migration of leukocytes into the DTH site may be blocked by the combined effect of an inhibition in HA matrix deposition (edema) and the lack of CD44 (HA receptor). Our studies distinguish the role of CD44 from that of LFA-1 since mAbs against LFA-1 block both the early and late phases of the DTH response and inhibit the extravasation of cells into both normal and draining lymph nodes (19, 31).

The wide-spread expression of HA and CD44 suggests that CD44–HA interactions must be regulated to prevent nonspecific cell adhesion. Indeed, the CD44 expressed on many cells, including all primary leukocytes tested, fails to bind HA in vitro. However, CD44 can be induced to bind HA by treating cells with the anti-CD44 mAb IRAWB14.4, suggesting that the conformation and/or aggregation of CD44 is important for its adhesive capacity (22). The physiologic signals involved in initiating CD44/HA binding are unknown; however, it is possible that cytokines released at a site of inflammation induce CD44 to bind HA. In addition, receptor interactions between extravasating leukocytes and the vascular endothelium may trigger CD44 function, and thus prepare the leukocyte for its subsequent transmigration through the underlying HA-rich extracellular matrix. The induction of CD44 function may be effected through increases in the level of surface expression, and/or changes in the phosphorylation and cytoskeletal association of CD44, all of which are associated with leukocyte activation (22, 50, 51).

In summary, our data indicate that CD44 plays a critical role in the generation of the early phase of an inflammatory response involving nonlymphoid tissue, but does not appear to be required for extravasation of cells from the blood into lymph nodes.

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