Prevention of Tumor Metastasis Formation by Anti-Variant CD44

By S. Seiter,* R. Arch,* S. Reber,* D. Komitowski,‡ M. Hofmann,§ H. Ponta,§ P. Herrlich,§ S. Matzku,‖ and M. Zöller*

*From *Deutsches Krebsforschungszentrum, Institut für Radiologie und Pathophysiologie; ‡Deutsches Krebsforschungszentrum, Institut für Experimentelle Pathologie, D-6900 Heidelberg 1; §Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, D-7500 Karlsruhe 1; and ‖E. Merck & Co., D-6100 Darmstadt 1, Germany*

Summary

A splice variant of CD44 (CD44v) originally discovered on metastases of a rat pancreatic adenocarcinoma (BSp73ASML) has been shown by transfection to confer metastatic behavior to nonmetastatic tumor cells (Günthert U., M. Hofmann, W. Rudy, S. Reber, M. Zöller, I. Haussmann, S. Matzku, A. Wenzel, H. Ponta, and P. Herrlich. 1991. *Cell.* 65:13). A monoclonal antibody (mAb), 1.1ASML, to the metastasis-specific domain of the CD44v molecule retards growth of lymph node and lung metastases of the metastatic tumor line BSp73ASML, and can efficiently prevent formation of metastases by the transfected line. The antibody is only effective when given before lymph node colonization. Anti-CD44v does not downregulate the expression of CD44v, and prevention of metastatic growth by anti-CD44v is not due to activation of any kind of immune defense. We suggest that the mAb interferes with proliferation of metastasizing tumor cells in the draining lymph node, most probably by blocking a ligand interaction. The interference with metastatic spread will greatly facilitate the exploration of the function of CD44v and, in particular, may also open new strategies for the therapy of human metastases.

CD44 is an integral membrane glycoprotein (1–7), originally described as a homing receptor of lymphocytes (8–17). Beside the involvement of CD44 in adhesion of lymphocytes to specialized endothelial cells, CD44 is known to bind to hyaluronate and probably also to collagen (7, 18–30), suggesting roles in the interaction between cells and extracellular matrix. Moreover, several variants of CD44 with additional extracellular domains have been detected in a variety of tissues and frequently on tumor cells (1, 31–40).

The fact that metastatic spread involves interaction between tumor cells and extracellular matrix as well as between tumor cells and endothelial cells has led to the hypothesis that especially the variant forms of CD44 may be involved in the process of metastatic spread (32, 35). Starting from a metastasis-specific mAb (1.1ASML) (41) that has been raised against the highly metastatic pancreatic adenomcarcinoma line BSp-73ASML (42), we have cloned a splice variant of CD44 (CD44v)[1] (35). The splice variant carries an expanded extracellular domain. Overexpression of this variant in any one of several nonmetastatizing tumor cell lines confers full metastatic behavior to these cells (35, 42a).

We now report on the specific interference of mAb 1.1ASML with metastasis formation. Intravenous injections of 1.1ASML inhibited or prevented the colonization of lymph nodes and lung. No evidence for an antibody-mediated immune attack could be detected. Furthermore, 1.1ASML did not interfere with surface expression of CD44v. The data support the view that CD44v catalyzes embedding/outgrowth of tumor cells in the draining lymph nodes.

Materials and Methods

Rats and Tumor Lines. BDX and DA rats were obtained from Charles River (Sulzfeld, Germany). Rats were kept under specific pathogen-free conditions and were used for experiments at the age of 8–12 wk. BSp73AS (AS) and BSp73ASML (ASML) are the nonmetastasizing and metastasizing sublines, respectively, of a spontaneous pancreatic adenocarcinoma of the BDX rat strain (42). Both lines were cloned and maintained in vivo by subcutaneous passages. BSp73ASpSV14 (AS-14) cells were derived from AS cells after transfection with CD44v cDNA (pMetal) (35). BSp73ASpSVneo (AS-20) and BSp73ASpSVCD44 (AS-44) were derived from transfections with pSVneo and standard CD44 (CD44s) cDNA, respectively (35; M. Hofmann et al., manuscript in preparation). Tumor lines were grown in vitro as monolayers in RPMI 1640 supplemented with antibiotics, L-glutamine, and 8% FCS (RPMI-s). The transfected lines were intermittently passaged in RPMI-s containing 300 μg/ml

[1] *Abbreviations used in this paper:* CD44s, standard CD44; CD44v, splice variant of CD44.

neomycin. Confluent cultures were trypsinized (0.25% trypsin) and split. As revealed by fluorescence-activated cell scanning (Coulter EPICS; Coulter Electronics GmbH), ASML cells express CD44v (staining with 1.1ASML), whereas AS and AS-44 cells express exclusively CD44s (staining with Ox49 and Ox50) (both mAbs kindly provided by A. F. Williams, Institute of Pathology, University of Oxford), with AS-44 cells displaying a significantly higher amount of CD44s on the surface than AS cells (our unpublished results). AS-14 cells express CD44s and CD44v, the latter at a density of ~75% in comparison with ASML cells (42b).

Antibodies. The mAb 1.1ASML (mouse IgG1) recognizes an epitope on exon v6 (43), which was originally designated as domain III of CD44v (35, 41). mAbs Ox49 (mouse IgG2a) and Ox50 (mouse IgG1) recognize epitopes on both CD44s and CD44v; the epitopes of both mAbs have not been mapped, but binding of 1.1ASML, Ox49, and Ox50 is not competitive, indicating that the two epitopes of CD44s and the one epitope of CD44v are not in close proximity (42b). mAb 4.4ASML (mouse IgG1) has been derived from the same fusion as mAb 1.1ASML and recognizes an epitope expressed only on the metastatic subline of the rat pancreatic adenocarcinoma (41). mAb 3–9 (mouse IgG1) is an antigallium chelate antibody (44), used as an isotype-matched control for 1.1ASML. The mAbs 1.1ASML, 4.4ASML, and 3–9 were derived from culture supernatants and were purified by protein A–Sepharose chromatography, collecting the fraction eluted at pH 6.0 (45). Ox49 and Ox50 were used as ascitic fluid. DA anti-BDX serum was derived by repeated subcutaneous and intramuscular injections of BDX lymphocytes in adjuvant. The serum was purified by ammonium sulfate precipitation. Secondary antibodies, FITC-labeled goat anti–mouse IgG1 and IgG2a, were obtained from Southern Biotechnology Associates (Birmingham, AL).

Metastasis Assay. Tissue culture–derived tumor cells were injected intrafootpad at a dose of $5 \times 10^5/50$ μl, if not stated otherwise. Where indicated, the tumor and the draining lymph node were excised by amputation in the knee at day 7 (ASML) or 10 (AS-14) after tumor cell application. Rats were controlled for metastatic tumor growth by measuring draining lymph node diameter. Rats were killed upon reaching a moribund state, which was defined by weight loss (>30%), rigidity of the thorax, shortness of breath, anemia as judged by paleness of the eyes, and a mean diameter of >2 cm of palpable lymph node metastases. Draining lymph nodes were weighed and lung colonies were counted. When rats were treated with mAbs they received 200 μg 1.1ASML, 4.4ASML, or 3–9, respectively, twice per week, intravenously, starting on the day of tumor cell inoculation. In one experiment, mAb treatment was started after excision of the primary tumor. Differences in lymph node and lung colonization depending on mAb treatment were assessed using a paired student's *t* test.

Histology. Metastatic lymph nodes and lungs were fixed with 4% formalin, cut into 5-μm slices, and stained with hematoxilin-eosin. For immunohistology tissue was fixed in liquid nitrogen–chilled ethalan, cut, and stained with 1.1ASML. FITC-labeled anti–mouse IgG1 was used as second antibody. For quantitative immunofluorescence, metastatic cells were separated from remaining lymphocytes by a discontinuous Percoll gradient centrifugation (46, 47), collecting tumor cells from the interface at density (σ) 1.077. Cells were stained with 1.1ASML and FITC-labeled anti–mouse IgG1; intensity was quantified with a Coulter EPICS Profile (Coulter Electronics GmbH).

Soft Agar Colony Assay. Outgrowth of metastasizing tumor cells in draining lymph nodes was estimated by meshing draining lymph nodes through fine gauze and dispersing the cell suspension in soft agar (0.3%), which was layered on top of a 0.5% agar underlayer. Plates were incubated at 37°C for 1–2 wk. The number of colonies was counted and the size of the colonies was estimated using a plaque counter apparatus (Optilux, Quebec).

Effector Cells and Cytotoxicity Assays. Spleen, lymph node, and peritoneal exudate cells were collected as described (48). If required, lymph node cells were separated from tumor cells by Percoll gradient centrifugation, collecting T cells from the interface of σ 1.210. NK cell activity was determined with spleen cells of σ 1.077 (49). Cytotoxic potential of peritoneal macrophages (MΦ) was evaluated as described elsewhere (50). T cells from BDX rats or from DA rats, which had received two intravenous injections of irradiated (30 Gy) BDX lymphocytes, were collected from the interface of σ 1.210 after Percoll gradient centrifugation of lymph node cells. Cells were cultured with 10^6 irradiated (30 Gy) allogeneic lymphocytes or with irradiated (400 Gy) tumor cells and/or 50 μg affinity-purified mAbs for 7 d at a density of 2×10^7 cells/5 ml RPMI-s, supplemented with 10% conditioned medium containing IL-2 (51). Blast cells were collected and purified from debris by Ficoll gradient centrifugation (52).

Cytotoxicity assays were set up in triplicates plating 10^4 ^{51}Cr-labeled target cells (T) per well and adding titrated numbers of effector cells (NK/T = 100–12.5:1; MΦ/T = 50–6:1; CTL/T = 20–2:1) in a total volume of 200 μl RPMI-s. For determining antibody-dependent cellular cytotoxicity (ADCC), 100 μg/ml affinity-purified mAbs were added in addition. Complement cytotoxicity was evaluated by adding preabsorbed guinea pig complement (1:5 dilution) to ^{51}Cr-labeled target cells, which were preincubated with affinity-purified mAb. Complement assays were terminated after 2 h, NK and CTL assays after 6 h, and MΦ assays after 12 h. Spontaneous release was in the range of 2–7% (complement assay), 10–18% (NK and CTL assay), and 18–25% (MΦ assay). Specific release was calculated by the formula: percent cytotoxicity = 100× [(counts test well − counts medium control)/(total counts − counts medium control)]. Specific cytotoxicity values >5% were statistically significant (student's *t* test). Differences in cytotoxicity values depending on mAb treatment were assessed using a paired student's *t* test.

In Vivo Evaluation of Immune Effector Mechanisms. The presence or absence of tumor-specific and/or mAb 1.1ASML-specific effector T cells was evaluated upon transfer of lymph node cells from rats that were untreated, or remained free of metastases after they had received 5×10^5 AS-14 cells, intrafootpad, and multiple injections of 1.1ASML, intravenously. Irradiated recipient rats (8.5 Gy) received 5×10^5 BSp73ASML cells, intrafootpad, concomitantly with an intravenous injection of 5×10^7 lymph node cells. The diameter of draining lymph nodes was determined every 3 d. Animals were killed after 28 d, draining lymph nodes were weighed, and lung colonies were counted.

ADCC-like mechanisms functioning in vivo were estimated by application of 5×10^6 ^{125}I-UDR-labeled (3 μCi/culture for 72 h) tumor cells, intrafootpad, together or without intravenous injections of mAb. Tumor cell decay was determined by recording remaining radioactivity in a whole body counter, as described previously (53). Rats were killed after 10 days, the draining lymph nodes were excised, and the number of tumor cells was determined by cloning in soft agar, as described above.

Results

A Variant Form of CD44 Containing Additional Exons, but Not CD44s, Confers Metastatic Potential. The system used to investigate the function of CD44v in metastasis formation is based on cell lines derived from the pancreatic carcinoma

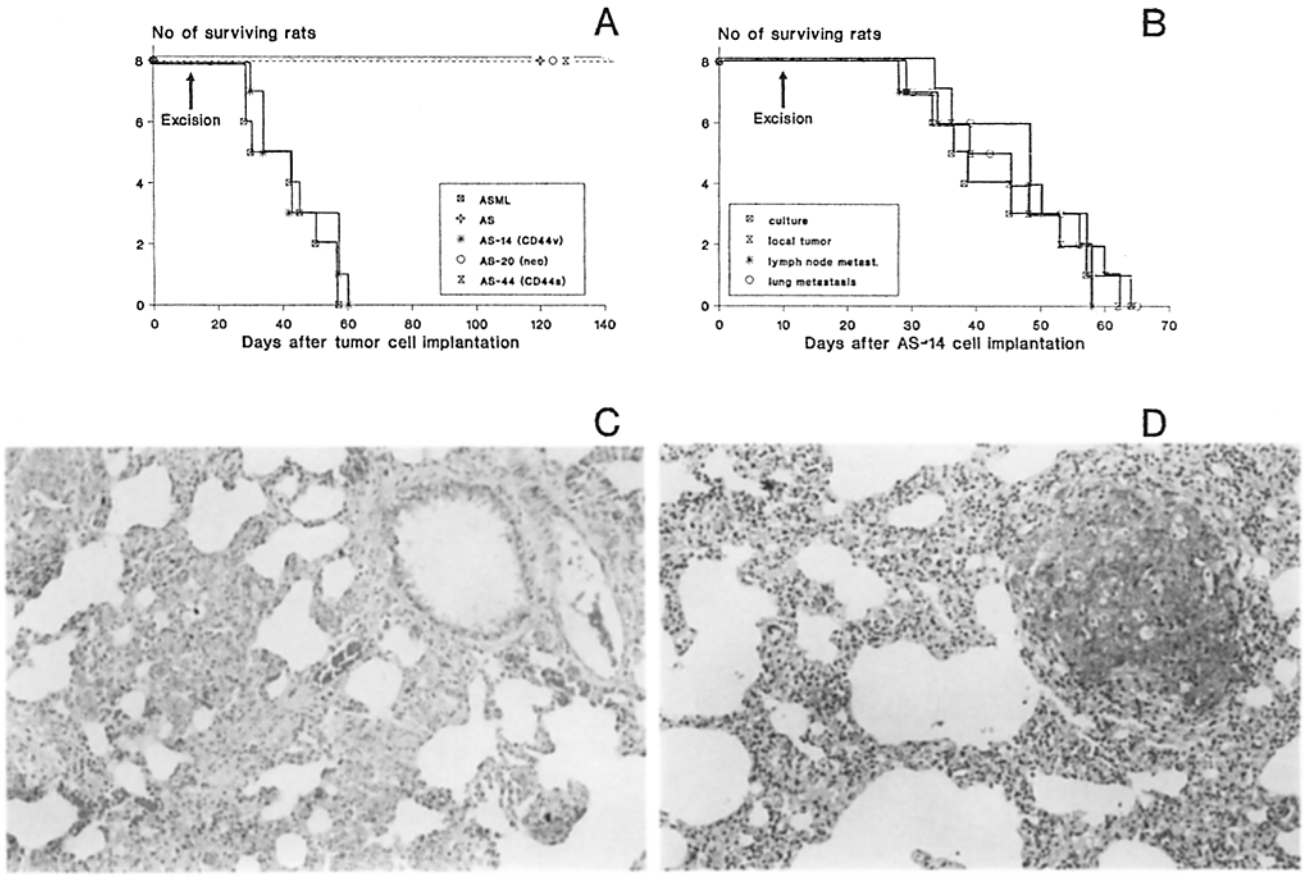


Figure 1. Metastatic progression of CD44v-positive tumor lines. (*A* and *B*) Expression of CD44v initiates metastatic progression. (*A*) BDX rats received 5 × 10^5 tumor cells, intrafootpad. The tumor and the draining popliteal lymph node were excised 10 d later by amputation in the knee. Rats were killed reaching a moribund state. Rats that had received ASML ($CD44v^+$) and AS-14 ($CD44v^+$ by transfection) became moribund between 30 and 60 d after tumor cell inoculation. Rats that had received AS ($CD44v^-$), AS-20 ($CD44v^-$, transfected with pSVneo), and AS-44 ($CD44v^-$, overexpression of CD44s by transfection) remained tumor free. (*B*) BDX rats received, intrafootpad, 5 × 10^5 AS-14 tumor cells that had been derived from either a primary nodule, or a lymph node or a lung metastasis. Rats were amputated and killed at a moribund state. (*C* and *D*) Metastasis formation in the lung. BDX rats received 5 × 10^5 ASML (*C*) or AS-14 cells (*D*), intrafootpad, and were killed 35 d later. In the lung of ASML-bearing rats a diffuse infiltration of alveolar walls by tumor cells was noted. AS-14 cells settled in nodular form in the lung; a single nodule is shown. ×40.

BSp73 (42). Animals that have been injected with the nonmetastatic cell line BSp73AS (AS) develop only a local tumor and remain healthy after excision of the local tumor. The highly metastatic cell line BSp73ASML (ASML) rapidly colonizes lymph nodes and lung and all animals become moribund within 60 d after intrafootpad application. The metastatic spread is complete at day 10, after which time excision of the local tumor together with the draining popliteal lymph node is inefficient. AS cells transfected with CD44v, as demonstrated for clone AS-14, are as metastatic as ASML cells, whereas G418-resistant control transfectants (clone AS-20) and, most importantly, transfectants with CD44s (clone AS-44) are nonmetastatic (Fig. 1 *A*).

To assure that it is solely expression of CD44v (and not an additionally property selected in vivo) that confers metastatic behavior to transfected AS cells, AS-14 cells were reisolated from local tumors as well as from lymph node and lung metastases. According to fluorescence analysis, AS-14 cells from all three sources expressed equivalent levels of CD44v (data not shown). Upon intrafootpad injection of the different reisolates, all animals developed lymph node and lung metastases with the same kinetics as the original AS-14 cell clone (Fig. 1 *B*).

Both, ASML and AS-14 cells metastasize via the lymphatic system to the lung. Yet, there are discrete differences between these lines, suggesting that CD44v cDNA does not confer all properties by which ASML cells differ from AS cells. (*a*) ASML cells do not form a local tumor in the footpad, the site of injection while after injection of AS-14 cells a locally growing encapsulated tumor is observed in some but not all rats. (*b*) Although the time course of lymph node and lung involvement is similar for ASML and AS-14 cells (data not shown), the morphology of the metastases is different. ASML cells diffusely infiltrate lymph nodes and lung giving rise to innumerable miliary nodules (Fig. 1 *C*), while with AS-14 cells the nodular form of metastatic settlement predominates (Fig. 1 *D*).

An Anti-CD44v mAb, 1.1ASML, Inhibits Metastasis Formation. The causal involvement of CD44v in metastatic behavior made us attempt to interfere with metastatic spread

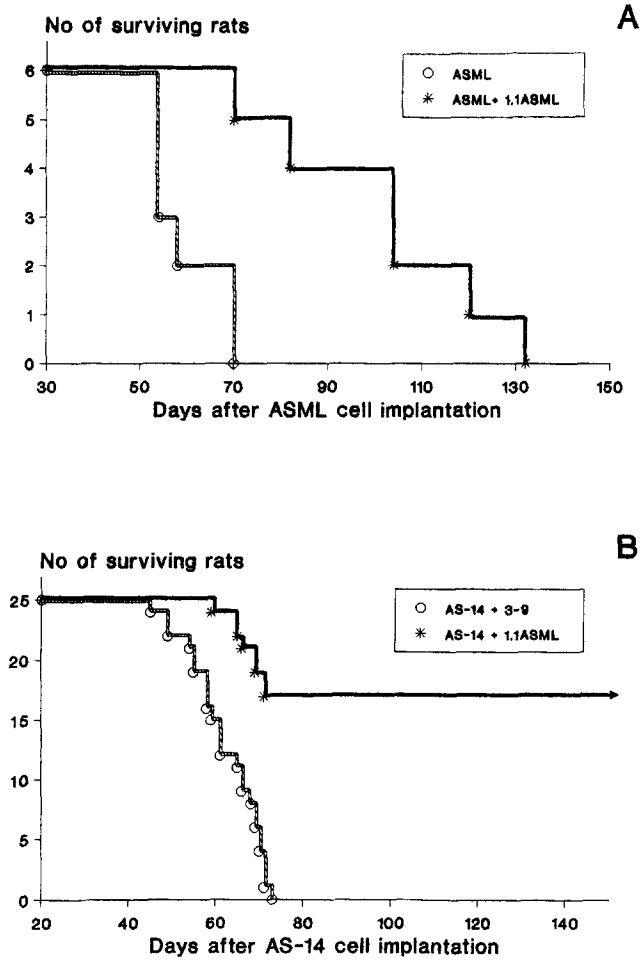


Figure 2. Retardation and prevention of metastasis formation. BDX rats received 5 × 10^5 ASML (*A*) or AS-14 cells (*B*), intrafootpad, and, where indicated, 200 μg 1.1ASML, intravenously, twice per week, starting at the day of tumor cell inoculation. The time for reaching a moribund state of six ASML-bearing rats per group and of 25 AS-14-bearing rats per group is shown.

by a mAb. Rats received a tumor dose of >10× TD_{100} (intrafootpad) together with the anti-CD44v mAb 1.1ASML (intravenously). Thereafter, mAb application (intravenous) was repeated twice per week. The effect of 1.1ASML was clearcut. The survival time of animals carrying ASML could be doubled, although the local depot of tumor cells at the site of injection was not excised. In animals injected with AS-14, metastasis formation could be completely prevented in 17 of 25 rats (Fig. 2). In this particular experiment, only two rats of the control group and two rats of the 1.1ASML-treated group developed a local tumor, which was excised when reaching a mean diameter of 0.3 cm. In the remaining rats no tumor developed at the injection site and, hence, the limb was not resected. After discontinuing injections of 1.1ASML at 140 d, animals remained healthy. When they were killed at day 240, histological examination of lymph nodes and lung did not reveal any sign of metastatic tumor cell growth (data not shown).

Complete inhibition of metastatic growth of AS-14 was

Table 1. *Tumor Takes under 1.1ASML Treatment*

Line	Dose	Metastatic growth (moribund state)	
		Control mAb	mAb 1.1ASML
	$\times 10^3$	*d*	*d*
ASML	500	5/5 (39)	5/5 (61)
	170	5/5 (42)	5/5 (86)
	60	5/5 (56)	5/5 (94)
	40	4/5 (66)	2/5 (144)
	20	3/5 (75)	0/5 (163)
AS-14	500	5/5 (54)	3/5 (66)
	170	5/5 (58)	2/5 (79)
	60	5/5 (66)	0/5 (-)
	40	5/5 (78)	0/5 (-)
	20	3/5 (98)	0/5 (-)

Rats received either 200 μg 3–9 (control mAb) or 1.1ASML, intravenously, twice per week.

obtained at a tumor dose of 1.5 × TD_{100} (6 × 10^4 tumor cells). In ASML-bearing rats the survival time could be prolonged by lowering the dose of tumor cells, but only at 1 × TD_{100}, 1.1ASML prevented metastasis formation (Table 1). The difference between the metastatic BSp73ASML line and the transfected line is an interesting feature: although CD44v expression suffices to confer metastatic behavior, ASML cells apparently have more complex properties.

The delay in metastasis formation by 1.1ASML treatment is reflected at the level of lymph node enlargement and of the number of lung colonies. When ASML cells reach the draining lymph node, a rapid onset of growth is observed within 20–30 d after injection. In 1.1ASML-treated animals, the enlargement of lymph nodes was delayed by 15–30 d (Fig. 3 *A*). After injection of AS-14, a longer time span of 30–55 d was required for outgrowth of metastases in the draining lymph node. In >80% of animals, the formation of lymph node metastases could be completely obliterated by 1.1ASML (Fig. 3 *B*). The failure of outgrowth of tumor cells in draining lymph nodes was confirmed by soft agar cloning of tumor cells out of draining lymph node suspensions. In the case of 1.1ASML-treated, ASML-bearing rats, the development of clones was delayed and remained reduced as compared with the number of clones in untreated rats (Fig. 3 *C*). In 1.1ASML-treated, AS-14-bearing rats, only occasionally were one to two clones obtained from draining lymph nodes (Fig. 3 *D*).

At 28 d after inoculation ASML cells showed massive settlement in the lung of untreated or mock-treated (mAb 3–9; data not shown) rats. In 1.1ASML-treated rats, however, the number of lung nodules were reduced to one to eight (Table 2). In AS-14-bearing rats, the development of lung metastases under the treatment with 1.1ASML was (if occurring at all) markedly reduced as judged from the very low number of lung nodules (Table 2).

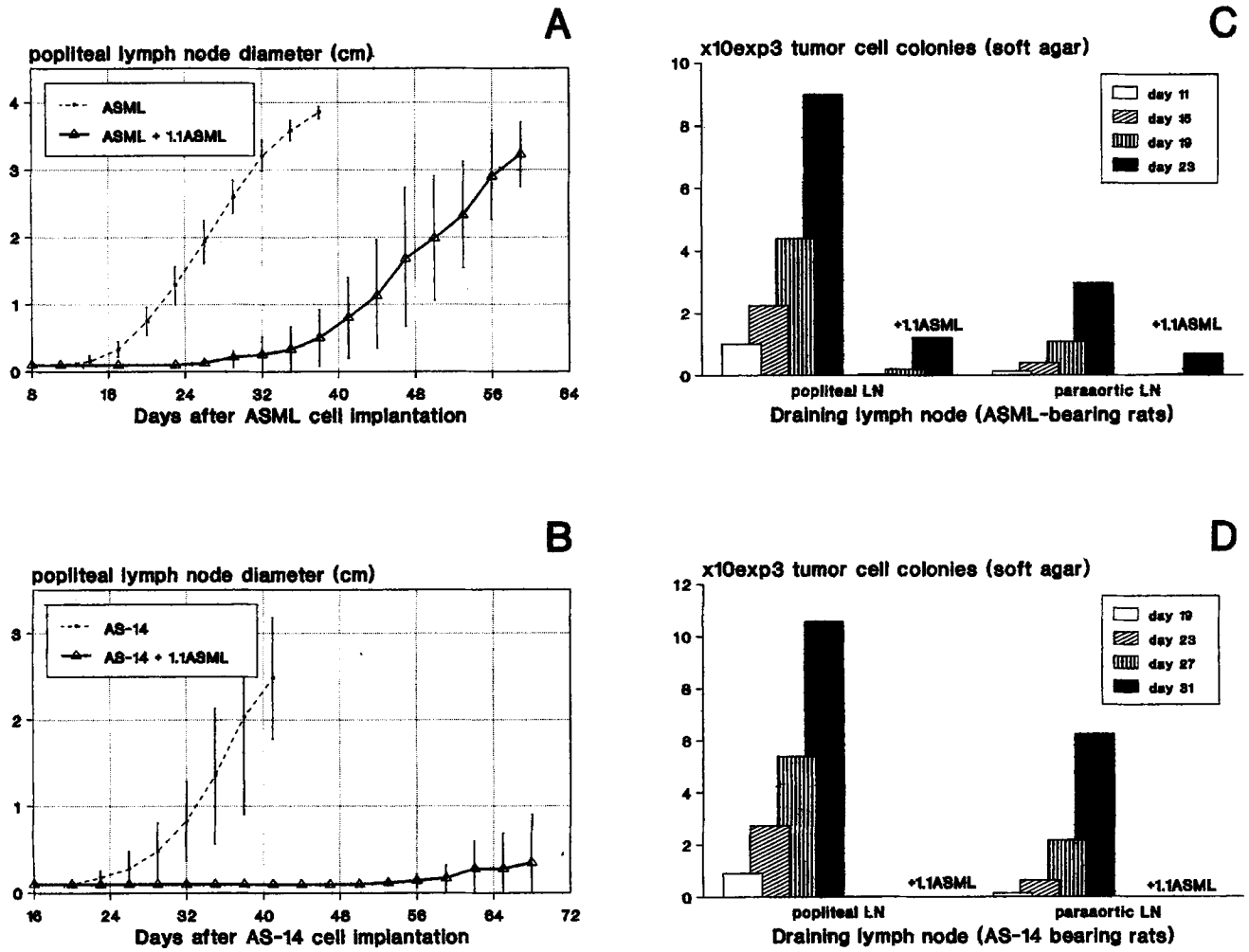


Figure 3. Retardation of metastatic lymph node colonization by anti-CD44v. (*A* and *B*) The mean diameter and standard deviations of the popliteal lymph nodes of 1.1ASML-treated and untreated ASML- and AS-14-bearing rats (six/group) are shown. It should be noted that with five of six mAb-treated, AS-14-bearing rats, the popliteal lymph node did not become palpable. (*C* and *D*) For the quantitation of metastasizing cells, draining lymph nodes of ASML- and AS-14-bearing rats, which were untreated or treated with 1.1ASML, were excised between 11 and 31 d after intrafootpad inoculation of tumor cells. Lymph nodes were meshed, plated in soft agar, and the number of tumor cell colonies was counted after 10 d of culture. Mean values of three to five lymph nodes per group are shown.

Finally, it should be noted that lymph node and lung colonization was retarded after only one injection of 1.1ASML. Yet, retardation of metastatic growth was vanishing at around 3 wk after cessation of 1.1ASML treatment. In addition, the effect of 1.1ASML on metastasis formation was highly specific for epitopes on CD44v. As a control antibody we used 4.4ASML. This antibody is of the same isotype as 1.1ASML, but binds to a different molecule on ASML (not on AS-14) cells. The epitope density for 4.4ASML is in the same range as CD44v expression (our unpublished finding). When ASML-bearing rats received intravenous injections of 4.4ASML, development of metastases in lymph nodes and lung was not retarded at all (Fig. 4).

The antibody experiments now permit us to explore the mechanism of antibody interference. The following possibilities will be considered. First, 1.1ASML could function as a trigger for immune effector mechanisms. Second, the antibody may interfere with the expression of CD44v, i.e., binding of 1.1ASML could result in downregulation of CD44v. Third, occupancy by the antibody inhibits the function of CD44v, e.g., a ligand interaction required for embedding of metastasizing tumor cells.

Reduction of Metastasis Formation by 1.1ASML Is Not Mediated by Immune Mechanisms. As described elsewhere (54), when using a protocol of preimmunization with a mAb explicitly adapted to initiate a cascade of immune responses, the appearance of antiidiotypic antibodies in response to 1.1ASML did not at all support a reduction of metastatic spread. Rather, an acceleration of metastatic spread was observed. In view of this finding, it was considered unlikely that immune mechanisms would account for the retardation of metastatic spread under 1.1ASML treatment. Since, however, the protocol of 1.1ASML application used here differed decisively from that described previously, the following im-

Table 2. *Reduction of the Number of Lung Colonies under 1.1ASML Treatment*

Treatment*	Day‡	Lung colonies	
		No.	Diameter
			cm
ASML	28	>1,000	0.1
ASML + 1.1ASML	28	1–8§	0.1
ASML	50	>2,000	0.1
ASML + 1.1ASML	100	100–200§	0.1
AS-14	50	30–80	0.3–1.0
AS-14 + 1.1ASML	100	0–8§	0.8–1.0

* BDX rats (seven per group) received 5 × 10^5 ASML or AS-14 cells, intrafootpad; where indicated, rats were treated with 200 μg 1.1ASML, intravenously, twice per week.
‡ Time after injection of tumor cells.
§ Significant differences ($p < 0.05$) in the numbers of lung colonies in untreated vs 1.1ASML-treated rats.

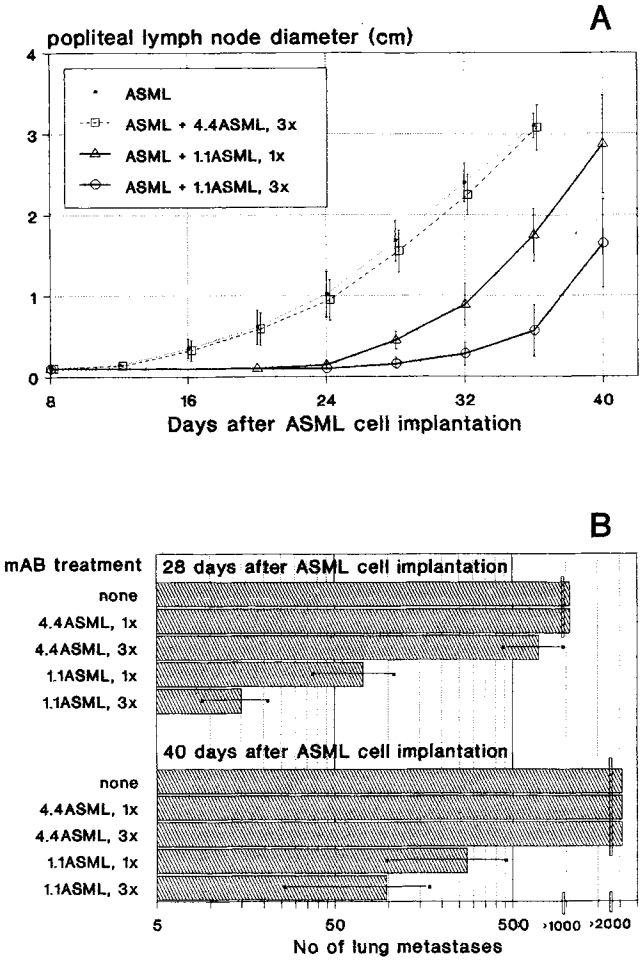


Figure 4. mAb-induced retardation of metastasis formation requires binding to CD44v. Rats (five/group) received 5 × 10^5 ASML cells intrafootpad, concomitantly with 200 μg 1.1ASML or 4.4ASML, intravenously. Where indicated, mAb application was repeated 3 and 6 d later. The mean diameter and standard deviations of the draining popliteal lymph nodes (*A*) and the number of lung metastases (*B*) are shown.

mune mechanisms had to be excluded: (*a*) cytotoxic activity of the antibody by itself or via complement; (*b*) antibody-dependent cellular cytotoxicity and (*c*) activation of Ig (1.1ASML)-specific T cells.

(*a*) The antibody is not cytotoxic by itself. When ASML and AS-14 cells were cultured for 4–96 h in the absence of mAb or presence of control mAbs 3–9, Ox50 (anti-CD44s), or 1.1ASML, thymidine incorporation was not reduced at all in cultures containing 1.1ASML as compared to cultures

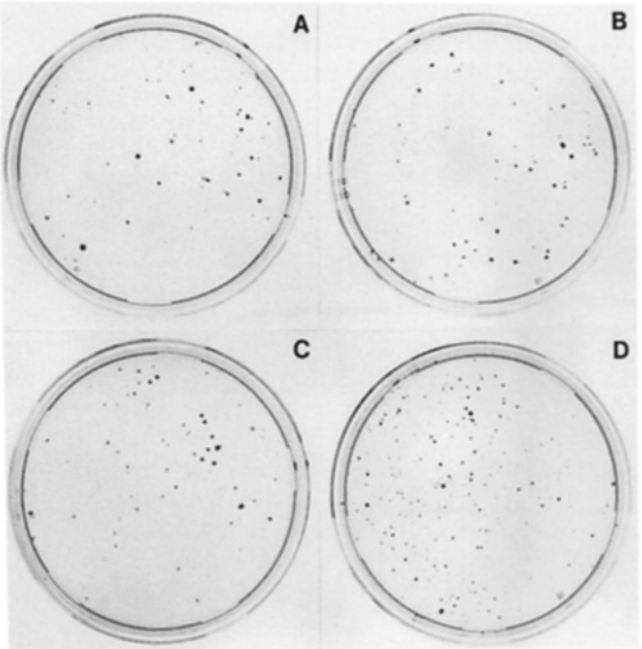


Figure 5. mAb 1.1ASML is neither cytotoxic nor cytopathic. ASML cells (800) were seeded in soft agar containing no mAb (*A*), 10 μg/ml 3-9 (*B*), Ox50 (*C*), and 1.1ASML (*D*), respectively. Plates were incubated for 7 d at 37°C, 5% CO_2 in air. Thereafter colonies were counted: 144 (*A*), 163 (*B*), 152 (*C*), 738 (*D*).

Table 3. *Antibody- and Complement-mediated Lysis*

Target	Antibody*	Percent cytotoxicity	
		No C‡	C‡
ASML	-	3.2	5.7
	3-9	2.2	5.3
	1.1ASML	1.9	7.4
	Anti-BDX	2.3	21.2§
AS-14	-	4.1	8.1
	3-9	4.1	7.9
	1.1ASML	2.8	9.6
	Anti-BDX	3.4	78.3§

* Target cells were preincubated with the mAb 3-9 (control IgG1) or 1.1ASML (IgG1), or with DA anti-BDX IgG at a concentration of 10 μg/ml.
‡ Guinea pig serum as source of complement, 1:5 diluted in HBSS.
§ A significant increase ($p < 0.05$) in cytotoxicity in the presence of antibody.

where 3-9, Ox50, or no antibody was added. Interestingly enough, addition of 1.1ASML, but not of 3-9 or Ox50, reproducibly enhanced the cloning efficiency of ASML (and AS-14; data not shown) cells (Fig. 5). Thus, at least in vitro, any cytopathic effect of 1.1ASML could be excluded.

In addition, neither in the absence nor in the presence of complement was a considerable increase in the lysis of ^{51}Cr-labeled target cells observed by addition of 1.1ASML, although tumor cells were lysed in the presence of complement and an alloantiserum directed against the MHC haplotype of the tumor (Table 3).

(*b*) Antibody-dependent cellular cytotoxicity is a priori unlikely because of the IgG1 isotype of 1.1ASML. With peritoneal macrophages and NK-enriched spleen cells, the cytotoxic potential of the effector cells was only insignificantly increased for 1.1ASML-loaded vs. unloaded tumor cells. This applies to ASML as well as AS-14 target cells, with lysis in the presence of 1.1ASML being augmented by only 2-10% (Table 4). Antibody-dependent cytotoxicity by unseparated spleen cells, including granulocytes, and by LAK cells could not be detected at all (data not shown). It should, however, be noted that AS-14 cells are highly susceptible to lysis by NK cells, LAK cells (data not shown), and macrophages. At a 50:1 ratio of E/T cells, 36.4 and 49.6%, respectively, of AS-14 cells are lysed, while ASML cells are rather resistant (NK lysis, 2.4%; and MΦ lysis, 16.3%). Furthermore, in the presence of an alloantiserum AS-14 cells were lysed at a considerably higher degree than ASML cells. However, as will be discussed below, there is no evidence that the high susceptibility of AS-14 cells for immune effector mechanisms is relevant for the more efficient eradication by 1.1ASML treatment of AS-14 than of ASML cells.

(*c*) Finally, the possibility had to be taken into account that mouse Ig-specific cytotoxic T cells may take part in the prevention of metastatic spread in 1.1ASML-treated rats. Although Ig-specific helper T cells were present after application of 1.1ASML in adjuvant (54), neither Ig-specific helper (data not shown) nor cytotoxic T cells could be detected after intravenous application of 1.1ASML. After in vitro restimulation with tumor cells plus 1.1ASML, T cells from draining lymph nodes of tumor-bearing rats did not reveal any significant degree of cytotoxic potential for 1.1ASML-coated or noncoated target cells. This was irrespective of whether the T cells were derived from 1.1ASML-treated or untreated rats. Since AS-14 cells and, to a lower degree, ASML cells were lysed by cytotoxic T cells from DA rats, the experiment excludes activation of tumor cell-specific as well as Ig-specific cytotoxic T cells (Table 5).

The idea that retardation/prevention of metastasis forma-

Table 4. *Antibody-dependent Cellular Cytotoxicity*

Effector cells*	E/T	Percent cytotoxicity					
		ASML +			AS-14 +		
		None	1.1ASML	Anti-BDX	None	1.1ASML	Anti-BDX
NK	100:1	3.8	5.4	19.7‡	50.6	60.5	78.5‡
	50:1	2.4	4.1	13.2‡	36.4	44.1	59.3‡
	25:1	1.1	2.3	7.9‡	28.2	34.3	42.2‡
MΦ	50:1	16.3	23.0	37.8‡	49.6	53.1	85.6‡
	25.1	10.2	14.6	23.1‡	38.3	42.1	63.7‡
	12.1	6.5	11.2	19.4‡	24.2	31.7	48.5‡

* NK, spleen cells, Percoll fraction of σ 1.077; MΦ, adherent peritoneal cells.
‡ A significant increase ($p < 0.05$) in cytotoxicity in the presence of antibody.

Table 5. *Ig-specific Cytotoxic T Cells*

Lymphocyte donor	Stimulus‡	Percent cytotoxicity*			
		ASML	ASML + 1.1ASML	AS-14	AS-14 + 1.1ASML
BDX	ASML	6.1	3.2		
BDX	ASML + 1.1ASML	5.3	5.2		
DA	BDX lymphocytes	23.1			
BDX	AS-14			14.0	12.9
BDX	AS-14 + 1.1ASML			14.1	11.2
DA	BDX lymphocytes			68.5	

* Cytotoxicity values are shown for a ratio of E/T cells of 10:1.
‡ BDX lymph node cells were derived from tumor-bearing rats that had or had not received 1.1ASML treatment. They were restimulated in vitro under the same conditions. DA lymph node cells were derived from DA rats that had received two injections of irradiated BDX lymphocytes and were restimulated in vitro with BDX lymphocytes.

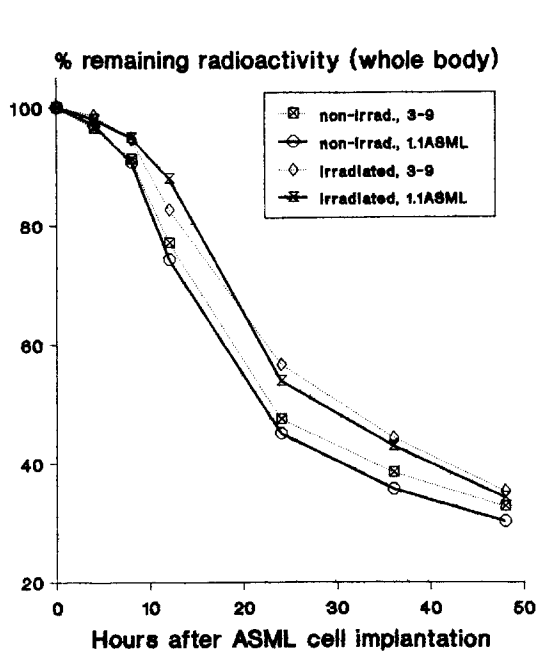
tion by 1.1ASML is not based on immune effector mechanisms was further supported by the following in vivo experiments. The possibility of an ADCC-like mechanism functioning in vivo was evaluated by simultaneous injections of ^{125}I-UDR-labeled ASML cells (intrafoodpad) and 1.1ASML or 3-9 (intravenous) in nonirradiated and sublethally irradiated rats. The survival rate of tumor cells was recorded during the following 48–240 h by whole body counting. Throughout the first 48 h, the tumor cell decay (ASML and AS-14) was totally independent of 1.1ASML application. This accounted for nonirradiated as well as irradiated rats (Fig. 6 *A*). Only thereafter was tumor cell decay slightly accelerated in 1.1ASML- as compared with mock-treated rats, the accelerated decay again being independent of immunocompetence of the host (Fig. 6 *B*). Thus, it appears unlikely that retardation/prevention of metastases formation under 1.1ASML treatment relies on an ADCC-like mechanism.

In conformity with the in vitro results, there was no evidence for a tumor-specific and/or 1.1ASML-mediated T cell response in vivo. Lymph node cells from rats, which had received AS-14 cells and 1.1ASML treatment and remained free of metastases, were transferred into irradiated rats that received concomitantly 3×10^5 AS-14 cells. Metastases formation was not at all retarded or inhibited in comparison with rats that had received lymph node cells from untreated rats (data not shown). We conclude that prevention of metastatic spread by 1.1ASML is not due to activation of any kind of immune defense mechanisms.

Exclusion of 1.1ASML-mediated Modulation of Expression of CD44v. Since a defined threshold level of surface expression is probably essential to initiate metastatic spread of non-metastasizing cells transfected with CD44v cDNA (35; Hofmann et al., manuscript in preparation), we now investigated whether anti-CD44v treatment results in downregulation of CD44v expression. In fact, this was not the case. Fluorescence staining of ASML and AS-14 cells, which were cultured in the presence or absence of 1.1ASML for 1 wk, revealed no decrease in the intensity of staining with 1.1ASML (Fig. 7, *A* and *B*). Also, in vivo, expression of CD44v was not downmodulated under 1.1ASML treatment (shown for ASML cells isolated from draining lymph nodes; Fig. 7 *C*). Accordingly, AS-14 lung nodules of 1.1ASML-treated and untreated rats were stained with 1.1ASML at comparable intensity (data not shown).

Expression of CD44v Is Required during Embedding and/or Outgrowth of Metastatic Cells in Lymph Nodes. A further hint at the possible function of CD44v during metastatic spread was derived from experiments in which the application of anti-CD44v was delayed. Starting 1.1ASML treatment at the time of tumor cell inoculation prevented metastasis formation and/or prolonged the survival time. Even after a single injection of 1.1ASML, formation of metastasis was delayed. However, 1.1ASML was inefficient when injected 10–14 d after tumor cell inoculation, irrespective of excision of the local tumor and the draining popliteal lymph node (Table 6).

The finding that expression of CD44v is required early during metastasis formation was further supported by excision of the popliteal lymph node after intrafootpad application of 5×10^6 ^{125}I-UDR-labeled tumor cells into irradiated rats (8.5 Gy) and determining the level of radioactivity in parallel with the number of tumor cell colonies in soft agar. A peak of radioactivity was detected 4 d after tumor cell inoculation. This was independent of the concomitant application of 3-9 or 1.1ASML. Thereafter, radioactivity declined more rapidly in the draining lymph node of 1.1ASML- as compared with mock-treated rats, indicating a 1.1ASML-dependent, decreased survival rate of tumor cells in the draining lymph node. Lymph nodes excised at 10 d after tumor cell inoculation were dispersed and cloned in soft agar. In the lymph node of 3-9-treated rats, >2,000 ASML and >1,000 AS-14 colonies were detected. In 1.1ASML-treated rats, 51 ASML and 19 AS-14 colonies appeared. This indicates that



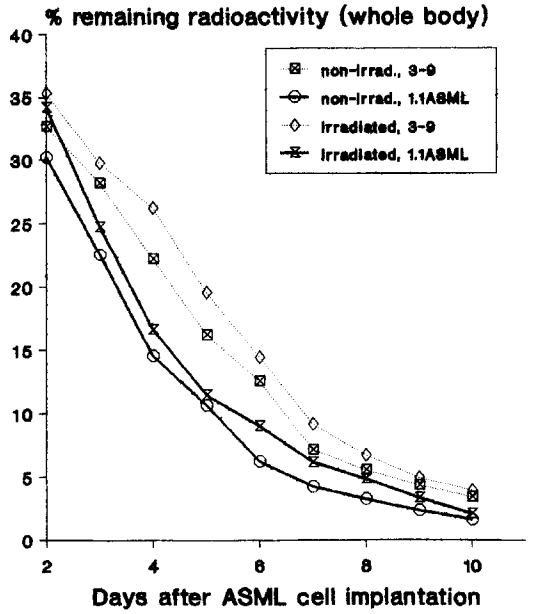


Figure 6. In vivo elimination of ^{125}I-DUR-labeled ASML cells in immunocompromised rats. Nonirradiated and irradiated (8.5 Gy) BDX rats received 5 × 10^6 ^{125}I-UDR-labeled ASML cells, intrafootpad, together with 200 μg 3-9 or 1.1ASML, intravenously. Antibody application was repeated every 3 d. Mean percent of retention of radioactivity (whole body counts, five rats/group) was determined after 4–240 h.

Table 6. *Delayed Injection of 1.1ASML Does Not Inhibit Metastatic Spread*

Line*	1.1ASML‡ treatment (day)	Excision (day)	Tumor takes	Moribund state
				d
ASML	–	–	5/5	55
	–	10	5/5	57
	0	–	5/5	95
	0	10	2/5	120
	10	–	5/5	59
	10	10	5/5	67
AS-14	–	–	5/5	59
	–	14	5/5	63
	0	–	2/5	109
	0	14	0/5	–§
	14	–	5/5	68
	14	14	5/5	95

* 5 × 10^5 ASML or AS-14 cells, intrafootpad.
‡ 200 μg 1.1ASML, intravenously, twice per week, starting at the indicated day after tumor cell application.
§ Rats were killed at day 150 and were tumor free.

tumor cells had started to divide in mock-treated, but not in ASML-treated rats (Fig. 8). Thus, expression of CD44v is required early in metastasis formation. In particular, CD44v appears to be important for settlement and outgrowth of tumor cells in the lymph node.

Discussion

Overexpression of CD44v is sufficient to confer metastatic potential to nonmetastasizing tumor cells (35). Since several tumor lines, irrespective of their histology, acquired metastatic potential with CD44v expression (M. Hofman et al., manuscript in preparation), it appears that CD44v implements a common limiting function for tumor cell progression. So far, the smallest version of CD44v conferring metastatic properties carries only 85 amino acids (including the epitope for 1.1ASML) of extra sequence in the variant part of the molecule (42a). We have shown now that mAb 1.1ASML binding within this variant part of CD44 interferes strongly with the outgrowth of metastases. The following features could be instructive as to the basic function of the CD44v molecule.

(*a*) 1.1ASML affects lymphatic spread of both ASML and AS-14 cells. Yet, metastasis formation of AS-14 cells is more efficiently inhibited. This indicates that ASML and AS-14 cells differ in properties not transferred by CD44v. We know that ASML and AS cells, AS-14 cells being derived from the latter, differ in susceptibility to NK cells and macrophages, in the morphology of tumor nodules and in their cytoskeletal

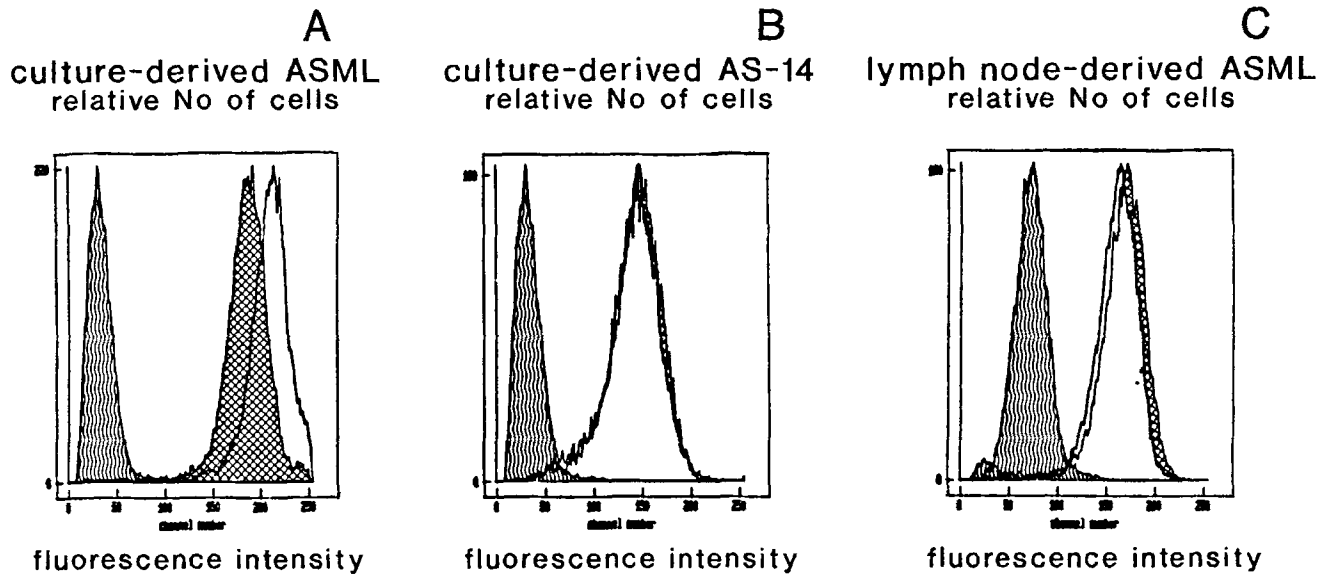


Figure 7. Anti-CD44v does not modulate expression of CD44v. ASML (*A*) and AS-14 (*B*) cells were cultured for 1 wk in the absence or presence of 10 μg/ml 1.1ASML. (*C*) ASML cells were derived from the draining lymph node of ASML-bearing untreated and 1.1ASML-treated rats. Tumor cells were stained with 1.1ASML as first, and FITC-labeled anti-mouse IgG1 as second, antibody. (*Striped*) Negative control; (*blank*) cultured or grown in vivo in the absence of 1.1ASML; (*crosshatched*) cultured or grown in vivo in the presence of 1.1ASML.

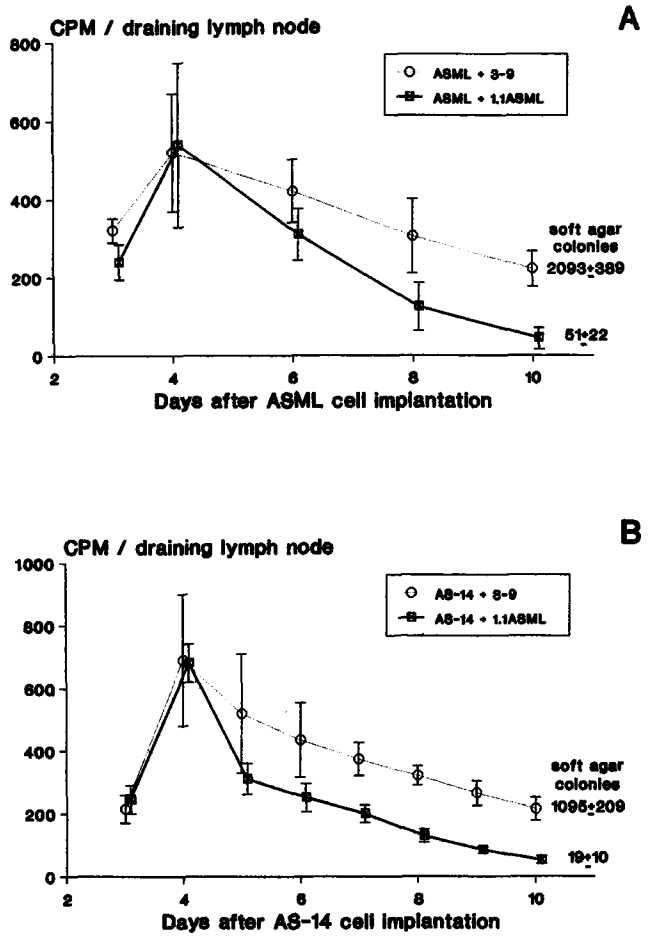


Figure 8. Influence of anti-CD44v on tumor cell decay and proliferation in the draining lymph node. Sublethally irradiated (8.5 Gy) BDX rats received 5 × 10^6 ^{125}I-UDR-labeled ASML (*A*) and AS-14 (*B*) cells, intrafootpad, and every 3 d, 200 μg mAb, intravenously, starting at the time of tumor cell inoculation. The draining and the contralateral popliteal lymph nodes of three rats per group were excised at the indicated time points, and radioactivity was determined in a gamma counter. Mean cpm of the draining lymph node minus mean cpm of the contralateral node (12–37 cpm) are shown. At 10 d after tumor cell application, the excised lymph nodes were meshed and plated in soft agar. The number of colonies visible after 7 d of culture is reported.

structure (42, 47, 55–58). It is plausible that molecular functions in addition to CD44v influence invasiveness and metastatic potential. Obviously, however, CD44v represents a decisive element in metastasis formation of both ASML and AS-14 cells.

(*b*) 1.1ASML does not function by implementing immune effector cells or by initiating an Ig-specific T cell response. The whole array of antibody-mediated or antibody-induced immune defense mechanisms was tested in vitro and/or in vivo and found to be inefficient. On the contrary, under in vivo conditions, 1.1ASML supports the cloning efficiency of tumor cells. In addition, the higher susceptibility of AS-14 cells for nonadaptive immune effector cells (47) could facilitate tumor cell eradication. Yet, this is not a decisive factor, since we observed in sublethally irradiated rats, as well, a blockade of AS-14 outgrowth in the presence of 1.1ASML (Fig. 8). Finally, 1.1ASML failed to mount an efficient anti-mouse Ig cytotoxic T cell response (59). This was not actually surprising, if one is aware of an important physiological function of CD44v in lymphocyte activation. During the activation process, T cells express CD44v, and activation can be inhibited efficiently by anti-CD44v (43), i.e., anti-CD44v displays immunosuppressive properties.

(*c*) 1.1ASML is only efficient when present before the establishment of metastatic colonies. In addition, evaluation of remaining radioactivity in the draining lymph node after injection of ^{125}I-UDR-labeled tumor cells indicates that comparable numbers of tumor cells reach the draining lymph node in 1.1ASML- and mock-treated rats. Yet, outgrowth of tumor cells appears to be inhibited. These findings, together with the observation that 1.1ASML does not downregulate expression of CD44v, provide the basis of our working hypothesis on the function of CD44v and on the interference by 1.1ASML. CD44v is required early in lymphogenic spread. The antibody appears to cover efficiently CD44v-positive cells during migration and prevents CD44v from passing through a decisive interaction with an as yet unknown ligand. Ligand binding could be an initial step for signal transduction and/or activation of an enzymatic machinery, which allow for selective survival and expansion of tumor cells in lymph nodes and lung. If this interaction is blocked long enough, the cells cannot survive and the animal remains free of metastases.

Irrespective of the functional definition of CD44v at the molecular level, the observation that by an antibody blockade metastatic spread can be obliterated has importance in itself. Although retardation of metastatic spread by mAbs directed against cell surface receptors required for metastatic progression has been described in several systems (54, 60–65), prevention of metastasis formation by a mAb, to our knowledge, was observed for the first time. This finding undoubtedly is most promising for the development of new therapeutic strategies.

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Address correspondence to Margot Zöller, Deutsches Krebsforschungszentrum, Institut für Radiologie und Pathophysiologie, Im Neuenheimer Feld 280, D-6900 Heidelberg 1, Germany.

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