Efficient Inhibition of Intra-Peritoneal Tumor Growth and Dissemination of Human Ovarian Carcinoma Cells in Nude Mice by Anti-L1-Cell Adhesion Molecule Monoclonal Antibody Treatment

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Abstract
The L1 cell adhesion molecule is implicated in the control of proliferation, migration, and invasion of several tumor cell types in vitro. Recently, L1 overexpression was found to correlate with tumor progression of ovarian carcinoma, one of the most common causes of cancer-related deaths in gynecologic malignant diseases. To evaluate L1 as a potential target for ovarian cancer therapy, we investigated the effects of anti-L1 monoclonal antibodies (e.g., 7E7 and L1-11A) on proliferation and migration of L1-positive human SKOV3ip ovarian carcinoma cells in vitro and the therapeutic efficacy of L1-11A against i.p. SKOV3ip tumor growth in nude mice. In vitro, both anti-L1 antibodies efficiently inhibited the proliferation of SKOV3ip cells as well as other L1-expressing tumor cell lines (renal carcinoma, neuroblastoma, and colon carcinoma). On two cell lines, hyper-cross-linking of L1-11A with a secondary antibody was necessary for significant inhibition of proliferation, indicating that cross-linking of L1 is required for the antiproliferative effect. L1-negative prostate carcinoma cells were not influenced by antibody treatment. Biweekly treatment of ovarian carcinoma-bearing mice with L1-11A led to a dose-dependent and significant reduction of tumor burden (up to ~63.5%) and ascites formation (up to ~75%). This effect was associated with reduced proliferation within the tumors. L1-directed antibody-based inhibition of peritoneal growth and dissemination of human ovarian carcinoma cells represents important proof-of-principle for the development of a new therapy against one of the leading gynecologic malignant diseases. (Cancer Res 2006; 66(2): 936-43)

Introduction
Ovarian carcinomas are one of the most highly malignant cancers in women and are prevalently characterized by extremely poor prognosis for the patients. One major problem is the absence of clear symptoms and the marginal number of predictive markers. Consequently, at the point of diagnosis, most patients reveal late-stage ovarian cancer, which is characterized by widespread peritoneal dissemination and ascites (1). All established therapies reveal a poor efficiency in the late stage of the disease, and although therapies have been further optimized in the last decade, the mortality due to ovarian cancer remains uncharged (2). Therefore, new therapeutic strategies for ovarian cancer treatment are urgently needed.

In a variety of human malignancies, tumor progression is associated with changes in expression of cell adhesion molecules (CAMs; ref. 3). One of these membrane-bound CAMs, L1, was recently detected in ovarian and uterine carcinomas in a stage-dependent manner and its expression was found to be a valuable marker for poor prognosis (4). L1 expression is also correlated with tumor progression and metastasis of several other types of cancer, including malignant gliomas (5), recurrent neuroblastoma (6), cutaneous malignant melanoma (7), renal cell carcinoma (8), and colon carcinoma (9). L1 promotes many cellular activities by interacting through its extracellular domain with other CAMs, extracellular matrix molecules, and cell surface receptors (10, 11) leading to direct or indirect transmission of signals regulating cell differentiation, proliferation, migration, and invasion (12-14). In carcinomas, ectopic expression of L1 enhanced cell motility and invasiveness in cell culture as well as tumor growth in nude mice (9, 15). The extracellular domain of L1 can be released from the cell surface via proteolytic cleavage involving proteinases, such as plasmin and ADAM10 (15-18). Soluble L1 can stimulate cell migration and survival through autocrine/paracrine binding to integrins (15, 19). In addition, shed L1 can also serve as a substrate for adhesion and migration of tumor cells (20). Soluble L1 is also found in the serum and ascites of uterine and ovarian carcinoma patients (4).

Because L1 was recently identified as a potentially novel drug target (21), we wanted to investigate the effects of anti-L1 monoclonal antibodies (mAb) on proliferation of different L1-positive tumor cell lines in vitro and on ovarian tumor growth in vivo. We have used an i.p. tumor model of SKOV3ip-lacZ ovarian carcinoma cells in athymic immunodeficient nude mice. With this model, we mimic late-stage ovarian cancer, with both extensive dissemination of ovarian carcinoma cells to the peritoneal wall and organ surfaces and the development of massive ascites. lacZ tagging and subsequent staining with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosid (X-gal) allows straightforward distinction between tumor and host tissue (22), facilitating quantification of therapy effects with high resolution in this model. We show that L1 represents a valuable therapeutic target as we could show the potential of an anti-L1 mAb to suppress tumor growth, local dissemination, and ascites formation in this xenograft human ovarian carcinoma model.
Materials and Methods

Cell lines and cell culture. SKOV3ip human ovarian carcinoma cells (kindly provided by Ellen Vitetta, University of Texas, Dallas, TX) and HCT116 human colon carcinoma cells were grown in DMEM (Biokrom, Berlin, Germany) with 10% FCS under cell culture conditions (5% CO₂, 95% relative humidity, 37°C). For identification and quantification of tumor mass, the SKOV3ip cells were stably transduced with a lacZ-encoding retroviral vector (GeneSuppressor Retroviral System, Biocarta, Hamburg, Germany). HEK293-hL1 cell generation and cultivation was described previously (14).

The following cell lines were cultured in medium and supplements were supplied by Bioconcept (Allschwil, Switzerland; 1 mmol/L glutamine, 10% FCS, and penicillin-streptomycin-fungizone). SK-N-AS [DMEM, 1% nontoxic amino acids (NEAA)] and SK-N-BE2c (Ham’s F-12/MEM, 1% NEAA) human neuroblastoma cells were obtained from the Institute of Pathology University Hospital Zurich (Zurich, Switzerland), respectively. PC3 (DMEM) human prostate carcinoma were obtained from American Type Culture Collection (Manassas, VA).

Antibodies. The mAb L1-11A to the ectodomain of human L1 (subclone of UJ 127.11) was used for Western blot analysis as described before (15). For in vitro and in vivo use, L1-11A was purified by Invivo BioTech Services GmbH (Henningsdorf, Germany). chCE7, a chimeric IgG1 directed against EpCAM, was described before and binds to all human adenocarcinomas (24). HEA125, a mouse IgG1 directed against EpCAM, was described before and binds to all human adenocarcinomas (25). Anti-His antibody (tetra-His antibody) was purchased from Qiagen AG (Hombrechtikon, Switzerland). Goat anti-mouse IgG was affinity purified and absorbed to human serum proteins (Zymed Laboratories, Inc., San Francisco, CA).

Cell proliferation assay. Cells (8,500-56,000, depending on the cell lines used) were seeded in quadruplicate in six-well dishes (Falcon, Milian SA, Geneva, Switzerland) in 2 ml medium containing 10% FCS. After 6 hours, when cells had attached to the plates, antibodies (sterile filtered, in azide-free PBS) were added at concentrations of 5 μg/ml. For hyper-cross-linking of L1-11A via anti-mouse IgG, equimolar concentrations of both antibodies were added to Caki-2 renal carcinoma and SK-N-AS neuroblastoma cells. For comparison, treatments were also done with individual antibodies alone. After 48 hours, antibody additions were repeated. Cells were grown for up to 96 hours, detached in PBS containing 1 mM EDTA, washed twice in PBS, and counted in a hemocytometer. Statistical significance of data was evaluated with Student’s t test (two-tailed, unequal variance).

Viability of cells was tested by a dye exclusion assay. SKOV3ip cells were grown in six-well plates in DMEM, as described, in the presence of 10% FCS controls (or) 5 μg/ml mAb L1-11A, 5 μg/ml mAb chCE7, or 5 μg/ml mAb HEA125. After 48 and 72 hours, cells were detached using PBS, including 1 mM EDTA, washed twice in PBS, and resuspended in 1 mL PBS. Cell suspension (100 μL) was incubated with 100 μL trypan blue solution for 4 minutes at 37°C. Cells from quadruplicate samples were counted with a hemocytometer. Viability [% Live cells = Unstained cells / (Stained cells + Unstained cells)] ranged between 88% and 95% and no significant difference was found between the different samples.

For cross-linking experiments, SKOV3ip cells were seeded in quadruplicate into six-well plates. After 6 hours, chCE7 antibody (5 μg/ml), chCE7-F(ab)’ fragments (15 μg/ml), or anti-His antibody (10 μg/ml) were added; after 48 hours, antibodies additions were repeated and cells were grown for up to 96 hours.

Transmigration assay. The haptotactic cell migration assay was done in Transwell chambers (Corning Costar, Corning, NY) and was described in detail elsewhere (15). After 16 hours, the transmigrated cells at the backside of the filter were stained with crystal violet solution as described (15). The eluted dye was measured at 595 nm in an ELISA reader. Each determination was done in quadruplicate and data are shown as mean ± SE.

Positron emission tomography imaging for in vivo detection of tumor growth. Nude mice (CD-1 nu/nu, female, 5 weeks) from Charles River, Inc. (Sulzfeld, Germany) were used for the experiments. In a pilot experiment, growth of SKOV3ip tumors was monitored by sequential positron emission tomography (PET) imaging with [18F]Cu-CPTA-labeled mAb chCE7. A nude mouse, which had been injected i.p. with 5 × 10⁶ SKOV3ip human ovarian carcinoma cells, and a control mouse, which did not receive SKOV3ip cells, were imaged once weekly for a period of 3 weeks. For the PET experiment, tumor mice were imaged using the dedicated small animal PET tomograph NanoPET (Oxford Positron Systems, Oxford, United Kingdom). Animals were injected with 25 Mbq (150 μg) [18F]Cu-CPTA-chCE7, prepared as described (26), via a lateral tail vein. Animals were anesthetized 2 hours later and scanned as described before. PET data were acquired in list mode for 60 to 90 minutes and reconstructed in a single time frame using the OPL-EM algorithm (0.5 mm bin size, 200 × 240 × 240 matrix size). Image files were analyzed using the dedicated software Pmod (27).

Tumor model and therapy. Pathogen-free, female athymic CD1 nu/nu mice (7-9 weeks old; 20 g on average; Charles River) were inoculated with 5 × 10⁶ human lacZ-tagged ovarian carcinoma cells (SKOV3ip-lacZ) into the peritoneal cavity at day 0, leading to i.p. tumor formation within 5 weeks. Anti-L1 mAb L1-11A was diluted in sterile PBS to the concentrations needed for treatment. Tumor-bearing mice were treated i.p. twice weekly with a 300 μl solution of the respective L1-11A dosage (1, 5, or 10 mg/kg per application, respectively), vehicle (PBS), or unlabeled IgG antibody control. Antibody treatments started from day 3 after tumor cell injection to give the tumor cells time to attach to the inner side of the abdominal wall and the surfaces of the i.p. organs. At autopsy (day 38), ascites was sampled from all mice, the volume was determined and aliquots were preserved for L1-ELISA. A small piece of visible tumor mass was cut off, weighed, and preserved for Western blot analysis of proliferating cell nuclear antigen (PCNA). Following this, all i.p. organs (including tumor mass), the abdominal wall, and the diaphragm were removed, stained with β-galactosidase substrate (X-gal; Roche-Diagnostics, Penzberg, Germany), photographed, and weighed. The indigo blue tumor mass between the organs, on the diaphragm and the inner site of the abdominal wall, was removed and weighed alone. The relative tumor burden in each mouse was calculated by dividing tumor mass weight by total situs weight.

Western blot analysis and ELISA. Western blot analysis of tumor tissue samples for PCNA and α-tubulin was done as described previously (28). L1 expression levels in ascites samples of representative mice from treatment groups and the control were analyzed by L1 ELISA as described previously (26).

Statistical analysis. Throughout the study, results are presented as mean ± SE. In the in vitro proliferation studies, differences between groups were analyzed using the Student’s t test (two-tailed, unequal variance). In the mouse studies, differences between groups were analyzed using the Mann-Whitney rank-sum test.

Results

Inhibition of tumor cell proliferation in vitro by anti-L1 mAbs. As L1 is discussed to be involved in the regulation of tumor growth, we tested if tumor cell proliferation can be inhibited by anti-L1 mAbs in vitro. Treatment with the anti-L1 mAb chCE7 led to a significant (P < 0.05) reduction of proliferation of all L1-expressing tumor cells tested in this experiment by ~40% (Föhn renal carcinoma) to ~61% (SK-N-BE2c neuroblastoma) compared with the respective untreated controls (Table 1). The second anti-L1 mAb L1-11A also significantly inhibited (P < 0.05) the proliferation of the ovarian carcinoma SKOV3ip, the renal carcinoma Föhn, the neuroblastoma SK-N-BE2c, and the colon carcinoma HCT116 by ~35% (HCT116) to ~60% (Föhn; Table 1). Proliferation of the renal carcinoma cell line Caki-2 and the neuroblastoma cell line SK-N-AS was not influenced significantly by this mAb. However, hyper-cross-linking of L1-11A with anti-murine IgG led to significantly (P < 0.05) reduced proliferation of
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these cells (Table 1). Treatment with the anti-EpCAM mAb HEA125 inhibited only the neuroblastoma cell line SK-N-BE2c significantly ($P = 0.0005$) by $\sim 31\%$, whereas the proliferation of all other L1-positive cell lines remained unaffected by the antibody when compared with the respective untreated control cells (Table 1). Proliferation of the L1-negative prostate carcinoma cell line PC3 was not inhibited by the anti-EpCAM mAb HEA125 or the anti-L1 mAbs (L1-11A and chCE7; Table 1). All three antibodies did not affect the viability of cell lines as tested with the trypan blue exclusion assay (data not shown).

Because the focus of our study was on L1-dependent ovarian cancer progression, we investigated the antiproliferative effect of the three antibodies (L1-11A, chCE7, and HEA125) on SKOV3ip cells more closely over a longer period of time up to 96 hours (Fig. 1A). When SKOV3ip cells were grown in medium containing 10% FCS, the growth inhibitory effect of the anti-L1 antibodies was found to be significant at 48 hours and at later times (Table 1; data not shown). When cells were grown in medium containing low FCS (0.5%), the extent of inhibition by the mAbs was similar, but the effect was delayed and was significant after 72 hours ($P < 0.02$) and 96 hours ($P < 0.01$; Fig. 1A). The mAb HEA125 had no significant influence on growth over the whole time course of the experiment.

To examine whether cross-linking of L1 on the cell surface of SKOV3ip cells is required for the growth inhibition observed in the presence of anti-L1 antibodies, we next compared the antiproliferative activity of the His-tagged L1-specific monovalent F(ab') fragment of chCE7 with complete mAb chCE7 (Fig. 1B). Using equimolar concentrations of both antibodies, only the intact chCE7 inhibited the growth of SKOV3ip cells. However, when the F(ab') fragments were cross-linked by an anti-His antibody, a significant ($P < 0.05$) growth inhibition of SKOV3ip cells was observed in the same range as the complete chCE7 antibody (Fig. 1B).

Inhibition of L1-mediated haptotactic cell migration in vitro by anti-L1 mAb L1-11A. L1 is involved in the enhanced migration of ovarian carcinoma cells in vitro (14). Therefore, we wanted to investigate if mAb L1-11A could inhibit this process. We studied the haptotactic migration of the two L1-positive ovarian cancer cell lines, SKOV3ip and OV-MZ-6, using Transwell chambers. Treatment with mAb L1-11A led to a substantial inhibition of haptotactic migration of SKOV3ip cells (~51% reduction; Fig. 2A, column 1) and OV-MZ-6 cells (~34% reduction; Fig. 2B, column 2) on fibronectin compared with the untreated control (Fig. 2A and B, column 1). In contrast, treatment with an isotype control antibody did not influence migration of the SKOV3ip cells (Fig. 2A, column 3) and only minimally affected the migration of OV-MZ-6 cells on this substrate (~9% reduction; Fig. 2B, column 3). Treatment with an anti–integrin $\alpha_5$ antibody, used as positive control, potently inhibited haptotactic cell migration of both cell lines on fibronectin (SKOV3ip ~70% reduction; OV-MZ-6 ~86% reduction; Fig. 2A and B, column 4).

To confirm that the observed reduction in cell migration was specific for L1, we compared the migration of HEK293-hL1 cells with the parental HEK293 cell line. As shown in Fig. 2C (column set 1), expression of L1 in the HEK293-hL1 cells significantly enhanced their haptotactic migration by ~3 times compared with parental HEK293 cells in agreement with previous results (14). Treatment with the anti-L1 mAb blocked almost completely this enhancement of migration of the L1-expressing transfectants, whereas the basal L1-independent migratory activity of HEK293 cells was not influenced (Fig. 2C, column set 2). The treatment with anti–integrin $\alpha_5$ antibody revealed again a strong inhibitory effect on the migration of L1-expressing cells but was only marginally effective on the basal migration of the parental HEK293 cells (Fig. 2C, column set 3). Monitoring of i.p. ovarian carcinoma tumor progression with radiocopper-labeled anti-L1 mAb chCE7. To investigate

<table>
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<tr>
<th>Human tumor cell lines</th>
<th>Time (h)</th>
<th>% Increase in cell number compared with control (100%)</th>
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<td></td>
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<td>mAb L1-11A</td>
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<td>SKOV3 ovarian carcinoma</td>
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<td>61.8 ± 13.0, significant ($P = 0.009$)</td>
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<td>64.9 ± 9.6, significant ($P = 0.009$)</td>
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<td>PC3 (L1-negative) prostate carcinoma</td>
<td>48</td>
<td>93.4 ± 18.8, not significant ($P = 0.708$)</td>
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NOTE: Cells were grown in the presence of 10% FCS and 5 $\mu$g/mL antibodies as described in Materials and Methods. In addition, Caki-2 and SK-N-AS cells were also incubated with anti-L1 mAb L1-11A hyper-cross-linked with anti-mouse IgG (*). Data are cell numbers after 48 or 72 hours expressed as percentage of controls in the absence of antibodies (mean ± SD of quadruplicate samples).
the i.p. tumor growth progression over time course and to show the tumor-targeting capability of L1 antibodies in our SKOV3ip-lacZ xenotransplantation mouse model, we monitored SKOV3ip tumor growth and dissemination in vivo via PET imaging using a radioactively labeled anti-L1 mAb (64Cu-CPTA-chCE7). One day after inoculation of 5 × 10^6 SKOV3ip cells into the peritoneal cavity of a nude mouse, PET imaging revealed only nonspecific background signals in the lymph nodes and some organs (Fig. 3, top left, left mouse). The same background pattern was detected in the tumor-free control mouse over the whole time course of the experiment (Fig. 3, right mouse in all four images). Eight days after i.p. inoculation of SKOV3ip cells, formation of tumor nodules near the left ovary were clearly detectable (Fig. 3, top right, arrow). At day 16, the size of these tumor nodules was significantly increased and further tumor mass could be detected beneath the liver and near the right ovary (Fig. 3, bottom left, arrows). One week later (day 23), the size of all detected tumor mass revealed a substantial further increase (Fig. 3, bottom right, arrows), indicating a continuous progression of SKOV3ip ovarian cancer tumor growth in nude mice. Concerning the tumor-targeting ability of L1 antibodies in vivo, images showed that the highest concentration of antibody was found in the tumor (20% ID/g of tumor 24 hours after i.v. application of the tracer as determined by measuring radioactivity of dissected tumor samples). Similar results were obtained with the anti-L1 mAb L1-11A (data not shown).

Inhibition of i.p. ovarian carcinoma tumor growth and ascites formation by L1-11A treatment. Next, we investigated whether i.p. SKOV3ip ovarian carcinoma growth in mice could be inhibited by treatment with the mAb L1-11A. SKOV3ip-lacZ cells were injected into the peritoneal cavity of female nude mice 2 days before the onset of therapy. To investigate possible dose-dependent
effects of L1-11A, biweekly i.p. treatments were done using three
different antibody concentrations (1, 5, and 10 mg/kg). Control mice
were treated with PBS or an IgG control antibody (biweekly 1 mg/kg
i.p.). The study was stopped at day 38 when the majority of mice in
the control groups were moribund. In contrast in the L1-11A
treatment groups, the mice were all vital and showed no signs of
ascites or other impairments. Explantation and X-gal staining of
the total situs at the end of the study revealed a widespread
dissemination of the tumor mass in the peritoneal cavity of control
mice (Fig. 4A, left). Tumor was detectable on the diaphragm, peritoneum parietale, and peritoneum viscerale, with a main
proportion beneath the liver, between the spleen, stomach, and
the cranial part of the intestine (Fig. 4A, left). In all anti-L1 mAb
treatment groups, the tumor sites revealed the same pattern of
localization as in the control (Fig. 4A, middle 1, middle 2, and right).
However, a substantial decrease in the amount of tumor mass
was visible in all three treatment groups compared with the control (Fig. 4A). The PBS-treated control mice revealed a mean tumor mass of 2.07 ± 0.35 g (equivalent to a proportion of 23.6 ± 3.0% of the total situs weight; Fig. 4B). Compared with the control, all
dosages of anti-L1 mAb led to a dose-dependent reduction of i.p.
tumor burden [L1-11A (1 mg/kg), −25.6%; L1-11A (5 mg/kg), −35.9%; L1-11A (10 mg/kg), −63.5%; Fig. 4B]. Tumor reduction in
the group treated with the highest dosage of L1-11A (10 mg/kg) was statistically significant ($P_{1-11A} (10 \text{ mg/kg}) = 0.01$) compared with the control. Differences between the control and the groups treated
with 1 and 5 mg/kg, respectively, showed a clear dose-dependent
trend although statistically not significant ($P_{1-11A} (1 \text{ mg/kg}) = 0.476$ and $P_{1-11A} (5 \text{ mg/kg}) = 0.111$). Mice treated with the IgG control
antibody revealed no detectable reduction of SKOV3ip-lacZ i.p.
tumor burden compared with the PBS-treated group (Fig. 4B). In
addition, in an additional experiment, treatment with an anti-
EpCAM mAb (HEA125) was not sufficient to reduce the tumor
burden in mice compared with the PBS-treated control (data not shown), although EpCAM is present on the SKOV3 cells; therefore,
HEA125 binds to the tumor cells. PBS-treated control mice as well
as mice treated with the IgG control antibody revealed massive
tumor-related ascites formation in the last week of the study,
whereas all L1-11A-treated mice did not show prominent swelling of the abdomen until the end of the experiment. This was confirmed
during autopsy, where large volumes of ascites could be collected
from PBS- and IgG-treated control mice (3.25 ± 0.80 mL = 100%; Fig. 4C), whereas the amount of ascites in all L1-11A-treated mice
was substantially reduced in a dose-dependent manner at the highest
dosage significantly [L1-11A (1 mg/kg), −19%; L1-11A (5 mg/kg), −48%; L1-11A (10 mg/kg), −75%; Fig. 4C]. Treatment of tumor-bearing mice with anti-L1 mAb also led to a treatment-correlating
decrease of L1 protein levels in ascitic fluid at all three dosages
[L1-11A (1 mg/kg), −34%; L1-11A (5 mg/kg), −49%; L1-11A (10 mg/kg), −64%; Fig. 4D] compared with the control (12.76 ng/mL = 100%; Fig. 4D). No side effects or severe toxicity of mAb L1-11A treatment
was observed during the whole course of treatment.

Detection of suppression of tumor cell proliferation in vivo.
The dose-dependent inhibitory effect of anti-L1 mAb on SKOV3ip-
lacZ cell proliferation in vitro (Fig. 1) and the dose-related
reduction of tumor growth in the anti-L1 mAb treatment groups
(Fig. 4B) led to the assumption that inhibition of proliferation
of SKOV3ip-lacZ cell by anti-L1 mAb may be an important
mechanism in this model. To assess antiproliferative activity, we
determined protein levels of PCNA, an established proliferation
marker, in representative tumor sample pools (four samples per
group) of each group by Western blot analysis (Fig. 5A) with
subsequent densitometric quantification (Fig. 5B). In all treatment
groups, the signal of PCNA was reduced compared with the control (Fig. 5A). Densitometric quantification of the bands (normalized to α-tubulin) revealed a dose-dependent decrease of PCNA protein
level in the anti-L1 mAb treatment groups [L1-11A (1 mg/kg),
−16.1%; L1-11A (5 mg/kg), −41.5%; L1-11A (10 mg/kg), −61.2%; Fig. 5B] compared with the control (ratio PCNA/α-tubulin = 0.52
equivalent to 100%), indicating an antiproliferative effect.

Discussion
The present study shows, for the first time, that the L1-CAM may
represent an important target for ovarian cancer therapy and can
be used to image tumor progression in vivo. Treatment with a mAb
against L1 (L1-11A) led to a substantial and dose-dependent
inhibition of i.p. SKOV3ip-lacZ human ovarian carcinoma tumor
growth, dissemination, and ascites formation in a xenotransplantation
mouse model and was associated with a substantially
improved health status of the treated mice compared with the
control. This antitumor effect correlated with suppression of tumor
cell proliferation in vivo. Treatment with two independent L1-
directed antibodies (L1-11A and chCE7) in vitro significantly
inhibited the proliferation of six L1-positive human tumor cell lines
of different origin, including the human ovarian carcinoma cell
line SKOV3ip, whereas proliferation of the L1-negative prostate
carcinoma cell line PC3 was not influenced. These findings are in
accordance with previous studies (9, 21) describing L1 as an
important positive regulator of cell growth of several cancer cell
lines, including breast, colon, and cervical carcinoma cells (21).
The scope of the present study was to examine, for the first time, the feasibility of L1-directed interference as therapeutic strategy. The extracellular domain of L1 can mediate growth factor signaling events by direct interaction with growth factor receptors (29), by serving as a cellular ligand for integrins (15), and by enhancing cell adhesion (30). Although we found that the tumor cells were distributed and attached to the same areas in the peritoneum upon treatment with the L1-directed antibody as in the controls, the striking difference lies in the mass of disseminated tumor cells. So far, we cannot rule out that this effect is based not only on the lower number of tumor cells but also on different adhesive properties on tumor or host cells. L1-related cell adhesion has been reported (31) and can influence important tumor-associated processes, including tumor growth, angiogenesis, and stimulation of vascular permeability leading to ascites formation. The apparent dose-dependent decrease of ascites formation in our model can likely be attributed to such functions.

Another important function of L1 in tumor progression, related to adhesion, is its involvement in migration mediated by cell-cell interactions via homophilic L1 ligation (32) or by interaction with integrins via its Arg-Gly-Asp integrin recognition motif in the ectodomain (33). Recently, L1 was proposed to influence cell motility and invasion also by a direct translational mechanism via the extracellular signal-regulated kinase (ERK) pathway (13). In the

Figure 4. Dose-dependent inhibition of SKOV3ip-lacZ i.p. tumor growth and ascites formation by treatment with L1-11A anti-L1 mAb. A, tumor spread on the peritoneal organs, diaphragm, and abdominal wall of representative mice of the control group and groups treated with different dosages of anti-L1 mAb. B, dose-dependent inhibition of SKOV3ip-lacZ i.p. tumor growth in nude mice by anti-L1 mAb L1-11A. Compared with the PBS-treated control (tumor burden, 23.6 ± 3.0%; n = 6), biweekly treatments with anti-L1 mAb led to a reduced tumor burden in the peritoneal cavity of mice at all dosages used [L1-11A (1 mg/kg), 17.5 ± 4.9%; n = 6; L1-11A (5 mg/kg), 15.1 ± 3.5%; n = 6; L1-11A (10 mg/kg), 8.6 ± 2.3%; n = 6], whereas administration of IgG control antibody revealed no effect on i.p. tumor growth (IgG antibody, 23.7 ± 5.9%; n = 4). C, reduced ascites formation in L1-11A-treated tumor-bearing mice. Compared with the massive ascites formation in mice of the control group (3.25 ± 0.80 mL), all L1-11A treatment groups revealed a reduced amount of ascites in the peritoneal cavity of mice [L1-11A (1 mg/kg), 2.60 ± 0.55 mL; L1-11A (5 mg/kg), 1.69 ± 0.93 mL; L1-11A (10 mg/kg), 0.81 ± 0.25 mL]. D, L1 protein levels in ascites of treated and untreated tumor-bearing mice. Compared with the control (12.76 ± 1.73 ng L1/mL ascites), all anti-L1 mAb treatment groups revealed reduced L1 levels in the ascites [L1-11A (1 mg/kg), 8.30 ± 5.61 ng/mL; L1-11A (5 mg/kg), 6.45 ± 1.63 ng/mL; L1-11A (10 mg/kg), 4.49 ± 0.18 ng/mL]. ELISA for detection of protein levels was done in triplicate for the ascites of three different mice per group. Columns, mean; bars, SE. *, P < 0.05 (Mann-Whitney rank-sum test; B and C).
present study, treatment with the anti-L1 mAb L1-11A significantly blocked the haptotactic migration of SKOV3ip and OV-MZ-6 ovarian cancer cells and almost completely inhibited the L1-mediated motility of HEK293-hL1 transfectants, indicating that the inhibitory effect of L1-11A on cell motility is due to inhibition of L1. In addition, in several other studies, interference with L1 (e.g., overexpression or knockdown) was shown to influence the motility of different cell lines \( \text{in vivo} \) (9, 15). However, \( \text{in vivo} \), an obvious influence of L1-11A treatment on migration of SKOV3ip cells in the peritoneal cavity of mice (influencing the pattern of tumor dissemination) was not detectable at time point of autopsy. However, this does not exclude that inhibition of L1 by L1-11A had an effect on migration of SKOV3ip cells \( \text{in vivo} \) in an earlier phase of the study.

Recently, it has been suggested that L1 is linked to prevention of cell death because inhibition of L1 was shown to promote apoptosis of human umbilical vein endothelial cells \( \text{in vitro} \) (34) as well as in the nervous system (35). This is another possible mechanism by which L1-directed antibodies could contribute to the reduction of tumor mass at the different mesothelial linings in the peritoneal cavity. However, in the \( \text{in vitro} \) experiments, treatment with two independent anti-L1 mAb (L1-11A and chCE7) over 3 days had no influence on viability of six different L1-positive tumor cell lines, taking into account that appropriate factors are lacking in the \( \text{in vitro} \) situation.

In our \( \text{in vivo} \) study, treatment with anti-L1 mAb L1-11A significantly reduced the i.p. tumor burden in mice in a dose-dependent manner and this therapeutic effect could be linked to inhibition of L1-function, because treatment with an anti-EpCAM mAb (HEA125) revealed no effect on tumor progression. The inhibitory effect of L1-11A on L1 was associated with reduced tumor cell proliferation \( \text{in vivo} \). This is consistent with previous studies, in which manipulation of L1 expression levels was shown to influence tumor growth in mice (9, 14).

It is presently unclear whether the therapeutic effect of anti-L1 mAb L1-11A \( \text{in vivo} \) is due to interference with the membrane-bound or the soluble form of L1 or both. The mAb L1-11A binds both membrane-associated L1 and the soluble form and was shown previously to inhibit L1-mediated adhesion and migration as well as the autocrine/paracrine binding of shed L1 (15). In the present study, shed L1 was found in the ascites of mice in correlation to tumor burden. This is consistent with previous clinical data in which soluble L1 was also detected in ascites of ovarian carcinoma patient in a stage-dependent manner (4). Soluble L1 from human ascites was shown recently to be a potent trigger of cell migration as well as ERK phosphorylation (36), indicating an essential role in tumor progression of ovarian carcinomas. Although we should not rule out the possibility that also soluble L1 can induce tumor cell proliferation, our \( \text{in vitro} \) experiments indicate that antibody binding and cross-linking of membrane-bound L1 may be an important mechanism responsible for the antiproliferative effect. We underlined this point by showing that proliferation of Caki-2 and SK-N-AS could only be significantly inhibited by L1-11A when this antibody was hyper-cross-linked with an anti-mouse IgG antibody. These results are in accordance with other publications in which the weak antitumor activity of several antibodies against different antigens was dramatically enhanced by hyper-cross-linking with secondary antibodies (37) or by homodimerization (38).

The present study is the first report on efficient and dose-dependent inhibition of i.p. tumor growth and dissemination of a highly aggressive ovarian cancer cell line by antibody treatment directed against L1-CAM. These findings may open a new perspective in the development of strategies for ovarian cancer treatment by evaluating L1-CAM as a potential and promising target.

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