Protection from Lymphoma Cell Metastasis in ICAM-1 Mutant Mice: A Posthoming Event

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It has been hypothesized that the intercellular adhesion receptors used by normal cells could also be operative in the spreading of circulating malignant cells to target organs. In the present work, we show that genetic ablation of the ICAM-1 gene confers resistance to T cell lymphoma metastasis. Following i.v. inoculation of LFA-1-expressing malignant T lymphoma cells, we found that ICAM-1-deficient mice were almost completely resistant to the development of lymphoid malignancy compared with wild-type control mice that developed lymphoid tumors in the kidneys, spleen, and liver. Histologic examinations confirmed that ICAM-1-deficient mice, in contrast to wild-type mice, had no evidence of lymphoid infiltration in these organs. The effect of ICAM-1 on T cell lymphoma metastasis was observed in two distinct strains of ICAM-1-deficient animals. Nonetheless, lymphoma cells migrated with the same efficiency to target organs in both normal and ICAM-1-deficient mice, indicating not only that ICAM-1 expression by the host is essential in lymphoma metastasis, but also that this is so at stages subsequent to homing and extravasation into target organs. These results point to posthoming events as a focus of future investigation on the control of metastasis mediated by ICAM-1. *The Journal of Immunology,* **1998, 161: 2333–2338.**

The extravasation of normal leukocytes is critically dependent upon tightly regulated interactions between multiple cell adhesion molecules that are expressed on both lymphocytes and endothelial cells (1). Integrins are heterodimeric proteins containing a noncovalently associated α and β subunit (2); these proteins are essential for leukocyte migration, since they bind to proteins of the extracellular matrix, such as fibronectin and laminin, and to the Ig-like cell surface proteins that are expressed at the surface of the endothelial and stromal cells. Since lymphoma cells often express the same adhesion molecules as their normal counterparts, it has been postulated that cell adhesion molecules may participate in the dissemination of lymphoma cells to target organs in the late stages of the metastatic process by mediating firm cell to cell contact to arrest in the circulation (3, 4). Studies demonstrating that LFA-1 expression at the surface of tumor cells correlates with metastatic behavior (5) and showing the high concentration of circulating ICAM-1 in patients with malignant lymphoma support the idea that ICAM-1 is involved at some point in tumor development (6). However, numerous experiments designed to establish whether LFA-1/ICAM-1 interactions are critical in lymphoma cell invasiveness were inconclusive. In vivo, Hamann and Thiele (7) showed that blocking mAbs that are specific for LFA-1 could not prevent the entry of Moloney-transformed lymphoma cells into the spleen, while other studies showed that blocking mAbs reduced significantly but partially the metastatic potential of lymphoma cells to the liver (8, 9). Using a similar approach, Zahalka et al. (10) have found that while anti-β2 mAbs injected i.v. could not block the infiltration of their lymphoma cells into the lymph nodes, a local s.c. injection of this Ab near the lymph nodes could, albeit partially, inhibit lymphoma invasion into these organs.

To date, two of the three ligands interacting with LFA-1, ICAM-1 and ICAM-2, are expressed on the vascular endothelium (11–13). However, the expression of these molecules on the endothelium is very distinct. The expression of ICAM-1 is tightly regulated by the presence of inflammatory cytokines, and therefore occurs mostly in inflamed areas, whereas ICAM-2 is constitutively expressed on endothelial cells and is believed to be the major receptor for lymphocyte LFA-1 in the resting endothelium. These distinct expressions of ICAM-1 and ICAM-2 on vascular endothelial cells along with the inability of anti-ICAM-1 to block lymphoma invasiveness has led to the hypothesis that ICAM-2, rather than ICAM-1, may be operative in lymphoma invasiveness (14). However, ICAM-1 appears to be expressed at appreciable levels in the absence of inflammation in some tissues, and it is noteworthy that these tissues are the preferred target sites for lymphoma metastasis (14, 15).

The idea that a blockage of lymphoma dissemination by blocking mAbs is due to their ability to inhibit the homing of tumor cells to target organs has recently been challenged by studies that have demonstrated a lack of correlation between the expression of cell adhesion molecules, homing, and the metastatic potential of tumor cells. The blocking of integrin function by disintegrins does not seem to affect the extravasation and homing of melanoma cells; rather, this blocking affected their subsequent tumor growth (16). In addition, the hemogenous spreading as well as the peripheral node invasion of lymphoma-derived leukemic cells may occur independently of the expression of the lymphocyte homing receptor (15). Furthermore, in vivo studies using intravital videomicroscopy have shown that most tumor cells entering the circulation extravasate efficiently into tissues regardless of their metastatic potential (17, 18). More recently, we have shown that the critical step for the successful metastasis of the lymphoma cell was determined in the final steps of the disseminating process, namely after homing (19). The lack of correlation between the ability of a tumor cell to migrate specifically to a target organ and its ability to

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form a tumor in this organ raise the question again of whether cell adhesion molecules are essential for the dissemination of lymphoid tumors to peripheral organs.

The effect of the mutation or inactivation of a targeted gene is the most definitive and comprehensive assay for the biologic role of that gene in the context of a living organism. Therefore, we investigated whether the absence of ICAM-1 could prevent the dissemination of LFA-1-expressing lymphoma cells to peripheral organs. Using ICAM-1-deficient mice, we show that the expression of ICAM-1 by the host plays a critical role in lymphoma dissemination in the liver, spleen, and kidneys following i.v. inoculation of malignant T lymphoma cells. Furthermore, we found that resistance to lymphoma metastasis in these mice is manifested at stages that are subsequent to homing and extravasation in target organs.

Materials and Methods

Mice

Mutant ICAM-1-deficient mice were generated by gene targeting in 129-derived (H-2^b) J1 embryonic stem cells that had been injected into C57BL/6 (H-2^b) blastocysts (20). This strain carries a mutation in the fourth exon of the ICAM-1 gene. A second strain of ICAM-1 mutant mice (ICAM1^{tm1Bay}) that was backcrossed onto a C57BL/6 background was obtained from The Jackson Laboratory (Bar Harbor, ME). Mice of this strain carry a deletional mutation in the fifth exon of the ICAM-1 gene (21). Mice were age- and sex-matched with their wild-type (wt)[3] mates. All mouse colonies were maintained in the specific pathogen-free animal facility at the Institut Armand-Frappier.

Antibodies

mAbs were purified from hybridoma culture supernatants by chromatography on protein G-Sepharose (Pharmacia, Piscataway, NJ). The following mAbs were used: M17/4.2 (anti-mouse LFA-1) (22), PS/2 (anti-mouse α_4 chain) (23), 145-2C11 (anti-mouse CD3ϵ chain) (24), and YN1/1.7.4 (anti-mouse ICAM-1) (25). The fluoresceination and biotinylation of Abs were carried out using standard protocols. FITC-conjugated anti-mouse ICAM-2 (clone 3C4) was obtained from PharMingen (San Diego, CA). This mAb reacts against the murine ICAM-2 molecule (26). The streptavidin (SA)-RED670 conjugate was obtained from Life Technologies (Grand Island, NY). Phycoerythrin-conjugated anti-mouse CD4 was obtained from Boehringer Mannheim (Laval, Canada). FITC-conjugated anti-CD8 was obtained from Becton Dickinson (Mountain View, CA).

Flow cytometry

Cells were stained at 4°C and washed with PBS containing 0.5% BSA and 0.2% sodium azide. Before staining, cells were incubated with 10 μg/ml of human IgG (Sigma, St. Louis, MO) for 20 min at 4°C to block nonspecific binding. Next, fluorochrome- or biotin-labeled mAbs were added at appropriate concentrations and incubated for another 20 min. Cells were subsequently washed four times with PBS containing 0.5% BSA and 0.2% sodium azide. For indirect staining with SA-RED670, cells were washed three times following the reaction with the first mAb and then incubated for 20 min on ice with the fluorescent conjugate. The results shown are representative of at least three independent experiments. Flow cytometric analyses were performed on a Coulter XL-MCL flow cytometer (Hialeah, FL). Analyses were conducted on a logarithmic scale on 1 to 5×10^5 events.

T cell lymphoma invasiveness

The malignant 164T2 T lymphoma line was obtained from an in vitro culture of radiation-induced thymic lymphoma in C57BL/Ka mice (27). The C57BL/Ka mice were originally derived from a C57BL/6 colony maintained at Stanford University (Stanford, CA) by Dr. Henry Kaplan. To induce tumor spreading to target organs, 1 to 5×10^5 malignant T lymphoma cells were injected i.v. via the tail vein to 6- to 10-wk-old mutant and wt mice. Animals were observed at regular intervals for clinical signs of lymphoma development, including dyspnea, runting, and splenomegaly. Unless otherwise indicated, all animals were sacrificed at 6 to 8 wk postinjection and examined for the presence of lymphoid tumors at necropsy.

[3] Abbreviations used in this paper: wt, wild-type; SA, streptavidin; KO, knockout; MMP, matrix metalloproteinase.

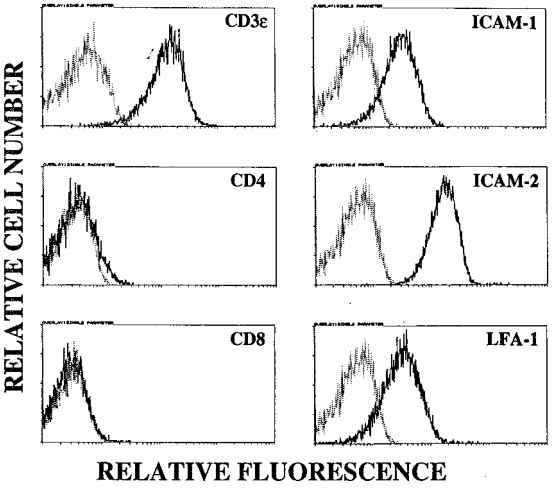

FIGURE 1. Flow cytometric analysis of the cell surface phenotype of 164T2 cells. Staining with mAbs (——) and autofluorescence controls is shown (. . . . .). The expression of ICAM-1, ICAM-2, LFA-1, and CD8 was shown using FITC-labeled mAbs. CD4 and CD3ϵ expression was shown using phycoerythrin-conjugated mAbs and a biotin-SA-RED670 combination, respectively. The results are representative of three independent experiments.

Mice were examined macroscopically, and their kidneys, liver, spleen, lungs, and thymus were harvested and fixed in 10% formalin for histologic examination.

In vivo migration assays

The migration of 164T2 lymphoma cells was analyzed using standard indium-111 labeling of lymphoma cells as described previously (19). Briefly, 10^7 cells were labeled with 10 mCi of indium-111 in 0.5 ml RPMI 1640 for 15 min at room temperature. The cells were washed four times with RPMI 1640 containing serum and resuspended in PBS. The viability of the labeled cells was >95% as determined by trypan blue exclusion. Each mouse was injected i.v. with 10^6 cells (0.5 to 1×10^6 cpm). Animals were sacrificed at various times (five mice for each time point), and the kidneys, spleen, liver, and thymus as well as heparinized blood samples were recovered. The total radioactivity in circulating blood was estimated in 400-μl aliquots of blood, assuming a total volume of 2 ml of circulating blood per mouse.

Results

A malignant T cell lymphoma line (164T2) that had been derived from a radiation-induced thymic lymphoma in a C57BL/Ka (H-2^b) mouse was used for testing lymphoma invasiveness. The T cell origin of the 164T2 cell line was confirmed by the expression of CD3ϵ at the cell surface (Fig. 1). The cells also expressed a repertoire of cell adhesion molecules that were similar to those found on normal thymocytes, including ICAM-1, ICAM-2, and LFA-1.

Since the 164T2 lymphoma cells expressed the lymphocyte integrin LFA-1 and could form solid tumors in the peripheral organs of mice following i.v. injection (19), we tested whether these cells could also spread in the absence of ICAM-1 in vivo. ICAM-1 knockout (KO) mice (129 × C57BL/6) (20) and wt controls, were injected i.v. with 10^5 164T2 lymphoma cells. All animals were sacrificed after 6 wk and examined for the presence of tumors by macroscopic and microscopic observations. We found that 17 of 32 (53%) injected normal mice developed solid tumors in the kidneys and spleen following i.v. injection of 164T2 lymphoma cells (Table I), whereas only 1 of 32 ICAM-1 KO mice (3%) developed such tumors. A massive perivascular infiltration of lymphoma cells in the liver was also detected by a histologic examination of normal mice with lymphoid tumors (Fig. 2). In ICAM-1 KO mice, no

Table I. *Frequency of mice with solid tumors in peripheral organs*[a]

Treatment	Normal Mice	ICAM-1 Deficient Mice
Expt. 1:[b]		
10^5 lymphoma cells	9/17	1/17
Expt. 2:[b]		
10^5 lymphoma cells	8/15	0/15
Expt. 3:[c]		
5×10^5 lymphoma cells	5/5	0/5

[a] i.v. inoculation was carried out in the tail vein. Mice were considered positive if at least one solid tumor was found in either the kidney, spleen, thymus, or liver.
[b] Animals were sacrificed and examined histologically at 6 wk postinjection.
[c] Animals were sacrificed and examined histologically at 9 wk postinjection.

infiltration by lymphoma cells was detected in any of these organs. The only ICAM-1 KO mouse with lymphoid tumors had a massive infiltration in both kidneys, precluding the possibility that infiltration was due to a local trauma. Resistance was not dose-dependent, as demonstrated by injecting a fivefold higher dose of lymphoma cells (Table I). The resistance of ICAM-1 KO mice was also not time-dependent, since these mice did not develop tumors for up to 24 wk postinjection of lymphoma cells (data not shown). In three different experiments, only 1 of 37 ICAM-1 KO mice developed a lymphoid malignancy, compared with 22 of 37 wt control mice.

Although C57BL/6 and 129 mice are histocompatible (H-2^b), the fact that lymphoma 164T2 originated from the C57BL/Ka (H-2^b) strain raised the concern that the resistance of ICAM-1-deficient mice to 164T2 lymphoma metastasis observed in the first series of experiments using C57BL/6 $\times$ 129 mice could be due to differences in the genetic background provided by the 129 embryonic stem cells used for the generation of the mutant mice. To verify this hypothesis, we tested the metastatic potential of 164T2 lymphoma cells in an independently derived ICAM-1-deficient strain (ICAM1^{tm1Bay}) that was maintained on the C57BL/6 background (21, 28). When 5×10^5 of 164T2 were injected i.v., seven of nine normal C57BL/6 mice developed lymphomas in peripheral organs within 6 wk of postinjection; at 12 wk postinjection, all ICAM1^{tm1Bay} mice were still resistant to lymphoma metastasis (Table II). These results establish that an absence of ICAM-1 in mice confers resistance to the development of lymphoma to target organs. The resistance to lymphoma metastasis that was observed in ICAM-1-deficient mice was specific, since an i.v. injection of H59 carcinoma cells (19) resulted in tumor development in both

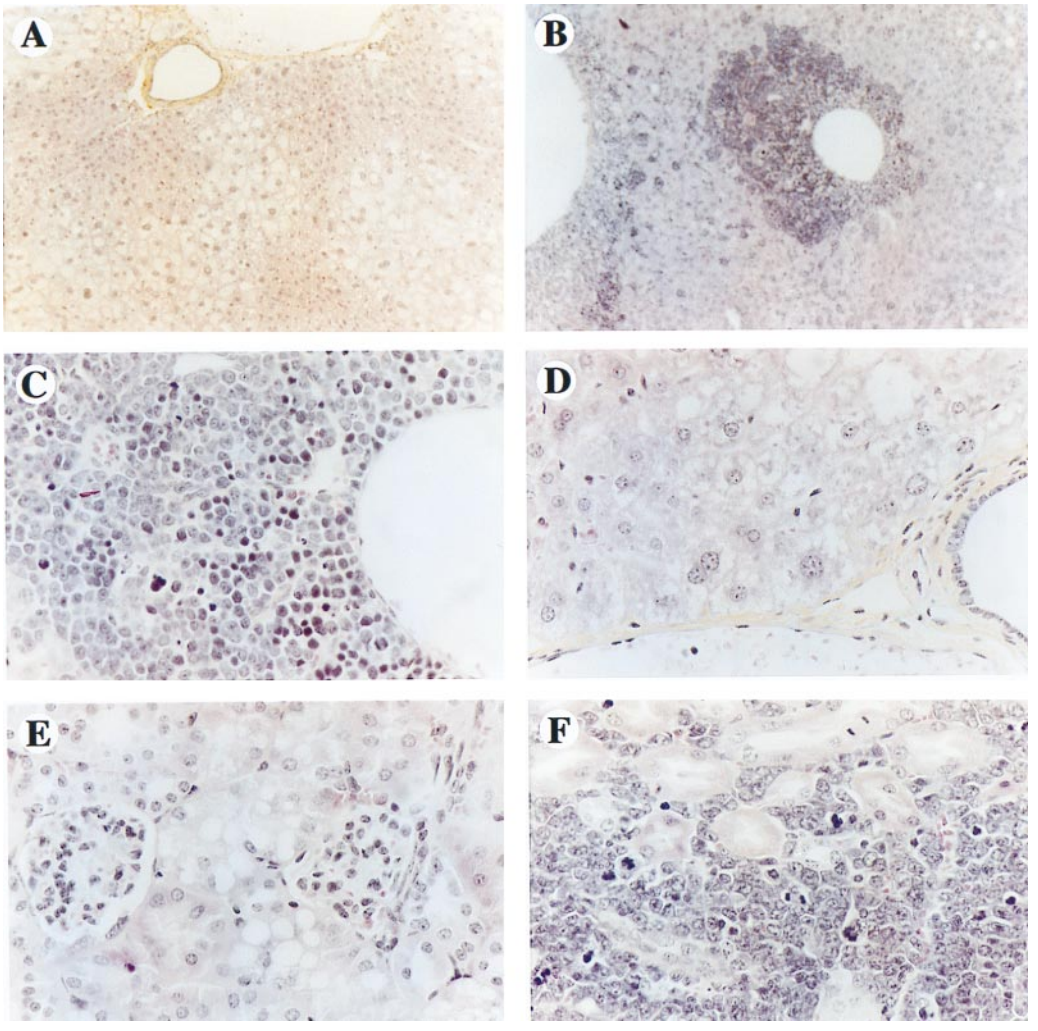

FIGURE 2. Histologic examinations of wt and ICAM-1-deficient mice following i.v. inoculation of 164T2 T lymphoma cells at 6 wk postinjection. An examination at low ($\times$40) and high ($\times$400) magnitudes of the livers of normal mice (*B* and *C*) showed massive lymphoma cell infiltration, with an emphasis on the perivascular areas. No indication of lymphoma cell infiltration could be detected in the livers of ICAM-1-deficient mice (*A* and *D*) following the same treatment. A histologic examination of kidneys at high magnitude ($\times$400) shows massive infiltration in intertubular areas in normal mice (*F*) but not in ICAM-1-deficient mice (*E*). These results are representative of $>$50 histologic examinations on wt and ICAM-1-deficient mice.

Table II. *Frequency of mice with solid tumors in peripheral organs of C57BL/6 ICAM^{tm1Bay} mice*[a]

Treatment	Normal C57BL/6 Mice	ICAM^{tm1Bay} Mice
Expt. 1:[b]		
5×10^5 lymphoma cells	5/5	0/5
Expt. 2:[c]		
5×10^5 lymphoma cells	2/4	0/4

[a] i.v. inoculation was carried out in the tail vein. Mice were considered positive if at least one solid tumor was found in either the kidney, spleen, thymus, or liver.
[b] The experiment was terminated at 9 wk postinjection.
[c] The experiment was terminated at 12 wk postinjection.

normal and ICAM-1-deficient mice at the same rate and in all animals of both groups (data not shown).

To investigate whether the resistance of ICAM-1-deficient mice to lymphoma metastasis might be caused by the inability of 164T2 lymphoma cells to migrate into target organs, lymphoma cells were labeled with indium-111, and the homing of T lymphoma cells was compared in both normal and ICAM-1-deficient mice at different times postinjection. The results indicated that the majority of cells had left the blood circulation to migrate to the lung and liver at 1 h postinjection (Fig. 3). While the homing to the lung was transient, as indicated by the decreasing amount of radioactivity recovered after 3 and 24 h postinjection, the amount of radioactivity recovered in the liver, spleen, and kidneys remained constant. No significant homing was detected in the thymus. This pattern of homing indicated that T lymphoma cells migrate to and remain in organs in which lymphoma development is observed in susceptible mice. Most importantly, we found no significant differences in tumor-homing kinetics and organ specificity between susceptible normal mice and resistant ICAM-1-deficient mice. By 24 h postinjection, only 0.21 and 0.27% of cells were retained in the lungs of normal and ICAM-1-deficient mice, respectively. At 3 h postinjection, a similar pattern of migration was obtained in the liver, spleen, and kidneys of normal and ICAM-1-deficient mice. By 24 h, injected cells had left the circulation; the majority of these cells had migrated to the livers of both wt and mutant mice. These results indicate that lymphoma cells, regardless of ICAM-1 expression on the endothelium, have the same capacity to migrate to target organs with a similar fate and at the same rate, indicating that ICAM-1-resistance to lymphoma dissemination is manifested after homing.

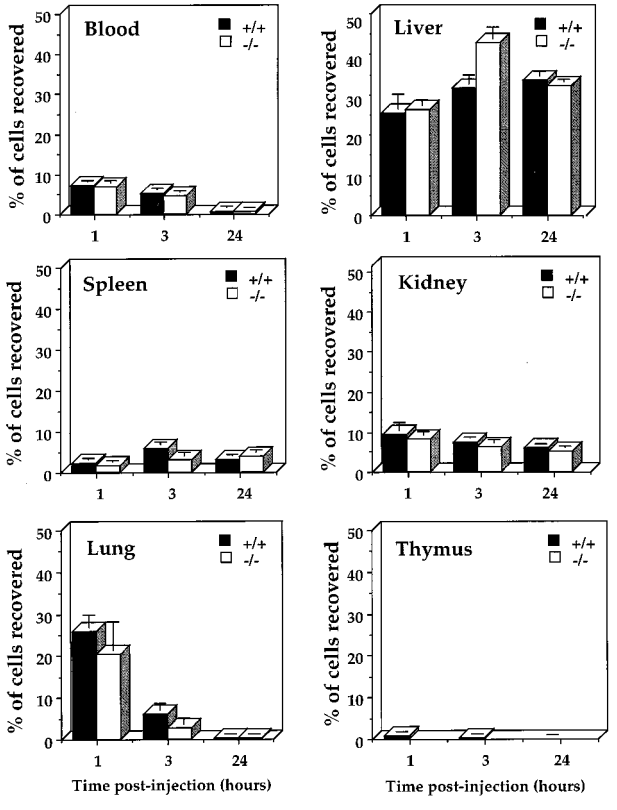

FIGURE 3. In vivo migration assays of 164T2 lymphoma cells in normal (+/+) and ICAM-1-deficient mice (−/−). Cells were labeled in vitro with indium-111, and 10^6 cells were subsequently injected i.v. Radioactivity was counted in the organs at different times and expressed as the percent of total radioactivity injected as described previously (19). Data represent the mean values ± SDs of five determinations based on two independent experiments.

Discussion

The data show that the absence of ICAM-1 in the host prevents T cell lymphoma metastasis. In vivo migration assays revealed that lymphoma cells migrated with a similar fate and at the same rate in both normal and ICAM-1-deficient mice, indicating that ICAM-1 expression in tissue affects lymphoma metastasis at a stage that is subsequent to homing to target organs. The fact that it is in the postextravasational events of lymphoma metastasis that ICAM-1 exerts its function supports the idea that, in addition to extravasation, adhesion molecules may also be implicated in the control of tumor progression and growth after the cells pass from the blood into the target organs (19). The recent observations that the expression of α_4 integrins inhibits lymphoma metastasis without affecting homing (29) and that the very late Ag-2 adhesion molecule is involved in rhabdomyosarcoma cell metastasis during the migration through the perivascular tissue support this idea (30). The metastatic potential of lymphoma cells may be affected by their susceptibility to CTL and NK cell lysis in vivo. It is unlikely, however, that these effector functions are responsible for the resistance of ICAM-1-deficient mice to lymphoma metastasis, because: 1) CTLs from ICAM-1 mutant mice are not hindered as responders when compared with lymphocytes from normal mice (20); 2) in contrast to YAC-1 lymphoma cells, the 164T2 lymphoma cells are not sensitive to NK cell lysis, at least when tested in a standard in vitro cytotoxicity assay (data not shown); and 3) we found that the splenic NK cell activity in ICAM-1-deficient mice is not significantly different from that observed in normal C57BL/6 mice (our unpublished observations). Alternatively, it is possible that cell to cell interactions between tumor cells and host stromal cells (endothelial and fibroblastic cells, macrophages, etc.) via ICAM-1 regulate the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of MMP, both of which are abundantly expressed in malignant lymphomas (31, 32). MMPs have been shown to play a crucial role in the invasive behavior of normal and tumor cells (33), whereas tissue inhibitors of MMP, whose expression often mirrors that of MMPs, can promote cell growth under certain conditions (34). In vitro, we have recently found that the LFA-1/ICAM-1 but not the LFA-1/ICAM-2 interaction is involved in the induction of MMP-9 expression during the adhesion of 164T2 lymphoma cells to endothelial cells (35). Thus, it is possible that contact between lymphoma cells and the vascular endothelium via LFA-1/ICAM-1 interactions induces the expression of MMP-9 which, in turn, allows lymphoma cells to migrate through the extracellular matrix to reach the metastatic sites in which they grow into secondary tumors. Alternatively, the interaction of LFA-1/ICAM-1 may also mediate interactions with stromal cells that

promote the growth of lymphoma cells or resistance to the antitumoral immune response elaborated by the host. The migration of lymphocytes or lymphoma cells involves a complex cascade of intracellular activation events that are thought to depend upon integrin-mediated adhesion to endothelial cells. Adhesion molecules that are known to support firm adhesion to the vascular endothelium have been proposed as candidate molecules that could engage transmigration processes and the survival of lymphocytes into tissues (36, 37). These possibilities are now under investigation in our laboratory.

The accumulation of T lymphoma cells in specific target organs, compared with the transient passage into the lungs, is in agreement with previous studies showing that the liver, spleen, and kidneys are the preferred target sites for lymphoma metastasis (14, 15). This pattern of migration has also been observed with circulating lymphoblasts and activated mature lymphocytes, which show significant affinity for the liver and lungs, although retention in the lungs appears to be transient for most circulating blasts (38). Since ICAM-1 is not essential in the homing of T lymphoma cells to specific target organs, one might suppose that other adhesion molecules might be involved during that process. For instance, the ubiquitous and constitutive expression of ICAM-2 on the vascular endothelium has led to the suggestion that ICAM-2, rather than ICAM-1, would be the likely receptor used for homing of lymphoma cells if LFA-1 is involved in lymphoma invasiveness. Other adhesion molecules of the integrin and selectin families may also be implicated. Alternatively, differential responses among cancer cells to organ-specific soluble factors and/or local concentrations of specific chemoattractants may also explain the differences observed in the migration pattern. The availability of several genetically engineered KO mouse models will certainly be helpful in elucidating whether other cell adhesion molecules are involved in the homing of T lymphoma cells in our model.

Our data showed that the development of LFA-1-negative carcinoma was identical in normal and ICAM-1-deficient mice, with a 100% incidence, suggesting that resistance was specific to LFA-1-bearing lymphoma cell lines. Whether all LFA-1-bearing lymphoma cell lines are also dependent upon the presence of ICAM-1 by the host remains to be tested. In the case of T cell lymphomas, we have observed that the resistance of ICAM-1 mutant mice was not unique to 164T2 lymphoma cells, since these mice were also resistant to the metastasis induced by i.v. injection of 267 T lymphoma cells, another malignant T lymphoma cell line. Other models of T cell lymphoma are currently being tested. It will also be interesting to test whether B cell lymphomas also require ICAM-1 to disseminate. However, there are very few in vivo models of B cell lymphomas that are H-2^b histocompatible. We are currently generating a panel of syngeneic B leukemic cell lines that could eventually be tested in our model, the ICAM-1-deficient mouse.

In summary, we show that genetic ablation of ICAM-1 confers resistance to lymphoma cell metastasis in peripheral organs, and that lymphoma metastasis is controlled by ICAM-1 at a stage that is subsequent to homing to target organs. These results point to postextravasational events as a focus of future investigation on metastasis control.

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