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Anti-LFA-1 antibodies enhance metastasis of ocular lymphoma to the brain and contralateral eye

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Abstract

Previously we demonstrated that intraperitoneal (IP) inoculation of Rev-2-T-6 mouse lymphoma into syngeneic Balb/c hosts resulted in brain metastasis, migration along the optic nerve sheath, and ocular infiltration. In a second model: intravitreal inoculation of Rev-2-T-6 cells, the developing lymphoma was largely confined within the eye, seldom breaching the retinal pigment epithelium to reside in the choroid and sclera. There was no retrograde infiltration into the brain. Here, we describe a third, complementary model, whereby intravitreal inoculation of Rev-2-T-6 cells into Balb/c mice, followed by repeated IP inoculations of anti-LFA-1/CD11a monoclonal antibodies, results in extensive infiltration of the choroid, sclera, conjunctiva, eyelids and orbit. Furthermore, the lymphoma cells metastasize along the optic nerve sheath into the brain, and through the contralateral optic nerve tract into the contralateral eye. There is no systemic involvement of the lymphoma. Furthermore, anti-LFA-1 treatment results in elevated levels of serum anti-Rev-2-T-6 antibodies. Inoculation of Rev-2-T-6 cells into the vitreous of severe combined immune deficient mice demonstrates a course of clinical signs and histopathological findings similar to those in immune-competent mice treated with anti-LFA-1 antibodies, including invasion of the contralateral eye. Taken together, these findings suggest that confinement of Rev-2-T-6 lymphoma cells to the eye depends on active immune surveillance using a population of effector cells expressing the cell surface integrin LFA-1. Impairing this protection enhances tumor aggressiveness within the eye, and the likelihood of early retrograde lymphoma metastasis into the brain and the contralateral eye.

Keywords

Metastasis; Lymphoma; Eye; Brain; LFA-1

Introduction

The heterodimeric LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) is a member of the integrin superfamily of adhesion molecules and is expressed on the cell surface of all leukocytes. The alpha chain (CD11a) is specific to LFA-1, and the beta chain (CD18) is shared by the heterodimeric integrins Mac-1 ($\alpha_M\beta_2$), p150.95 ($\alpha_X\beta_2$) and the $\alpha_D\beta_2$ heterodimer [1–3]. Its major ligand is intracellular adhesion molecule-1 (ICAM-1/CD54), but it binds also to ICAM-2, ICAM-3 and junctional adhesion molecule-1, JAM-1 [4].

LFA-1 plays a role in the regulation of inflammation and the immune response including: adhesion to endothelial cells and transendothelial migration [5–7]; key participation in the immune synapse structure and function [8, 9]; target cell killing by CD8⁺ cytotoxic T cells [10]; costimulation [11]; differentiation of naïve T cells to Th1 effector lymphocytes [12, 13], and antiapoptotic activity, similar to that described for VLA-4 [14], and trafficking [8, 15]. These findings led to the use of LFA-1 as a therapeutic target in pre-clinical [16–21] as well as in clinical studies [11, 22–27].

Primary CNS lymphoma and vitreoretinal lymphoma (or primary intraocular lymphoma) are usually high-grade B cell lymphomas, limited to the eye and CNS and rarely spreading systemically. The disease is rare but has a high mortality rate. Immunosuppression is a risk factor for CNS lymphoma and also vitreoretinal lymphoma [28–30].

Previously we have developed two experimental mouse models for the study of brain and ocular lymphoma. In the first model, intraperitoneal (IP) inoculation of Rev-2-T-6 lymphoma cells (a T cell lymphoma of Balb/c origin) into young (day 7 post-natal) syngeneic Balb/c mice, results in metastasis of these cells to the brain and eyes [31] (IP inoculation into mature mice does not result in brain or ocular metastasis). Major routes of infiltration include the choroid plexus, cranial nerves and cranial nerve ganglia [32]. The lymphoma cells then, spread within the brain, and migrate along the optic nerve sheath into the eye. The orbit is also infiltrated [31, 32]. In the second model [33], Rev-2-T-6 cells are inoculated directly into the eye (vitreous of mature Balb/c mice). In general, the lymphoma cells are confined inside the eye, and while homing from the vitreous to the retina, they do not cross the retinal pigment epithelium (RPE) to the choroids throughout the course of the experiments [33]. There is no involvement of the ocular adnexa (conjunctiva, eyelid and orbit) and no retrograde metastasis of the lymphoma cells into the brain. Although they are T cell lymphoma models, they provide a unique opportunity for evaluating molecular mechanisms involved in tumor metastasis between brain and eye.

Rev-2-T-6 cells express LFA-1 (but not ICAM-1) on their surface [31]. They also produce the cytokine TNF-alpha in response to Con A, LPS and PMA (unpublished data). Previously, we have found that LFA-1 mediates the adhesion of Rev-2-T-6 cells to brain endothelial cells. This adhesion is mediated through activation of the endothelial cells (ICAM-1 up-regulation) by TNF-alpha, and impaired by anti-LFA-1 antibodies [34]. Taken together with the findings that LFA-1 mediates a variety of immune related processes (see above), the aim of our study was to assess the effect of systemic injection of anti-LFA-1 antibodies on the ocular distribution and progression of Rev-2-T-6 lymphoma following intravitreal transplantation of these cells into mature mice. This approach was further prompted by recent findings demonstrating that intraperitoneal as well as intraocular administration of an anti-ICAM-1 (the LFA-1 receptor) monoclonal antibody, impaired the intraocular proliferation of human melanoma cell lines expressing ICAM-1 in SCID mice [35]. Our findings paradoxically demonstrate that intraperitoneal administration of anti-LFA-1 antibodies exert a stimulatory, rather than an inhibitory effect on intraocular Rev-2-

T-6 lymphoma providing an experimental model for analyzing lymphoma spreading within the eye, into the brain and, eventually, into the contralateral eye.

Materials and methods

The studies conformed to the principles for laboratory animal research outlined by the Animal Welfare Act (NIH/DHHS) and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the National Eye Institute Animal Care and Use Committee.

Cells

Rev-2-T-6 cells were derived from substrate-adherent, non-tumorigenic (immunogenic) variants of the S49 mouse T cell lymphoma, as previously described [31]. They produce progressive, solid abdominal tumors following intraperitoneal inoculation into mature syngeneic Balb/c mice. These cells demonstrate cell–cell adhesion, growing in suspension culture as cell aggregates. Rev-2-T-6 cells were maintained in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10 % horse serum, 50 U/ml penicillin, and 50 µg/ml streptomycin.

Monoclonal antibody

The monoclonal antibody M17/4.2 [36] (anti-LFA-1/CD11a) was used in our study. This antibody was previously shown, in vivo, to inhibit ocular inflammatory diseases (uveitis) in experimental mouse models [20, 21, 37]. Daily anti-LFA-1/CD11a antibody was injected intraperitoneally.

Mice

Balb/c mice and immune deficient SCID mice (both, 6–8 weeks old) were obtained from Jackson Laboratories (Bar-Harbor, ME). Mice were provided with food and water ad libitum and kept on a 12-h light–dark cycle.

Intravitreal inoculation

Twenty-five Balb/c and 20 SCID mice were anesthetized with an intraperitoneal injection of a combination of 44 mg/kg ketamine and 10 mg/kg xylazine. Proparacaine ophthalmic solution was administered topically to the right eye. Under a dissection microscope, 1×10^5 Rev-2-T-6 cells in 3 µl PBS were injected through the pars plana into the vitreous of the dilated eye with a 32-gauge needle attached to a syringe (Hamilton, Ren, NV). The injection was performed using an aseptic technique throughout. After the injection, erythromycin ophthalmic ointment was applied.

Treatment with anti-LFA-1 antibodies

Fifteen Balb/c and 10 SCID mice received a daily IP inoculation of anti-LFA-1 (CD-11a) monoclonal antibody M17/4.2 (5 mg/kg/0.2 ml) for 21 days, starting at the day of intravitreal lymphoma inoculation. The dosage was chosen based on our previous publication [21] and information from Dr. Robert Rothlein who kindly provided the antibody for our study. Ten Balb/c and 10 SCID mice received a daily IP inoculation of PBS (0.2 ml/mouse) for 21 days, starting at the day of intravitreal lymphoma inoculation. All mice were followed individually and evaluated for clinical signs of tumor development and invasion. The experiments with Balb/c mice were carried out three times and the experiments with SCID mice were carried out twice.

Clinical evaluation of Rev-2-T-6 tumor growth

Mice were monitored every other day and underwent fundoscopic examination once a week. Clinical signs were graded from 0 to 6, according to the following progressive scores:

- 0** Clear; no tumor cells detected by fundoscopy.
- 1+** Tumor cells in the anterior chamber (AC) and vitreous.
- 2+** Up to 1/2 AC and 1/2 Vitreous filled with tumor cells.
- 3+** More than 1/2 AC and 1/2 vitreous filled with tumor cells.
- 4+** AC and vitreous filled with tumor cells.
- 5+** Conjunctiva and eyelid invasion by tumor cells.
- 6+** Orbital invasion by tumor cells.

Mice that reached stage 6 of the clinical signs, namely: a large accumulation of numerous cells in the anterior chamber and periorbital swelling (see also Fig. 1b, c), were euthanized. None of the mice reached any other humane endpoints during the course of the experiments [(such as ataxia, spinning when held by the tail, arched backs (CNS involvement signs) or restricted water and food intake or weight loss].

Histopathologic evaluation of Rev-2-T-6 tumor growth

Animals were euthanized at various time points based on clinical signs and fundoscopic findings. The collected eyes and brains were either embedded in OCT- optimal cutting temperature compounds (Sakura Finetek, Torrance, CA) and snap frozen, or fixed in 10 % formalin. The formalin-fixed tissues including the eyes, ocular adnexa, brain, liver, lung and spleen were embedded in paraffin. Paraffin embedded sections were cut through the papillary-optic nerve plane and stained with H&E. Severity of the histopathologic findings was graded from 0 to 6, according to the following progressive scores:

- 0** No tumor cells;
- 1+** Intraocular tumor cells: AC, vitreous and retina involved.
- 2+** Tumor cells in the subretinal space; retinal necrosis.
- 3+** Tumor cells in the choroid and ciliary body.
- 4+** Tumor cells in the sclera and cornea.
- 5+** Tumor cells in the orbit and ipsilateral optic nerve sheath.
- 6+** Tumor cells in the brain, contralateral optic nerve sheath and eye.

Western blot analysis

Rev-2-T-6 cells were lysed in RIPA Lysis Buffer (Upstate, Lake Placid, NY). The protein concentration of the cell lysate was adjusted to 1 mg/ml. Two hundred micrograms of proteins were separated by 8–16 % gradient prep gel (Bio-Rad, Hercules, CA) under reducing conditions and transferred to Immobilon-P Transfer Membrane (Millipore, Bedford, MA). Membrane was cut to 0.4 cm wide strips and processed according to the instruction manual of Western Breeze Immunodetection kit (Invitrogen, Carlsbad, CA). Briefly, membranes were blocked with blocking solution for 30 min, and then, incubated with 1:500 diluted mice sera for one hour after washing. The membranes were next incubated with secondary (alkaline phosphatase-conjugated goat anti-mouse) antibody for 30 min after washing. The signals were visualized by immersing the membrane in

Chromogenic Substrate for several minutes and the reaction was stopped by washing with water.

Results

As previously reported [33], intravitreal inoculation of Rev-2-T-6 cells into mature, syngeneic mice, resulted in development of lymphoma that was largely localized inside the eye and seldom breached the RPE. During the course of those experiments (7 weeks), none of the inoculated mice have shown any clinical or histological manifestations of lymphoma spread outside of the ocular compartment. Repeated experiments (see “Materials and methods” section) demonstrated similar findings (not shown).

Anti-LFA-1 antibodies enhance ocular aggressiveness and metastasis of Rev-2-T-6 cells to the contralateral eye

Following inoculation into the right vitreous, extensive right orbit involvement in Balb/c mice treated with anti-LFA-1 (Fig. 1A—b, c) is evident. PBS treated mice (Fig. 1A—a) are devoid of orbital involvement as previously reported [33]. Figure 1B demonstrates the increase of clinical scores in individual mice belonging to both groups. While the anti-LFA-1 treated group shows clinical signs of orbital infiltration as early as 4 weeks post inoculation and all mice reach the maximal score of 6+ (Mean $\pm$ SE: 5.9 ± 0.3) within 3–6 weeks, none of the PBS treated mice reached a clinical score above 4+ (Mean $\pm$ SE: 3.2 ± 1.1) throughout the entire experiment (7 weeks). Thus, anti-LFA-1 treated mice demonstrate a more aggressive tumor phenotype including more mitoses than PBS treated mice.

At different times post inoculation, mice were euthanized, and their eyes and brains were either frozen, or embedded in paraffin. H&E stained slides were used for histopathological analysis and grading, as described under “Materials and methods” section. When compared to the control (PBS treated) group, treatment with anti-LFA-1 antibodies increased the histopathology scores (Mean $\pm$ SE) from 1.5 ± 0.8 ($n = 5$) to 5.5 ± 1.0 ($n = 8$), respectively (Table 1). These findings suggest that LFA-1 expressing cells play a decisive role in confining Rev-2-T-6 lymphoma within the intraocular compartment and that anti-LFA treatment impairs this function. Despite the increased aggressiveness of tumors (infiltration of the orbit, brain, and contralateral eye) in anti-LFA-1 treated mice (compare Fig. 2A to 2B and C), none of these, or of the PBS injected mice, demonstrated any metastases to other organs (liver, lungs, kidney, or spleen) during the course of the experiments, up to 50 days post inoculation (not shown). These findings are in accordance with our previous experiments, in which a total of 95 Balb/c mice were inoculated intravitreally with Rev-2-T-6 cells followed by PBS, none of these mice demonstrated extraocular infiltration of the lymphoma (maximum histopathology score <3) (not shown).

Anti-LFA-1 antibodies exacerbate intraocular Rev-2-T-6 lymphoma in SCID mice

Since LFA-1 is an immune regulator, and since the intraocular restriction of Rev-2-T-6 cells was impaired in immune competent Balb/c mice by anti-LFA-1, we studied whether immune-effector cells are involved in this process. To that effect, an experimental setting, identical to the intravitreal inoculation of Rev-2-T-6 cells into Balb/c mice (Fig. 1B), was carried out using SCID mice devoid of B and T lymphocytes. Figure 3 shows the increase of clinical scores in individual mice. In this case, both anti-LFA-1 treated and PBS treated groups reached maximal clinical scores of 6+ (5.9 ± 0.3 and 5.7 ± 0.5 , respectively) within 4 weeks post inoculation. The severity of clinical signs was such that it mandated euthanasia of all mice of the former group, and most of the latter group by 4 weeks post inoculation. Although both groups achieved a maximal clinical score, it still took an additional week for the PBS control group to reach this score (Fig. 3), thus demonstrating a somewhat less

aggressive phenotype. Seven of the PBS treated SCID mice and six of the anti-LFA-1 treated SCID mice were further subjected to histopathological grading. The scores (Mean $\pm$ SE) were 5.9 ± 0.3 for the former and 6.0 ± 0.0 for the latter. Despite the identical scores, in three of the PBS treated mice it took an additional week to reach them (Fig. 3). Thus, even in SCID mice the anti-LFA-1 treatment seems to exercise some enhancing effect over control mice. Figure 4A shows an example of the histological findings in SCID mice depicting the wide spread of Rev-2-T-6 cells within, and outside of the ocular compartment, and Fig. 4B and C shows the effect that anti-LFA-1 antibodies exert on this process. Table 2 summarizes the sites infiltrated by Rev-2-T-6 cells according to the histopathological analysis in Balb/c and SCID mice. It is evident that anti-LFA-1 treated Balb/c mice (column 2), as well as immune deficient SCID mice (columns 3 and 4) are more sensitive to infiltration of lymphoma cells to the ocular adnexa and the brain, when compared to non-treated Balb/c mice (column 1). Furthermore, in both Balb/c and SCID mice, anti-LFA-1 administration enhances contralateral eye involvement. None of the mice demonstrated systemic metastasis by the lymphoma other than the brain and eye during the course of the experiments (6–7 weeks post inoculation).

As intravitreal inoculation of Rev-2-T-6 cells into SCID mice resulted in extensive ocular and brain metastasis we asked whether intraperitoneal inoculation of these cell into SCID mice might result in ocular and brain metastasis. As mentioned before, this does not occur in syngeneic, mature, immune competent hosts. To that effect, 10 mature SCID mice and 10 mature Nude mice were inoculated IP with Rev-2-T-6 lymphoma cells and followed thereafter. None of the mice demonstrated any signs of brain or ocular involvement (according to our established criteria). All mice developed progressive solid abdominal tumor masses (As Rev-2-T-6 cells usually develop).

Serum antibodies against Rev-2-T-6 lymphoma cells are elevated in anti-LFA-1 treated Balb/C mice

It was further determined whether the sera of anti-LFA-1 and control (PBS treated) Balb/C mice contain antibodies that recognize Rev-2-T-6 cells. Individual sera from the control (PBS) group ($n = 10$) and experimental (anti-LFA-1) group ($n = 15$) as well as sera from naïve Balb/C mice ($n = 2$) were subjected to Western blot analysis using Rev-2-T-6 cell extract. Figure 5 shows that in both PBS and anti-LFA-1 treated mice, serum antibodies to Rev-2-T-6 cells are evident. These antibodies seem to recognize similar antigens in both groups. Naïve Balb/C mice sera contain no IgGs (or degraded fragments thereof) that react with Rev-2-T-6 cells. The concentration of antibodies produced by the anti-LFA-1 treated mice is higher than in the PBS treated group. In all lanes, sera were used at a dilution of 1:500, which in the case of the PBS-treated group was also their titer (highest dilution giving a positive response in western blot analysis). In the sera of anti-LFA-1 treated mice, the antibody titer for mice No. 3 and 8, for example, reached a value of 1:16,000 and 1:32,000, respectively (not shown). Although Rev-2-T-6 cells develop progressive solid abdominal tumors, following intraperitoneal inoculation, they are also immunogenic (increased median survival relative to their parental lymphoma), causing the generation of specific serum antibodies, the Western blotting pattern of which is identical to the results in Fig. 5. The tumor-associated antigens were previously defined by us as mouse mammary tumor virus proteins, a retrovirus that Rev-2-T-6 lymphoma cells harbor [38].

While antibodies to LFA-1 increased the ocular aggressiveness and CNS metastasis of Rev-2-T-6 cells, at the same time, introduction of these antibodies also resulted in an increase in the level of serum anti-Rev-2-T-6 antibodies, up to 64 fold over control mice. Taken together with the findings that no systemic metastasis was found in these mice, it is conceivable that such an immune response could play a role in impairing development of systemic, non-CNS metastases throughout the course of the experiments.

Discussion

Adhesion molecules, involved in normal lymphocyte trafficking, have an essential role in lymphoma dissemination. It appears likely that Abs to adhesion receptors may block lymphoma dissemination. Indeed, this has been demonstrated in experimental models [39–41], emphasizing the clinical potential of such Abs. The recent findings by Wang et al. [35] with regards to melanoma (see “Introduction” section) are in line with this notion. However, since normal cells express a variety of adhesion molecules as well, treatment with Abs against adhesion molecules may also block normal function [42–44]. Due to the important roles that LFA-1 plays for, example, in formation of the immune synapse and differentiation of Th1 lymphocytes, as mentioned above, it can not be excluded that long-term treatment with anti-LFA-1 blockers may lead to adverse effects.

We previously demonstrated that systemic introduction of Rev-2-T-6 mouse lymphoma cells into syngeneic Balb/c mice resulted in metastasis of these cells into the brain, followed by infiltration of the eyes [31, 32]. Subsequently, we have reported that intravitreal inoculation of these cells into Balb/C mice resulted in lymphoma proliferation, albeit confined within the eye [33]. No retrograde metastasis to the brain was observed under these conditions. Here we characterized the consequences of continuous systemic anti-LFA-1 monoclonal antibody treatment on ocular proliferation and spread of Rev-2-T-6 cells, following their intravitreal inoculation into Balb/C mice. Under these conditions, extensive spread of the lymphoma within the eye, into the ocular adnexa, into the brain (through the optic nerve sheath), and finally, into the contralateral eye (through the contralateral optic nerve tract) was observed.

Clinical and histological signs, similar to those in the anti-LFA-1 treated Balb/c mice, were observed in immune deficient SCID mice following intravitreal inoculation of Rev-2-T-6 cells. Furthermore, in SCID mice too, the onset of these signs started somewhat earlier when exposed to anti-LFA-1 treatment. These findings indicate that metastasis of lymphoma from the eye into the brain and contralateral eye is enhanced when the immune system is compromised. The downward gradient of sites involved, from the injected eye towards the contralateral eye, taken together with the findings that those mice with contralateral eye involvement always demonstrated involvement of the brain/optic nerve, orbit and the injected eye, strongly supports the ocular-optic nerve/brain-contralateral optic nerve-contralateral eye route of lymphoma spread in SCID mice and anti-LFA-1 treated Balb/c mice. This analysis is similar to the one carried out previously where metastasis from the brain to the eye was determined [32].

The confinement of Rev-2-T-6 lymphoma cells within immune privileged compartments, like the eye and brain, depends, to a large extent, on active immune surveillance, (both adaptive and innate) that uses effector cells such as T, NK, or macrophages expressing the cell surface integrin LFA-1. These cells serve as the keepers of the ocular gate. When the activity of this population is compromised via anti-LFA-1 treatment, or the elimination of T and B cells (in SCID mice) tumor aggressiveness within the eye is enhanced, as well as the likelihood of early retrograde lymphoma dissemination from the eye into the brain.

Inhibition of Th1 phenotype by LFA-1 mAb can shift tumor-associated macrophages to an alternatively activated M2 phenotype. M2 macrophages express arginase-1, IL-10 and transforming growth factor-beta (TGF- β); these molecules reduce the anti-tumor activity of Th1 cells and natural killer cells resulting in lower tumor-specific immune response and less in favor of a Th1/CTL response. Recently, van der Burg's group demonstrated that CD4 Th1 cells can switch tumor-induced M2 to activated M1 macrophages [45]. M2 macrophages are also found to produce anti-inflammatory cytokines in uveal melanoma, which contributes to

the inflammatory microenvironment in tumor development and progression [46]. In addition, M2 macrophages release many pro-angiogenic cytokines and growth factors, such as vascular endothelial growth factor, IL-8 and basic fibroblast growth factor, which can help tumor growth [47, 48].

The findings that even in SCID mice Anti-LFA-1 antibodies enhance (albeit to a lesser extent than in Balb/C mice) lymphoma aggressiveness is consistent with a role that NK cells play in the ocular confinement of Rev-2-T-6 cells.

Enhanced aggressiveness of the lymphoma is manifested not only by the increased tumor load, but also by the migration (and its rate) to the contralateral eye. The migratory rate of Rev-2-T-6 cells (with an approximate size of 10 μm) to the contralateral eye in SCID mice is roughly 20 $\mu\text{m}/\text{h}$, based on: 12,000 μm (distance [49])/25 days (average time for migration)/24 h. This rate is in the range of lymphocyte migration (5–40 $\mu\text{m}/\text{min}$) and similar to the rate of fibroblast migration (15 $\mu\text{m}/\text{h}$) [50–53].

It is noteworthy that despite increased aggressiveness of the lymphoma cells within the ocular-brain axis of treated mice, no systemic infiltration of these malignant cells could be detected. In the case of SCID mice it cannot be excluded that lymphoma cells might have metastasized systemically with longer follow-up. However, since the experiments had to be terminated within 4 weeks due to the severity of clinical ocular signs, not enough Rev-2-T-6 cells accumulated in sites where they could be detected histologically. This is apparently not the case for Balb/C mice, as these mice were followed for an additional 2–3 weeks. Thus, in immune competent mice it is conceivable that a systemic immune response is involved in impairing lymphoma dissemination outside of the eyes and brain. Indeed, both anti-LFA-1 treated, and control (PBS treated) mice, generated anti-Rev-2-T-6 antibodies, the titer of which was higher (at least by an order of magnitude) in the anti-LFA-1 treated mice.

The enhanced generation of serum anti-Rev-2-T-6 antibodies, in spite of daily treatment with anti-LFA-1, demonstrates that while the ocular confinement of lymphoma is broken, the anti-LFA-1 levels are apparently not sufficient to inhibit the systemic immune response initiated by antigen presenting cells migrating from the eye to the spleen. Thus, in the case of Rev-2-T-6 cells, by enhancing the humoral response, anti-LFA-1 could act in an overall capacity of adjuvant. As Rev-2-T-6 cells express LFA-1 on their surface [31], we would have expected anti-LFA-1 antibodies to impair their infiltration (and aggressiveness), both within the eye as well as into the brain. While systemic antibodies do have the capability to enter the eye [54], our findings suggest that the systemic anti-Rev-2-T-6 antibodies have no down regulating effect on intraocular tumor growth even though they reach a relatively high blood titer.

Our findings demonstrate for the first time conditions whereby intraocular lymphoma migrates to the contralateral eye. Furthermore these findings represent a situation whereby treatment with anti-LFA-1 antibodies may be detrimental rather than beneficial. To circumvent adverse effects of anti-adhesion antibodies on normal cells, Cohen et al. [44] proposed the use of bi-specific antibodies that bind simultaneously to a particular adhesion molecule and to a tumor-specific antigen. These bind more avidly to tumor cells that express both target antigens than to normal cells that express only the adhesion receptor. Thus, bi-specific antibodies against adhesion molecules and against tumor-specific antigens may selectively block tumor metastasis while leaving normal functions intact. In this context it is noteworthy that statins, used to lower cholesterol levels have been reported to bind LFA-1 [55], thus, having the potential of affecting the immune system and aiding in the treatment of inflammatory/autoimmune diseases, and even cancer [56, 57]. At the same time, based on the present study, it is speculated that such compounds could adversely affect developing

tumors in immune privileged sites as the eye and the brain. It is therefore of interest to study the effect of these, and other drugs, that interfere with LFA-1 binding to its receptor, on the current mouse model as well as on other tumor types that target the eye and brain.

The present findings, taken together with our previous ones, namely the brain to eye metastasis [31, 32] and the ocular confinement of lymphoma cells in untreated mice [33], constitute a comprehensive in vivo model system for analyzing and comparing the molecular basis of brain to eye infiltration and the retrograde eye to brain dissemination of lymphoma, as well as for developing novel experimental therapeutic approaches towards impairing lymphoma metastasis to, and within these compartments. These animal models can also be used to better define the immune mechanisms involved.

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Abbreviations

ICAM-1	Intracellular adhesion molecule-1
LFA-1	Lymphocyte function-associated antigen-1
SCID	Severe combined immune deficient
IP	Intraperitoneal
APCs	Antigen presenting cells

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Fig. 1. **A** Clinical manifestations following intravitreal (*right eye*) inoculation of Rev-2-T-6 lymphoma cells into Balb/c mice, and subsequent IP injections of monoclonal anti-LFA-1 antibodies. Photographs were taken at 4 weeks post inoculation. *a* Control (PBS treated) mouse; *b, c* anti-LFA-1 treated mice. The right orbits show severe proptosis, erythema and edema in *b, c*. **B** Development of clinical signs of ocular involvement in Balb/c mice following intravitreal inoculation of Rev-2-T-6 cells and subsequent IP injections of anti-LFA-1 monoclonal antibodies ($n = 15$). Control mice ($n = 10$) received PBS in place of antibodies. Mice were followed individually until euthanization (end point of each curve). Number of mice observed at each time point is given in *parentheses*

Fig. 2.

Photomicrographs of eyes from Balb/c mice following intravitreal inoculation of Rev-2-T-6 cells and subsequent IP injections of PBS (**A**) or anti-LFA-1 monoclonal antibody (**B**, **C**).

A Lymphoma cells (*arrows*) reside mainly in the vitreous (*right and upper left*).

Lymphocytes (*white broken arrow*) are surrounding the retinal vessels (*lower left*).

Pathology score—1+ (see “Materials and methods” section for score definition) (H&E, *upper left* ×25, *lower left* ×200, *right* ×100).

B Lymphoma cells extensively invade and destroy the entire right eye with only remnants of the retina (*asterisk*). The eyelids and orbit are also packed with lymphoma cells, showing mitotic figures (*arrow*). Pathology score—6+ (H&E, *left* ×25, *right* ×200).

C Lymphoma cells (*broken arrows*) are observed in the meninges and subarachnoid space (*upper right*). The contralesional left eye is devoid of lymphoma cells but shows a focal aggregate in the optic nerve sheath (*arrows, left and lower right*). (H&E, *left* ×50, *right* ×200)

Fig. 3. Development of clinical signs of ocular involvement in SCID mice following intravitreal inoculation of Rev-2-T-6 cells and subsequent IP injections of anti-LFA-1 monoclonal antibodies. Control mice received PBS. Mice were followed individually until euthanization (end point of each curve). Number of mice observed at each time point are given in *parentheses* ($n = 10$ in both groups)

Fig. 4.

Photomicrographs of eyes from SCID mice following intravitreal inoculation of Rev-2-T-6 cells and subsequent IP injections of PBS (**A**), or anti-LFA-1 monoclonal antibody (**B, C**). **A** Lymphoma cells are evident in the conjunctiva and eyelid (*arrows*), as well as within the eye. The anterior chamber is filled with tumor cells (*asterisk*). In addition, large masses of tumor cells infiltrate and destroy the choroid (C) and sclera (S), and invade the extra ocular compartments of the orbit (O). Pathology score—5+ (H&E, left, $\times 25$, right, $\times 100$). **B** Lymphoma cells are packed inside and outside the right eye including the eyelids, cornea, anterior chamber, uvea, retina, sclera, and orbit. Pathology score—6+ (H&E, $\times 25$). **C** Lymphoma cells infiltrate the optic nerve tract (ONT), meninges (*arrow*), and brain parenchyma (P) and the anterior chamber (AC) of the contralateral eye. (H&E, *upper* $\times 100$, *lower* $\times 200$)

Fig. 5. Serum antibodies in Balb/C mice following intravitreal inoculation of Rev-2-T-6 cells and subsequent IP injections of either PBS or anti-LFA-1 monoclonal antibodies. Mice were bled and sera were applied for western blotting using Rev-2-T-6 cell extract. *NMS* normal mouse serum

Table 1

Pathology scores of individual Balb/C mice inoculated with Rev-2-T-6 cells and subsequently treated with either anti-LFA-1 or PBS

Treatment	Weeks post inoculation	No. of mice scored	Pathology score
Anti-LFA	4	4	6+
Anti-LFA	4	1	5+
Anti-LFA	5	1	6+
Anti-LFA	5	1	3+
Anti-LFA	6	1	6+
Control (PBS)	4	1	3+
Control (PBS)	5	1	1+
Control (PBS)	6	1	1+
Control (PBS)	6	1	1.5+
Control (PBS)	6	1	1+

Table 2

Sites invaded by Rev-2-T-6 cells following intravitreal inoculation into Balb/C and SCID mice and subsequent treatment with anti-LFA-1 monoclonal antibodies

Mice	Balb/C		SCID	
Treatment	<i>n</i> = 10 PBS (i.p)	<i>n</i> = 15 Anti-LFA (i.p)	<i>n</i> = 10 PBS (i.p)	<i>n</i> = 10 Anti-LFA (i.p)
Sites involved				
Injected eye	10/10 (100 %)	15/15 (100 %)	10/10 (100 %)	10/10 (100 %)
Orbit	0/10 (0 %)	14/15 (93.3 %)	9/10 (90 %)	10/10 (100 %)
Brain/ON	0/6 ^a (0 %)	7/8 ^a (87.5 %)	6/7 ^a (85.7 %)	6/6 ^a (100 %)
Contralateral eye	0/10 (0 %)	2/15 (13.3 %)	0/10 (0 %)	3/10 (30 %)
Systemic metastasis	0/10 (0 %)	0/15 (0 %)	0/10 (0 %)	0/10 (0 %)
Maximal scores (Mean ± SEM)				
Clinical	4 (3.2 ± 1.1)	6 (5.9 ± 0.3)	6 (5.7 ± 0.5)	6 (5.9 ± 0.3)
Histological	3 (1.5 ± 0.8) <i>n</i> = 5	6 (5.5 ± 1.0) <i>n</i> = 8	6 (5.9 ± 0.3) <i>n</i> = 7	6 (6.0 ± 0.0) <i>n</i> = 6

Control mice received PBS instead of anti-LFA-1 antibodies ON- optic nerve

^aSome brains from these groups were frozen and not used for histological evaluation