Pathologic Correlates of Primary Central Nervous System Lymphoma Defined in an Orthotopic Xenograft Model

Cigall Kadoch,1 Eduard B. Dinca,2 Ramona Voicu,2 Lingjing Chen,1 Diana Nguyen,1 Seema Parikh,3 Juliana Karrim,1 Marc A. Shuman,1,5 Clifford A. Lowell,4 Patrick A. Treseler,3,5 C. David James,2,5 and James L. Rubenstein1,5

Abstract

Primary central nervous system lymphoma (PCNSL) is defined as a high-grade lymphoma presenting in the brain or spinal cord in the absence of systemic lymphoma. Although the predominant histology of PCNSL is large B-cell lymphoma, Burkitt’s, Burkitt-like, lymphoblastic, and T-cell lymphomas may also manifest and be confined to the brain in a manner consistent with PCNSL (1, 2).

There is significantly less information regarding the molecular pathogenesis of PCNSL in comparison with systemic non-Hodgkin’s lymphoma. Generally, PCNSL appears to be derived from mature B cells, which have been exposed to antigen and have undergone T-cell-dependent affinity maturation in a germin center microenvironment (3). In support of this conclusion is the fact that the majority of non-AIDS PCNSL tumors express Bcl-6, a germin center marker. Other characterizations of the immunophenotype of PCNSL have revealed that these tumors exhibit overlapping features of germinal center and activated B-cell differentiation: immunohistochemical analysis shows that the majority of non-AIDS PCNSL tumors express MUM-1, a marker of activated B cells, and Bcl-6, a marker of germinal centers (4, 5). This latter conclusion is additionally supported by the results of microarray analyses of PCNSL gene expression (6, 7). This immunophenotypic stage of differentiation provides a logical basis to explain how a variant of large B-cell lymphoma that usually exhibits germinal center features, including Bcl-6 expression, is generally associated with an inferior outcome relative to systemic lymphomas of the same histology and stage (8, 9).

In addition to Bcl-6 and MUM-1, gene expression analyses have revealed ectopic expression of interleukin-4 (IL-4), a B-cell growth factor, by tumor cells and endothelium in PCNSL specimens (7). Moreover, the activation of STAT6, a transcriptional mediator of IL-4-dependent gene expression, was also shown to be expressed by PCNSL cells and tumor endothelia, providing evidence for active IL-4 signaling in CNS lymphoma. Elevated expression of activated STAT6 by lymphoma cells was associated with adverse prognosis in patients treated with
Translation of primary nervous system (CNS) lymphoma is a rare variant of non-Hodgkin's lymphoma and is associated with a distinctly poor prognosis. Here, we report the first intracerebral model of CNS lymphoma, which recapitulates several defined aspects of its molecular pathogenesis. These include the relevant immunophenotype of B-cell differentiation as well as the expression of interleukin-4 (IL-4) by malignant B cells in lymphoma xenografts. IL-4 is known to potentiate B-cell survival and to mediate the polarization of tumor macrophages to a M2 or alternative activation state associated with enhanced tumorigenesis. We provide the first evidence for expression of macrophages with M2 features in non-Hodgkin's lymphoma both in the model and in diagnostic specimens of CNS lymphoma. These results have implications regarding the potential effect of therapies, which target the IL-4 pathway, as well as the prognostic significance of macrophage subpopulations within non-Hodgkin's lymphoma.

Materials and Methods

Tissue culture. Raji, Ramos, 2F-7, and EBV-transformed peripheral blood lymphocytes were obtained from the American Type Culture Collection. These were cultured at 37°C and 5% CO₂ in high-glucose DMEM-21 supplemented with 1% penicillin-streptomycin, 10% FCS, and 1% nonessential amino acids. Raji cells were selected for high-dose methotrexate-based chemotherapy, supporting a significant role for IL-4 signal transduction in CNS lymphoma pathogenesis (7). To date, no animal model of PCNSL has been developed, which recapitulates both histopathologic and molecular features of this disease, including the relevant immunophenotypic state of differentiation of tumor cells and the reactive microenvironment. Given the poor prognosis of PCNSL as well as the severe toxicities associated with established genotoxic therapies, there is a significant need for such models to be applied in the systematic evaluation of novel agents for treating this cancer.

The development of bioluminescence imaging (BLI) has facilitated the noninvasive evaluation of tumor growth and therapeutic response in a variety of cancer xenograft models, including for brain tumors (10, 11). However, this technology has not been applied previously to the study of lymphomatous microenvironment. Given the poor prognosis of PCNSL and the severity of its molecular pathogenesis, there is a significant need for such models to be applied in the systematic evaluation of novel agents for treating this cancer.

In vivo assessment of temozolomide response. Mice with intracranial Raji-fl cells were imaged 4 days after injection and distributed into two groups (10 each) with equivalent average BLI signal. The temozolomide treatment group received five daily doses of 50 mg/kg by oral gavage for a total dose of 250 mg/kg temozolomide between days 4 and 8 after tumor engraftment. The control group received similar volumes of OraPlus, the methylcellulose vehicle used for temozolomide solubilization. Kaplan-Meier survival curves were generated using Prism3 software and the statistical difference between curves was derived with a log-rank test.

RNA isolation and quantitative reverse transcription-PCR. Lymphoma cell nuclei accounted for at least 70% of all cells in each tumor specimen used in this analysis as determined by histopathologic analysis of H&E-stained sections used in parallel. Tissue sections (14 μm thick) were fixed in 2% formaldehyde, blocked, and subjected to antibody staining with α-IL-4, α-VWF, α-CD68, or α-CD31 (Invitrogen). Secondary antibodies conjugated to Alexa Fluor 488 and 594 (Molecular Probes) were used for detection with an Olympus AX70 upright microscope. 4',6-Diamidino-2-phenylindole counterstain was used to visualize cell nuclei. Brightness and contrast of images were modified for publication.

Results

Development of an intracerebral model of invasive CNS lymphoma enabled for BLI. Raji cells were selected for stereotactic frame (Stoelting Just-For-Mice) and a Hamilton syringe with 26-gauge needle was used for injection at a volume of 5 μL and at a rate of 1 μL/min, with the needle left in place for 5 min after injection. All animal experiments were done following institutional guidelines.

BLI was conducted using a Xenogen Lumina optical imaging system (Caliper). Mice were anesthetized before intraperitoneal injections of luciferin (10 mg/kg, Orbital Biotechnology) at a dose of 150 μg/kg, providing a saturating substrate concentration for luciferase enzyme. Peak luminescent signals were consistently recorded 12 min after luciferin injection. Regions of interest encompassing the intracranial axis of signal were defined using Living image software, and the total photons/steradian/cm² were recorded.

In vivo assessment of temozolomide response. Mice with intracranial Raji-fl cells were imaged 4 days after injection and distributed into two groups (10 each) with equivalent average BLI signal. The temozolomide treatment group received five daily doses of 50 mg/kg by oral gavage for a total dose of 250 mg/kg temozolomide between days 4 and 8 after tumor engraftment. The control group received similar volumes of OraPlus, the methylcellulose vehicle used for temozolomide solubilization. Kaplan-Meier survival curves were generated using Prism3 software and the statistical difference between curves was derived with a log-rank test.

Immunohistochemistry, immunofluorescence, and in situ hybridization. For immunohistochemical studies, frozen or paraffin sections (8 μm thick) were fixed, blocked, and immunostained with the appropriate antibody: α-CD20 (BD Biosciences), α-Bcl-6 (CellMarque), α-MUM-1 (DAKO), monoconal α-IL-4 (Santa Cruz Biotechnology), rabbit polyclonal α-VWF (Santa Cruz Biotechnology), α-STAT6 (Upstate), α-CD11b (BD Biosciences), α-CD68 (clone KPI; DAKO), and monoclonal α-factors (Biogenex). Affinity-purified polyclonal rabbit α-Ym1 antibody was a kind gift of Dr. Richard Locksley. Immunoreactivity was visualized using the biotin-streptavidin peroxidase method. Immunofluorescence microscopy was done with frozen sections (6-8 μm) that were fixed in 2% formaldehyde, blocked, and subjected to antibody staining with α-IL-4, α-VWF, α-CD68, or α-factor XIIIa. Secondary antibodies conjugated to Alexa Fluor 488 and 594 (Molecular Probes) were used for detection with an Olympus AX70 upright microscope. 4’,6-Diamidino-2-phenylindole counterstain was used to visualize cell nuclei. Brightness and contrast of images were modified for publication.

For in situ hybridization, full-length human IL-4 cDNA in pDNDR-Dual (6971781; American Type Culture Collection) was subcloned into pBlueScriptSK(-). In vitro transcription was done using T3 and T7 polymerase to generate digoxigenin-labeled riboprobes. All tissues used for in situ hybridization were snap-frozen in OCT, sliced into frozen sections (16 μm thick), and stored at -80°C. Sections were then fixed in 4% paraformaldehyde and subjected to proteinase K digestion followed by incubation in 1% Triton X-100. Hybridization was done at 60°C with 400 ng/mL digoxigenin-labeled antisense and sense riboprobes to human IL-4 and the bound probe was detected using alkaline phosphatase-labeled anti-digoxigenin F(ab) (1:5,000 dilution) and NBT/BCIP tablets (Roche Applied Science).

RNA isolation and quantitative reverse transcription-PCR. Lymphoma cell nuclei accounted for at least 70% of all cells in each tumor specimen used in this analysis as determined by histopathologic analysis of H&E-stained sections used in parallel. Tissue sections (14 μm thick) were prepared using a cryostat. RNA was quantified based on absorbance at 260 nm, and quality was assured by measuring absorbance 260:280 ratios. Quantitative reverse transcription-PCR (RT-PCR) for determination of human IL-4 and mouse Ym1 (Chi3-L3) RNA expression was done using primer probe sets (Applied Biosystems).

Results

Development of an intracerebral model of invasive CNS lymphoma enabled for BLI. Raji cells were selected for...
intracranial injection based on their tumorigenicity in mouse models of systemic non-Hodgkin’s lymphoma and because of their high relative expression of oncogenes, such as Pim kinases, which are associated with poor prognosis in systemic lymphoma and refractory disease in CNS lymphoma (7, 12–14). The Raji cells were modified to stably express firefly luciferase by means of lentiviral transduction as described (11). Modified cells implanted in the brain (5 × 10⁶ per mouse) were found to be highly tumorigenic and exhibited an invasive phenotype with molecular characteristics of PCNSL: CD20⁺/Bcl-6⁺/MUM-1⁺, the predominant immunophenotype of PCNSL (refs. 4, 5; Fig. 1).

Raji lymphoma cells implanted in the brain are extremely infiltrative, with evidence for leptomeningeal invasion and dissemination into the contralateral hemisphere in nearly every xenograft, replicating this quintessential feature of PCNSL (Fig. 1). Immunohistochemistry and quantitative RT-PCR showed that Raji cells express cathepsin D, a protease shown previously to be up-regulated in PCNSL pathologic specimens compared with nodal non-Hodgkin’s lymphoma specimens and a candidate mediator of CNS lymphoma invasiveness (7). We also used immunohistochemistry and quantitative RT-PCR to show lymphoma xenograft expression of Pim-2 kinase, a prosurvival gene associated with refractory CNS lymphoma and which has also been linked to prostate cancer invasiveness (refs. 13, 15, 16; data not shown).

**IL-4 expression in PCNSL.** We previously showed significant expression of IL-4 within PCNSL tumors by quantitative RT-PCR and noted a trend between high intratumoral expression of IL-4 and shorter overall survival. We have also applied in situ hybridization to investigate intratumoral IL-4 expression within PCNSL diagnostic specimens, the results of which showed that IL-4 RNA was reproducibly expressed by lymphoma cells in PCNSL (7) as well as in cases of nodal large B-cell lymphoma.⁶

We therefore evaluated the expression of IL-4 by Raji lymphoma cells in vitro using quantitative RT-PCR and determined constitutive IL-4 expression in Raji cells under tissue culture conditions. Similar results were detected in other B lymphoma cell lines analyzed for IL-4 expression including Ramos cells (Fig. 2A) and 2F-7 and EBV-transformed human B-lymphocytes (data not shown). Using species-specific primers for human IL-4, we used quantitative RT-PCR to show that IL-4 expression is induced by >3-fold in Raji cells on intracerebral implantation (P < 0.03), suggesting that this multifunctional cytokine participates in CNS lymphomagenesis.

Relative IL-4 RNA levels in Raji intracerebral xenografts were similar to levels in PCNSL tumors, particularly in cases with short survival in which normalized IL-4 RNA expression was between 0.03 and 0.332 (relative to hGUS), supporting the physiologic relevance of these measurements. To our knowledge, this is the first direct demonstration of IL-4 expression by malignant B lymphoma cells in vitro or in vivo.

We confirmed the intratumoral expression of IL-4 by Raji lymphoma cells in xenografts both by in situ hybridization and by immunohistochemical detection methods (Fig. 2B). IL-4 protein was detected predominantly on tumor cells, tumor vasculature, and occasionally tumor-associated macrophages.

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⁶ Unpublished observations.
but not within areas of normal brain devoid of tumor infiltration.

Further evidence for IL-4-mediated autocrine as well as paracrine signaling within the Raji xenograft-based CNS lymphoma model was indicated by the expression of activated STAT6 by tumor cell and tumor vasculature, consistent with our demonstration of the expression of IL-4 (and activated STAT6) selectively by tumor vessels in human PCNSL tumors (ref. 7; Fig. 3A). By contrast, tumor angiogenesis in response to intracerebral implantation of human glioblastoma cells was not associated with expression of IL-4 or activated STAT6 (Fig. 3B).

**M2 macrophage polarization in PCNSL.** A recent analysis of the cerebrospinal fluid proteome in brain tumor patients showed that tryptic peptides derived from CD14, a marker of activated macrophages, are significantly up-regulated in patients with CNS lymphoma (17). Based on this observation, which suggests that macrophage activation may be a prominent feature of the disease, as well as the evidence for a significant pathologic role of tumor-associated macrophages in cancer (18–22), we pursued the characterization of the phenotypic differentiation of intratumoral macrophages within our model.

We determined that the intracerebral implantation of Raji cells results in profound intratumoral infiltration of macrophages, often concentrated around blood vessels, as shown by staining for CD11b (Mac-1), a monocye/macrophage marker (Fig. 4A and B). There was minimal or absent CD11b immunoreactivity in areas of normal brain.

Given our demonstration of the endogenous expression of IL-4 by tumor cells and by other cells within the microenvironment in human PCNSL, we hypothesized that a significant proportion of intratumoral macrophages in the xenograft model would express Ym1 (chitinase-3-like 3), one of the most highly induced IL-4 target genes and an established marker of IL-4-induced M2 polarization of murine macrophages (23,24). Immunohistochemical analysis revealed significant expression of Ym1 by the majority of tumor-associated macrophages in Raji intracranial xenografts as evidenced by colocalization with CD11b (Fig. 4). Quantitative RT-PCR confirmed the intratumoral induction of mouse Ym1 RNA within Raji xenografts after intracranial tumor implantation in athymic mice.

In parallel, we hypothesized that intratumoral macrophages in human PCNSL would also exhibit features of M2 polarization, induced by IL-4. Given that Ym1 is a mouse-specific...
macrophage marker and that only a subset of macrophage polarization markers apply from one species to another (23), we focused on the identification of macrophage polarization markers, which are relevant in human lymphoma. Recent studies have suggested that factor XIIIA is an IL-4-induced marker of M2 activation of human macrophages in vitro (25, 26). Our own studies of the gene expression of tumor-associated CD14+ macrophages, positively selected from the cerebrospinal fluid from patients with CNS lymphoma, suggested that factor XIIIA is a candidate marker of tumor macrophages in this disease. In the present study, we used immunohistochemistry to examine the expression of factor XIIIA within PCNSL specimens. Both immunoperoxidase and dual-color immunofluorescence strategies with a monoclonal antibody against factor XIIIA revealed its colocalization with CD68+ macrophages in 15 of 20 diagnostic specimens of large cell PCNSL (Fig. 5). As expected, factor XIIIA+ cells did not coexpress glial fibrillary acidic protein (not shown). Factor XIIIA+/CD68+ cells also did not express S100, suggesting that these macrophages have not undergone dendritic cell maturation. In addition, factor XIIIA+ macrophages were often detected in close proximity to the tumor vasculature (Fig. 5A). By contrast, significant numbers of factor XIIIA+ macrophages were rare in eight consecutive specimens of nodal large B-cell lymphomas as deduced by a parallel immunohistochemical analysis. These data are consistent with the
hypothesis that IL-4, produced by lymphoma cells, tumor endothelia, and/or T cells within PCNSL tumors, stimulates macrophage programming toward a M2 phenotype. We believe this to be the first evidence of macrophages displaying M2 features in large B-cell lymphoma.

Monitoring intracerebral tumor growth and therapeutic response using BLI. To assess the utility of this CNS lymphoma xenograft model for studying intracranial tumor response to therapy, Raji cells were modified with luciferase lentivirus and thereby enabled for in vivo BLI. Results in Fig. 6 show a significant correlation between bioluminescence signal and Raji cell number in vitro ($R^2 = 0.98$). Analysis of the kinetics of intracranial xenograft growth indicated two phases: a slow growth phase between days 0 and 10 followed by rapid, log-phase growth. Untreated, the median survival of mice bearing intracranial lymphoma in this model was only 13 days.

We next investigated the response of intracranial luciferase-modified Raji xenografts to temozolomide, an alkylating agent now commonly used in PCNSL therapy, both during induction therapies, either as monotherapy or in combination with methotrexate and as means of salvage (27–30). A short course of temozolomide (250 mg/kg/d × 5 days), orally administered, was reproducibly associated with significant delay in tumor progression compared with mock-treated control mice as shown both by BLI and by the delayed onset of neurologic symptoms and prolongation of survival in mice bearing intracranial lymphoma ($P < 0.0001$; Fig. 6). Whereas Raji cells in vitro were found to be sensitive to temozolomide in a dose-dependent manner, in vivo Raji tumors rapidly exhibited resistance to this agent, and overall survival of treated mice bearing CNS lymphoma xenografts was not extended beyond 23 days even when the dose of temozolomide was increased to 300 mg/kg/d.

We hypothesized that high constitutive expression of the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) could account for the relative resistance to temozolomide (11). Given that methylation of the MGMT promoter has been shown to predict favorable response to temozolomide in glioblastoma (31), we tested for this possibility using methylation-specific PCR and were unable to detect DNA methylation of the MGMT promoter in Raji cells. The lack of MGMT promoter methylation was consistent with the results of immunoblot analysis for MGMT protein, which revealed high constitutive expression of MGMT protein by Raji lymphoma cells, compared with four of five glioblastoma xenografts derived from distinct patient tumors (data not shown). These results suggest that lack of MGMT promoter methylation, high constitutive MGMT expression, and possibly other survival signals including the activation of STAT6 signaling may contribute to relatively high alkylator resistance in this CNS lymphoma model.

Discussion

We show that the intracerebral growth of Raji lymphoma cells in athymic mice recapitulates several molecular and
histopathologic features of PCNSL pathogenesis, supporting the use of this model for studies of novel agents that selectively address resistance mechanisms identified in this rare variant of non-Hodgkin’s lymphoma. In comparison with prior studies of orthotopic glioma xenografts (11), intracerebral Raji xenografts are moderately sensitive to the alkylating agent temozolomide and exhibit expression of CD20, Bcl-6, MUM-1, IL-4, activated STAT6, Pim-2 kinase, and cathepsin D as well as tumor macrophages displaying M2 features, reflecting molecular and cellular characteristics defined in PCNSL. We believe this to be the first model of CNS lymphoma to recapitulate multiple molecular and histopathologic features of the disease. Further, we show the first application of bioluminescence to monitor tumor progression and response in a highly infiltrative orthotopic xenograft model of CNS lymphoma.

In this study, we confirm our original observation regarding the expression of IL-4 by B lymphoma cells in CNS lymphoma and directly show its constitutive expression by lymphoma cells in tissue culture and in vivo. We propose a model in which IL-4 expression by lymphoma cells has multiple prosurvival functions in these tumors: (a) IL-4 expression may potentiate antiapoptotic mechanisms in B lymphoma cells (32, 33); this is supported by our prior demonstration that PCNSL tumors with foci of strong STAT6 activation are associated with resistant.
disease and short survival (7). (b) IL-4 may regulate tumor angiogenesis (34–36). (c) IL-4 may regulate macrophage differentiation to a M2 phenotype, which promotes tissue remodeling, tumor progression, and tumor-associated immunosuppression (18, 26, 37).

Macrophages exhibit diverse functional programs in response to environmental signals. Classically activated macrophages, denoted as M1, are characterized by high intrinsic cytotoxic activity against tumor cells by the release of toxic intermediates such as nitric oxide, reactive oxygen intermediates, and tumor necrosis factor. There is increasing evidence that anti-inflammatory molecules such as IL-4, IL-13, IL-10, glucocorticoids, and transforming growth factor-β induce a distinct alternative activation of macrophages, generally denoted as M2. M2 macrophages are involved in Th2 responses and promote wound healing, angiogenesis, and tumor progression. The generation of M2 macrophages attenuates adaptive immunity to environmental signals. Classically activated macrophages, generally denoted as M1, produce nitric oxide, are proinflammatory, and drive chronic inflammation. There is increasing evidence that anti-inflammatory microenvironments are maintained within the brain under normal conditions and the potential pathophysiological mechanisms that may be subverted by IL-4-producing tumors such as PCNSL to suppress the immune response.

It is particularly noteworthy that Raji cell growth within the brains of athymic mice is highly aggressive in the absence of additional, exogenous immunosuppression. To prevent xenograft rejection, Clynes et al. used total body irradiation (3.0 cGy) to facilitate the tumorigenesis of 5 × 10^6 Raji cells subcutaneously implanted in athymic mice (43). By contrast, we determined that the intracerebral growth of a smaller inoculum of Raji cells (5 × 10^5 cells) produced tumors in 100% of athymic mice without immune suppression induced by chemotherapy or irradiation before tumor implantation (n = 50 mice). The parallel, subcutaneous, flank implantation of the same inoculum of Raji lymphoma cells failed to elicit tumor growth in three of three immunosuppressed mice.

We hypothesize that, based on its capacity to promote B-cell survival, to stimulate angiogenesis and to suppress the immune response through the M2 polarization of tumor macrophages, intratumoral IL-4 expression may significantly contribute to CNS lymphomagenesis. For these reasons, we suggest that pharmacologic antagonists of IL-4 signaling may facilitate apoptotic response in the treatment of CNS and systemic B-cell malignancies as well.

The evidence for M2 differentiation of a subset of intratumoral macrophages in CNS lymphoma, as shown by their expression of the IL-4 target genes, Ym1 or factor XIIIA, has implicatons regarding the clinical evaluation of macrophage content in pathologic specimens as a predictor of prognosis in non-Hodgkin’s lymphoma (44–46). Because CD68 is expressed by multiple subpopulations of tumor-associated macrophages in humans, including M1 and M2 phenotypes, the utility of this antigen as a prognostic marker may be limited given that it reflects the total macrophage population, including subtypes that are divergently polarized with different functional properties. Clearly, additional studies are needed to identify phenotypic markers that delineate M1-type versus M2-type macrophage polarization in human cancer. Further studies are needed to evaluate the significance of these macrophage phenotypes as well. The determination of the relative proportion of intratumoral subpopulations of M1 versus M2 macrophages will likely provide more accurate insight into their prognostic significance than are current approaches based on the immunohistochemical detection of CD68 expression alone.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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