## 2008 Research Grant Program Winning Abstract

### Tumor Macrophage Polarization

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Tumor-induced immune suppression is a significant impediment to innate immunosurveillance and immunotherapy of cancer. Macrophages are a critical component of anti-tumor immunity but may be subverted from the classically-activated, or M1 phenotype, which mediates tumor elimination, to an alternatively-activated, M2 phenotype, which promotes tumor progression. Interleukin-4 (IL-4) signaling is a pivotal regulator of macrophage polarization to the M2 phenotype.

There is an unmet need for the identification of biomarkers which delineate the phenotypic subpopulations of human macrophages within clinical specimens. Such biomarkers may enhance clinical prognostication, provide insight into relevant pathways of immune suppression utilized by the cancer, and significantly impact the development of strategies to potentiate the anti-tumor immune response. A number of recent studies have demonstrated that tumor-associated macrophages within pathologic specimens of cancer impacts prognosis; some of these conclude that intratumoral macrophages portend adverse prognosis, for example in follicular lymphoma, while others suggest that intratumoral macrophages predict favorable outcome. The basis for these conflicting results may lie in the fact that, in clinical practice, tumor-associated macrophages are identified and quantified by the immunohistochemical detection of the total population of CD68+ cells. Because CD68 is expressed by multiple subpopulations of tumor-associated macrophages, including M1 and M2 phenotypes, the use of CD68 as a prognostic marker may be unreliable given that it reflects the total macrophage population including subtypes which are divergently polarized with different functional properties. The identification of selective biomarkers of the M2 phenotype of macrophages in human tumors may lead to advances in tumor prognostication and therapeutic strategies.

The efficacy of rituximab, an anti-CD20 monoclonal antibody that has become a cornerstone of treatment for B-cell lymphoma, appears to be mediated predominantly by antibody-dependent cellular cytotoxicity (ADCC) with tumor-associated macrophages being the dominant effector cell type. There is an extreme paucity of molecular information regarding the role and phenotype of tumor macrophages in human brain tumors, including CNS lymphoma. Moreover, the phenotype of tumor-associated macrophages (M1 vs. M2) has not been evaluated as a potential determinant of rituximab efficacy or resistance, within any intratumoral compartment; this is largely due to the absence of reliable biomarkers of macrophage polarization in humans.

While macrophages from the cerebrospinal fluid (CSF) are routinely quantified in cytospin analyses which are performed during standard clinical practice, to date there is no established information regarding the phenotype and state of differentiation of macrophages within the leptomeningeal compartment. We have developed a novel flow-cytometrry-based protocol for the isolation and phenotypic characterization of macrophages from the cerebrospinal fluid (CSF) of patients with CNS lymphoma.

We are using BD FACSAria and Aria II instrumentation for sorting as well as a set of BD fluorescence-conjugated antibodies to define these subpopulations of human tumor macrophages. We are using a set of candidate markers of M2 differentiation of macrophages which we predicted to be relevant based upon gene expression studies of CD14+ activated CSF macrophages isolated by positive selection from the CSF: our main candidates of M2 markers include CD206 and Factor XIII (each of which have previously been shown to be induced in macrophages upon IL-4 stimulation and are candidate markers of M2 programming).

We are currently evaluating the phenotypes of tumor macrophages within the leptomeningeal compartment in patients with recurrent central nervous system lymphoma who are participating in a Phase I clinical trial with intraventricular rituximab plus methotrexate (MTX). This study represents an extension of our initial Phase I trial of rituximab monotherapy (J Clin Oncol, 2007). A major correlative aim of this trial is to test the hypothesis that M2 macrophage polarization contributes to acquired rituximab resistance. Thus far our data demonstrate that rituximab treatment results in a strong M2-skewing effect, with a five-fold increase in the proportion of macrophages which express M2 markers such as CD206 within four weeks of repeat rituximab administration. During weeks three and four of twice-weekly treatment with intraventricular rituximab plus MTX, CSF macrophages continued to exhibit progressive M2-skewing, with a greater than 10-fold increase in the proportion of M2 macrophages. We are also currently using this flow-cytometric strategy as a means to sort distinct macrophage subpopulations for gene expression analyses using Affymetrix microarrays to further define the phenotypic features of M2 macrophages.

While preliminary, we believe this to be the first data depicting the polarization states of intratumoral macrophages in humans and the first description of dynamic changes in macrophage phenotypes during the evolution of resistance to rituximab therapy. While FACS is used to detect lymphoma or leukemic cells in CSF, its application to evaluate and discover macrophage subpopulations in brain tumors is novel. The elucidation of distinct macrophage subpopulations based upon the expression of candidate markers of M1 vs. M2 phenotypes may provide insight into tumor pathogenesis and prognosis and may yield the development of a novel flow-cytometric test which predicts the onset of rituximab resistance, based upon the detection of the ratio of M1 vs. M2 macrophages. Such a test could impact clinical decision-making, not only in CNS lymphoma, but could also be translated to the analysis of circulating macrophage subpopulations in the blood.

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