Glioblastoma derived exosomes contribute to tumor immune evasion

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Glioblastoma derived exosomes contribute to tumor immune evasion
IR-01. CYTOMEGALOVIRUS SUBVERTS THE MONOCYTE LINEAGE TO BECOME GLIOMA PROPAGATING
Krystine Dzurzynska1, Jun Wei1, Wei Qiao1, Mustafa A. Hattaboglu2, Daniel P. Cahill3, Nicolas B. Levine1, Sujit S. Prabhu1, Ganesh Rao1, Raymond Sawaya1, and Amy B. Heimberger1; 1The University of Texas MD Anderson Cancer Center, Houston, TX; 2Royal University Hospital, Saskatoon, SK, Canada

We have identified a mechanism by which cytomegalovirus (CMV) interleukin-10 (IL-10) is utilized by glioblastoma multiforme (GBM) to maintain the immunosuppressive microenvironment. CMV has been ubiquitously detected within high-grade gliomas, and the aggressive nature not only has been cited. GBMs harvested ex vivo were analyzed by flow cytometry to determine CMV antigen expression. Distinct expressing subsets of cells, such as the myeloid lineage and CD133+ cells, were identified. CMV antigens US28, pp63, IE1, and IE2 were present within all four individual and fully charactered human GBM cell lines. GBM-associated monocyte/macrophages and glioma-associated microglia, were exposed to CMV-conditional medium or recombination of cocultured monocyte/macrophages and glioma cells, respectively. Exposure of CD14+ cells to CMV IL-10 induced the up-regulation of IE1, a marker of transcriptional activation. Distinct expression of CD14+ cells was observed. GBM IL-10 was able to induce the migration of gCSCs compared with the supernatant from CD14+ cells cultured in medium alone. This result indicates that CMV subverts GBM-associated microglia/macrophages to support the immune-suppressive microenvironment by shifting their phenotype to the immune-suppressive M2. The shift is subsequent to activation of the STAT3 pathway, resulting in the propagation of glioma angiogenesis via an increase in VEGF and by increasing glioma invasion. Therapeutic strategies involving immune-mediated cytotoxic responses now include strategies to reverse tumor-mediated immune suppression. This study suggests that including CMV as a target could enhance the effectiveness of immunotherapy.

IR-02. IMMUNE-MODULATORY PROPERTIES OF GLOBLASTOMA MULTIFORME EXOSONES
Jerome de Vrij1, Kitty M.C. Kwappenberg2, Syben L.N. Maas3, Anne Kleijn1, Martine L. Lamfers1, Clemens M.F. Dirven1, Marco W. Schilliam, and Marike L.D. Broekman4; 1Erasmus Medical Center, Rotterdam, the Netherlands; 2Leiden University Medical Center, Leiden, the Netherlands; 3University Medical Center Utrecht, Utrecht, the Netherlands

The cellular immune response in patients with glioblastoma multiforme (GBM) is severely impaired, and the aggressive nature not only has been cited. GBMs harvested ex vivo were analyzed by flow cytometry. We observed higher CD14 expression and lower HLA-DR expression on monocytes after 3 days of exposure of the peripheral blood mononuclear cells to GBM exosomes than on monocytes exposed to medium alone. The effect on monocytes was strengthened with a purified monocyte population, indicating a direct effect. These results correspond to the changes in the phenotype of monocytes in peripheral blood of GBM patients and suggest an important immunomodulatory role for brain tumor exosomes.

IR-03. INTENSE HUMAN CYTOMEGALOVIRUS (HCMV) IMMUNE RESPONSE IN GLIOBLASTOMA PATIENTS: A PROGNOSTIC FACTOR FOR SURVIVAL
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INTRODUCTION: HCMV is a ubiquitous human herpesvirus found in nearly all humans worldwide, with persistent infection occurring in over 70% of adults. The virus has been implicated in the development of several human malignancies owing to the oncomodulatory effects of HCMV infection. There is growing scientific evidence about an association between HCMV and malignant gliomas. To study the prognostic value of the anti-HCMV immune response, we prospectively assessed the levels of serum HCMV immunoglobulins M and G (IgM and IgG) in newly diagnosed glioblastoma patients and correlated the results with the clinical course.

METHODS: We analyzed serum from 32 glioblastoma patients treated with standard chemoradiotherapy at our institution between November 2008 and October 2010. Serum serologies were obtained by chemiluminescent quantitative analyses. HCMV IgM >0.5 UAI/ml was considered diagnostic for acute HCMV infection, and HCMV IgG >16 UAI/ml was regarded as positive for latent infection. Intense HCMV immune response was defined as HCMV IgG >100 UAI/ml. All clinical and pathological data were recorded in a database system using the SPSS version 13.0 statistics package. RESULTS: After a median follow-up of 18.2 months, 24 patients (75%) have died. HCMV IgG was positive for latent infection in 23 patients (72%), 10 of whom had an immune response (31%). Two patients had an acute HCMV reactivation with positive values for IgM. In univariate analysis, HCMV IgG >100 UAI/ml demonstrated a strong significant association with a longer overall survival (p = 0.0087). Positive HCMV IgG was found to be marginally associated with survival (p = 0.07). In multivariate analysis, HCMV IgG >100 UAI/ml retained statistically significant as a prognostic factor for longer survival (hazard ratio, 0.18; 95% CI 0.04-0.81; p = 0.02). CONCLUSION: Intense HCMV IgG immune response is significantly associated with longer overall survival in our series. Larger studies are required to validate HCMV IgG as a prognostic factor for survival in glioblastoma patients.

IR-04. TUMOUR-INFILTRATING T-CELL SUBPOPULATIONS IN GLOBLASTOMAS
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This study was designed to determine the incidence and prognostic value of various populations of tumour-infiltrating T cells in glioblastomas. We also evaluated the difference in T-cell populations after conventional treatment. Sixty-seven patients with glioblastomas underwent surgery between 2003 and April 2009. Immunohistochemical staining was performed for CD3, CD4, CD8, and FoxP3, and the average number and percentage of positive cells were calculated. From eight patients, the average number of CD3, CD4, CD8, and FoxP3, and FoxP3+ T cells, 6.8 ± 1.3 CD8+ T cells, 1.5 ± 0.5 CD4+ T cells, and 0.6 ± 0.2 FoxP3+ T cells. The percentage of positive T-cell subpopulations was 89.6%, 22.4%, 77.6%, and 34.3% for CD3, CD4, CD8, and FoxP3, respectively. Among the eight patients there was no difference in the subpopulations between the first and second operations. The median progression-free survival was 7.0 months (95% CI, 5.2-8.9 months) and the overall survival was 14.8 months (95% CI, 11-18.7 months). Univariate analysis showed a statistically significant difference in progression-free survival for CD8 (p = 0.02) and in overall survival for

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IR-05. INTERLEUKIN-4 RECEPTOR ALPHA CHAIN
(4-ARPHA) PROMOTES THE IMMUNOSUPPRESSIVE ACTIVITY OF GLIOMA-INFILTRATING MONOCYTES
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4-AR-alpha is expressed on immunosuppressive cells of monocyte lineage and mediates its production of transforming growth factor (TGF)-beta in response to interleukin-13. We thus hypothesized that 4-AR-alpha expression on monocytes plays a significant role in glioma development. Analyses of human glioma-infiltrating leukocytes revealed that glioma-infiltrating monocytes, but not peripheral blood CD14+ monocytes, express high levels of 4-AR-alpha, suggesting the unique up-regulation of HLA-DR and tumor suppressor. Analysis of in vitro cultured bone marrow cells demonstrated that compared with cells derived from WT mice, H4-AR+/- mouse-derived cells contained lower numbers of CD11b+Gr1+ monocytes with lower arginase and TGF-beta expression as well as a decreased ability to suppress T-cells in vivo and in vitro. Because type-1 skewed T-cells in H4-AR+/- animals could have contributed to the observed better survival compared with WT mice, we next depleted CD4+ and CD8+ T-cells by using antibodies. Although T-cell depletion shortened the overall survival of WT and H4-AR +/- mice, T-cell-depleted H4-AR +/- mice still exhibited enhanced survival over T-cell-depleted WT mice. These data suggest that 4-AR-alpha expression on glioma-infiltrating monocytes promotes the immunosuppressive microenvironment of gliomas through a variety of mechanisms, including TGF-beta production and T-cell inhibition, thereby facilitating glioma development.

IR-07. THE RCAS/TW-A MODEL OF MURINE GLIOMA REPRODUCES IMMUNOSUPPRESSION PRODUCED BY MYELOID-DERIVED SUPPRESSOR CELLS IN HUMANS WITH GBM
Basant Raychaudhuri and Michael A. Vogelbaum; Cleveland Clinic, Cleveland, OH

Myeloid-derived suppressor cells (MDSCs) are a population of bone marrow-derived cells with potent immunosuppressive properties. We previously showed (Neuro-Oncology, 2011) that MDSCs are found at elevated levels in the circulation of patients with glioblastoma multiforme (GBM) and that they produce reversible T-cell dysfunction. We now show that MDSCs are present in GBM tumors and that the mouse model overexpressing platelet-derived growth factor subunit B in Nestin-tva/mk4a- arf/KO reproduces our observations with patients. We collected tumor tissue and blood from consenting patients (n = 5) with newly diagnosed GBM. Peripheral blood mononuclear cells were isolated, and MDSC subsets were detected by fluorescence-activated cell sorting analysis. From the murine glioma model (n = 18 mice) we harvested gliomas, normal brain tissue, and hematological tissues. Cells were dissociated, stained, and subjected to similar analysis. MDSCs were present in both human and murine gliomas. In murine tumors there were more monocytic MDSCs (Gr1low, > 5%) than neutrophilic MDSCs (Gr1high, > 3%), and both were present at much higher levels in the tumors than in normal brain tissue (p < 0.016). MDSCs were also higher in the circulation of mice with gliomas than of control mice, but there the Gr1high subset predominated. GBM patients also had MDSCs present in tumor tissue (70 ± 3.1%). Subclassification of the MDSCs in the human samples indicated that lineage-negative MDSCs (CD15-CD33+/HLA-DR-) were more prevalent than MDSCs with a neutrophilic subtype (CD15+ CD14-CD33+ HLA-DR-). We also found that the proliferation and intracellular interferon-gamma level of splenocytes isolated from normal mice were decreased in the presence of Gr1+ MDSCs from murine gliomas, indicating that this model reproduces the MDSC-induced immunosuppression seen in our GBM patients. We showed that MDSCs are present in both human and mouse gliomas and that the cells suppress T-cell function. Neutrophilic MDSCs predominate in the circulation of both species, whereas in the tumors the monocyte MDSCs dominate in mice and the lineage-negative subset dominates in humans.

IR-06. IMMUNOLOGICAL SOIL AND PREVENTION OF BREAST CANCER BRAIN METASTASIS
Yan Liu, Masasuke Ohno, and Hideho Okada; University of Pittsburgh Cancer Institute, Pittsburgh, PA

As therapies for surgical cancer improve and patients survive longer, the risk of cerebral metastases increases. Therefore, the cerebral metastasis of cancer is a major obstacle that must be overcome before cancers can be cured by any means. In our recent data, mice bearing 4T1 breast cancers in the primary site (mammary pad) showed accumulations of myeloid-derived suppressor cells (MDSCs) in the brain before their brains demonstrated any presence of metastatic tumor cells. These observations were accompanied by marked up-regulation of the inflammatory chemokines S100A8, S100A9, and serum amyloid A 3 (SAA3) in the pre-metastatic brains. Elevated levels of the cytokines tumor necrosis factor alpha and vascular endothelial growth factor, significantly promote MDSCs, were detected in the sera of 4T1-bearing mice. Chemokine CCL2 was also up-regulated in the pre-metastatic brain of 4T1-bearing mice, and anti-CCL2 treatment reduced MDSC infiltration. The cyclooxygenase-2 inhibitor celecoxib reduced MDSC infiltration as well as the expression of S100A8, S100A9, and SAA3 expression in the pre-metastatic brains of 4T1-bearing mice. On the other hand, neither MDSC accumulation nor up-regulation of S100A8, S100A9, or SAA3 was detected in the brains of mice bearing JC breast cancer cells, which are not metastatic. Our results suggest that tumor cells with high metastatic activity up-regulate proinflammatory cytokines and induce immunosuppressive conditions in the distant target organs, such as brain, and thereby promote metastasis. Anti-CCL2 and celecoxib treatments might be used to prevent the formation of pre-metastatic immunological soil. Further understanding of the mechanisms underlying the immunological soil will allow us to develop effective strategies to prevent cerebral metastasis of breast cancer.

IR-08. GLIOBLASTOMA-DERIVED EXOSOMES CONTRIBUTE TO TUMOR IMMUNE EVASION
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Glioblastoma multiforme (GBM) is the most frequent and lethal primary brain tumor in adults. Despite intense biomedical research, the median survival after diagnosis is 15 months. One factor contributing to this poor prognosis is the immune protection afforded by the tumor microenvironment. Tumors have a diverse repertoire of immune-evasive techniques. One method of evasion not well explored is the release of tumor-derived exosomes. Exosomes are tiny membrane-bound vesicles of endocytic origin that contain viable mRNA and functional proteins that can affect the physiology of recipient cells. Exosome release has been reported for numerous cancer types, including GBM. Exosomes from colon cancer have been shown to carry Fas ligand (FasL) and to induce apoptosis of activated T cells. The metastatic activity in their primary site induce immunosuppressive microenvironment of gliomas through a variety of mechanisms, including TGF-beta production and T-cell inhibition, thereby facilitating glioma development.

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IR-09. CHARACTERIZATION OF THE IMMUNE RESPONSE TO ONCOLYTIC ADENOVIRUS THERAPY FOR MALIGNANT GLIOMA
Angelie Kleiapp, Jannke Kloesemann, Elke Trefers-Westerlaken, Gulia Füls, Sieger Leenstra, Clemens Dirven, René Debeets, and Martine Lamfers
1Erasmus Medical Center, Rotterdam, the Netherlands; 2Massachusetts General Hospital, Harvard Medical School, Boston, MA

IR-11. MODULATION OF NEUTROPHIL ACTIVATION BY TUMOR AND TUMOR-ASSOCIATED NECROSIS IN Glioblastoma
Trisha R. Suppel, Jason White, Rae Russel, and Allen Waziri; University of Colorado Anschutz Medical Campus, Aurora, CO

We recently identified an immunosuppressive mechanism in GBM patients that results in such neutrophil activation and release of inflammatory cytokines and that may result in reversible T-cell dysfunction. We noted isolated instances of increased neutrophil infiltration of GBM, but the mechanism through which significant numbers of activated neutrophils persist within the peripheral circulation of these patients remains unclear. We hypothesized that tumor-associated factors are responsible for the activation and subsequent alteration of surface-binding proteins of neutrophils within the tumor microvasculature. To initially explore this hypothesis, normal donor neutrophils were incubated with tumor-conditioned medium or necrotic material. Induction of degranulation was measured via flow cytometric quantification of surface markers associated with neutrophil activation. Following incubation with conditioned medium or necrotic material, increased surface expression was observed for both CD11b (1.6- and 1.5-fold, respectively) and CD66 (1.7- and 1.4-fold, respectively), confirming tumor-specific induction. In further exploration of baseline characteristics of circulating neutrophils in GBM patients, resting and degranulated populations were purified from peripheral blood using a dual-density Histopaque gradient. Baseline expression patterns of CD66 on resting and CD11b on degranulated neutrophils expressed paradoxically low levels of CD11b and high levels of L-selectin (0.6- and 1.3-fold, respectively, compared with normal cells). Because CD11b is required for strong intravascular adhesion and L-selectin is normally expressed under low-grade stimuli, these data suggest that peritumoral activation may result in degranulation without effective intratumoral infiltration. These results provide a preliminary explanation for the prevalence of degranulated neutrophils within the peripheral circulation of GBM patients. In addition, we propose that factors present within the tumor microvasculature induce important changes in the functional binding characteristics of these cells.

IR-10. STEREOTACTIC RADIOSURGERY COMBINED WITH DOUBLE IMMUNOTHERAPY WITH ANTI-CTLA-4 AND ANTI-4-1BB YIELDS LONG-TERM SURVIVAL AND PROTECTIVE ANTITUMOR RESPONSE IN A MOUSE ORTHOTOPIC Glioblastoma MODEL
Zanech Belcaid, Jillian A. Phallen, Jing Zeng, Alfred P. See, Emilia Albesiano-Nicholas M. Durham, Betty Tyler, Henry Brem, Drew M. Pardoll, Charles Drake, and Michael Lim; Johns Hopkins University, Baltimore, MD

Several studies have demonstrated a significant immunological impact of single nucleotide polymorphisms (SNPs) in innate immune response-related genes, such as Toll-like receptors 3 and 4 (TLR3 and TLR4). We recently reported that among patients with WHO grade 2 and 3 gliomas, those with the AA genotype of the rs12553612 SNP in the interferon-alpha-8 (IFNA8) promoter region had better overall survival than those with the AC genotype. On the basis of these observations we hypothesized that compared with the C allele, the A allele in the IFNA8 promoter allows for enhanced transcription factor binding and expression levels of IFNα. Analyses of THP-1 cells transfected with a luciferase gene downstream of a short sequence containing the SNP in the IFNA8 promoter demonstrated that the A allele results in enhanced promoter activity. In silico analysis suggested c-Krox and ELK-1 as likely transcription factors that bind to the IFNA8 polymorphic region. Co-transfection of plasmids encoding the c-Krox or ELK-1 and the luciferase constructs revealed that ELK-1 negatively regulates the promoter activity in the long-term survivors. A 17-mer was assessed using subcutaneous tumor re-challenge compared to a group of naive animals. The median survival time was 18 days for control animals, 19 days in the anti-4-1BB and anti-CTLA-4 arm, and 23 days in the stereotactic radiation arm. As of day 100 post-implantation, 50% of mice in the treatment arm combining stereotactic radiation with anti-4-1BB and anti-CTLA-4 antibodies are classified as long-term survivors; thus, the median survival for this treatment group has not been reached. Treatment of multiple naïve animals at day 17, whereas the long-term survivors had no sign of tumor growth by day 50. Our study shows that double immunotherapy using anti-4-1BB agonist and anti-CTLA-4 blockade combined with stereotactic radiosurgery results in long-term survival with development of a protective memory response.

IR-12. ELK-1 REGULATES INTERFERON-ALPHA-8 EXPRESSION VIA A POLYMORPHIC REGION IN INTERFERON-ALPHA-8 PROMOTER ASSOCIATED WITH THE PROGNOSIS OF GLIOMA PATIENTS
Gary Kohanbash, Ichic Ishikawa, Mitsuaki Fujita, Masasuke Ohno, Yan Lin, Masahiro Sasaki, Maki Ikura, and Michael Scheuer; Melissa Bondy, and Hideho Okada
1Erasmus Medical Center, Rotterdam, the Netherlands; 2Massachusetts General Hospital, Harvard Medical School, Boston, MA; 3University of Pittsburgh, Pittsburgh, PA; 4University of Tsukuba, Ibaraki, Japan; 5Aichi Cancer Center, Nagoya, Japan; 6Baylor College of Medicine and The University of Texas MD Anderson Cancer Center, Houston, TX; 7University of Texas, Houston, TX

Despite the best available therapies for glioblastoma multiforme, prognosis for patients remains poor. We tested an immunotherapeutic approach using two monoclonal antibodies in combination with stereotactic radiosurgery: anti-CTLA-4 blockade and anti-4-1BB agonist. CTLA-4 downregulates pathways of T-cell activation, while signaling through 4-1BB triggers T-cell expansion. Evidence that radiation alters the tumor microenvironment to alter pathways of T-cell activation, while signaling through 4-1BB triggers T-cell expansion. We recently performed to gain more insight into this response and to develop strategies to enhance the anti-tumor immune response.

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IR-09. CHARACTERIZATION OF THE IMMUNE RESPONSE TO ONCOLYTIC ADENOVIRUS THERAPY FOR MALIGNANT GLIOMA

IR-11. MODULATION OF NEUTROPHIL ACTIVATION BY TUMOR AND TUMOR-ASSOCIATED NECROSIS IN Glioblastoma

IR-10. STEREOTACTIC RADIOSURGERY COMBINED WITH DOUBLE IMMUNOTHERAPY WITH ANTI-CTLA-4 AND ANTI-4-1BB YIELDS LONG-TERM SURVIVAL AND PROTECTIVE ANTITUMOR RESPONSE IN A MOUSE ORTHOTOPIC Glioblastoma MODEL

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Several studies have demonstrated a significant immunological impact of single nucleotide polymorphisms (SNPs) in innate immune response-related genes, such as Toll-like receptors 3 and 4 (TLR3 and TLR4). We recently reported that among patients with WHO grade 2 and 3 gliomas, those with the AA genotype of the rs12553612 SNP in the interferon-alpha-8 (IFNA8) promoter region had better overall survival than those with the AC genotype. On the basis of these observations we hypothesized that compared with the C allele, the A allele in the IFNA8 promoter allows for enhanced transcription factor binding and expression levels of IFNα. Analyses of THP-1 cells transfected with a luciferase gene downstream of a short sequence containing the SNP in the IFNA8 promoter demonstrated that the A allele results in enhanced promoter activity. In silico analysis suggested c-Krox and ELK-1 as likely transcription factors that bind to the IFNA8 polymorphic region. Co-transfection of plasmids encoding the c-Krox or ELK-1 and the luciferase constructs revealed that ELK-1 negatively regulates the promoter activity in the long-term survivors. A 17-mer was assessed using subcutaneous tumor re-challenge compared to a group of naive animals. The median survival time was 18 days for control animals, 19 days in the anti-4-1BB and anti-CTLA-4 arm, and 23 days in the stereotactic radiation arm. As of day 100 post-implantation, 50% of mice in the treatment arm combining stereotactic radiation with anti-4-1BB and anti-CTLA-4 antibodies are classified as long-term survivors; thus, the median survival for this treatment group has not been reached. Treatment of multiple naïve animals at day 17, whereas the long-term survivors had no sign of tumor growth by day 50. Our study shows that double immunotherapy using anti-4-1BB agonist and anti-CTLA-4 blockade combined with stereotactic radiosurgery results in long-term survival with development of a protective memory response.
HCMV is the leading viral cause of birth defects, affecting primarily the central nervous system. It is also strongly associated with glioma in adults and plays a role via oncomodulation. Previously we created an HCMV-infected T98G cell line long-term infection model (J Virol 2007). We investigated HCMV infection by culturing infected cells longer term without passaging and viral genome persistence in infected cells with continuous passaging. HCMV Ag+ cells formed clusters during long-term culture without passaging. Maintenance of the viral genome in T98G cells was determined by PCR, nested PCR, and FISH, and viral genome copy number was analyzed by qPCR. PCR and nested PCR results showed that viral genome lasted to passage 7 (24 days post-infection) and to at least passage 13 (42 days post-infection), respectively; there was a high copy number of the viral genome at earlier passages, and the copy number was maintained around 400 from passages 10 through 13. To confirm that the viral genome was retained in Ag- (GFP-IE2-) cells, GFP-fused virus (WT-J-eGFP) was used for infection, purified Ag- cells were collected and cultured with continuous passaging, and viral genomes were detected by FISH. All cells had about 30 spots of HCMV genome in the nuclei. To investigate the HCMV infection feature in T98G cells, cells were transfected with p53 (host factor) and the IE1 protein-positive rate increased one-fold; for viral factors, pp71 mutant virus (T223A) and clinical isolates (TR and Tollelo) were used to infect cells. Cells with the mutant virus infection had a higher IE1 protein-expressing rate; clinical isolates went into latency directly or faster. Thus, HCMV infection in T98G cells without the disturbance of passaging forms Ag+ clusters, indicating that infection in the brain without interference may become worse. High copy number, HCMV genome persistence, and cellular and viral factors affected HCMV gene expression but could not change the infection, indicate a promising latent infection model that would be useful in glioma oncomodulation studies.