



# Neuroimmune mechanisms of behavioral alterations in a syngeneic murine model of human papilloma virus-related head and neck cancer



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## ABSTRACT

Patients with cancer often experience a high symptom burden prior to the start of treatment. As disease- and treatment-related neurotoxicities appear to be additive, targeting disease-related symptoms may attenuate overall symptom burden for cancer patients and improve the tolerability of treatment. It has been hypothesized that disease-related symptoms are a consequence of tumor-induced inflammation. We tested this hypothesis using a syngeneic heterotopic murine model of human papilloma virus (HPV)-related head and neck cancer. This model has the advantage of being mildly aggressive and not causing cachexia or weight loss. We previously showed that this tumor leads to increased IL-6, IL-1 $\beta$ , and TNF- $\alpha$  expression in the liver and increased IL-1 $\beta$  expression in the brain. The current study confirmed these features and demonstrated that the tumor itself exhibits high inflammatory cytokine expression (e.g., IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ) compared to healthy tissue. While there is a clear relationship between cytokine levels and behavioral deficits in this model, the behavioral changes are surprisingly mild. Therefore, we sought to confirm the relationship between behavior and inflammation by amplifying the effect using a low dose of lipopolysaccharide (LPS, 0.1 mg/kg). In tumor-bearing mice LPS induced deficits in nest building, tail suspension, and locomotor activity approximately 24 h after LPS. However, these mice did not display an exacerbation of LPS-induced weight loss, anorexia, or anhedonia. Further, while heightened serum IL-6 was observed there was minimal priming of liver or brain cytokine expression. Next we sought to inhibit tumor-induced burrowing deficits by reducing inflammation using minocycline. Minocycline (~50 mg/kg/day in drinking water) was able to attenuate tumor-induced inflammation and burrowing deficits. These data provide evidence in favor of an inflammatory-like mechanism for the behavioral alterations associated with tumor growth in a syngeneic murine model of HPV-related head and neck cancer. However, the inflammatory state and behavioral changes induced by this tumor clearly differ from other forms of inflammation-induced sickness behavior.

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## 1. Introduction

Patients with cancer often report high levels of symptom prior to the start of cancer therapy. It is these symptoms that often drive patients to seek medical attention. While disease-related symptom expression is diverse, patients often report centrally mediated symptoms such as fatigue (Jacobsen et al., 1999; Stone et al., 2000). For example, it has been reported that up to 75% of patients with

advanced cancer report severe fatigue (Stone et al., 1999). Unfortunately, the mechanisms by which the tumor induces fatigue is still unclear. A better understanding of these mechanisms may allow us to provide more direct symptom relief to advanced cancer patients as well as reduce the additive neurotoxic effects of cancer therapy.

Neuroinflammatory processes are well known to underlie symptoms of sickness and fatigue in inflammatory disorders and immune challenges (Dantzer et al., 2008; Morris et al., 2015). Many tumors induce local inflammation, which promotes tumor progression and dissemination (Hanahan and Weinberg, 2011). Based on these known factors, the primary hypothesis concerning disease-driven symptoms in patients with cancer is that tumor-induced

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inflammation propagates to the brain where it affects the neural networks responsible for the feelings of sickness and fatigue. While this hypothesis is well-accepted, the supporting data is rather scarce. Specifically, there is limited data indicating that local tumor-induced inflammation can propagate from the tumor to the brain to induce symptoms. Additionally, while there are studies showing a relationship between serum cytokine levels and symptom expression in cancer patients (Bower et al., 2011; Bower et al., 2009; Mills et al., 2005; Wang et al., 2010, 2012), other studies report high symptom expression in the absence of detectable cytokines (Ahlberg et al., 2004; Geinitz et al., 2001; Pusztaï et al., 2004; Ray et al., 2011). Given the limitations of clinical research and the difficulty in studying treatment naïve patients, studies with animal models can be informative. Previous research has shown that tumor-bearing laboratory animals have elevated expression of IL-1 $\beta$  mRNA expression within the brain (Braun et al., 2011; Norden et al., 2015; Pyter et al., 2014; Vichaya et al., 2016). Further, experimentally-induced tumors can induce depressive-like behavior, reduce voluntary wheel running, and decrease home cage behavior although many of these effects are difficult to dissociate from the anorexia/cachexia syndrome that develops often in tumor bearing animals (Braun et al., 2011; Norden et al., 2015; Ropelle et al., 2007; Wood et al., 2006). There is also evidence that reducing central inflammation via administration of minocycline may reduce depressive-like behavior but this was observed in the context of possible disease-related alterations in physiology and metabolism (Norden et al., 2015).

In the current study we explored the hypothesis that tumor growth is associated with inflammation within the brain and that this brain inflammation is responsible for disease-related behavioral changes using a syngeneic heterotopic murine model of human papilloma virus (HPV)-related head and neck cancer. We selected this tumor model based on its mild aggressiveness despite its inflammatory nature, its lack of associated cachexia/anorexia, and its association between increased expression of liver and brain inflammatory cytokines and reduced motivated behavior (as evaluated by burrowing, an important species specific behavior in the repertoire of rodents) (Vichaya et al., 2016).

## 2. Methods

### 2.1. Experimental subjects

Male C57BL/6 mice housed in temperature controlled environments on 12 h light-dark cycles (light on from 7:00 to 19:00 h) were used for all experiments. Food and water were available ad libitum. The Institutional Animal Care and Use Committees of the University of Texas MD Anderson Cancer Center and Sanford Research approved all procedures described.

### 2.2. Tumor model

These studies used a heterotopic syngeneic murine model of HPV-related head and neck cancer (Spanos et al., 2008; Spanos et al., 2009). Tumors were induced by injecting mice intramuscularly with  $1 \times 10^6$  tumor cells into the right hind leg. These tumor cells were derived from C57BL/6 male mouse oropharyngeal epithelial cells transfected with HPV E6/E7 oncogenes and hRAS. Tumor volume was estimated from three mutually orthogonal tumor diameters assessed by Vernier calipers [volume =  $\pi/6(d1*d2*d3)$ ] as previously described (Mason et al., 2005; Suit et al., 1976).

### 2.3. Drugs and treatments

Lipopolysaccharide (LPS; serotype 0127:B8) was purchased from Sigma-Aldrich (St. Louis, MO). It was prepared in PBS vehi-

cle and administered by intraperitoneal (IP) injection at a dose of 0.1 mg/kg between day 27–29 post tumor implantation. After treatment mice were monitored at 2, 6, and 24 h.

Minocycline (M-9511; Sigma-Aldrich, St. Louis, MO) was administered orally via the drinking water. It was dissolved in water at a concentration of 0.375 mg/mL to achieve a dose of approximately 50 mg/kg/day. The minocycline was introduced to the home cage, in place of regular drinking water, starting on day 10 after tumor implantation. Due to an observed decrease in drinking immediately following concurrent cisplatin and radiation treatment (CRT) after the first cycle, mice were injected with minocycline the day of and the day after the second and third treatment (tumor day 18, 19, 25, and 26), at which time the mice were provided with regular drinking water. To assess the specificity of the minocycline effect, a second experiment was conducted in which mice were treated with vehicle or minocycline in the context of CRT, previously shown to suppress inflammation (Vichaya et al., 2015). CRT was administered on day 12, 19, and 26 post-tumor implantation in mice used for the follow-up minocycline experiment. The regimen of cisplatin plus localized radiation selected for this study has previously been used to effectively treat MEER tumors (Spanos et al., 2008). Cisplatin (Calbiochem, EMD Millipore, Billerica, MA) was dissolved in a vehicle of sterile saline and administered at a dose of 5.28 mg/kg by IP injection once weekly for 3 weeks. Local radiation was administered immediately following cisplatin treatment at a dose of 8 Gy via a small animal cesium<sup>137</sup> irradiator that collimates the parallel opposed radiation beams to a 3 cm field and, thereby, avoids irradiation of surrounding tissue.

### 2.4. Behavioral assessments

General health was evaluated by assessing body weight, food consumption, and locomotor activity. To assess locomotor activity mice were placed in new environment (i.e., an empty 18.4  $\times$  29.2 cm translucent shoebox cage) and video recorded from above for 5 min. Distance traveled was quantified using Ethovision XT Software (Noldus Information Technology, Leesberg, VA). Burrowing and nest building were used as measures of motivated behavior. In the burrowing task mice were provided access to a slightly elevated tube constructed from PVC and filled with 230  $\pm$  1 g of standard chow for a 30 min time period. The amount of food displaced from the tube during this time was recorded (Deacon, 2009). In the nest building task we used a procedure adapted from that described by Negus et al. (2015); a nestlet was divided into 6 pieces that were placed in 6 areas of the cage. The number of pieces consolidated by the mouse were evaluated at 1, 3, 5, 10, 30, and 60 min. Finally, depression-like behavior was evaluated by measuring duration of immobility in the tail suspension task. In this task mice were suspended by their tail for 6 min using tape placed approximately 2 cm from the tip of their tail. The time immobile was scored by a trained researcher blinded to experimental conditions.

### 2.5. Tissue collection and analysis

At the end of the study mice were euthanized with CO<sub>2</sub>, blood was collected by cardiac puncture, mice were perfused intracardially with saline, and tissue was collected. Samples were stored at  $-80^\circ\text{C}$  until analysis. Serum was analyzed for IL-6 levels using an IL-6 ELISA (cat number: 431304; BioLegend, San Diego, CA) according to manufacturer's instructions. Brain (or microdissected brain regions), liver, and tumor samples were evaluated for changes in inflammation-related gene expression. To ensure a representative sample, whole brain and tumor tissue were crushed with a mortar and pestle on liquid nitrogen prior to RNA extraction using the E.Z.N.A. RNA Isolation Kit II (Omega Bio-Tek, Norcross, GA). A High Capacity cDNA Reverse Transcription Kit (Applied Biosys-

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