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Alcohol exposure differentially effects anti-tumor immunity in females by altering dendritic cell function

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ABSTRACT

Dendritic cells (DCs) are a critical component of anti-tumor immunity due to their ability to induce a robust immune response to antigen (Ag). Alcohol was previously shown to reduce DC ability to present foreign Ag and promote pro-inflammatory responses in situations of infection and trauma. However the impact of alcohol exposure on generation of an anti-tumor response, especially in the context of generation of an immune vaccine has not been examined. In the clinic, DC vaccines are typically generated from autologous blood, therefore prior exposure to substances such as alcohol may be a critical factor to consider regarding the effectiveness in generating an immune response. In this study, we demonstrate for the first time that ethanol differentially affects DC and tumor Ag-specific T cell responses depending on sex. Signaling pathways were found to be differentially regulated in DC in females compared to males and these differences were exacerbated by ethanol treatment. DC from female mice treated with ethanol were unable to activate Ag-specific cytotoxic T cells (CTL) as shown by reduced expression of CD44, CD69, and decreased production of granzyme B and IFNγ. Furthermore, although FOXO3, an immune suppressive mediator of DC function, was found to be upregulated in DC from female mice, ethanol related suppression was independent of FOXO3. These findings demonstrate for the first time differential impacts of alcohol on the immune system of females compared to males and may be a critical consideration for determining the effectiveness of an immune based therapy for cancer in patients that consume alcohol.

Introduction

The impact of alcohol on cancer risk remains inconclusive due to seemingly conflicting data in several reports (Allen et al., 2009; Brandon-Warner, Walling, Schrum, & McKillop, 2012; Fowke, Howard, Andriole, & Freedland, 2014; Garcia-Lavandeira, Ruano-Ravina, & Barros-Dios, 2016; Gong, Kristal, Schenk, Tangen, Goodman, & Thompson, 2009; Meadows & Zhang, 2015; Newcomb et al., 2013; Zhang, Zhu, Meadows, & Zhang, 2015). These conflicting reports may be partially due to the study of different types of cancer, such as prostate, liver, breast, lung and melanoma, but also are due in large part to direct effects of alcohol on the immune system. The immune system, especially the adaptive immune response, is critical for recognition and destruction of tumor cells. Cytotoxic T cells (CTL) recognize tumor associated antigens by major histocompatibility complex (MHC) I mechanisms which trigger release of Granzyme B, IFNγ and promote lytic responses to destroy tumor cells. Alcohol has been shown to have dramatic effects on the adaptive immune response including inhibiting T and B cell development (Zhang, Zhu, Zhang, & Meadows, 2015). Alcohol was also found to have substantial effects on macrophages (Goral, Choudhry, & Kovacs, 2004) and in DC populations harvested from ethanol fed mice (Heinz & Waltenbaugh, 2007; Lau, Abe, & Thomson, 2006). However, studies have not examined the impact of alcohol on the efficacy of an immune therapy which includes dendritic cell (DC) activation and function of tumor antigen (Ag) specific T cells. Alcohol can have dramatic effects on the immune cells directly or mediate changes in the tumor microenvironment (TME) that lead to immune tolerance in favor of immune activation. For example, increases in indolamine 2-3-dideoxygenase (IDO),

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while considered pro-inflammatory in some instances, is immune suppressing in the TME. IDO catabolizes tryptophan and leads to inhibition of T cell proliferation and activation (Platten, von Knebel Doeberitz, Oezen, Wick, & Ochs, 2014; Xiao, Liu, & Link, 2004). Additionally, alcohol can up-regulate signaling pathways which can be detrimental in the generation and activation of immune responses providing protection from tumor development such as MAPK, AKT and SGK (Ebert et al., 2016; Goral et al., 2004; Ho et al., 2012; Huang et al., 2015; Morelli et al., 2010). Given the recent rise in use and promise of immune therapies for cancer, this may be an important aspect to consider when deciding on an appropriate treatment regimen (Boudewijns, Bloemendal, Gerritsen, de Vries, & Schreibelt, 2016; Linch & Redmond, 2016). In the current study, we demonstrate for the first time that ethanol has a significant influence on DC from female but not male activation and effector function of tumor-Ag specific T cells.

Methods

Experimental mice

C57BL/6, male and female mice aged 6–8 weeks were obtained from Jackson laboratories. FOXO3$^{-/-}$ were bred from previously acquired generous gift from Dr. Karen Arden (UCSD). Tyrosinase related protein 2 (TRP2) Ag-specific T cells were obtained from 24H9 mice, obtained from NCI-Frederick. For *in vitro* studies, C57BL/6 mice were used as a source of bone marrow derived dendritic cells (BMDC). Mice were housed under specific pathogen-free conditions and were treated in accordance with NIH guidelines under protocols approved by the animal care and use committee (IACUC) of Loyola University Chicago. (Maywood, IL).

Cell isolations

TRP2 T cells were isolated by gently rolling lymph nodes harvested from 24H9 mice between frosted glass slides into PBS +2% FBS. The single cell suspension generated by this method is 99% CD8$^+$ TRP-Ag specific T cells as 24H9 mice are on a Rag2−/− background, thus they only have CD8 cells with the specific T cell receptor (TcR) (Singh, Ji, Feigenbaum, Leighty, & Hurwitz, 2009).

Bone marrow cell differentiation

Bone marrow was taken from the tibia and fibula of 6–8 week old male or female mice and treated with AKC lysis buffer to remove red blood cells. Remaining cells were plated in complete RPMI supplemented with 20 ng/mL GM-CSF (Thompson et al., 2015). Media was changed every other day for 9 days to direct differentiation of dendritic cells. After nine days of differentiation BMDCs were plated at a concentration of 1×10^6/ml and were treated for 3 h with 50 mM ethanol. After ethanol pre-treatment cells were stimulated with 100 ng/mL LPS for 12 h.

Ethanol treatments

Cells were plated in RPMI media +5% FBS and left untreated or treated with ethanol at 2.5, 25 and 50 mM concentrations. To avoid evaporation during treatment cells cultures were placed in plastic sealed containers with additional ethanol maintained in beaker outside of the well. (Garcia-Lavandeira et al., 2016). Absolute ethanol was purchases from Sigma-Aldrich and used for all ethanol treatments (D'Souza El-Guindy et al., 2010).

Western blots and immunoprecipitations

Whole cell lysates were generated from bone marrow derived DCs using lauryl-maltoside (Sigma Aldrich) immunoprecipitation buffer supplemented with protease inhibitor (Roche). Proteins were run by electrophoresis on a 4–15% gradient polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad) Membrane were blotted for FOXO3 using anti-FOXO3 antibody, anti-MAPK p42/44 (ERK1/2), anti-AKT, anti-AKTp308, anti-SGKp (Cell Signaling) or for β-Actin (Sigma-Aldrich) used to normalize protein loading. Densitometry was calculated using Bio-rad Image Lab software.

Flow cytometry

Cell suspensions were blocked with Fc block, washed, and incubated with antibodies anti-CD11c, CD317 (PDCA-1), CD11b, CD4, CD8, CD80, anti-CD86 and anti-MHCII (BD Pharmingen) or anti-CD44, and anti-CD69 eBioscience for surface staining and intracellular staining was performed on cells following surface staining and treatment with fix-perm buffer on ice for 30 min for IL-12 and granzyme B, (eBioscience) (Thompson et al., 2015).

Proliferation

T cells were isolated from transgenic mice containing the TRP2 Ag T cell receptor. T cells were stimulated with control or ethanol pre-treated DC loaded with TRP2 peptide for 48 h in RPMI supplemented with 50 μM β-ME. Cultures were then stained for surface markers indicated above and CFSE dilution was measured on CD3$^+$/CD8$^+$ cells by Flow cytometry detection as a measure of proliferation (Thompson et al., 2015).

qRT-PCR

Cells were cultured as indicated above and DCs were harvested for mRNA purification using the Bio-Rad Aurum total RNA kit per the manufacturer's instructions. RNA was isolated from DCs purified from tumors by RNAeasy Spin Columns (Qiagen) per manufacturer's instructions. RNA quality was determined by analysis on an Agilent bioanalyzer 2000. PCR reactions were run on a QuantStudio 6 using a Taqman assay system with primers for IDO, IL-10, IL-6, TNF-α, β-actin, and GAPDH (Thompson et al., 2015). C_T values were determined by the Applied Biosystems 7300 SDS software. Data were analyzed by the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ uses the C_T values of the gene of interest (GOI) and housekeeping gene (HKG). ΔC_T is determined by subtracting the C_T (HKG) from the C_T (GOI), and then the $\Delta\Delta C_T$ is determined by subtracting the ΔC_T (control) from the ΔC_T (experimental). Fold change is equal to $2^{(-\Delta\Delta Ct)}$.

ELISPOT

Multiscreen plates (Millipore) were coated with 100 μl of capture antibody (R&D Systems) overnight at 4 °C. IFN-γ (1×10^4) purified TRP2 T cells were added to increasing concentrations of peptide loaded control or ethanol pre-treated DC (WT or FOXO3$^{-/-}$). After incubation, plates were washed and processed.

Statistical analysis

To determine statistical significance, data was analyzed using descriptive and graphical techniques. Statistical analysis for differences between groups for cytokine production or gene expression used the unpaired Student's *t*-test. Data generated with multi variables was transformed to their common logarithms to satisfy