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Chronic alcohol consumption enhances iNKT cell maturation and activation

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ABSTRACT

Alcohol consumption exhibits diverse effects on different types of immune cells. NKT cells are a unique T cell population and play important immunoregulatory roles in different types of immune responses. The effects of chronic alcohol consumption on NKT cells remain to be elucidated. Using a mouse model of chronic alcohol consumption, we found that alcohol increases the percentage of NKT cells, especially iNKT cells in the thymus and liver, but not in the spleen or blood. Alcohol consumption decreases the percentage of $NK1.1^-$ iNKT cells in the total iNKT cell population in all of the tissues and organs examined. In the thymus, alcohol consumption increases the number of $NK1.1^+CD44^{hi}$ mature iNKT cells but does not alter the number of $NK1.1^-$ immature iNKT cells. A BrdU incorporation assay shows that alcohol consumption increases the proliferation of thymic $NK1.1^-$ iNKT cells, especially the $NK1.1^-CD44^{lo}$ Stage I iNKT cells. The percentage of $NKG2A^+$ iNKT cells increases in all of the tissues and organs examined; whereas $CXCR3^+$ iNKT cells only increases in the thymus of alcohol-consuming mice. Chronic alcohol consumption increases the percentage of IFN-γ-producing iNKT cells and increases the blood concentration of IFN-γ and IL-12 after *in vivo* α-galactosylceramide (αGalCer) stimulation. Consistent with the increased cytokine production, the *in vivo* activation of iNKT cells also enhances the activation of dendritic cells (DC) and NK, B, and T cells in the alcohol-consuming mice. Taken together the data indicate that chronic alcohol consumption enhances iNKT cell maturation and activation, which favors the Th1 immune response.

Introduction

NKT cells are a unique population of T cells, and most of these cells express the NK cell receptor, NK1.1. For that reason, they are called NKT cells. Unlike conventional αβ T cells, which recognize peptide antigens presented by MHC I and MHC II molecules, NKT cells only recognize lipid antigens presented by the non-classic MHC I molecule, CD1d. Thus, NKT cells are CD1-restricted T cells. The major population of NKT cells expresses an invariant T cell receptor (TCR) alpha chain, which is Vα14Jα18 in mice and Vα24Jα18 in humans. These NKT cells are designated as invariant NKT (iNKT) or type I NKT cells. Type II NKT cells comprise the remainder of the NKT cells, and they exhibit diverse TCR (Bendelac et al., 2007).

Although NKT cells are a small population of lymphocytes, they comprise many functionally distinct subsets (Godfrey et al., 2010). One of the unique features for iNKT cells is that they exhibit a broad cytokine profile. They can produce Th1 cytokines such as IFN-γ and TNF-α; Th2 cytokines including IL-4, IL-10, and IL-13; and the Th17 cytokines, (Coquet et al., 2008; Watarai et al., 2012). Due to the broad spectrum of cytokines produced, these cells play important roles in the regulation of immune responses associated with antitumor immunity, autoimmune diseases, and liver injury (Hong et al., 2001; Jahng et al., 2001; Crowe et al., 2005; Terabe and Berzofsky, 2008; Park et al., 2009; Jin et al., 2011; Kumar, 2013; Wen et al., 2013). iNKT cells are more like immunoregulatory cells than immune effector cells. Once activated, they quickly produce large amount of cytokines, chemokines, and effector molecules, which further shape the downstream innate and adaptive immune responses. Consequently, these cells act more like innate immune cells than adaptive immune cells, and they form a bridge between innate and adaptive immunity (Brennan et al., 2013).

Like conventional T cells, iNKT cells develop in the thymus (Benlagha et al., 2002). Based on the expression of the cell surface markers, NK1.1 and CD44, the development of iNKT cells in the thymus can be divided into three developmental stages: Stage I: $NK1.1^-CD44^{lo}$; Stage II: $NK1.1^-CD44^{hi}$; and Stage III: $NK1.1^+CD44^{hi}$. Stages I and II iNKT cells are immature iNKT cells, and Stage III cells are mature iNKT cells. iNKT cells egress from the thymus to the periphery at Stage II where they further mature into Stage III in the peripheral organs (Benlagha et al., 2002; Pellicci et al., 2002). Some of the iNKT cells stay in the thymus and mature into Stage III cells (Benlagha et al., 2002; Berzins et al., 2006). iNKT cells distribute into many lymphoid and non-lymphoid organs and tissues, including the spleen, lymph nodes (LN), bone marrow (BM), lungs, and liver. Around 10–30% of liver lymphocytes are NKT cells.

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Most of the iNKT cells in the lymph nodes are NK1.1$^-$ (Brennan et al., 2013).

The development and maturation of iNKT cells require the activation of TCR stimulated by endogenous lipids. It still remains unknown what specific mammalian endogenous lipids work as self-antigens to control iNKT cell development and activation, although several mammalian lipids were identified as agonists of iNKT cells. Ganglioside GD3 produced by some cancer cells can modulate iNKT cell activation (Wu et al., 2003; Webb et al., 2012). Isoglobotrihexosylceramide (iGB3) can activate iNKT cells and was proposed as mammalian endogenous self-antigen that governs iNKT cell development (Zhou et al., 2004). Further research indicated that iGB3 is not crucial for iNKT cell development and maturation (Porubsky et al., 2007, 2012). β-D-Glucopyranosylceramide, a glycosphingolipid, was identified as a potent endogenous agonist of iNKT cells in mouse and human (Brennan et al., 2011). The production of this lipid was up-regulated during the microbe infection (Brennan et al., 2011). Recently, thymic peroxisome-derived ether-bounded mono-alkyl glycerophosphates lipids were found to play important roles in iNKT cell development and maturation as well as the control of peripheral iNKT cell numbers (Facciotti et al., 2012). These plasmalogen lipids could be the natural ligands of iNKT cell receptors that control iNKT cell development and maturation *in vivo*.

One marine sponge-derived lipid, α-galactosylceramide (αGalCer), is a potent agonist of iNKT cell TCR (Kawano et al., 1997). Studies on iNKT cell biological function have heavily relied on the activation of these cells through αGalCer stimulation. Once activated by αGalCer, iNKT cells rapidly produce a large amount and a broad spectrum of cytokines. These cytokines subsequently activate other immune cells such as dendritic cells (DC), NK, T, and B cells. The balance between the Th1 and Th2 cytokines produced by iNKT cells determines the downstream immune response. If iNKT cells produce Th1-dominant cytokines, they will activate DC to produce IL-12, which, in turn, will activate NK, NKT, and T cells to produce IFN-γ and induce Th1 immune responses (Fujii et al., 2003). If iNKT cells produce Th2-dominant cytokines, such as IL-4 and IL-10, they will induce DC to produce IL-10, which inhibits Th1 immune responses, and enhances Th2 immune responses (Kojo et al., 2005). Activated iNKT cells produce IFN-γ, which triggers the MHC/NKG2A inhibitory signaling pathway to prevent further activation of iNKT cells (Ota et al., 2005). The repeat activation of iNKT cells with αGalCer leads to iNKT cell anergy (Chang et al., 2008; Parekh et al., 2009). The anergic iNKT cells temporarily lose the function to produce IFN-γ but still retain the ability to produce IL-4. As a result, anergic iNKT cells exhibit a Th2-dominant cytokine profile (Parekh et al., 2005).

A large body of research indicates, convincingly, that chronic alcohol consumption increases the incidence of infectious diseases, cancer, and liver injury (Takada and Tsutsumi, 1995; Nelson and Kolls, 2002; Horie et al., 2003; Miller et al., 2011; Nelson et al., 2013). The deterioration of the immune system likely plays a key role in these illnesses in alcoholics. The effects of chronic alcohol consumption on NKT cells are largely unexplored as compared to the intensive studies on the effects of alcohol on other immune cells. Although it is reported that alcohol consumption increases NKT cells in the liver (Minagawa et al., 2004), the underlying mechanism is not known. Using a murine chronic alcohol consumption model that does not induce liver injury, we found that alcohol increases iNKT cells not only in the liver but also in the thymus. Alcohol consumption enhances immature iNKT cell proliferation and maturation in the thymus and increases IFN-γ-producing iNKT cells. The *in vivo* activation of iNKT cells induces a Th1-dominant immune response.

Materials and methods

Animals and alcohol administration. Female C57BL/6 mice, at 6–7 weeks of age, were purchased from Charles River laboratories (Wilmington, MA). Breeders of IFN-γ knockout (KO) mice with a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). The KO mice were bred and maintained in the Wegner Hall Vivarium, College of Pharmacy, Washington State University, which is accredited by the American Association for Assessment and Accreditation of Laboratory Animal Care. Only female offspring were used in experiments. Mice in experiments were single-housed in plastic cages with microfilter tops and allowed free access to Rodent Lab chow 5001 and sterilized Milli-Q water. Mice were randomly divided into two groups after 1 week of acclimation to the new environment. One group was provided 20% w/v alcohol (Everclear, St. Louis, MO) as the sole drinking fluid, while the other group continued to be given Milli-Q water as a control. Both groups were allowed free access to chow. Mice were used in experiments after 3–6 months of alcohol consumption, which is a time frame when the immune responses are relatively stable (Zhang and Meadows, 2008). In this model, mice consume at least 30% of their caloric intake from alcohol, the blood concentration of alcohol is around 0.03%, and no liver injury is observed in the alcohol-consuming mice (Blank et al., 1991). All of the experimental protocols were approved by the Institutional Animal Care and Use Committee at Washington State University.

Antibodies and reagents. The following anti-mouse monoclonal antibodies used in the experiments were conjugated with PE, FITC, PerCP, PE-Cy5.5 or allophycocyanin (APC) and were purchased from BioLegend (San Diego, CA). These include the following: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11c (N418), anti-CD19 (6D5), anti-CD44 (IM7), anti-CD69 (H1.2 F3), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-NK1.1 (PK136), anti-NKG2A (16A11), anti-CCR6 (29-2 L17), anti-CXCR3 (CXCR3-173), anti-CXCR4 (2B11/CXCR4), anti-IFN-γ (XGM1.2), anti-IL-4 (11B11). Anti-mouse CXCR6-PerCP (Clone 221002) was purchased from R&D systems (Minneapolis, MN). BrdU was purchased from Sigma (St. Louis, MO). Anti-BrdU antibody was purchased from eBiosciences (San Diego, CA). Mouse CD1d/PBS57-tetramer was synthesized by NIH tetramer facility (Atlanta, GA). Fixation and permeabilization wash buffer for intracellular staining was purchased from BioLegend (San Diego, CA).

Cell isolation and phenotyping. The isolation of bone marrow (BM) cells, splenocytes, thymocytes, and peripheral blood lymphocytes (PBL) followed the methods established in our laboratory as reported previously (Zhang et al., 2012). Mechanical disruption-based intrahepatic immune cell isolation followed the methods of Blom et al. (2009). Multicolor flow cytometry-based cell phenotype analysis followed our previously reported methods (Zhang et al., 2012). CellQuest software (BD Biosciences, San Jose, CA) was used to analyze the data.

BrdU in vivo incorporation assay. Each mouse was injected i.p. with 2 mg of BrdU in 200 μl of sterilized PBS. Mice were euthanized 3 h after BrdU injection. BM cells, splenocytes, thymocytes, and liver leukocytes were isolated as described above and then suspended in PBS + 0.1% BSA. An appropriate amount of isolated cells from each organ was incubated with anti-CD16/32 antibody on ice for 5 min to block Fc receptors, followed by incubation with either anti-CD3-PerCP/CD1d-PBS57-tetramer-PE or anti-NK1.1-APC/anti-CD44-PE-Cy5.5/CD1d-PBS57-tetramer-PE at room temperature for 30 min. Cells were washed twice with 200 μl of FACS buffer (PBS + 0.1% BSA + 0.1% NaN3), resuspended in 100 μl of fixation buffer and incubated on ice for 30 min. The fixed cells were washed twice with 200 μl of permeabilization wash buffer. The cells were incubated with anti-BrdU antibody on ice for 30 min and washed twice with permeabilization wash buffer. BrdU-positive cells were analyzed by flow cytometry as described above.

In vivo activation of iNKT cells by αGalCer. αGalCer was dissolved into DMSO at 1 mg/ml and stored at -20 °C as a stock solution. Each mouse was injected i.p. with 4 μg of αGalCer in 200 μl of sterilized PBS. Mice