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Research paper

Ex-vivo α-Galactosylceramide activation of NKT cells in humans and macaques

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ABSTRACT

NKT cells are key mediators of antiviral and anticancer immunity. Experiments in mice have demonstrated that activation of NKT cells *in vivo* induces the expression of multiple effector molecules critical to successful immunity. Human clinical trials have shown similar responses, although *in vivo* activation of NKT cells in humans or primate models are far more limited in number and scope. Measuring *ex vivo* activation of NKT cells by the CD1d-restricted glycolipid ligand α-Galactosylceramide (α-GalCer) through cytokine expression profiles is a useful marker of NKT cell function, but for reasons that are unclear, this approach does not appear to work as well in humans and non-human primate macaque models in comparison to mice. We performed a series of experiments on human and macaque (*Macaca nemestrina*) fresh whole blood samples to define optimal conditions to detect NKT cell cytokine (TNF, IFNγ, IL-2) and degranulation marker (CD107a) expression by flow cytometry. We found that conditions previously described for mouse splenocyte NKT cell activation were suboptimal on human or macaque blood NKT cells. In contrast, a 6 h incubation with brefeldin A added for the last 4 h, in a 96-well plate based assay, and using an α-GalCer concentration of 1 μg/ml were optimal methods to stimulate NKT cells in fresh blood from both humans and macaques. Unexpectedly, we noted that blood NKT cells from macaques infected with SIV were more readily activated by α-GalCer than NKT cells from uninfected macaques, suggesting that SIV infection may have primed the NKT cells. In conclusion, we describe optimized methods for the *ex vivo* antigen-specific activation of human and macaque blood NKT cells. These assays should be useful in monitoring NKT cells in disease and in immunotherapy studies.

1. Introduction

NKT cells are lymphocytes of the innate immune system that are important in viral and antitumor immunity through their ability to be rapidly activated and express a wide range of effector molecules (Lindqvist et al., 2009; Motohashi et al., 2009; Brigl and Brenner, 2010; Fujii et al., 2010). NKT cells are activated via an antigen-specific T cell receptor (TCR). Improved methodologies to assess the activation of NKT cells *ex vivo* should assist dissecting the importance of these immune cells in humans and non-human primates.

Type I or semi-invariant NKT cells express an invariant TCR-α chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) and are restricted by the MHC-class Ib molecule, CD1d, expressed on APCs presenting lipid based antigens such as glycolipid antigens (Exley et al., 1997). Lipid based antigens are presented to NKT cells by direct ligation onto surface CD1d molecules or internalized into endosomes where loading onto CD1d occurs and subsequent membrane surface presentation on a lipid raft (Venkataswamy and Porcelli, 2010). NKT cells respond to a broad variety of lipid-based antigens including self and foreign glycolipid and phospholipid antigens (Venkataswamy and Porcelli, 2010). α-galactosylceramide (α-GalCer) is a synthetic glycosphingolipid derived from the marine sponge, *Agelas mauritianus*, and is commonly used in mice and human NKT studies as a potent activator of NKT cells *in vivo* or *in vitro*

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(Kawano et al., 1997). α-GalCer has immunomodulatory effects in cancer immunotherapy (Giaccone et al., 2002; Chang et al., 2005; Uchida et al., 2008; Kunii et al., 2009; Motohashi et al., 2009; Schneiders et al., 2011; Yamasaki et al., 2011; Vivier et al., 2012), autoimmunity (Novak and Lehuen; Hong et al., 2001; Wu and Van Kaer, 2009), bacterial (Brigl and Brenner, 2010; Emoto et al., 2010), and viral infections (Guillonneau et al., 2009; Lindqvist et al., 2009; Schneiders et al., 2011).

Upon activation with α-GalCer, NKT cells produce large amounts of Th1, Th2 and Th17 cytokines such as IFNγ, TNF, IL-2, IL-4, IL-10, IL-13, IL-17, IL-21 and IL-22 (Godfrey et al., 2010). The cytokines produced by NKT cells trigger the activation of other cells of the immune system such as NK cells, T and B cells and DCs (Fujii et al., 2003; Cerundolo et al., 2009). The detection of the expression of these cytokines by intracellular cytokine staining and flow cytometry permits an analysis of NKT cell functional status.

Studies on α-GalCer activation of mice NKT cells often involve *in vivo* activation, where α-GalCer induced cytokine production by NKT cells, and indirectly downstream by NK cells, is measured in serum (Sullivan et al., 2010), or directly *ex-vivo* following α-GalCer administration *in vivo* (Wilson et al., 2003; Uldrich et al., 2005). In the latter case mice are administered α-GalCer, organs such as spleen, liver or lymph node are harvested a few hours after challenge and the relevant cells placed in culture with a protein transport inhibitor, without any further activation. Alternatively, the global mitogen-induced activation of NKT cells *ex vivo* is often assessed by PMA/ionomycin stimulation. Such mitogenic stimulation, while providing information on the total functional potential of NKT cells, may not reflect the *in vivo* capacity of NKT cells when interacting with antigen presented by CD1d. Furthermore, activation with PMA/ionomycin poses difficulties in enumerating intracellular cytokine production from NKT cell subsets as the CD4 surface marker is downregulated upon mitogenic stimulation (O'Neil-Andersen and Lawrence, 2002). Many other studies have assessed activation of mouse NKT cells *ex vivo* with α-GalCer in combination with protein transport inhibitors such as monensin to detect intracellular cytokines produced in vitro (Uldrich et al., 2005; Patel et al., 2011).

It is important to translate advances in understanding NKT cell biology in mice towards more directly relevant non-human primate models and humans. For example, there is considerable interest in harnessing the antiviral activity of NKT cells in the setting of chronic viral infections such as HIV infection of humans or SIV infection of macaques (Fernandez et al., 2009; Snyder-Cappione et al., 2009). However, such studies require *ex vivo*/*in vitro* analysis of NKT cells, yet at present the literature is lacking in clearly defined optimized methods for acute *ex-vivo* activation of NKT cells with α-GalCer either in humans or macaques. The relative ability of fresh blood NKT cells to respond to acute *ex vivo* activation with cognate ligands such as α-GalCer has not been studied in HIV infection, or SIV infection, compared to naïve SIV uninfected macaques. Such assays would be useful in immunotherapy or vaccine clinical or pre-clinical trials designed to harness NKT cell based immunity. In this study we assessed *ex vivo* NKT function upon stimulation of human and macaque blood with α-GalCer under a variety of experimental conditions to optimize methods for NKT cell activation.

2. Methods

2.1. Healthy human subjects and animals

For all studies on optimized NKT cell activation methods (Figs. 2–5) we studied subjects and animals as detailed below. Healthy HIV uninfected human volunteers (n = 5 aged 23–48 years; four females, one male) with varying frequencies of peripheral NKT cells (range of 0.03%-0.23%) were recruited for this study. We also studied a total of 9 juvenile pigtail macaques (*Macaca nemestrina*) aged 2 to 6 years. Five macaques were infected with SIV_{mac251} intravaginally and were 21–28 weeks post infection at the time of this study. Four SIV-uninfected healthy pigtail macaques were also studied. Macaques were sedated with ketamine (10 mg/kg monkey weight) and intravenous peripheral blood was drawn from macaques or humans. Spleens from two naïve C57BL/6 mice were also harvested for mononuclear cells. All studies were approved by the relevant institutional human and animal research ethic committees.

2.2. Activation of macaque or human NKT cells with α-GalCer or PMA/ionomycin

Lyophilised α-Galactosylceramide (Sapphire Biosciences, catalog number SL-232) was resuspended to 200 μg/ml in PBS containing 56 mg/ml sucrose, 7.5 mg/ml L-histidine, 0.5% tween-20 by step-wise addition of the buffer interspersed with sonication, vortexing and heating at 60 °C. Unless otherwise stated NKT cells within macaque or human peripheral blood (200 μl) were activated with 5 μg/ml α-GalCer for 6 h at 37 °C, 5% CO_2 in a 96-well U-bottom plate followed by the addition of brefeldin A (BFA) at 10 μg/ml for the last 4 h of activation. Mouse anti-human CD107a (clone H4A3, BD) was added at the beginning of the activation period. PMA/ionomycin activation of macaque NKT cells was carried out with 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 3 μM ionomycin calcium salt (Sigma) for 4 h at 37 °C, 5% CO_2 with the addition of a protein transport inhibitor for the last 2 h of activation. Unstimulated samples (Figs. 2–5) were incubated in PBS buffer containing sucrose, histidine and tween at concentrations given above. Selected experiments used mouse IgG1, κ isotype control antibody (APC-H7-clone MOPC-21, BD) as negative staining control for the CD107a surface stain (Supplementary Fig. 1).

2.3. α-GalCer activation of mouse splenic NKT cells

Spleens were harvested from mice into PBS containing 2% fetal bovine serum, filtered through 70 μm filters and centrifuged at 350 *g* for 4 min. Red blood cell lysis was conducted using 4 ml Red Blood Cell Lysing Buffer (Sigma) at room temp for 4 min. Live splenocytes were enumerated with trypan blue dye exclusion in a heamocytometer. NKT cell activation was performed as previously described in the literature (Uldrich et al., 2005; Patel et al., 2011). Splenocytes were incubated with 0.1 μg/ml α-GalCer in 4 wells of a 96-well U-bottom plate at 0.5×10^6 splenocytes per well for 8 h