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Development of a qPCR method to rapidly assess the function of NKT cells

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Abstract

NKT cells comprise a rare, but important subset of T cells which account for ~0.2% of the total circulating T cell population. NKT cells are known to have anti-tumor functions and rapidly produce high levels of cytokines following activation. Several clinical trials have sought to exploit the effector functions of NKT cells. While some studies have shown promise, NKT cells are approximately 50% lower in cancer patients compared to healthy donors of the same age and gender, thus limiting their therapeutic efficacy. These studies indicate that baseline levels of activation should be assessed before initiating an NKT cell based immunotherapeutic strategy, thus the goal of this study was to develop a sensitive method to rapidly assess NKT cell function. We utilized artificial antigen presenting cells in combination with qPCR in order to determine NKT cell function in peripheral blood mononuclear cells from healthy donors and breast cancer patients. We found that NKT cell activation can be detected by qPCR, but not by ELISA, in healthy donors as well as in breast cancer patients following four hour stimulation. This method utilizing CD1d-expressing aAPC will enhance our knowledge of NKT cell biology and could potentially be used as a novel tool in adoptive immunotherapeutic strategies.

Keywords

NKT cells; aAPC; CD1d

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Conflicts of Interest

The authors declare no conflict of interest.

1. Introduction

Natural killer T (NKT) cells recognize lipid antigen presented in the context of the non-classical major histocompatibility class I molecule, CD1d (Dellabona et al., 1994; Lantz and Bendelac, 1994; Berzins et al., 2011; Brennan et al., 2013). Upon activation, NKT cells produce high amounts of cytokines which can stimulate other immune cells and initiate both innate and adaptive immune responses. While NKT cells comprise a relatively small percentage of lymphocytes (1-2% of mouse splenocytes and 0.01-2% of human peripheral blood mononuclear cells), they have been demonstrated to play important roles in autoimmune disease (Illes et al., 2000), tumor surveillance (Terabe and Berzofsky, 2008; Swann et al., 2009), hematological cancers (Neparidze and Dhodapkar, 2009), infectious disease and inflammatory conditions such as ischemia reperfusion injury (Kinjo et al., 2005). These effects are mediated through two defined subsets of NKT cells. Type I NKT cells (also known as invariant NKT cells, or *i*NKT cells) express an invariant V α 14J α 18 TCR in mice and V α 24J α 18 TCR in humans. Type II NKT cells are CD1d restricted T Cells that express a more diverse set of α chains in their TCR. The two types of NKT cells often exert opposing effects especially in tumor immunity where Type II cells generally suppress tumor immunity while Type I NKT cells enhance anti-tumor immune responses (Ambrosino et al., 2007). In this study, we focus on Type I NKT cells.

α -Galactosylceramide (α -GalCer) is a potent activator of *i*NKT cells (Burdin et al., 1998) (Carnaud et al., 1999; Fujii et al., 2003b; Hermans et al., 2003; Seino et al., 2005). It was discovered during a screen for anti-tumor reagents derived from the marine sponge *Agelas mauritanus* (Kawano et al., 1998). Now α -GalCer is the most extensively utilized and best-characterized antigen used to study NKT cell activation. Following stimulation with antigen presenting cells pulsed with α -GalCer, NKT cells produce T helper 1 (Th₁), Th₂ and Th₁₇ type cytokines (Cerundolo and Kronenberg, 2010; Monteiro and Graca, 2014; Singh et al., 2014). Because NKT cells can activate different immune cells and produce high amounts of immune cell stimulating cytokines like interferon- γ (IFN- γ), interleukin- 4 (IL-4) and IL-10 they are considered an important immunoregulatory cell type that plays a pivotal role in modulating the immune responses (Godfrey et al., 2004; Sun et al., 2012).

Given that NKT cells can mediate potent anti-tumor immune responses, they have been considered as a novel immunotherapeutic target (Matarollo et al., 2006; Fujii, 2008). In two studies, patients with advanced cancers were injected with either α -GalCer (Giaccone et al., 2002) or α -GalCer loaded immature dendritic cells (Nieda et al., 2004) in order to modulate NKT cell responses. Chang and colleagues showed that multiple injections of α -GalCer loaded mature dendritic cells lead to sustained expansion of NKT cells and antigen specific T cells (Chang et al., 2005). However, these expanded NKT cells from cancer patients still exhibited reduced capacity for IFN- γ secretion compared to NKT cells from healthy controls. Recent clinical trials evaluating the effectiveness of NKT cell based immunotherapy in treating patients with solid tumors in the liver or lung, as well as unresectable head and neck cancers, have shown some promise (Ishikawa et al., 2005; Uchida et al., 2008). Collectively, these studies and others (Kawano et al., 1999b; Tahir et al., 2001; Fujii et al., 2003a) have demonstrated that many cancer patients have a deficiency

in both NKT cell number and function, which suggests that *in vivo* NKT cell modulation would only be effective in patients with sufficient numbers of functional NKT cells.

Several groups have shown that soluble forms of CD1d molecules loaded with lipid antigen are directly able to target NKT cells *in vitro* (Naidenko et al., 1999; Schumann et al., 2003; Sriram et al., 2005), and we have used this to develop a method to *ex vivo* activate and expand NKT cells using CD1d-based artificial antigen presenting cells (aAPC) (Webb et al., 2009; East et al., 2012; Sun et al., 2012). The beads are loaded with CD1d dimers that bind α -GalCer and anti-CD28 antibodies to also activate the costimulatory molecule on the cell surface of NKT cells. The complex of α -GalCer/CD1d binds to the NKT cell TCR. The use of aAPC allows a rapid, reproducible, and standardized method to examine NKT cell function. NKT cell function is typically assessed by enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT) and intracellular staining (ICS). A disadvantage of these methods is that a large blood volume is needed in order to obtain a sufficient number of peripheral blood mononuclear cells (PBMC) to assess the activation of specific T cell subsets. To circumvent these issues, Ndhlovu et al. developed an assay to detect low-frequency measles virus-specific CD8⁺T cells in whole blood (Ndhlovu et al., 2009). This highly sensitive assay only requires a minimal amount of blood. Given that cancer patients also have low circulating levels of NKT cells in their blood, the goal of this study was to determine if a similar assay could be developed to rapidly assess the function of NKT cells in breast cancer patients. Herein, we demonstrate that stimulation with CD1d-aAPC in combination with real time quantitative PCR (qPCR) can be used to rapidly assess the function of NKT cells within the peripheral blood.

2. Materials and Methods

2.1. Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotek, Uppsala, Sweden) density gradient centrifugation or with BD Vacutainer PPT Tubes for Molecular Diagnostics (20-959-51D; Fisher Scientific, Suwanee, GA.) All donors gave written informed consent before enrolling in the study. The Institutional Review Board at the University of Maryland School of Medicine approved this investigation. To optimize the protocol, leukocyte paks were purchased from a commercial vendor, Biological Specialty Corp., Colmar, PA. The percentages of NKT cells were assessed in newly diagnosed patients, prior to treatment and healthy donors.

2.2. Preparation of artificial antigen presenting cells (aAPC)

CD1d-based aAPC were prepared as previously described (Shiratsuchi et al., 2009; Webb et al., 2009). In brief, to conjugate hCD1d-Ig dimer molecules to beads, 50 μ g of hCD1d-Ig (Pharmingen) was added to 0.5ml of epoxy beads (Dynal, product #140.01, Dynabeads, M-450, Epoxy, 4 $\times$ 10⁸ beads/ml) in sterile 0.1M Borate buffer, pH 7.0-7.4, in the presence of anti-CD28 mAb (Biolegend). The bead protein combination was mixed with rotation and incubated for 24h at 4°C. The beads were subsequently washed and the hCD1d molecules were loaded with 40 $\times$ molar excess lipid antigen (α -GalCer, Enzo) in PBS, calculated based on the amount of hCD1d-Ig protein added to the beads. For anti-CD3/CD28 microbeads,

20µg of each mAb (Biolegend) was added to 4×10^8 beads. The beads were washed and used as described above.

2.3. Stimulation of PBMC

Human PBMC were cultured in complete medium: RPMI 1640 medium supplemented with non-essential amino acids (Sigma-Aldrich), sodium pyruvate (Gibco, Invitrogen Corporation), vitamin solution (Gibco), 2-mercaptoethanol (Gibco), 10% fetal calf serum (Gibco), and Pen/Strep (Gibco). PBMC (10^6) were added to borosilicate glass vials (Wheaton Science Products) and different stimuli were added: empty beads as a negative control, anti CD3/CD28 beads, CD1d/CD28 beads and PMA (50 ng/ml) and ionomycin (1 µM) was used as a positive control. The total volume of cell culture was 1 ml and a 1:1 (PBMC: beads) ratio was maintained for each experiment.

For the time course studies, the PBMCs stimulated for 0, 30, 60, 120 or 240 minutes at 37°C. After the incubation the beads were removed via an EasySep magnet (Invitrogen) and the cells were transferred into 1.5 ml eppendorf tubes and centrifuged for 5 minutes at 5000 rpm. The supernatants were collected in separate 1.5 ml eppendorf tubes for further use and stored at -20°C, the cells were washed with $1 \times$ PBS, and the cell pellets were stored at -20°C or immediately used for RNA isolation.

2.4. ELISA

To detect the cytokine production following stimulation of PBMC, standard sandwich ELISAs (IFN-γ, TNF-α, GM-CSF, all purchased from Biolegend) were performed according to the manufacturer's instructions. The plate was read by Synergy H1 Hybrid reader from BioTek and the data recorded. The data were analyzed by Excel and GraphPad Prism.

2.5. RT-PCR

RNA was isolated using the RNA Easy Plus Kit (Qiagen) according to the manufacturer's protocol. After isolation the RNA concentration and purity was determined using the Take 3 plate and the Synergy H1 Hybrid reader. The purity was also checked by 1% agarose gel. Reverse transcription PCR performed by the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's directions. The PCR was done with primers using proprietary sequences generated by Qiagen that were specific for IFN-γ (cat. # PPH00380C) and 18S (cat. # PPH05666E). Primers for Vα24 (Va24_CCY9XUZ, cat. # 4400294) were purchased from Applied Biosciences by Life Technologies. For PCR, the HotStarTaq Plus Master Mix kit (Qiagen) was used and the PCR protocol included 35 cycles. For each sample: 10µl master mix, 2µl Coral load, 6µl Nuclease free water, 1µl cDNA and 1µl primer set were used.

2.6. Real-time quantitative PCR (qPCR)

To measure the induction of IFN- γ mRNA in the stimulated PBMC qPCR was performed. The ABI Sybr Green master mix and HotStarTaq master mix kit from QIAGEN was used. qPCR was performed using primers specific for 18S, Vα24 and IFN-γ, as described above. The total volume of the reaction mix was 20µl and consisted of 10µl master mix, 1 µl primer

mix(3μM), 5μl H₂O and 4μl cDNA (diluted 1:10). The Applied Biosystems 7500 Fast Real Time PCR system was used. The C_T values were collected and the fold increase calculated as follows: n-fold increase in IFN-γ mRNA = $2^{[-(C_{T\text{sample}} - C_{T\text{18S rRNA}}) - (C_{T\text{empty beads}} - C_{T\text{18S rRNA}})]}$ where C_T is the threshold cycle.

2.7. Antibodies and flow cytometry

Data were acquired with a BD LSR II Flow Cytometer (BD Biosciences) and analyzed with FCS Express V3 (De Novo Software, Los Angeles, CA). Doublets were excluded with FSC-A and FSC-H linearity. Human antibodies were as follows: anti-TCR Vα24-Jα18 (clone 6B11), anti-CD3 (clone UCHT1) - all purchased from BD Biosciences, anti-TCR Vα24 (clone C15) and anti-TCR Vβ11 (Beckman Coulter), and CD1d tetramers loaded with PBS57, an analog of α-GalCer (National Institutes of Health Tetramer Core Facility, Atlanta, GA).

2.8. Statistical analysis

An unpaired two-tailed Student t test was performed by Prism software (version 5.02 for Windows; GraphPad) to compare healthy donors to cancer patients. A p value <0.05 was considered significant. The error bars in the bar graphs show the S.E.M.

3. Results

3.1. Circulating NKT cells levels are low

Despite the importance of NKT cells in regulating immune responses, their low frequency significantly restricts their potential for clinical application. As a precursor to developing a method to rapidly assess their function, we first examined the percentage of NKT cells in healthy donors and newly diagnosed breast cancer patients prior to treatment or surgery (Fig 1A). As shown in Fig 1B, the percentage of circulating NKT cells within the lymphocyte population was 0.17 ± 0.06 in breast cancer patients (N=28) compared to 0.65 ± 0.23 in healthy donors (N=11). NKT cells were significantly reduced in breast cancer patients compared to healthy donors (p=0.0016).

3.2. Human T cells produce cytokine within hours following stimulation

Given the low frequency of NKT cells within the peripheral blood, the goal of this study was to develop a sensitive assay in order to assess NKT cell function. First, we conducted time course studies to determine the kinetics of cytokine production. PBMC were stimulated for 0, 1, 2 and 4 hours with PMA and ionomycin. As shown in Figure 2A, cytokine production could be detected by ELISA within four hours following stimulation. It was found that non-specific stimulation with PMA and ionomycin resulted in the rapid production of TNF-α, GM-CSF, IFN-γ and IL-17A (data not shown). Therefore, we next assessed T cell activation by stimulating the PBMC with anti-CD3/CD28 microbeads (Fig 2B). We found that TNF-α and IFN-γ were quickly and reproducibly induced following stimulation. Notably, it was found that IFN-γ was consistently produced at high levels and could be used to measure T cell function in our studies.

We conducted studies to determine the sensitivity of qPCR compared to flow cytometry for examining the NKT cell population. In order to assess the sensitivity of each assay, we sought to determine the lowest percentage of NKT cells that could be accurately measured by qPCR. To do these studies, purified NKT cells were serially diluted into a million PBMC and IFN- γ and V α 24 transcripts were measured by qPCR. We were able to detect a clear linear relationship between 100-10⁴ NKT cells (V α 24J α 18) and IFN- γ mRNA (data not shown). We also compared the V α 24 transcripts by qPCR results to α -GalCer tetramer⁺ NKT cells by flow cytometry (Fig. 3). We were able to detect low frequencies of NKT cells by flow cytometry; however, we found that qPCR was extremely sensitive in detecting increases in V α 24 transcripts.

3.3. aAPC-qPCR can be used to assess NKT cell function

Studies from our laboratory and others have shown that α -GalCer artificial antigen presenting cells (aAPC) can be used to activate NKT cells (Shiratsuchi et al., 2009; Webb et al., 2009; Sun et al., 2012). Thus, after our initial studies defined the optimal incubation time for assessing T cell activation, PBMC of healthy donors were incubated for four hours with various stimuli (see schematic of experimental design in Figure 4). The percentage of NKT cells from each donor is shown in Fig 5A. Healthy donors with relatively low circulating numbers of NKT cells were chosen to assess the sensitivity of this assay. To specifically activate NKT cells, α -GalCer loaded aAPC were used to stimulate the PBMC, empty beads were used as a negative control, anti-CD3/CD28 microbeads were used to measure total T cell function, and PMA/ionomycin was used as a positive control. The induction of IFN- γ was assessed as an indication of T cell activation and was measured by ELISA, conventional RT-PCR, and qPCR (Figures 5B-E). Following activation, there was a clear induction of IFN- γ production in NKT cells following α -GalCer-aAPC stimulation by RT-PCR and qPCR. Data shown in Figure 5C is from Donor 1. As shown in the ELISA data (Figure 5D), IFN- γ induction was not as profound in all donors and an extremely sensitive method is required to specifically detect NKT cell activation. The qPCR-aAPC method was able to detect NKT cell activation in 3 out of 4 donors, as shown in Figure 5, whereas NKT cell activation was undetectable by ELISA. We were able to detect NKT cell responses, as assessed by IFN- γ induction, in all healthy donors with high percentages of circulating NKT cells (> 0.5%; data not shown).

3.4. NKT cell function can be rapidly assessed in Breast cancer patients

We have shown that aAPC-qPCR can be used to measure circulating NKT cell responses in the PBMC from healthy donors, but NKT cells have been reported to be reduced in number and function in cancer patients. As shown in Figs 1 and 6A, the circulating percentage of NKT cells in the peripheral blood of breast cancer is low. Thus, we next sought to determine if this qPCR-aAPC method can be used as a tool to determine baseline NKT cell function in breast cancer patients. In these studies, PBMC from breast cancer patients were stimulated with α -GalCer loaded aAPC and then functional studies were performed. We found it difficult to assess NKT cell activation by both classic RT-PCR (Figure 6B) and ELISA (Figure 6C); however, we were able to detect NKT cell activation by qPCR (Figure 6D). Taken together our data shown that α -GalCer loaded aAPC can be used in combination with qPCR to investigate baseline NKT cell function in healthy donors and cancer patients.

4. Discussion

NKT cells comprise a rare, but important subset of T cells that are activated following the recognition cognate lipid antigen presented in the context of CD1d (Godfrey et al., 2004). Following activation, NKT cells can directly lyse tumors and rapidly produce a plethora of cytokines (Fowlkes et al., 1987; Berzins et al., 2011). Thus, NKT cells are considered to be important for immune surveillance (Prigozy et al., 2001). NKT cells have been demonstrated to play a role in autoimmune disease (Illes et al., 2000), tumor surveillance (Terabe and Berzofsky, 2008; Swann et al., 2009), hematological cancers (Neparidze and Dhodapkar, 2009), infectious disease (Prigozy et al., 2001), and inflammatory conditions such as ischemia reperfusion injury (Kinjo et al., 2005).

Despite the importance of NKT cells in regulating immune responses, their low number diminishes their potential for clinical application. Previous studies have shown that circulating NKT cell numbers are reduced in cancer patients (Kawano et al., 1999a; Tahir et al., 2001; Giaccone et al., 2002; Motohashi et al., 2002; Crough et al., 2004; Nieda et al., 2004; Chang et al., 2005; Molling et al., 2005). While NKT cells are low in cancer patients, their strong anti-tumor functions have lead several groups to attempt to activate NKT cells in cancer patients (Nieda et al., 2004; Ishikawa et al., 2005; Uchida et al., 2008). These studies showed promise for NKT cell based immunotherapeutic strategies, because it was found that cancer patients that received α -GalCer-pulsed dendritic cells had enhanced NK and CD8⁺ T cell responses. However, the best responses were observed in patients with detectable baseline levels of NKT cells. Thus, in the current study we have developed a novel method to assess baseline NKT function in healthy donors and breast cancer patients using aAPC in combination with qPCR, in order to help to determine which patients may benefit most from NKT cell-based therapies.

As expected, we found that induction of NKT cells in breast cancer patients was reduced compared to healthy donors. Specifically, there was a modest 3 fold increase in IFN- γ by qPCR in one of the breast cancer patients following stimulation with α -GalCer-loaded aAPC, compared to cells incubated with the control beads. Notably, the lymphocytes from healthy donors and breast cancer patients were activated by anti-CD3/CD28 and PMA/ionomycin and this could be observed by ELISA as well as by conventional RT-PCR. These data highlight the need for a sensitive method for NKT cell activation because we were able to detect NKT cell activation in very few patient samples. Likewise, our data show that the mRNA fold change is much higher in healthy donors, compared to breast cancer patients for each type of stimulation.

It has been recently reported by Schneiders, et al. that levels of NKT cells were strong predictors of clinical outcome in patients with HNSCC treated with curative-intent radiotherapy (Schneiders et al., 2012). In this study, the authors further highlight the prognostic potential of NKT cells since their group and others have established a direct relation between a low frequency of intratumoral NKT cells and poor prognosis (van der Vliet et al., 2006). We have validated our method using fresh and frozen PBMCs and are currently working to optimize the conditions needed to assess NKT cell function using whole blood.

In summary, herein we show that NKT cells function can be rapidly assessed *in vitro* after stimulation with aAPC in healthy donors and breast cancer patients. Although activation levels were highly donor specific, we were able to detect a clear induction in IFN- γ mRNA following stimulation. Ongoing studies will help us to address the mechanisms by which NKT cells are numerically reduced and functionally impaired in breast cancer patients; however, this method has the potential to provide a better understanding of which patients may benefit from NKT cell-based immunotherapeutic strategies.

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Abbreviations used in this paper

NKT	Natural killer T
aAPC	artificial Antigen Presenting Cells
α-GalCer	α -galactosylceramide
PBMC	peripheral blood mononuclear cells

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Highlights

- Circulating NKT cells are low in number and function in cancer patients.
- It is currently difficult to determine which patients would benefit from NKT cell-based immunotherapy.
- Here, we describe a qPCR-based method to rapidly assess baseline NKT cell function.

Figure 1. Circulating percentages of NKT cells are low in healthy donors and breast cancer patients

Peripheral blood mononuclear cells (PBMC) were collected from healthy donors and breast patients. Cells were stained for Vα24⁺Vβ11⁺ or iNKT (6B11)/CD3 to determine the percentage of NKT cells within the lymphocyte population and analyzed by FACS. (A) Representative dot plots are shown from healthy donors (HD) and breast cancer patients (BC). (B) Scatter plots demonstrate the variation in the percentages of NKT cells.

Figure 2. PBMC produce multiple cytokines within hours following stimulation

(A) PBMC were stimulated with PMA and ionomycin for the indicated time period. Culture supernatants were harvested and standard sandwich ELISA was used to measure TNF- α , GM-CSF, or IFN- γ production. (B) Human T cells rapidly produce cytokines following ex vivo stimulation. PBMC were stimulated with anti-CD3/CD28 microbeads and cytokines were measured by ELISA. PBMC were stimulated with anti-CD3/CD28 microbeads for 0, 2 and 4 hours. This is a representative experiment of eight similar experiments.

Figure 3. qPCR provides a sensitive method for detecting NKT cells in the peripheral blood Healthy human NKT cells were added in increasing concentrations to donor PBMCs. The cells were stained with PE- conjugated CD3 and APC-conjugated α -GC tetramer antibodies, with unloaded tetramer antibody serving as a negative control. The cells were analyzed by flow cytometry and percentage of NKT cells was calculated by subtracting unloaded tetramer from α -GC tetramer staining. Loaded tetramer percentage is listed on the left y-axis, while V α 24 mRNA fold change by qPCR is shown on the right y-axis. The total NKT cell number is displayed on the x-axis. The data shown are the average of three independent experiments.

Figure 4. Schematic diagram of experimental design

In this study, PBMC from healthy donors and breast cancer patients were stimulated with various stimuli: empty beads, CD1d-aAPC, anti-CD3/CD28 microbeads, or PMA and ionomycin for four hours. In this system, empty microbeads served as a negative control. To specifically activate NKT cells using aAPC, CD1d-Ig is used to provide the cognate antigen specific signal through the TCR (signal 1) and anti-CD28 mAb provides signal 2. These aAPC were pre-loaded with α -GalCer in order to induce cytokine production by classic type I NKT cells. Simulation with PMA and ionomycin was used as the positive control. Following stimulation, the supernatants were collected for ELISA and the RNA was extracted from the cell pellets and used for RT-PCR and qPCR.

Figure 5. CD1d-based aAPC in combination with qPCR can be used to assess NKT cell activation

(A) A representative dot plot is shown from HD1 and a scatter plot indicates the %NKT cells from each of the donors shown in panels C-E. (B) Schematic of CD1d-based aAPC. Stimulation with α -GalCer loaded CD1d-Ig aAPC induces IFN- γ expression in NKT cells in healthy donor PBMC. PBMC were incubated for 4 h with either empty beads, CD1d-aAPC, anti-CD3/CD28 microbeads, or PMA/ionomycin. NKT cell activation was assessed by measuring IFN- γ (C) mRNA levels by RT-PCR (D) production in the supernatants by ELISA (E) induction by qPCR. RT-PCR data in (C) is from donor 1, the other panels show data from four different healthy donors. Data shown are from one experiment and are representative of 3 independent experiments.

Figure 6. NKT cell function in breast cancer patients can be assessed using CD1d-based aAPC in combination with qPCR

(A) Flow cytometric analysis of circulating NKT cells from breast cancer (BC) patients shown in panels B-D. PBMC from breast cancer patients were incubated for 4 h with empty beads, CD1d-aAPC, anti-CD3/CD28 microbeads, or PMA/ionomycin. (B) NKT cell activation was assessed by measuring IFN- γ mRNA levels by RT-PCR (C) ELISA and (D) qPCR. RT-PCR data in (B) is from donor 3.