In-vivo stimulation of macaque NKT cells with α-Galactosylceramide

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Abstract

NKT cells are a potent mediator of antiviral immunity in mice but little is known about the effects of manipulating NKT cells in non-human primates. We evaluated the delivery of the NKT cell ligand, α-Galactosylceramide, in 27 macaques, by studying the effects of different dosing (1-100ug), and delivery modes (directly IV or pulsed onto blood or PBMC). We found that peripheral NKT cells were transiently depleted from the periphery following α-GalCer administration across all delivery modes, particularly in doses of ≥10ug. Furthermore, NKT cell numbers frequently remained depressed at IV α-GalCer doses of >10ug. Levels of cytokine expression were also not enhanced after α-GalCer delivery to macaques. To evaluate the effects of α-GalCer administration on antiviral immunity, we administered α-GalCer either together with live attenuated influenza virus infection or prior to SIV infection of two macaques. There was no clear enhancement of influenza-specific T or B cell immunity following α-GalCer delivery. Further, there was no modulation of pathogenic SIVmac251 infection following α-GalCer delivery to a further 2 macaques in a pilot study. Accordingly, although macaque peripheral NKT cells are modulated by α-GalCer in vivo, at least for the dosing regimens tested in this study, this does not appear to have a significant impact on antiviral immunity in macaque models.
**Introduction**

NKT cells are a small subset of T cells that provide an immune defense against viral infections and cancer. In human and macaque peripheral blood, NKT cells typically comprise 0.01-0.1% of the lymphocyte population [1]. NKT cells respond to glycolipid antigens presented by CD1d, a non-classical MHC-like molecule, localized on antigen presenting cells such as dendritic cells, monocytes and B cells [2]. Type I NKT cells are referred to as semi-invariant NKT cells due to their common usage of the TCR chain Vα24-Jα18, paired with a limited repertoire of Vβ chains [2-4]. They have a memory phenotype [5], and upon stimulation NKT cells produce large amounts of cytokines including IFNγ, TNF, IL-2, IL-4, IL-10, IL-13, IL-17, IL-21 and IL-22 [6, 7]. The cytokines produced by NKT cells leads to concomitant activation of other cells of the immune system such as NK cells, B cells and T cells, leading to a larger cascade of cytokine and chemokine production [2].

NKT cells are depleted during HIV infection of humans [8-10] or SIV infection of macaques [11, 12]. Depletion of the CD4+ subset of NKT cells is most marked and parallels depletion of the total conventional CD4+ T cells. Depletion of NKT cells correlates with disease progression in both HIV and SIV infection [12, 13]. Whether manipulation of NKT cell numbers or function could modulate HIV or SIV infection is not known.

The prototypical NKT cell ligand α-Galactosylceramide (α-GalCer) has been used extensively in mice and human studies to demonstrate immunomodulatory effects in cancers [14-20], microbial infections [21-23] and autoimmunity [24-26]. NKT cells have also been shown to promote immunity to influenza virus [27, 28], and α-GalCer has been used successfully in mice to enhance immunity to influenza virus A [29-31] or other viral infections [32, 33]. α-GalCer administration in combination with DNA vaccination of mice
was protective in microbial infections or potent in augmenting T cell responses [34, 35].

Furthermore, mice immunized with α-GalCer together with Herpes Simplex Virus-2 glycoprotein D via the intranasal or intravaginal routes were protected against lethal HSV-2 intravaginal challenge [36].

Many previous studies have used autologous or syngeneic dendritic cells pulsed with NKT cell ligands to activate NKT cells in vivo, reviewed in [37]. Such systems require specialized facilities and will not be widely applicable. In previous studies our lab has demonstrated the effectiveness of ex-vivo pulsing of CTL peptides onto macaque PBMC or whole blood [38, 39]. Presumably, immature blood DCs can effectively present the antigen following in vivo maturation. Whether a simpler system of whole blood or PBMC delivery of NKT cell ligands is effective in vivo is unknown.

Macaques are a useful primate model for a variety of infectious diseases. However, there is no information on effective conditions to activate or expand NKT cells in macaques and thereby modulate virus infections. We conducted a study of 27 SIV-uninfected macaques to determine conditions suitable for in vivo activation of NKT cells. We subsequently conducted studies to investigate the efficacy of α-GalCer administration in macaques to augment live-attenuated Influenza virus immunity. We then investigated SIV disease progression in macaques administered α-GalCer just prior to SIV infection.
Methods

Study animals

We studied a total of 42 pigtail macaques (*Macaca nemestrina*) for changes in peripheral NKT cell frequencies in response to α-GalCer administration combined with or without SIV\textsubscript{mac251} and/or Influenza A virus inoculations. Twenty-seven animals were administered with α-Galactosylceramide (α-GalCer) and NKT cell frequencies and function observed. Two animals were administered α-GalCer at the same time as live attenuated influenza virus infection along with 13 control animals that received Influenza only, and observed for anti-influenza virus immunity (Table 1). In another study 2 animals were administered α-GalCer prior to SIV infection and observed for SIV infection along with 5 controls that received SIV only (Table 1). All animals expressed the MHC Class I allele Mane-A1*08401 (previously named Mane-A*10) [40]. Prior to any procedure macaques were sedated intramuscularly with ketamine (10 mg/kg). All animals were monitored for any significant changes in weight or behavior, or toxic side effects during the respective studies. All studies were approved by the relevant institutional ethics committees.

Modes of delivery of α-Galactosylceramide

α-Galactosylceramide (KRN7000, Sapphire Biosciences) was prepared as previously described [41]. For studies on optimization of expansion of in vivo NKT cell levels we used a total of 27 healthy, SIV-uninfected macaques. Macaques were randomly assigned into 3 groups and administered α-Galactosylceramide either intravenously (IV), pulsed onto autologous ex vivo peripheral whole blood (WB), or pulsed onto autologous freshly prepared PBMC. Seven macaques were given α-GalCer IV at doses of 1ug, 10ug or 100ug each (Table 1 and Figure 1). Nine macaques were given α-GalCer pulsed onto WB. Peripheral blood (9 ml) was drawn into Na-heparin vacuette tubes from each macaque; incubated with 1ug or 10ug α-GalCer for 1h or 3h at 37°C with mixing every 15 min, and
re-infused into the respective animal. Eleven macaques were assigned to the PBMC group. PBMC, typically 10-20 x 10^6 cells, were prepared from ex vivo blood of the respective animal by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and incubated with 1ug or 10ug α-GalCer for 1h, 3h or 12h as above in 2ml serum-free, RPMI media. Following α-GalCer administration, sequential peripheral blood was drawn from each macaque according to a schedule shown in Figure 1 and monitored for NKT cell frequencies as described previously [12, 41].

PMA/ionomycin activation of NKT cells and intracellular cytokine expression.

Ex vivo NKT cells were activated for 4 hours with PMA (10ng/ml) in combination with ionomycin (3uM) in whole blood assays at day 0, 9 and 20 post α-GalCer administration as previously reported [41], with the addition of monensin (2uM) for the last 2 hours of the activation. Unstimulated controls, containing 0.41% DMSO, contained the same percentage of DMSO as stimulated samples and were treated as above. Intracellular IFNγ expression was enumerated as described [41].

Recombinant Influenza-SIV vaccination

Construction of 3 separate live-attenuated Influenza A (Flu) virus each encoding an SIV epitope restricted by Mane-A1*08401 is described previously [42, 43]. Animals (n=15) previously unexposed to α-GalCer or Flu were enrolled in this study. Flu-SIV (n=8), Flu-SIV in combination with a single dose of α-GalCer (5ug) pulsed onto WB for 2h (n=2) and control Flu (n=5) inoculations were given 4 times over 18 weeks (Figure 4). Flu specific antibody response was measured by hemagglutination inhibition assays as described [42], by Drs Karen Laurie and Aeron Hurt. A Flu specific CD8+ T cell response to a nucleoprotein epitope was assayed with the Mane-A*10-RA9 tetramer reagent on thawed
PBMC of the above 15 animals as well as those from Flu naïve macaques (n=13) as previously described [42].

SIV infection of macaques

Macaques (n=2) were given a single dose of α-GalCer (5ug) pulsed onto WB for 2h, 2 days prior to SIV\textsubscript{mac251} infection with $10^4$ TCID\textsubscript{50} (Fig 5). 5 control animals were also infected with SIV\textsubscript{mac251} at the same time. SIV viraemia and CD4 T cell counts were measured as previously described [43].

Flow cytometry analyses absolute counts of NKT cells

Peripheral blood isolated from macaques was stained with CD1d tetramer (CD1dtet) loaded with an α-GalCer analog, PBS-44, (kindly provided by Professor Paul Savage (Brigham Young University), CD3 (clone SP34-2, BD), CD4 (Clone L200, BD) and CD8 (clone SK1, BD). NKT cells were identified by flow cytometry as doublet excluded lymphocytes that are double positive for CD3 and CD1dtet.

NKT cell numbers were enumerated on a Coulter AC.T diff\textsuperscript{TM} Analyzer (Beckman Coulter) by measuring total peripheral blood lymphocyte counts.

Statistical analyses

Statistical analyses were performed using SPSS version 20 (IBM, Chicago, IL, USA). Data were analysed by two-way ANOVA in conjunction with Bonferroni post hoc test. Where necessary log\textsubscript{10} transformation before ANOVA was performed for the data to pass or tend towards Levene’s test for equal variances. Data that were not normally distributed were analysed by Kruskal-Wallis (Fig 4b) or Kruskal-Wallis with Mann–Whitney post hoc test (Fig 4c).
Results

α-Galactosylceramide administration modulates peripheral blood NKT frequencies

Enhancing NKT cell numbers or function is an important goal of immunotherapy strategies. Previous studies endeavoring to activate or expand peripheral blood NKT numbers in humans using α-GalCer directly have involved either IV injection of α-GalCer in solution [17], in vitro expansion of autologous PBMC with α-GalCer in the presence of IL-2 and GM-CSF [15, 16, 44, 45] or purified DCs pulsed with α-GalCer [46-48]. Macaques are a useful primate model to study NKT cells [11, 12, 49, 50], however, to our knowledge no studies have assessed NKT cell activation or expansion strategies using α-GalCer in macaques. Therefore, we sought to determine simple yet effective ways to achieve this with pigtail macaque NKT cells in vivo.

Twenty-seven healthy macaques were randomly assigned to be given 1-100ug α-GalCer (KRN7000) by IV injection, pulsed onto ex vivo whole blood or pulsed onto autologous PBMC for defined periods (Table 1, Figures 1 and 2). α-GalCer was well tolerated by all 27 macaques, as no systemic or local clinical manifestations were observed. CD1d tetramers loaded with the α-GalCer analog, PBS-44 were used to identify NKT cells from these animals. PBS-44 is used as it loads more efficiently into CD1d tetramers, and we determined that these tetramers stained NKT cells comparably to CD1d tetramers loaded with KRN7000 (the form of this glycolipid that was administered into the animals) (Supplementary Figure 1). A pronounced reduced detection of NKT cell frequencies occurred at day 2/3 post α-GalCer delivery, regardless of the mode of administration, in animals given 10-100ug α-GalCer (mean 0.012% NKT cells within the lymphocyte population, range 0.00%- 0.06% for all animals in the 10-100ug group; mean 0.02%, range...
0.00%-0.06% in PBMC group; mean 0.001%, range 0.00%-0.001% in WB group, and mean 0.002%, range 0.00%-0.003% in IV group). This effect was less pronounced when only 1ug α-GalCer was given (mean 0.071% NKT cells of lymphocytes, range 0.002%-0.45% for all animals within the 1ug group; mean 0.24%, range 0.03%-0.45% in PBMC group; mean 0.02; range 0.002%-0.08% in WB group; mean 0.04%, range 0.003%-0.09% in IV group). In some animals, particularly those administered α-GalCer pulsed onto PBMC, there was even a slight decline in detection of NKT cell frequencies within the circulating lymphocyte population within 10 minutes of administration (range 3-43% reduced detection compared to baseline in 19 of 27 animals, Figures 1a and 1b, Figure 2b).

Although NKT cell levels illustrated variable patterns across the animals, after α-GalCer administration, a common finding was that detectable NKT levels began to rise after the nadir at 2/3 days, reaching maximal levels between days 6-9, followed by a slight drop at day 20/21 before stabilizing. An interesting observation noted was that some animals that had higher baseline NKT frequencies achieved higher NKT cell expansion after α-GalCer delivery (Figure 1b). For example, one animal in the PBMC 1h 1ug group (top panel, leftmost graph) had a pre-administration NKT cell frequency of 0.4% that rose to 1.41% at day 9, while another animal, in the WB 1h 1ug group (centre panel, leftmost graph) had a starting NKT cell frequency of 0.13% that rose to 0.4% after α-GalCer delivery. However, in 13 of 19 animals NKT levels did not return to pretreatment baseline levels at the end of the observation period of 69 days (range 4-140% of baseline numbers; median 34%).

Pulsing either whole blood or PBMC for a longer duration (>1 hour) did not perceptibly increase NKT percentages. Interestingly, NKT levels in the two animals IV administrated 100ug α-GalCer remained at 4-8% of baseline frequencies through to day 69, the lowest of all the animals studied.
We also assessed absolute numbers of blood NKT cells over time after α-GalCer administration by concurrently measuring total lymphocyte counts. All animals in all groups had detectable, albeit variable, baseline NKT numbers (mean 5951 NKT cells/ml blood, range 145 - 21,200 NKT cells/ml blood) consistent with observations on peripheral NKT levels in humans, other macaque species and our previous data in pigtail macaques [1, 11, 12, 41, 49, 50]. On the whole, total NKT numbers in blood sampled over time mirrored NKT frequencies enumerated within the lymphocyte population by flow cytometry (Fig 1b). Across the whole cohort, average NKT cell percentages were 0.11 at 10 min and 0.03 at 2/3 days after α-GalCer administration.

Effect of dose and mode of α-GalCer administration and impact on NKT cell subsets

The PBMC mode of delivery of α-GalCer was better than IV delivery for enhancing NKT cell frequencies at day 9 and day 20, across all doses (Figure 2b; p=0.001 between PBMC group and IV group in a two-way ANOVA with Bonferroni post hoc test). Intravenous administration of α-GalCer more commonly resulted in sustained lower frequencies of NKT cells compared to PBMC administration (p=0.033 at day 20). Whole blood delivery of α-GalCer was not different to the other modes of delivery for NKT cell frequencies at day 9 and day 20.

A high dose of α-GalCer was detrimental for recovery of NKT cells. Animals given ≥10 ug α-GalCer had significantly lower NKT frequencies across day 9 and day 20 (p=0.004) compared to animals given 1 ug α-GalCer. NKT levels across all doses had expanded significantly by day 9 after the initial decline observed at day 2/3 (p < 0.001). α-GalCer administration, however, resulted in no overall expansion of NKT cells, from baseline levels, by day 9 or later (p=0.438) (Figure 2b).
To determine factors regulating the differences in NKT cell frequencies we studied the impact of delivery of α-GalCer on peripheral NKT cell subsets over time (Figure 2c). Our previous data in pigtail macaques showed that the major peripheral blood NKT subsets were CD4+CD8- and CD4-CD8+ and that the CD4+ NKT cell subset declined after SIV infection [12]. In this study ex vivo peripheral blood NKT cell subset frequencies across all 27 animals at day 0 were as follows: CD4+ NKT cells (mean 49%; range 16%-74%), CD8+ NKT cells (mean 40%; range 5%-77%); CD4+CD8+ double positive (DP) NKT cells (mean 7.9%; range 0%-29%) and CD4-CD8- double negative (DN) NKT cells (mean 1.8%; range 0%-7.5%). The CD4+, DP, or DN NKT subset frequencies at day 9 after α-GalCer administration were not statistically different from baseline levels (p ≥0.083). However, the CD8+ NKT subset frequency was significantly lower at day 9 compared to baseline values (p = 0.007).

Activation profile of NKT cells after α-GalCer administration

In addition to numerical levels of NKT cells, the capacity of NKT cells to be activated upon stimulation may be an important measure of NKT cell function. NKT cells from the baseline time-point (day 0) activated in vitro in whole blood assays with PMA/ionomycin, exhibited clear intracellular IFNγ staining (Figure 3) [41]. In the absence of mitogenic PMA/ionomycin stimulation, we detected very little IFNγ production from NKT cells at 9 and 20 days after in vivo α-GalCer administration (<4% IFNγ expression, data not shown). This is probably because at these later timepoints, the NKT cells are no longer in an activated state in response to α-GalCer challenge, which is consistent with results from studies in mice [51]. However, the peak of NKT cell expansion at day 9 did not coincide with higher levels of mitogen-induced IFN-γ expression. Furthermore, IFNγ production at days 9 or 20/21 was lower than baseline levels (p<0.001).
Effect of α-GalCer-pulsed blood as a vaccine adjuvant

α-GalCer has been shown to improve vaccine efficacy in murine studies of both Influenza and Herpes simplex virus vaccinations [29-31, 36]. No studies have analyzed the effects of α-GalCer as a vaccine adjuvant in macaques. We evaluated the effectiveness of α-GalCer as an adjuvant in a pilot trial of 2 macaques given 5 ug α-GalCer delivered via peripheral blood at the same time as the first dose of live attenuated Influenza virus A (Flu) carrying 3 SIV-specific CD8 T cell epitopes [42, 43]. Macaques were reinoculated with three further doses of Flu over a 19-week period, but without any further administration of α-GalCer (Figure 4a shows the vaccination schedule). A second cohort of 8 animals were given the above regimen but without α-GalCer, and a third cohort of 5 animals received live attenuated Flu without SIV epitopes inserted.

As observed with the administration of α-GalCer onto whole blood in the absence of vaccination, detection of peripheral blood NKT cell levels in the two immunized macaques declined to approximately 50% within a half hour of α-GalCer delivery, and these cells were essentially undetectable at 2 days, after which NKT cell levels begin to rise (Figure 4a). However, one animal never recovered its baseline frequency of NKT cells. In contrast, no apparent depletion of NKT cells occurred in animals immunized with Flu-SIV or Flu only but not given α-GalCer.

To assess whether α-GalCer had an impact on Influenza-specific immunity we studied both antibody response by haemagglutination inhibition (HI) antibody titration and influenza T cell response by MHC Class I tetramer (Figure 4b and c). HI antibodies were rarely observed prior to 14 days after the first Influenza inoculation, but thereafter HI antibodies to Influenza were present in all animals and, although variable across the small
numbers of animals, were of a similar magnitude in animals administered \( \alpha \)-GalCer compared to those not administered \( \alpha \)-GalCer.

We also studied an Influenza-specific T cell response late into the Flu infection, at 133 days after the first Flu inoculation. We previously mapped an Influenza nucleoprotein-specific CD8 T cell response (RA9) restricted by the MHC Class I allele Mane-A\textsuperscript{1}A1*08401 [42]. Since all animals expressed this MHC allele we compared the RA9-specific CD8 T cell response in animals given \( \alpha \)-GalCer to those not given \( \alpha \)-GalCer. The levels of RA9-specific CD8 T cells produced by animals given a single dose of \( \alpha \)-GalCer were relatively low (0.060-0.080% of CD8 T cells) and were within the range of animals that received either Flu-SIV or Flu only infections.

Pre-treatment with \( \alpha \)-GalCer does not alter SIV infection

NKT cells activated following \( \alpha \)-GalCer delivery in mice remain in an activated state, with production of IFN\( \gamma \) for the first 48 hours [52, 53]. Our studies showed that NKT cells are no longer detected in the peripheral blood 2 days post \( \alpha \)-GalCer delivery and we reasoned that at this time-point NKT cells may be in a more activated state and primed to respond to infection. We therefore evaluated whether SIV infection or disease progression was influenced by stimulation of NKT cells with \( \alpha \)-GalCer prior to SIV infection. We administered 5 ug \( \alpha \)-GalCer pulsed onto peripheral blood 2 days prior to intravenous SIV\textsubscript{mac251} infection in two naïve macaques, and followed markers of SIV infection systemically in peripheral blood (Figure 5). Peripheral blood NKT cells were depleted in both animals as observed in the earlier studies, although not completely in one animal (Figure 5). NKT cell proportions subsequently rose back to near baseline levels, 1-2 weeks after SIV infection. SIV infection subsequently resulted in near complete depletion of NKT cells in one of the 2 animals, consistent with our previous analyses of total NKT cell levels.
following SIV infection [12]. Depletion occurred primarily in the CD4+ subset of NKT cells (Figure 5b). The animal that showed near complete depletion of NKT cells (red filled square) had a significantly lower pretreatment baseline CD8+ NKT cell frequency of 0.61% compared to 29% in the other animal administered α-GalCer (blue filled square), and a higher CD4+ NKT cell frequency of 77% (c.f. 51%) (data not shown). As in our previous study [12], the proportion of CD8+ NKT cells rose concomitantly (Figure 5c), whilst variable depletion was observed in the CD4+CD8+ (DP) subset (Figure 5d). Despite NKT cell modulation after α-GalCer administration, peripheral SIV viraemia and CD4 T cell levels were very similar between the animals administered α-GalCer to those not administered α-GalCer (Figure 5e-f). In addition, preliminary data carried out on these two animals found no changes in levels of serum cytokines such as IL-2, IL-4, IFNγ or TNF at 2 weeks post α-GalCer administration compared to pre-administration levels (data not shown).
Discussion

NKT cells can be harnessed to modulate infections to microbes by exploiting their ability to enhance innate and adaptive immune responses [37]. NKT cell numbers vary widely in humans and this may influence the ability of these cells to influence immunity (Reviewed in [1, 7]), and similar variability in NKT cell numbers is observed in macaques [12, 49, 50]. Macaques represent an important model for the study of influenza and HIV infection and immunity [42, 43, 54-57], and may be a valuable means to study the impact of NKT cell activation on these diseases, and also whether NKT cell numbers can be enhanced by in vivo activation. Therefore, we undertook a study to determine the response of macaque NKT cell activation in vivo, as well as a pilot study into the ability of NKT cell activation to enhance immune responses and/or ameliorate infection. We observed a transient reduction in detectable NKT cells within 2 days of α-GalCer administration, followed by restoration of NKT cell numbers, the extent of which appeared to vary in an α-GalCer dose-dependent manner. Thus, a low dose of 1ug resulted in normal to increased NKT cell numbers, particularly when loaded onto PBMC, while higher doses (10-100ug) tended towards lower NKT cell numbers compared to the starting population, regardless of the mode of delivery. Our preliminary findings failed to demonstrate an enhanced immune response to influenza or SIV infection following α-GalCer co-administration.

Presentation of α-GalCer upon APC generated in vitro with IL-2 and GM-CSF from cultured autologous PBMC was successful in elevating baseline NKT cell levels in some cancer patients [14-16, 46-48]. We determined the efficacy of introducing α-GalCer-pulsed PBMC or peripheral blood into healthy macaques, without the use of long-term cultures or cytokines during the pulsing stage. Similar to the previous studies in humans, expansion of NKT cells above baseline levels was observed in some but not all animals after delivery of
α-GalCer either IV, pulsed onto PBMC or pulsed onto peripheral blood. At best, a 2.5 to 3.5 fold expansion of NKT cells was achieved. Interestingly the two animals that had the most expansion had one of the highest baseline NKT frequencies.

Previous studies in humans have shown a decrease in circulating numbers of NKT cells upon IV injection of α-GalCer, particularly at 2 days post administration, and a lack of rebound to baseline levels [17, 58]. Our data is partially consistent with this finding, although with the caveat that the extent of rebound appears to be α-GalCer dose-dependent. The early transient depletion of macaque peripheral NKT cells due to α-GalCer stimulation, noted in mice studies as well, most likely reflects TCR downregulation causing NKT cells to be undetectable by CD1dtetramers [52, 53, 59, 60]. However, we cannot exclude the possibility that some of the NKT cell disappearance was due to migration from blood to other tissues. We also observed gradual long-term depletion of blood NKT cells (<75% of baseline levels) in 13 of 19 animals administered 10-100ug α-GalCer, and followed out to 10 weeks after α-GalCer administration. Again, we were unable to determine if this was due to NKT cell death, or migration to other sites.

Given that in some studies, in vivo activation of NKT cells by α-GalCer results in an anergic response to rechallenge [51, 61], it was also important to determine whether in vivo activated NKT cells were still capable of producing cytokines at different timepoints after α-GalCer administration. While these previously stimulated NKT cells were clearly still capable of robust IFNγ production, the percentage of cells responding was slightly but significantly lower. At the time this study was conducted, optimal methods of ex-vivo glycolipid antigen-specific activation of macaque NKT cells [41] had not been developed so we were limited to mitogenic NKT cell activation to induce clear cytokine production. At this stage we cannot exclude the possibility that antigen-specific NKT cell activation
following in vivo α-GalCer stimulation may be different to mitogenic activation, or that expression of cytokines other than IFN-γ may have correlated with expansion.

In mice, NKT cells have pre-formed IL-4 and IFNγ mRNA transcripts poised for immediate secretion upon ligand stimulation [62, 63]. Similarly, in macaques, the peak of IFNγ expression may have occurred much earlier after in vivo α-GalCer stimulation, which would have been missed in this study since NKT cells were essentially undetectable at day 2/3. Furthermore in mice, in vivo α-GalCer stimulation results in peak secretion of various cytokines such as IL-4 and IFNγ, at different times after priming, which can precede peak NKT cell expansion [51, 63]. Future studies could assess additional time points for NKT cell activation after in vivo α-GalCer administration using both IFNγ expression, expression of other cytokines, and other NKT cell activation markers such as CD69, as well as the use of an exhaustion marker such as PD-1[64, 65] which may be useful in elucidating any apparent NKT cell dysfunction.

While it was encouraging that we detected a clear NKT cell response to in vivo administered α-GalCer, our pilot study of the impact of α-GalCer on immunity to influenza or SIV did not reveal an enhanced immune response to these viruses. It is possible that the timing of administration of α-GalCer together with Influenza vaccination (Figure 4) or prior to SIV exposure (Figure 5) in macaques was suboptimal. However, studies of α-GalCer therapy in mouse models of infections were successful in clearing infection and/or augmenting T cell responses using an α-GalCer delivery timing strategy similar to that done in our studies [29, 31, 32, 66-68]. For example, when α-GalCer was administered at the same time as irradiated sporozoites enhanced T cell immunity to the recombinant adenovirus vector was detected [67]. Another study by the same group found that α-
GalCer delivery 2 days before live sporozoite challenge completely cleared hepatic parasites [66]. In our study, we found no enhancement in CD8 T cell or antibody responses to live attenuated Influenza virus after inoculation. α-GalCer may play a more useful role in augmenting CD8 T cell responses to inactivated Influenza virus vaccination as noted in a mouse model[29], compared to the live influenza infection model we studied.

In our SIV infection study of two macaques, we reasoned that NKT cells and downstream activation of effector cells may be at their peak of activation 2 days after α-GalCer administration as evidenced by a loss of circulating NKT cells. Using this approach, however, we found no significant impact of α-GalCer delivery on the course of SIV infection. We cannot exclude the possibility that cytokine production, particularly IFNγ secretion, or bystander activation of other cell types such as antigen presenting cells, necessary to combat SIV replication, may have subsided within the 48 hours before infection.

There are several limitations to our study that warrant further attempts to modulate NKT cells in macaque models. First, we have not studied α-GalCer delivery via autologous dendritic cells. Although this method has been highly successful in both mice and small human cancer trials [14-16, 20, 46-48], such methods will not be practical in diverse clinical settings. Second, the timing of α-GalCer delivery may not have been optimal to influence Influenza or SIV-specific immunity. For example it remains possible that NKT cells stimulated by α-GalCer would be more effective at controlling SIV if timed to more closely coincide with peak viremia levels 2 weeks after infection. Third, we did not study tissue NKT cell levels or their activation status. We observed modest expansion of peripheral NKT cells and we know that substantial expansion of NKT cells can occur in the spleen and liver of mice following α-GalCer administration [51, 60]. Further studies are...
needed to determine if trafficking and expansion may have occurred in such sites. Taken together, a more extensive study comparing kinetics of α-GalCer administration and broader analysis of various cytokines, bystander cell types, and different tissue sources is required to properly determine the influence of α-GalCer on Influenza- of SIV-specific immunity.

In summary, we show that macaque peripheral NKT cells can be modulated safely with α-GalCer by pulsing onto PBMC or whole blood. Lower α-GalCer doses (<10 µg) appeared to result in better NKT cells expansion in peripheral blood than higher α-GalCer doses (10 µg or 100 µg). However, in preliminary experiments, we did not observe enhanced immunity to influenza or SIV infection associated with α-GalCer co-administration. Further studies are warranted to explore and optimize the impact of NKT cells on infectious diseases in macaque models.
Figure Legends

Figure 1:

**Transient depletion of peripheral NKT cells upon in vivo α-GalCer delivery.** (A)

Representative plots of flow cytometry analysis of pigtail macaque NKT cells within the lymphocyte population of peripheral blood stained ex vivo at different times following α-GalCer delivery; 5873 was administered 10ug α-GalCer pulsed onto PBMC for 12h, 36142 was administered 1 ug α-GalCer pulsed onto WB for 3h. NKT cell levels are enumerated as cells double positive for CD1d tetramers loaded with the α-GalCer analog, PBS-44 and CD3 as a proportion of gated lymphocytes. (B) Sequential blood samples were taken prior to delivery (0 min) and at 10 min, day 3, 9, 20 and 28 following 1ug α-GalCer administration (n=8) (Fig 1b, left column), or at 0 min, 10 min, day 2, 6, 9, 20, 69 following 1-100 ug α-GalCer delivery (n=19) (Fig 1b, columns 2-4). Each line represents the peripheral NKT cell frequency of one animal. Dose and mode of administration of α-GalCer are indicated above each individual graph.

Figure 2:

**Impact of dose and mode of delivery of α-GalCer, and impact on NKT subsets.** (A)

NKT cell levels were normalized to baseline levels and animals were segregated according to the dose of α-GalCer (A) and dose and mode of delivery (B). Groups contained variable numbers of animals: all animals/1ug (n=10), PBMC/1ug (n=2), WB/1ug (n=5), IV/1ug (n=3); all animals/10ug (n=17), PBMC/10ug (n=9), WB/10ug (n=4), IV/10-100ug (10ug, n=2; 100ug, n=2). 2-way ANOVA was carried out on data prior to normalization to baseline NKT frequencies. (C). NKT subsets defined as CD4+CD8-, CD4-CD8+, CD4+CD8+ double positive (DP) or CD4-CD8- double negative (DN) populations as indicated above each graph. Graphs show data normalized to the respective subset frequencies measured
at day 0 with some exceptions in the DP and DN graphs, where no DP or DN NKT subsets were detected at day 0, as follows: 4 animals shown in the DP graph were normalized to DP NKT cell frequencies measured at day 6, while one animal had no DP cells at any time-point; 8 animals in the DN graph were normalized to DN NKT frequencies detected between the 10 min – day 20/21 time-point, while 2 animals had no DN NKT cells at any time-point. 2-way ANOVA carried out on subset frequencies prior to baseline normalization (p ≥ 0.083 between day 0 and day 9 for each of CD4+, DP or DN subsets; p < 0.007 for CD8+ subset). Error bars, standard error of mean; # p=0.001 between PBMC and IV groups; ✝ p=0.03 between PBMC and IV groups; ● p=0.004.

**Figure 3:**

**Activation status of NKT cells.** Peripheral blood of macaques was activated ex vivo with PMA/ionomycin at day 0, 9 and 20 following α-GalCer administration. (A) Representative dot plots of NKT cell levels within the lymphocyte population following stimulation, and histograms of intracellular IFNγ production from NKT cells. NKT cells were stained with CD1d tetramers loaded with the α-GalCer analog, PBS-44. Cytokine frequencies shown are background-subtracted values. Grey shaded histograms are unstimulated controls containing 0.41% DMSO; black shaded histograms are mitogen activated. (B and C) Expression levels of IFNγ from NKT cells of macaques. Only animals that expressed IFNγ levels three times above background (unstimulated) at day 0, 9 and 20 are shown (B) and included in statistical analysis (C). Animals across all doses and modes of delivery were included in a two-way ANOVA with Bonferroni post hoc test (day 0, n=26; day 9, n=19; day 20/21, n=24); *** p<0.001. The mean of each group is represented by a horizontal bar.

**Figure 4:**
**Effect of α-GalCer on attenuated Influenza vaccination.** Live-attenuated Influenza A virus encoding three distinct SIV epitopes (Flu-SIV) was administered on the same day (Day 0) as 5 ug α-GalCer pulsed onto autologous peripheral blood (n=2), Flu-SIV without α-GalCer (n=8), and control Flu not containing exogenous antigens (Flu only; n=5). Further flu vaccinations were given to the respective groups on days 28, 56 and 119 after the first flu vaccination, without additional delivery of α-GalCer. (A) Peripheral blood NKT cell levels normalized to pre-Flu vaccination levels. (B) Serum antibodies to Flu was measured with a hemagglutination inhibition assay using macaque red blood cells. HI titres of the groups analysed by Kruskal Wallis non parametric test (C) A CD8 T cell response to a Mane-A1*08401 restricted Flu nucleoprotein epitope, RA9, was assessed by tetramer staining. An additional group of Flu naïve macaques (n=13) as previously described [42] was included in the analysis. Data analysed by Kruskal-Wallis with Mann-Whitney post hoc test. Error bars, standard error of mean.

**Figure 5:**

**Effect of α-GalCer on SIV infection of macaques.** Macaques were infected with SIV\textit{mac251} (control animals, n=5, clear circles) at day 0 or administered 5 ug α-GalCer pulsed onto peripheral blood 2 days prior to SIV\textit{mac251} infection (day α-GC; n=2, filled squares). (A) Peripheral blood NKT levels normalized to pre-α-GalCer NKT percentages (for animals given α-GalCer), or to peripheral NKT percentages prior to SIV\textit{mac251} infection (for control animals). (B), (C) and (D) NKT cell subset frequencies for each subset, normalized to pre-α-GalCer or pre-infection subset frequencies, as indicated in axes Values for red filled squares are off-scale and are indicated above the graph. (E) SIV viraemia and (F) peripheral total CD4 T cells levels, normalized to pre-SIV infection levels were measured.
Acknowledgments

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References


22. Emoto M, Emoto Y, Yoshizawa I, Kita E, Shimizu T, Hurwitz R, Brinkmann V, Kaufmann SHE. Alpha-GalCer ameliorates listeriosis by


35. Dondji B, Deak E, Goldsmith-Pestana K, Perez-Jimenez E, Esteban M, Miyake S, Yamamura T, McMahon-Pratt D. Intradermal NKT cell


<table>
<thead>
<tr>
<th>Study</th>
<th>No. of animals</th>
<th>Macaque ID</th>
<th>Details</th>
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<tbody>
<tr>
<td>α-GalCer delivery (27 macaques in total)</td>
<td>2</td>
<td>7448, C3752</td>
<td>1 ug α-GalCer pulsed onto PBMC for 1h</td>
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<td>10 ug α-GalCer pulsed onto PBMC for 1h</td>
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<tr>
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<td>36271, 45610</td>
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<tr>
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<tr>
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<td>2</td>
<td>C0942, 36121</td>
<td>IV 100 ug α-GalCer</td>
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</table>

α-GalCer administered at the same time as live attenuated Influenza A virus ¹ (28 macaques in total) ¹ Jegaskanda et al., PLoS One 2012

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of animals</th>
<th>Macaque ID</th>
<th>Details</th>
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<tr>
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<td>19341, 19351, 19530, B0508, C3754</td>
<td>Control Flu, not encoding SIV epitopes</td>
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α-GalCer administered 2 days prior to SIVmac251 infection (7 animals)

<table>
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<th>No. of animals</th>
<th>Macaque ID</th>
<th>Details</th>
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</thead>
<tbody>
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<td>*5873, *C3751</td>
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<td>**19341, **19351, **19530, **B0508, **B0547</td>
<td>Control animals not given α-GalCer, and infected with SIV at</td>
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<tr>
<td></td>
<td><strong>C3754</strong></td>
<td>the same time as above</td>
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<tr>
<td>--------</td>
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* Animals previously enrolled in "α-GalCer delivery" study; ** animals previously enrolled in "Control Flu, not encoding SIV epitopes" group.
1 µg α-GalCer pulsed PBMC, WB or IV

<table>
<thead>
<tr>
<th>Minute (')/Day</th>
<th>PBMC 1 ug</th>
<th>WB 1 ug</th>
<th>IV 1 ug</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>day 2/3</td>
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</tr>
<tr>
<td>day 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 20/21</td>
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10-100 µg α-GalCer pulsed PBMC, WB or IV

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<th>WB 10 ug</th>
<th>IV 10-100 ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
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<tr>
<td>day 2/3</td>
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<tr>
<td>day 9</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>day 20/21</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Figure 2**

b. Total NKT cell percentages post α-GalCer administration

- 1 ug
- 10-100 ug

C. %CD4+CD8- NKT cells

- 1 ug
- 10-100 ug

- CD4+CD8- NKT cells
- CD4-CD8+ NKT cells
- CD4+ (DP) NKT cells
- CD4-CD8- (DN) NKT cells

Time post α-GalCer delivery

- %NKT lymphocytes in blood normalized to pre-treatment level
- Total NKT cell percentages post α-GalCer administration
- %CD4+CD8- NKT cells
- %CD4-CD8+ NKT cells
- %CD4+ (DP) NKT cells
- %CD4-CD8- (DN) NKT cells

*Normalised to pre-treatment*
Figure 1

**a.** Animal 5873

<table>
<thead>
<tr>
<th>Time post α-GalCer delivery</th>
<th>% NKT lymphocytes in peripheral blood</th>
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<td>0 min</td>
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<tr>
<td>10 min</td>
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<td>day 20</td>
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**b.**

- PBMC 1h 1ug
- PBMC 1h 10ug
- PBMC 3h 10ug
- PBMC 12h 10ug
- WB 1h 1ug
- WB 3h 1ug
- WB 1h 10ug
- WB 3h 10ug
- IV 1ug
- IV 10ug
- IV 100ug

Minute ('')/Day post α-GalCer administration
Figure 3

(a.) Time post α-GalCer delivery

<table>
<thead>
<tr>
<th>Animal</th>
<th>Day 0</th>
<th>Day 9</th>
<th>Day 20</th>
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<tbody>
<tr>
<td>5873</td>
<td>0.53%</td>
<td>44%</td>
<td>0.40%</td>
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<tr>
<td>36142</td>
<td>0.07%</td>
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<td>34%</td>
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</table>

(b.)

- PBMC 1h 10ug
- PBMC 3h 10ug
- PBMC 12h 10ug
- WB 3h 1ug
- WB 1h - 3h 10ug
- IV 10 - 100ug

(c.)

Day post α-GalCer administration

**IFNγ+ NKT cells**

***
Figure 4  α-GalCer with Flu-SIV

**α-GalCer with Flu-SIV**

**Flu-SIV only**

**Flu only**

Day post initial Flu vaccination

b.

Flu-specific antibody response

- **α-GalCer with Flu-SIV**
- **Flu-SIV**
- **Flu only**

Mean HI Titre

Day post initial Flu vaccination

Day post initial Flu vaccination

C.

Flu-specific CTL response

- **α-GalCer with Flu-SIV**
- **Flu-SIV**
- **Flu only**
- **Flu naïve**
Figure 5

a. NKT cell frequencies normalized to pre-treatment

b. CD4+CD8- NKT cell frequencies normalized to baseline

c. CD4-CD8+ NKT cell frequencies normalized to baseline

d. CD4+CD8+(PP) NKT cell frequencies normalized to baseline

e. Viral load Log_{10} RNA copies/ml plasma

f. CD4 T cell counts normalized to baseline

Day post α-GalCer treatment/SIV_{mac251} infection
Supplementary Figure 1. Equivalent staining of pigtail macaque PBMC with CD1d tetramers loaded with either PBS-44 or KRN7000. Frozen PBMC from three macaques were singly stained with CD3 in combination with either CD1d tetramers loaded PBS-44 conjugated to PE fluorochrome, or CD1d tetramers loaded with KRN7000 conjugated to APC fluorochrome.
Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
Fernandez, CS; Jegaskanda, S; Godfrey, DI; Kent, SJ

Title:
In-vivo stimulation of macaque natural killer T cells with -galactosylceramide

Date:
2013-09-01

Citation:

Persistent Link:
http://hdl.handle.net/11343/44146