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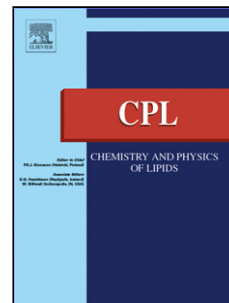
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Endocytic pH regulates cell surface localization of glycolipid antigen loaded CD1d complexes

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Running title: Endosomal pH regulates membrane localization of CD1d-antigen complexes

Graphical abstract

Highlights:

- Neutralization of endosomal pH increases presentation of Th2-biasing α GC analogues, and their association with CD1d in cholesterol rich “lipid raft” microdomains
- Pulsing the antigen presenting cells with α GC Th2-agonist in the presence of chloroquine induces higher NK cell transactivation

Abstract

Invariant natural killer T (iNKT) cells recognize glycolipid antigens presented by CD1d, an antigen presenting protein structurally similar to MHC class I. Stimulation of iNKT cells by glycolipid antigens can induce strong immune responses *in vivo*, with rapid production of a wide variety of cytokines including those classically associated with either T helper type 1 (Th1) or type 2 (Th2) responses. Alterations in the lipid tails or other portions of CD1d-presented glycolipid ligands can bias the iNKT response towards production of predominantly Th1 or Th2 associated cytokines. However, the mechanism accounting for this structure-activity relationship remains controversial. The Th1-biasing glycolipids have been found to consistently form complexes with CD1d that preferentially localize to plasma membrane cholesterol rich microdomains (lipid rafts), whereas CD1d complexes formed with Th2-biasing ligands are excluded from these microdomains. Here we show that neutralization of endosomal pH enhanced localization of CD1d complexes containing Th2-biasing glycolipids to plasma membrane lipid rafts of antigen presenting cells (APC). Transfer of APCs presenting these “stabilized” CD1d/ α GC complexes into mice resulted in immune responses with a more prominent Th1-like bias, characterized by increased NK cell transactivation and

interferon- γ production. These findings support a model in which low endosomal pH controls stability and lipid raft localization of CD1d-glycolipid complexes to regulate the outcome of iNKT cell mediated responses.

Keywords: CD1d, α GC, NKT cells, glycolipid, antigen presentation, NK activation, transactivation, IFN γ secretion, dendritic cells, endosomal presentation, endosomal pH, pH Neutralization

Introduction

Invariant natural killer T cells (iNKT cells) are a subset of T cells that also express various surface markers more typically associated with natural killer (NK) cells ¹. They are referred to as “invariant” because of the unusual structure of their T cell antigen receptors (TCRs), which are comprised of an invariant TCR α chain associated with TCR β chains that also have limited diversity ². These cells recognize and respond to glycolipid antigens presented in the context of CD1d, an antigen presenting molecule similar in overall structure to major histocompatibility complex (MHC) encoded class I proteins ³. Unlike classical T cells that initially exist in a naïve state that must undergo antigen induced activation and differentiation to become effector or memory cells, iNKT cells leave the thymus as a pre-programmed effector population ^{3a}. In addition, reporter mice developed to study IL-4 and IFN γ gene expression reveal that iNKT cells contain pre-formed mRNA transcripts for both of these cytokines, and start synthesizing and secreting cytokines within minutes of TCR engagement ⁴. This capability to respond rapidly and without prior antigen immunization makes this subset of T cells an attractive target for various approaches to immunotherapy or immunomodulation ⁵.

A large number of natural and synthetic glycolipid antigens that are presented by CD1d to iNKT cells have been described⁶. A common feature of most of these antigens is that they are composed of two lipid tails linked to a carbohydrate moiety or “head group”⁷. The best characterized of these antigens is a synthetic α -galactosylceramide with a long (C26) saturated acyl chain, initially designated as KRN7000 and also commonly referred to α GC C26:0⁸. Depending on the structure of the glycolipid antigen, iNKT cell stimulation may also be followed by the secondary stimulation or transactivation of bystander immune cells such as natural killer (NK) cells, B cells and additional subsets of T cells⁹. Agonists that fail to transactivate other types of leukocytes, especially NK cells, result in the secretion of higher amounts of serum IL-4 compared to IFN γ , and are called Th2-biasing agonists¹⁰. In contrast, agonists that strongly transactivate NK cells greatly amplify and prolong the accumulation of serum IFN γ , and these are often referred to as Th1-biasing agonists.

The mechanisms accounting for the different stimulatory activities of Th1- and Th2-biasing agonists are not completely understood, although several important correlations and potential mechanisms have been documented. For example, most and possibly all Th2-biasing agonists contain features that are likely to increase their aqueous solubility compared to the prototypical α -GC C26:0 compound. Such features include truncation of the sphingoid or acyl chain moieties, or introduction of double bonds at one or multiple locations in the lipid chain or other polar groups^{10c, 11}. Conversely, Th1-biasing agonists are relatively more hydrophobic than α -GC C26:0, and are generated by removal of polar groups. This includes substitution of the O-

glycosidic linkage by a C-glycoside, removal of hydroxyl groups or replacement of the monosaccharide head group with a carba-sugar analog^{6a, 11b, 12}.

Several studies using the monoclonal antibody L363, which specifically recognizes mouse CD1d/ α GC complexes^{10b, 13}, have revealed major differences in the site of formation, trafficking and cell surface localization of these complexes^{8a, 11b, 14}. In general, complexes containing Th1-biasing glycolipids are formed primarily in endocytic compartments in which the pH ranges from approximately 4.9 to 6.0¹⁵, and require assistance from lipid exchange proteins such as saposins for formation of complexes with CD1d¹⁶. Following transport to the cell surface, these complexes localize predominantly to cholesterol rich microdomains, usually referred as lipid rafts, in the plasma membrane^{8a, 11b}. These microdomains are enriched in GPI-linked and signal transduction proteins, and are believed to act as signaling platforms¹⁷.

In general, strong localization to lipid rafts of the complexes formed between CD1d and a particular glycolipid antigen is predictive of an increase in the Th1-bias resulting from iNKT cell stimulation. Presentation by lipid raft-localized CD1d results in the upregulation of costimulatory molecules on the surface of CD8a^{Pos} dendritic cells, the principal professional antigen presenting cell (APC) for glycolipid antigens^{8b}. The increase in costimulatory molecules correlates with higher transactivation of bystander cells such as NK cells, although the detailed mechanism remains unknown. In marked contrast to Th1-biasing glycolipids, the binding of Th2-biasing glycolipids to CD1d occurs predominantly on the cell surface, and the complexes formed are not localized in lipid rafts. Exclusion of these complexes from plasma membrane lipid rafts results in the upregulation of coinhibitory molecules on such as PD-L1 and PD-L2 on the surface

of glycolipid APCs. Of note, both of these co-inhibitory molecules are known to prohibit synapse formation between leukocytes¹⁸ and probably limit transactivation of bystander cells including NK cells, thus culminating in a “pure” iNKT responses characterized by a prominent Th2 cytokine bias.

Complexes of CD1d bound with Th2-agonist glycolipids are not normally detectable in endosomal compartments. However, neutralization of endosomal pH leads to their rapid accumulation in endosomes, consistent with an ability of low endosomal pH to destabilize these complexes^{14a}. In the current work, we have investigated whether endosomal pH also has a direct influence on cell surface levels and lipid raft association of CD1d/glycolipid complexes. Our findings confirm that neutralization of endosomal pH increases the cell surface levels of CD1d complexes containing Th2-biasing glycolipids, most likely by stabilizing these complexes in intracellular compartments to allow their transport to the plasma membrane. Interestingly, the stabilization of these complexes in endosomes led to their increased accumulation in plasma membrane lipid raft microdomains, and also resulted in enhanced NK transactivation. These results support a model for CD1d-mediated glycolipid presentation in which stable binding of glycolipid antigens in the endosome is the main determinant of subsequent transport to plasma membrane lipid rafts, leading to stimulation of Th1-biased iNKT cell responses.

Results

Effect of endosomal pH on CD1d expression and glycolipid presentation

The weak bases chloroquine (CQ) and ammonium chloride (NH₄Cl) have been used extensively to neutralize the pH of intracellular compartments such as late endosomes and lysosomes¹⁹. Using a well-defined transformed mouse dendritic cell line (JAWS II) which highly expresses CD1d as a model^{8a, 20}, we investigated the effects of these agents on CD1d expression and antigen presentation. We chose to use CQ and NH₄Cl, which accumulate in endosomes to neutralize their pH, rather than specific inhibitors of the vacuolar proton ATPase (e.g., Bafilomycin A and D, Concanamycin A and Concanolid A), since the latter can have additional effects on cells such as disruption of vesicular transport processes^{21, 22}. Both NH₄Cl and CQ diffuse rapidly into the lumen of endocytic vesicles and become trapped there in their protonated form, resulting in elevation of endosomal pH, and this effect has been shown to persist for up to several hours after removal of the drug from the extracellular medium^{19, 23}.

Dendritic cells are highly susceptible to changes in cell culture conditions, raising the potential concern that disruption of endosomal processing could cause cell death or alteration in the level of CD1d. We therefore tested CQ and NH₄Cl for their effects on cell viability and CD1d expression. We cultured JAWS II cells in media containing CQ or NH₄Cl for 4 hours, and then analyzed the cells for viability based on propidium iodide exclusion, and for surface expression of CD1d by staining with a CD1d-specific antibody. Treatment of these cells with either CQ or NH₄Cl at the concentrations used did not result in alteration of CD1d expression (Figure 1), and led to only minor reductions in cell viability and no visible alteration in cell morphology (not shown).

We focused on presentation of six well-characterized iNKT cell glycolipid antigens, which are illustrated in Figure 2. The α GC C26:0 glycolipid is a potent inducer of both IFN γ and IL-4 *in vivo*, and is described as a mixed (Th1 + Th2) or Th0 agonist^{8b}. The C-glycoside analogue α -C-GC C26:0, which stimulates much lower IL-4 and relatively higher and more prolonged IFN γ secretion, is the prototypical Th1-biasing agonist. The other glycolipids illustrated in Figure 2 (α GC C20:2, α GC C20:1, α GC C18:3 and α GC C10:0) belong to the class of Th2-biasing agonists, which stimulate strong IL-4 secretion relative to IFN γ ^{8a, 10a, 11a}. Taken together, these agonists are representative of the three currently classified categories of iNKT cell activating glycolipids, which are defined mainly by the relative Th1 versus Th2 cytokine biases of the responses they induce *in vivo* in mice. We included multiple Th2-biasing agonists in our analyses in order to identify general mechanisms that would apply to this class of agonists despite their marked structural dissimilarities.

Endosomal acidification is crucial to many normal cellular processing, and extended treatment with agents that neutralize endosomal pH may be toxic or lethal. To reduce the time of exposure, we first incubated the cells with glycolipids under normal culture conditions to allow the formation of glycolipid/CD1d complexes, and then treated them for a limited time with lysosomotropic agents. Thus, JAWS II cells were cultured with each glycolipid agonist for 16 hours, followed by treatment with NH₄Cl or CQ for an additional one or four hours, respectively. The efficiency of glycolipid antigen loading under these conditions was estimated by surface staining of the cells with L363, a monoclonal antibody specific for complexes formed by the binding of α GC glycolipids to mouse CD1d. A significant increase in the fluorescence intensity of cells loaded with

each of the four Th2-type glycolipid agonists was observed in response to treatment with either CQ or NH₄Cl. In contrast, the staining observed for the α GC C26:0 or α -C-GC treated cells was markedly reduced (Figure 3), highlighting the requirement of low endosomal pH for the loading of Th0 and Th1-biasing glycolipid analogues. In contrast, the increase in cell surface levels of CD1d bound with Th2-biasing glycolipids observed after NH₄Cl or CQ treatment suggested that low pH is non-permissive for intracellular loading of Th2-biasing agonists onto CD1d in endosomes. Another possibility would be that the binding of the Th2-biasing glycolipids to CD1d occurs on the cell surface, and is disrupted and lost during recycling through the acidic endosomal compartment under normal conditions. In this scenario, alkalinization of endosomal pH would reduce the extent of unloading of Th2-biasing glycolipids from CD1d and result in an accumulation of complexes on the cell surface.

Endosomal acidification and lipid raft localization of CD1d/glycolipid complexes

Since the effects of NH₄Cl and CQ on glycolipid presentation were similar in our initial experiments, we focused on NH₄Cl for further experiments to examine the effect of inhibiting endosomal acidification on plasma membrane localization of CD1d/ α GC complexes. Lipid rafts are enriched in cholesterol and contain tightly packed membrane lipids that make these microdomains resistant to extraction with low, sublytic detergent concentrations. Based on these properties, we previously developed a fluorescence-based method to estimate the lipid raft residency of cell surface CD1d/ α GC complexes^{11b}. Since plasma membrane lipid rafts are detergent resistant, CD1d/glycolipid agonist complexes localized in lipid rafts are not extracted by exposure to 0.06% Triton X-100,

and a minimal decrease in binding of fluorescent L363 antibody is observed over time. In contrast, for the CD1d/glycolipid complexes that are excluded from lipid rafts, the binding of fluorescent L363 decreases rapidly following the addition of detergent. Estimation of lipid raft residency can therefore be obtained based on the profile of decrease in L363 fluorescence after detergent exposure, with lower detergent sensitivity indicating a higher level of lipid raft occupancy.

We used this method to analyze the effect of NH_4Cl treatment of JAWS II cells on the lipid raft localization of CD1d/ αGC complexes formed by each of the six glycolipid antigens (Figure 4). As expected based on previous studies^{11b}, both αGC C26:0 (Th0) and $\alpha\text{-C-GC}$ (Th1) loaded CD1d complexes showed high levels of lipid raft localization, and this was not significantly changed by NH_4Cl treatment. In contrast, all of the Th2-biasing glycolipids showed much lower lipid raft residency. Strikingly, this was substantially increased in all four cases by NH_4Cl treatment (Figure 4a). Analysis of the percent of detergent resistant CD1d/ αGC complexes formed in the presence and absence of NH_4Cl was analyzed by two-way ANOVA, which confirmed the significant increase in lipid raft localization of CD1d complexes with Th2-biasing glycolipids in cells treated with NH_4Cl (Figure 4b). In addition, although there was a reduction in the number of CD1d/ αGC complexes formed with αGC C26:0 and $\alpha\text{-C-GC}$ C26:0 analogues, there was no difference observed in the lipid raft localization of these complexes after NH_4Cl treatment. These results indicated that endosomal acidification, while not required for trafficking of CD1d/ αGC complexes into lipid rafts for Th1-type glycolipids, has an inhibitory effect on the lipid raft localization of CD1d complexes

containing Th2-biasing glycolipids. This inhibitory effect can be at least partially overcome by inhibition of endosomal acidification by NH_4Cl .

Influence of endosomal acidification on NK cell transactivation

Previous studies have demonstrated that increased lipid raft residency of CD1d molecules presenting glycolipid antigens correlates with enhanced transactivation of bystander NK cells to trigger their $\text{IFN}\gamma$ secretion^{8a}. Since it is not feasible to treat mice with sufficient amounts of lysosomotropic bases to neutralize endosomal pH in APCs *in vivo*, we developed an *ex vivo* approach to study the effect of neutralization of endosomal pH on *in vivo* CD1d-mediated immune responses. For this, splenic dendritic cells (DCs) were isolated from Flt-3 melanoma bearing mice as previously described^{8b}. The purified DCs were pulsed with αGC C20:2 in the presence or absence of CQ for four hours. These cells were then transferred to naïve animals by intravenous injection, and the levels of NK cell transactivation were assessed 16 hours later by analyzing intracellular staining for $\text{IFN}\gamma$ in splenic NK cells. As a positive control, we also transferred $\alpha\text{-C-GC}$ C26:0 pulsed DCs, which have been previously shown to induce NK transactivation in similar cell transfer experiments^{8a}.

The NK cell transactivation was measured using a multiparameter flow cytometry approach to identify NK cells staining for intracellular $\text{IFN}\gamma$ as an index of transactivation, combined with a gating strategy to distinguish three different populations that express the cell surface marker NK1.1 (Figure 5a). These include a population of $\text{NK1.1}^{\text{Pos}} \text{TCR}\beta^{\text{Pos}}$ cells corresponding to NKT cells (R4), as well as the NK1.1 positive cells that lack $\text{TCR}\beta$ (R5). In addition, the R5 subset cells are further

split into classic NK cells that express NKp46, and a minor fraction of cells that are NKp46 negative. Induction of IFN γ production after injection of glycolipid loaded DCs was mainly observed in the NK1.1^{Pos} NKp46^{Pos} subset, and this showed the expected strong NK cell transactivation with α -C-GC compared to the weak transactivation with α GC C20:2 loaded DCs. Most notably, the treatment of α GC C20:2 loaded DCs with CQ prior to transfer to mice caused a significant increase in the number of IFN γ ⁺ cells detected in the NKp46^{Pos} subset, indicating increased NK cell transactivation *in vivo* (Figure 5b). Thus, loading of APCs with a Th2-biasing form of α GC in the presence of an inhibitor of endosomal acidification, which increases lipid raft localization of CD1d/glycolipid complexes, also correlated with increased transactivation of NK cells *in vivo*. We also analyzed the levels of serum IFN γ at 2 hours or 12 hours post injection, but did not observe a significant difference in these between the animals receiving CQ treated DCs versus untreated DCs (data not shown). Typically, the transactivation of NK cells following α GalCer administration results in a sustained elevation of serum IFN γ levels that peaks at 24 hours post glycolipid administration. Although we did not assess serum cytokine levels later than 12 hours after DC injections, the increase in NK cell transactivation that we observed would likely result in more sustained circulating IFN γ at later time points in the mice receiving CQ treated DCs compared to untreated DC group.

Discussion

CD1d-restricted iNKT cells are an intriguing subset of T cells that function at the interface between innate and adaptive immune responses. They are important for

mammalian immunity to microbial infections, and also are likely to contribute to autoimmune disorders and responses to cancers^{1b, 3a}. The ability to activate these cells by a single administration of a glycolipid agonist makes them an attractive target for development of immunotherapeutic applications^{5b}. The class of α GC analogues identified as Th1-biasing glycolipid antigens are extremely potent because of their capability to activate bystander cells and work as potent adjuvants for enhancing T cell priming applications such as vaccines against microbial pathogens as well as cancers^{6a}. On the other hand, the class of Th2-biasing glycolipid antigens are of potential interest as immunomodulators for controlling acute or chronic inflammation, and as adjuvants for enhancing antibody responses. It is therefore important to understand the detailed mechanisms that explain the structure-activity relationship for glycolipid activators of iNKT cells, which leads to their ability to mediate a wide range of different immunological outcomes.

We have previously demonstrated that Th1-biasing forms of α GC load efficiently onto CD1d only in acidified endosomal compartments, which also contain a number of lipid transport proteins that are likely to be important for this process^{8a, 24}. Furthermore, the association of glycolipids with CD1d in endosomes is strongly correlated with the trafficking of the resulting CD1d/glycolipid complexes to plasma membrane lipid rafts^{11b}. Although the mechanism accounting for the trafficking of these complexes into lipid rafts is not known, it is interesting to note that endosomal processing has also been associated with lipid raft localization of peptide-loaded MHC II complexes²⁵. It is also noteworthy that a substantial fraction of CD1d resides in late endosomal or lysosomal compartments, which is another feature that is shared with MHC class II molecules. We

therefore postulate that the intracellular loading of CD1d with Th0- and Th1-biasing glycolipids directs the newly formed complexes into trafficking pathways that lead to their localization into lipid raft domains on the plasma membrane. In contrast, the Th2-agonists that load CD1d on the cell surface do not have access to this machinery and are thus mostly excluded from the lipid rafts.

Previous studies by Bai *et al.* demonstrated that the uptake of both Th1-biasing and Th2-biasing α GC glycolipid antigens is very similar in dendritic cells, and that both classes of glycolipids are endocytosed and delivered to acidic endosomal compartments^{14b}. However, their studies showed that Th2-biasing analogues were unable to form stable complexes with CD1d in endosomes. Importantly, these studies also showed that neutralization of endosomal pH allows the detection of CD1d/Th2-biasing α GC complexes in late endosomal and lysosomal compartments. This effect of inhibition of endosomal acidification could be a result of enhanced intracellular loading of CD1d, or reduced dissociation of the glycolipid from CD1d under these conditions. In either case, this observation that neutralization of endosomal pH leads to the detection of CD1d with bound Th2-biasing glycolipids in endosomal compartments raised the issue of whether these complexes would be able to traffic into lipid rafts. This is the central question that we undertook to answer in the current study.

Our studies showed that there is indeed an increase in the localization of CD1d/Th2-biasing glycolipid complexes in lipid rafts after neutralization of endosomal pH. Localization in lipid rafts is linked with the transactivation of bystander cells such as B, T and especially NK cells. This motivated us to test if neutralization of endosomal acidity would enhance NK cell transactivation with Th2-biasing agonists under *in vivo*

conditions. However, administration of NH_4Cl or CQ to rodents directly for neutralizing endosomal pH *in vivo* presents difficult challenges. For example, NH_4Cl treatment in rodents induces metabolic acidosis²⁶ and CQ administration has been shown to inhibit secretion of cytokines by T cells²⁷. We therefore relied on the use of APCs pulsed with antigen *ex vivo* in the presence or absence of CQ to instigate the iNKT cell mediated responses following transfer into mice. One caveat of this approach is that the endosomal alkalization is reversible, and may be maintained for only a few hours after transfer of these cells into animal hosts. Nevertheless, our experiments using this method showed a modest but significant increase in NK cell transactivation when APCs were loaded in the presence of CQ with a representative Th2-biasing glycolipid.

The observed increase in NK cell transactivation in response to a Th2-biasing form of αGalCer with CQ treated APCs is an important finding, given that such transactivation is a characteristic feature of Th1-biasing glycolipids. We considered the possibility that the CQ treated DCs are more stable than the untreated DCs and are therefore able to activate NK cells for longer period of time. This is unlikely given that the DCs untreated with CQ can survive at least up to 24 hours in recipient hosts^{8b} and the peak of NK activation occurs at 16 hrs post DC transfer (unpublished data). In addition, although we did not see any difference in cell viability during the *ex vivo* pulsing of DCs with and without CQ before transfer into recipient animals, we expect the stability of cells to be decreased rather than increased by CQ treatment since endosomal acidification is essential for normal cell survival. Furthermore, the total number of cell surface CD1d/glycolipid complexes is generally higher on APCs exposed to Th2-biasing glycolipids compared to those exposed to similar concentrations of Th1-

biasing glycolipids, when measured by staining with the complex-specific L363 antibody^{8a, 11b, 14b}. This indicates that NK cell transactivation depends on a qualitatively different initial response of iNKT cells, and not merely a difference in the quantitative strength of TCR signalling. Our previous studies have revealed that upregulation of costimulatory molecules that can directly activate NK cells is orchestrated by reciprocal interactions between APC and iNKT cells^{8b}. Therefore, even the small but significant increase in NK cell transactivation observed with α GC pulsed DCs after treatment with CQ suggested that the quality of immune response initiated by the Th2-biasing glycolipid was altered to more closely resemble the response to a Th1-biasing glycolipid.

It is interesting to note that activation of iNKT cells is observed in infection models with several pathogenic bacterial species, including species of *Salmonella*, *Mycobacteria*, *Legionella* and *Chlamydia*. Most or all of these are intracellular bacteria that are known to block phagolysosomal fusion and reduce endosomal acidification²⁸. Activation of iNKT cells has been demonstrated in response to infections with several of these bacteria, even though the identification of a direct iNKT activating agonist produced by them has been elusive²⁹. Until recently, α -linked sphingolipids were thought to be unique to bacterial and plant species, but recent studies have shown that these glycolipids are indeed synthesized at low levels by mammalian cells and can be stimulatory for iNKT cells³⁰. Furthermore, infection of dendritic cells with mycobacteria results in the down regulation of fatty acid amide hydrolase (FAAH), an enzyme involved in the catabolism of sphingolipids that is active under low pH conditions³¹. These observations suggest that the ability to enhance stable loading of endogenous glycolipids in the endosomal compartment may occur during infections with organisms

that neutralize endosomal pH, leading to enhanced lipid raft localization of stimulatory CD1d/glycolipid complexes. In this model, activation of NKT cells during infection may not depend on the presentation of bacterial glycolipid antigens, but could instead result from presentation of endogenous glycolipids in response to loss of endosomal acidification. This hypothesis can also accommodate the common immune evasion strategies used by several viruses, such as herpes simplex and human immunodeficiency viruses, that interfere with CD1d recycling pathways³². Experimental modifications that disrupt the endosomal localization and recycling of CD1d, such as the deletion of its cytoplasmic tail endosomal targeting motif³³, would also be predicted to exclude CD1d/ α GC Th2 complexes from lipid raft microdomains, although our studies have not yet assessed this experimentally. Such a mechanism for generating or evading enhanced bystander cell activation could play an important role in recruiting and activating other immune cells during intracellular pathogen infection.

In conclusion, we have provided further evidence that low endosomal pH serves as a quality control checkpoint in the CD1d-dependent glycolipid antigen presentation pathway. In addition to confirming that neutralization of endosomal pH increases the intracellular association of Th2-biasing glycolipids to CD1d, we also show for the first time to our knowledge that this also enhances their presentation by lipid raft localized CD1d molecules. These findings provide further support for a model that explains the mechanism by which relatively subtle variations in glycolipid structure can lead to substantially different functional outcomes following iNKT cell activation.

Materials and Methods

Cell lines, antibodies and cytokines

The JAWS II cell line (derived from C57BL/6.p53^{-/-} mice) was obtained from the American Type Culture Collection (ATCC), and was cultured in alpha-MEM medium supplemented with 20% fetal calf serum (Atlanta Biologicals), 10 mM HEPES, 50 μ M β -mercaptoethanol, 50 μ g/ml gentamicin, 100 ng/ml GM-CSF (PeproTech), 0.1 mM of nonessential amino acids (NEAA) and essential amino acids (EAA) (complete α MEM). The mouse hybridoma line producing monoclonal antibody 2.4G2 specific for CD16/32 was also obtained from ATCC, and was cultured in RPMI-1640 medium supplemented with 10% FCS, 10 mM HEPES and 50 μ M β -mercaptoethanol. All media and additives were from Gibco-BRL unless otherwise indicated. The mCD1d/ α GalCer complex-specific mAb L363 was produced in our laboratory (Yu et al., 2007). Ammonium chloride (A9434) and chloroquine diphosphate (C 6628) were purchased from Sigma.

Preparation and solubilization of glycolipids

Synthesis and analysis of the analogues of α GC used in this study have been described previously^{8b, 11a, 34}. For *in vitro* assays, glycolipid stock solutions were prepared at 500 μ M in DMSO (Sigma). Immediately before use, these stocks were heated to 70°C, sonicated for 5 minutes and then diluted to 500 nM in pre-warmed (37°C) culture medium (complete α MEM). This stock was further diluted with complete α MEM culture media immediately before adding to cell cultures to give a final glycolipid concentration of 200 nM and a final concentration of 0.04% DMSO.

Estimation of CD1d/ α GC complexes

For all screening experiments, JAWS II cells were seeded at a density of 2.5×10^5 cells per well in 100 μ l culture media in flat bottom 96 well plates. Glycolipids were added to a final concentration of 200 nM. As an inert vehicle control, the cells were cultured in medium containing only 0.02% DMSO. After 16 hours of culture, the cells were detached using a cell scraper and washed 3 times with PBS containing 1% BSA and 0.1% sodium azide (wash buffer). After the final wash, the cells were resuspended in 50 μ l of FACS buffer (wash buffer with 1 μ g/ml of 2.4G2 mAb, which was added to block Fc receptors expressed on the surface of JAWS II cells). After 10 minutes of incubation at room temperature, 50 μ l of staining solution containing 5 μ g/ml of Alexa Fluor 647 conjugated mAb L363 in FACS buffer was added to the cells. After a further 10 minutes of incubation, cells were washed thrice with wash buffer and then resuspended in 300 μ l wash buffer. The cells were analyzed for L363 staining using an LSR II flow cytometer with FACS Diva software (BD Biosciences).

Estimation of lipid raft localization of CD1d/ α GC complexes

For estimation of lipid raft residency, the FACS analysis was performed in a kinetic mode. At the start of data acquisition, the initial fluorescence level was recorded for approximately 10 seconds. Triton X-100 was then added to a final concentration of 0.06% followed by brief (~1 sec) vortexing to mix the sample. Data collection was then resumed and fluorescence intensities were monitored for another 30 seconds. Data were collected as Flow Cytometry Standard (FCS) files and analyzed using FlowJo software vx10.07 (Treestar, Ashland, OR). MFI values at time 0 (prior to addition of Tx-

100) were normalized to 100 and the relative decrease in MFI values for different agonists after addition of Tx-100 were compared.

Isolation of splenic dendritic cells

Splenic dendritic cells (DCs) were isolated using the Miltenyi CD11c-positive magnetic beads (130-152-001). The frequency of these cells in naïve murine spleen is very low, so we used a model based on the expansion of these cells in mice implanted with Flt-3 expressing melanoma cells as described previously^{8b, 35}.

Adoptive transfer of glycolipid loaded dendritic cells

Female C57BL/6 mice, 6-8 weeks of age, were injected intravenously with purified CD11c+ dendritic cells pulsed with vehicle or α GalCer analogues in the presence or absence of CQ. For this, purified splenic DC were cultured in complete medium with 200 nM of the indicated glycolipids for 1 hour, followed by incubation with 20 μ M CQ for four hours in the groups as indicated. After extensive washing, 1×10^6 cells/mouse were injected into naïve mice. Animals were bled at 2 hours and 12 hours post DC transfer for measurement of serum IL-4 and IFN γ by ELISA, and then were sacrificed sixteen hours post DC transfer for analysis of activation of NK and NKT cells.

Measurement of NK cell transactivation

Single cell suspensions of splenocytes were generated as described previously. In brief, the tissue samples were cut in approximately 10 mm² pieces with a scalpel. The tissues pieces were digested with Liberase plus DNase, and passed through a 70

micron filter. For intracellular staining, the cells were first stained with antibodies to cell surface markers, washed three times with FACS buffer and stained with live/dead viability dye diluted in PBS, for 15 minutes at 20° C. Excess dye was quenched by adding complete RPMI media containing 10% FCS. After extensive washing, the cells were fixed with 2% paraformaldehyde. Cells were then permeabilized with buffer containing 1% BSA, 0.1% sodium azide, 0.05% glycine and 0.05% Triton X-100 (Buffer P). Cells were blocked with 10% rat serum in buffer P, followed by staining for intracellular IFN γ . After washing, samples were analyzed using an LSR II flow cytometer (BD Biosciences) and Flowjo software vx10.07 (Treestar, Ashland, OR).

Statistical analysis. One way ANOVA with Dunnet's correction was used for comparing groups of three or more when there was only one independent variable. Two way ANOVA with Holm-Sidak correction was used to analyze the effect of CQ or NH $_4$ Cl treatment on the loading and localization of CD1d/ α GC complexes as two independent factors. Statistical tests were performed using Prism 6 software (GraphPad).

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Figure legends

Figure 1: Effect of inhibitors of endosomal acidification on CD1d expression.

JAWS II cells were incubated with either ammonium chloride (NH₄Cl, 10 mM) for one hour, chloroquine diphosphate (CQ, 20 μM) for 4 hours or with medium only, following which cells were stained with anti-CD1d antibody (1B1) conjugated to FITC or a similarly labeled isotype control antibody. Cells were then analyzed by flow cytometry. Histograms showing the fluorescence intensities corresponding to CD1d expression are shown in grey, with various treatments of the cells indicated on the right. The white histogram at the bottom is the background fluorescence profile of JAWS II cells stained with the isotype control antibody. (b) The bar graphs show the median fluorescent intensity as mean ± SD for triplicate samples. The data were analyzed by one-way ANOVA using Graphpad Prism (NS, not significant, $P > 0.05$).

Figure 2: Chemical structures of the glycolipids used in this study.

All of these αGC analogues have been previously characterized as iNKT cell activators (see text for references).

Figure 3: Effect of neutralization of endosomal pH on CD1d loading with αGC

agonists. (a) JAWS II cells were incubated with 200 nM of various αGC agonists for 16 hours. After washing to remove unbound glycolipid, the cells were further incubated with CQ or NH₄Cl as in Figure 2, and stained with monoclonal antibody L363 specific for

CD1d/ α GC analogue complexes. The cells were then analyzed by flow cytometry.

Fluorescence histograms show the effect of different treatments (as labeled on the right hand side) on L363 staining levels. The bottom histogram shows the background staining with L363 (Bkgrd) of JAWS II cells cultured without α GC or either of the inhibitors of endosomal acidification. (b) Median fluorescent intensities of L363 staining for CD1d/ α GC complexes observed with individual glycolipid agonists with or without treatment with CQ or NH_4Cl . The black bars correspond to the standard culture conditions without inhibitors of endosomal acidification, while white and grey bars correspond to cells treated with CQ and NH_4Cl respectively. Data is mean \pm SD for triplicate samples and was analyzed by two way ANOVA with the glycolipid agonists and endosomal acidification inhibitors as independent variables. Effect of CQ and NH_4Cl treatment are highly significant for all the glycolipids tested (** $P < 0.01$ and **** $P < 0.001$).

Figure 4: Effect of ammonium chloride on the lipid raft localization of CD1d and CD1d/ α GC complexes. JAWS II cells were incubated with 200 nM of various α GC agonists for 16 hours, and then harvested and washed to remove unbound glycolipid. The cells were then incubated at 37°C for 1 hour in culture media with or without 10 mM NH_4Cl , and then stained with fluorescent L363 antibody. Analysis of L363 binding and its detergent sensitivity was carried out by flow cytometry using the kinetic mode for data acquisition. (a) Plots show the initial level of fluorescence at time 0 (mean fluorescence intensity (MFI) normalized to 100%), and the change in fluorescence relative to the starting level (expressed as percent of initial MFI) over time following

addition of detergent (0.06% Triton X-100, addition indicated by the break in the X-axis). Solid symbols represent cells cultured in medium with 10 mM NH₄Cl, and open symbols are medium without NH₄Cl. Symbols are mean values from three replicates, and error bars are 1 SD. (b) Bar graph showing the residual detergent resistant L363 binding (percent of starting MFI) at 20 seconds after addition of detergent for cells incubated with (filled bars) or without (open bars) 10 mM NH₄Cl. Data shown are means of 3 replicate values and error bars show 1 SD. **** $P < 0.001$ (two way ANOVA).

Figure 5: Effect of endosomal Alkanization on NK transactivation. Splenic DCs were pulsed with medium containing inert vehicle (Veh) or with 200 nM of α -C-GC C26:0, α GC C20:2 or α GC C20:2 plus 20 μ M chloroquine for four hours. These were then injected intravenously into mice, and the frequency of splenic NK1.1 positive cells producing IFN γ was analyzed by intracellular staining using flow cytometry. (a) Representative plots show the gating strategy used to identify NK and NKT cells. Viable cells (R1) were gated to exclude B220^{Pos} cells, and the B220^{Neg} cells (R2) were gated for NK1.1^{Pos} events (R3). These were further gated for TCR β , and gated for NK1.1 TCR β double positive as NKT cells (R4). The TCR β ^{Neg} (R5) events were analyzed for expression of NKp46 and IFN γ . (b) Scatter plots show mean \pm SD for frequencies of IFN γ positive cells for the various cell populations in groups of 5 mice (NS, not significant, ** $P < 0.01$, one way ANOVA).

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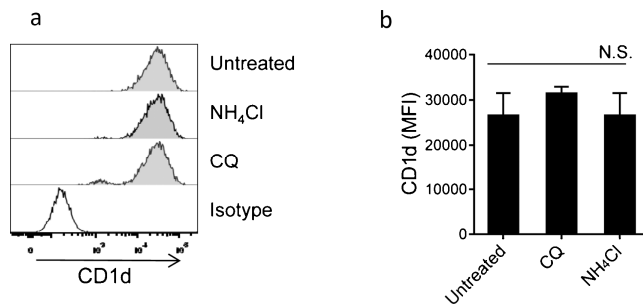


Fig. 1

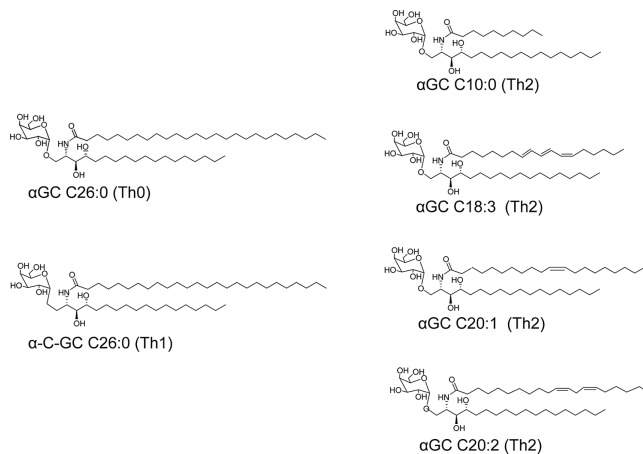


Fig. 2

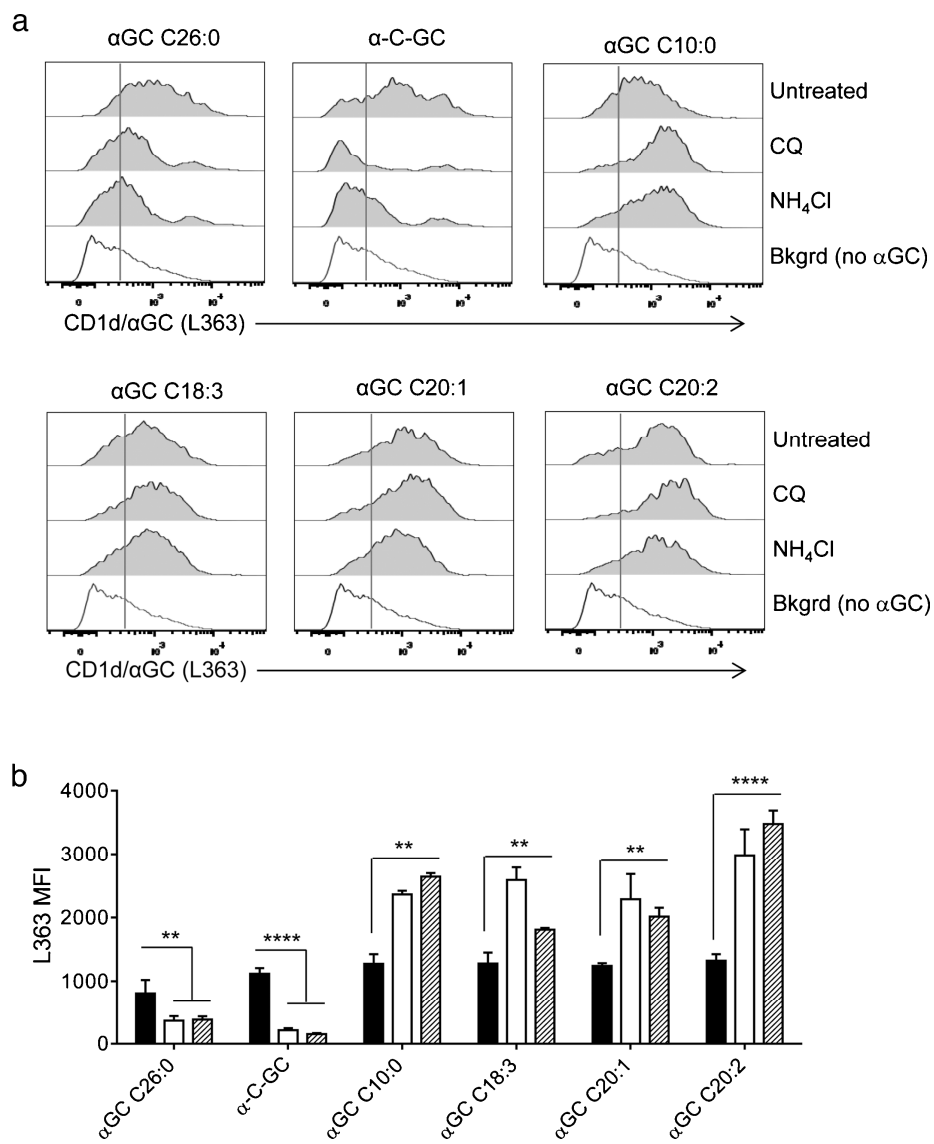


Fig. 3

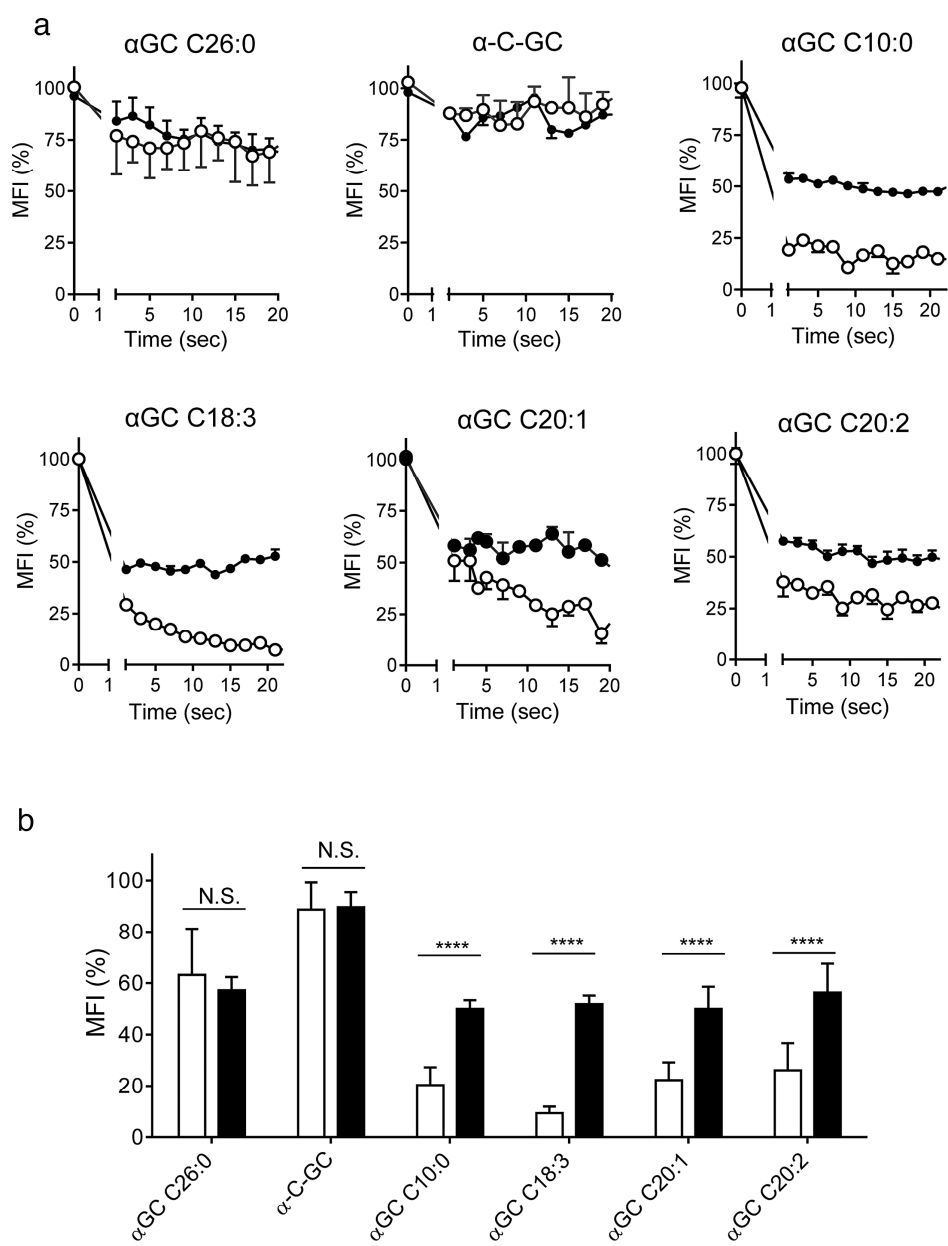


Fig. 4

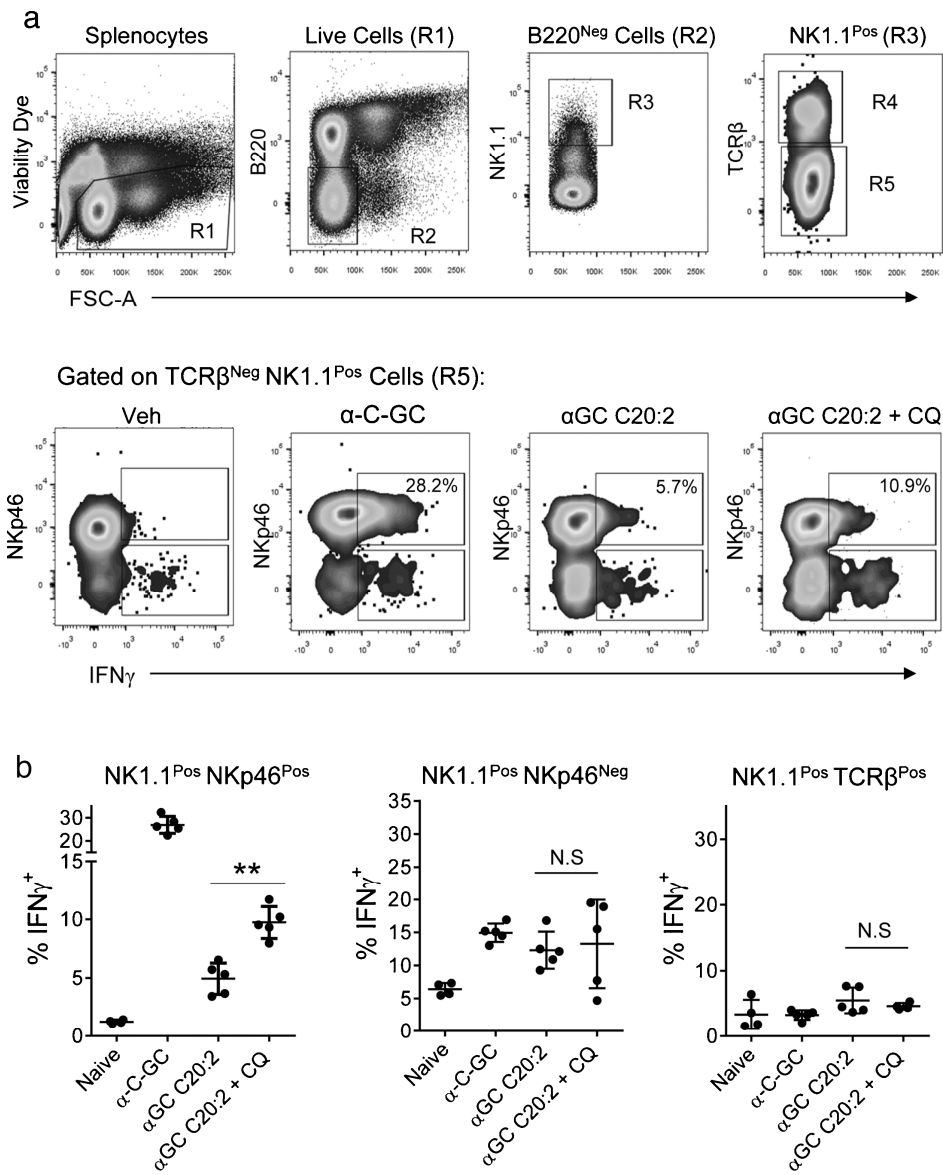


Fig. 5