Published in final edited form as:

Immunobiology. 2015 January ; 220(1): 32–41. doi:10.1016/j.imbio.2014.09.008.

All-*trans*-retinoic acid and CD38 ligation differentially regulate CD1d expression and α -galactosylceramide-induced immune responses

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Abstract

The MHC class-I like molecule CD1d presents glycolipid antigens and thereby activates invariant natural killer-T (NKT) cells. However, little is understood regarding the regulation of its expression. All-*trans*-retinoic acid (RA) and CD38, which is itself a target of RA, both independently regulate the differentiation of antigen presenting cells. In the current study, we treated human THP-1 cells and murine splenic cells with RA, with and without antibody-mediated ligation of cell-surface CD38. Whereas a physiological concentration (20 nM) of RA alone rapidly and markedly increased CD1d protein in THP-1 cells, there was a marked synergy between RA and ligation of CD38 with antibody to CD38. Moreover, RA and CD38 ligation differentially regulated CD1d protein distribution between the cell surface and intracellular compartments, as, whereas RA mainly increased intracellular CD1d protein, ligation of CD38 increased CD1d protein both at the cell surface and intracellularly. By confocal microscopy, CD1d was located close to the plasma membrane but only partially overlapped with LAMP1, a late endosomes/lysosomal marker. Furthermore, RA and/or CD38 ligation increased splenocyte proliferation and differentiation after treatment with the CD1 ligand α -galactosylceramide (α GalCer), evidenced by an increase in the number of splenic dendritic cells, NKT cells, and germinal center plasmacytes. RA also differentially regulated α GalCer-induced cytokine expression, increasing IL-4 and decreasing IFN γ production by total spleen cells and the NKT cell population. Our results indicate a previously unknown mechanism in which RA and CD38 differentially yet cooperatively regulate CD1d expression and antigen-presenting function, which could be important for the enhancement of immunity.

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Disclosure

The authors declare that they have no conflict of interest.

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Keywords

α -galactosylceramide; CD1d; CD38; monocyte; retinoic acid; NKT cell; IL-4 production; IFN γ production

Introduction

CD1d is a MHC-class I like molecule expressed on various antigen presenting cells (APC), including dendritic cells, macrophages, and splenic B cells (Brigl and Brenner, 2004). CD1d processes and presents glycolipid antigens that in turn activate invariant NKT cells, which themselves are regulators of both innate and adaptive immune responses (Brennan et al., 2013; Rossjohn et al., 2012). The prototypic CD1d ligand, α -galactosylceramide (α GalCer), isolated from a marine sponge (Morita et al., 1995) as well as other glycolipids isolated from bacteria and mammalian cell components are recognized by CD1d and thereby elicit anti-bacterial, autoimmune, and anti-tumor responses (Chen and Ross, 2012; Mattner et al., 2005; Sriram et al., 2005; Terabe and Berzofsky, 2014; Zhou et al., 2004). As is also the case for other antigen-presenting molecules, such as MHC class I and II, CD1d expression is subject to regulation by cytokines and antigenic stimulation, and is regulated epigenetically through histone modification (Skold et al., 2005; Yang et al., 2012). The level of CD1d on APC is likely to affect their capacity for antigen presentation and therefore may determine the efficacy of NKT or T cell-mediated immune responses.

Retinoic acid is a quantitatively minor but very active metabolite of vitamin A. The requirement for RA as a regulator of innate and acquired immunity has been demonstrated in a variety of animal experiments and clinical settings (Cui et al., 2000; Glasziou and Mackerras, 1993; Ross et al., 2011). T cell, NK cell, and B cell functions all require RA (Chen and Ross, 2005; Seguin-Devaux et al., 2005; Zhao and Ross, 1995). RA potentiates T cell-dependent antibody production (DeCicco et al., 2001; Ma et al., 2005), regulates NKT cell activity and cytokine production (Lee et al., 2012), and promotes the homing of T cells and B cells, which is necessary for immune protection in the gut (Hammerschmidt et al., 2011; Iwata et al., 2004). RA and other retinoids also induce differentiation and maturation of monocytic cells, implicating RA in the regulation of APC functions (Chen and Ross, 2004; Estey et al., 1999; Gaboli et al., 1998). Moreover, RA is a potent direct inducer of CD1d gene expression in monocytic cells, as we have shown previously (Chen and Ross, 2007), which could explain one way in which RA contributes to promoting the immune response.

CD38 is a type II membrane-associated glycoprotein that is expressed in almost all cells of the immune system. CD38 is most often used as a marker to define the developmental stage and differentiation status of cells (Donis-Hernandez et al., 2001; Reinis et al., 1997; Ridderstad and Tarlinton, 1998; Vences-Catalan and Santos-Argumedo, 2011). However, decades of research suggest that CD38 is not only a marker but also a dual-functional cell surface receptor and enzyme involved in cell signaling and intracellular calcium trafficking. CD38 has been shown to interact with the BCR, TCR/CD3 complex, CD16, and MHC class II molecules on various lymphoid and myeloid cells respectively, to facilitate the activation

process as appropriate to these different types of cells (Deaglio et al., 2002; Lund et al., 1996; Morra et al., 1998; Zilber et al., 2005). Moreover, the intrinsic enzymatic activity of the CD38 extracellular domain functions in the metabolism of NAD⁺, which is associated with calcium mobilization from intracellular storage and calcium-dependent intracellular signaling (Flora et al., 2004; Franco et al., 1998; Shubinsky and Schlesinger, 1997). RA has been shown to be a potent inducer of CD38 expression (Kishimoto et al., 1998; Mehta and Cheema, 1999), which occurs concomitantly with the cell differentiation process (Chen and Ross, 2004).

Our current studies began with knowledge that RA is a regulator of both CD1d gene expression (Chen and Ross, 2007), and of CD38 expression and monocytic cell differentiation (Chen and Ross, 2004; Chen et al., 2002; Fedele et al., 2004). We hypothesized that RA together with stimulation of CD38 might drive CD1d expression and function to a higher level than by either stimulus alone. We first observed that although RA alone was a potent regulator of CD1d protein expression in THP-1 cells, the combination of RA and CD38 ligation acted synergistically to up-regulate CD1d protein accumulation. We therefore conducted further experiments to test whether RA together with engagement of CD38 enhanced α GalCer-stimulated splenocyte activation. Together, these results provide evidence for a new retinoid-CD38-inducible pathway of CD1d/ α GalCer-mediated antigen presentation, which may have important implications for the immune response of NKT and B cells to lipid antigens.

Materials and Methods

Cell culture and treatments

THP-1 monocytic cells were propagated in RPMI-1640 medium supplemented with 10% FBS and 5×10^{-5} M β -mercaptoethanol (Invitrogen, Grand Island, NY), as described in (Chen and Ross, 2004). During each experiment, THP-1 cells were cultured in medium with 3% FBS to reduce exposure to RA and growth factors which may be present in serum while providing sufficient serum for cell maintenance (Chen and Ross, 2004).

Spleen cells were prepared from 8 week-old female Balb/c or C57BL/6 mice (Charles River, Wilmington, MA) that were fed a standard diet and maintained in plastic cages in temperature and humidity controlled rooms with 12-h light/dark housing. Spleens were dissociated on a wire mesh and mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma, St. Louis, MO). All-*trans*-RA (Sigma) was dissolved as a stock in absolute ethanol and diluted prior to each experiment so that the final concentration in the cell culture medium was 20 nM, a physiological concentration similar to that in plasma (Eckhoff and Nau, 1990). α GalCer (Alexis Biochemicals, San Diego, CA) and β GalCer (Sigma) as a negative control (Morshed et al., 2009) were prepared in PBS with 0.5% Tween 20 and used at a final concentration of 100 ng/ml in cell cultures. THP-1 cells were treated with 1 μ g/ml of anti-human CD38 antibody (AT1, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse cells with 1 μ g/ml of anti-mouse CD38 antibody (NIMR-5, Southern Biotech, Birmingham, AL).

Western blot analysis

THP1 cells were cultured in 6-well plates (2×10^6 cells/2 ml per well) with RA (20 nM) and/or α -CD38 (1 μ g/ml) for various times. At the time of harvesting, cells were washed with PBS and the cell pellets were lysed in RIPA buffer (follow the instruction from Santa Cruz) with the presence of 0.1 mM Sodium orthovanadate and complete proteinase inhibitor cocktail (Roche laboratory). Fifty μ g of cellular protein was applied to SDS-PAGE and subjected to western blot analysis as described previously (Chen et al., 2002). An anti-CD1d monoclonal antibody (C3D5, Santa Cruz Biotechnology) was used to detect the CD1d protein, followed by incubation with an anti-mouse antibody conjugated with horseradish peroxidase (Sigma) and chemiluminescence detection (Pierce Biotechnology, Inc., Rockford, IL).

Flow cytometry and confocal microscopic analysis

To determine the relative amount of cell surface CD1d protein, THP-1 cells after culture were incubated with anti-CD1d antibody for 30 min at 4° C, then incubated with Alexa 488-conjugated secondary antibody (Invitrogen Corp., Carlsbad, CA) for another 30 min. The fluorescence output was analyzed by flow cytometry using isotype-matched control antibody for background subtraction. Intracellular CD1d staining was performed by first fixing and permeabilizing cells according to the manufacturer's instructions (BD Biosciences, San Jose, CA), and then the primary antibody was incubated with cells overnight at 4° C, followed by addition of fluorochrome-conjugated secondary antibody for an additional 30 min at 4° C. Lysosomal-associated membrane protein 1 (LAMP1), a marker of the late endosome/lysosome compartment, was detected using a rabbit anti-LAMP1 antibody (H-228, Santa Cruz) plus Alexa 568-conjugated secondary anti-rabbit antibody. The cells were then washed and subjected to analysis by flow cytometry (BD Accuri C6 flow cytometer) and confocal microscopy (Olympus Fluoview 1000). Flow cytometric and confocal microscopy data were analyzed by FlowJo (Tree Star, Ashland, OR) and Flowview software (Olympus optical company, Olympus America, Melville, NY), respectively.

To determine the cell cycle activity, THP-1 cells cultured for 24 h were subjected to propidium iodide staining (Chen and Ross, 2004), and analyzed by flow cytometer to determine the cell cycle distribution of the cells.

To trace cell proliferation, splenocytes were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 10 nM, Life technologies, Grand Island, NY) for 30 min and followed by 4 days of culture, following protocols described in (Chen and Ross, 2005). Cells were then stained with fluorescent antibodies, including CD11c, CD3, CD49b, CD19, CD23 and CD138, all from BD Biosciences. Details regarding the combination of fluorescent-labeled antibodies for each experiment are described in the figure legends.

For NKT cell cytokine staining, spleen cells were cultured in the absence of Golgi Plug (Brefeldin A, BD Biosciences, San Diego, CA) for 4 days. Cells were first stained with TCR V β -FITC antibody and murine α GalCer-loaded CD1d tetramer stained with Alexa 647 (NIH Tetramer Facility, Emory University), along with an unloaded stained tetramer control, and cells were then fixed, followed by intracellular cytokine staining as described above. IL-4

and IFN γ were detected by anti-IL-4-PE or anti-IFN γ -PE antibody (BD Biosciences), respectively.

Cell proliferation assay

THP-1 cells or splenocytes were cultured in 96-well plates in the presence and absence of treatment for times as indicated in the figure legend. [3 H]-Thymidine was added to cells (0.2 μ Ci/well) for the last 6 h of culture. Cells were then harvested and incorporated radioactivity was measured using a liquid scintillation counter.

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR)

Total cellular RNA was isolated from cells at the time of harvesting (RNeasy mini kit, Qiagen, Valencia, CA). One μ g of RNA was subjected to reverse transcription (Promega, Madison, WI) and qPCR analysis (Biorad, Hercules, CA). Primers for IL-4 and IFN- γ mRNAs were as described previously (Ma et al., 2005). Both glyceraldehyde-3-phosphate dehydrogenase, GAPDH (Chen et al., 2002) and HPRT (forward: 5-AAGGACCTCTCGAAGTGTGGATA, reverse: 5-CATTTAAAAGGAAGTGTGACA ACG) served as internal controls for RNA integrity and qPCR amplification. PCR conditions were optimized as denaturing at 94°C, 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min for 35 cycles. The PCR product was also subjected to agarose gel electrophoresis to confirm the specificity of the amplification product.

ELISA to measure cytokine production

Supernatants from splenocytes cultured in the absence of Golgi Plug for 4 days were collected and tested for the IL-4 and IFN γ production using ELISA kits in accordance with the manufacturer's instructions (BD Biosciences).

Statistics

Results are presented as mean $\pm$ SEM. A two-tailed Student's *t*-test, one-way or two-way ANOVA was performed, and *P* < 0.05 was considered significant.

Results

RA and CD38 increase CD1d protein expression in THP-1 cells

To determine if RA and CD38-crosslinking regulate CD1d protein expression, western blot analysis was performed on THP-1 cells that had been treated with RA (20 nM) and α -CD38 (1 μ g/ml), an agonistic antibody known to engage cell surface CD38 and activate signaling pathways (Munoz et al., 2008; Munoz et al., 2003). CD1d is known as a highly modified membrane protein after its original synthesis in the ER, with molecular weights in the range of 38 kDa to 50 kDa after glycosylation (Kim et al., 1999). As shown in Fig. 1A, CD1d protein increased steadily over time in the presence of RA. The protein was first detectable at 6 h as a band of approximately 38 kDa, which became more obvious after 48 h of incubation, consistent with the results from transcriptional regulation at the mRNA level shown previously (Chen and Ross, 2007). At the later times (24 and 48 h), several protein bands were evident with a dominant band at about 50 kDa, suggesting the existence of

posttranslational protein modification such as glycosylation, as has been described for CD1d in other cells (Kim et al., 1999).

We also tested the effect of CD38 engagement on CD1d protein expression. It was surprising to observe that even though CD38 ligation did not alter the levels of either CD1d mRNA expression or promoter activity (data not shown), it significantly increased the level of CD1d protein, with a predominant band at 50 kDa (Fig. 1B). This suggests that both the amount of CD1d protein amount and its processing were augmented by CD38 signaling. Moreover, treatment with RA synergized with CD38 ligation to greatly increase the level of CD1d protein, observed for both the 38 kDa and 50 kDa bands at both 24 and 48 h.

RA and CD38 differentially regulate CD1d protein localization in THP-1 cells

Since CD1d is an antigen-presenting molecule, we further analyzed its expression and cellular distribution by flow cytometry and confocal microscopy. Fig. 2A and B illustrate representative histograms of the expression of CD1d protein on the cell surface and in intracellular compartments in THP-1 cells that were treated with RA and/or α -CD38 for 48 h. Both RA and CD38 engagement were factors for the intensity of CD1d protein, consistent with immunoblot analysis of total CD1d protein; however, the amount and distribution of CD1d protein were different for each treatment. As summarized in Fig. 2C–F, RA did not increase the cell surface CD1d protein level, but in contrast it significantly reduced cell surface CD1d after 48 h of culture, as illustrated by the percentage of positive cells and mean fluorescent intensity (MFI) (Fig. 2C). However, RA markedly increased intracellular CD1d (Fig. 2D), which was already evident by 6 h after addition of RA and remained high after 48 h. Moreover, ligation of CD38 dramatically increased CD1d protein on both the cell surface and intracellularly (Fig. 2E and F). Whereas the addition of RA did not further increase the percentage of CD1d-positive CD38-stimulated cells, which was already close to 100%, it significantly increased the MFI of CD1d staining induced by CD38 ligation on both the surface and intracellular locations.

Confocal microscopy was also used to detect CD1d expression and localization in THP-1 cells. Consistent with the flow cytometry analysis, the intensity of intracellular CD1d staining was increased by RA and/or engagement of CD38, compared with control cells, and the combination of RA and α -CD38 resulted in much brighter signals than by either stimulus alone (Fig. 3A). Co-staining for LAMP1, a marker of the late endosome/lysosome compartment, was performed to identify the location of CD1d molecules. As shown in Fig. 3B, while a partial colocalization of these two proteins was observed, more of the CD1d protein was distributed close to the surface of RA-treated and α -CD38-stimulated cells. These results suggest that the expression and cellular location of CD1d protein were both altered by RA and α -CD38. CD1d was mostly located on or near the plasma membrane and within the late endosome/lysosome compartments, also located close to the cell surface.

RA and CD38 ligation differentially regulated THP-1 cell proliferation

To examine whether RA and CD38 ligation would affect the THP-1 cell proliferation, which is a process associated with THP-1 cell activation and differentiation, THP-1 cells were treated with or without RA/ α -CD38 for 24 h, DNA synthesis was measured as well as cell

cycle progression. Whereas RA decreased the THP-1 proliferation (Fig. 4A), CD38 ligation increased cell proliferation. This was consistent with the results of cell cycle analysis (Fig. 4B), where CD38 ligation increased the percent of cells in the G₂/M phase, and RA tended to decrease these cells. The lesser reduction of cells in the G₂/M phase by RA compared to the greater effect on DNA synthesis might be due to asynchrony in the THP-1 cell culture. There was no difference of CD1d expression between G₁ and G₂/M cells, and the CD1d ligand α GalCer also could not affect the THP-1 cell proliferation (data not shown). Since RA and CD38 ligation differentially regulated THP-1 cell proliferation, the mechanisms underlying the cell proliferation may be different to that of the CD1d expression, as the later showed a strong synergy between these two treatments.

RA and CD38 ligation increase α GalCer-mediated spleen cell proliferation and differentiation

It is well-documented that α GalCer, a widely used glycosylceramide ligand of CD1d, is a potent and specific activator for both human and murine NKT cells (Morita et al., 1995), and, moreover, that NKT cell activation results in cell proliferation and the prompt expression and release of cytokines, including IL-4 and IFN γ by these cells (Skold and Behar, 2003). Having observed that RA and ligation of CD38 act synergistically in monocytic cells, we next explored their regulatory effects on primary splenic lymphocytes. To determine if RA or CD38 ligation can regulate CD1d/ α GalCer-mediated NKT activity, mouse spleen cells were treated with α GalCer, or with the inactive stereoisomer β GalCer, used as negative control (Morshed et al., 2009), concomitantly with RA and/or α -CD38. Whereas α GalCer, as expected, substantially increased spleen cell proliferation, in a time dependent manner, treatment with RA alone did not increase the rate of cell proliferation. However, RA significantly augmented cell proliferation induced by α GalCer (Fig. 5A and B). Furthermore, whereas CD38-crosslinking by itself rapidly increased spleen cell proliferation, which peaked at 48 h, it further increased α GalCer-induced cell proliferation, which was evident at 24 h (Fig. 5B).

Spleen cells were then analyzed by flow cytometry to determine the CD1d expression level on splenic dendritic cells (DC, marked by CD11b⁺CD11c⁺). As shown in Fig. 5C, while RA or α -CD38 alone showed the trend to increase the CD1d expression, combination of both significantly increased the CD1d expression on splenic DC. It is worthy of note that the CD38 expression level showed the similar pattern in terms of the regulation (Fig. 5C). We then examined the proliferation of dendritic cells and NKT cells (analyzed as CD3^{dim}, CD49b⁺ cells) after treatments, where splenocytes were first labeled with CFSE and then subjected to treatment for 4 days. Whereas we observed that RA and α -CD38 antibody by themselves did not affect the cell population (data not shown), however, in the presence of α GalCer, RA or CD38 ligation increased DC proliferation independently, while the combination of RA with α -CD38 further increased the DC population (Fig. 5D). A similar pattern was observed for NKT cells (Fig. 5E), where the greatest percentage of NKT cells was observed after treatment with RA, α -CD38 and α GalCer in combination.

Since B cells are APC and the expression of CD1d is upregulated by RA (Chen et al., 2013), B cell differentiation was also examined after this treatment (Fig. 5F). RA, α -CD38 and

α GalCer all increased the plasmacyte population (CD19⁺CD23⁺CD138⁺ cells), which are characterized as germinal center B cells with antibody secreting potential (Shinall et al., 2000). Moreover, whereas α GalCer alone was a reasonably strong stimulus for this cell population, the addition of RA, α -CD38 and both combined significantly increased the percentage of plasmatic cells (Fig. 5F). These data suggest that RA and CD38 ligation facilitate α GalCer-mediated antigen presentation in both DC and B cells, leading to NKT cell stimulation and B cell differentiation.

RA regulates α GalCer-induced IL-4 and IFN γ production by splenocytes and NKT cells

To determine whether RA and/or CD38 ligation influences the synthesis and secretion of cytokines produced in response to α GalCer, the production of IL-4 and IFN γ , two major NKT cell secreted cytokines, was measured at both the mRNA and protein levels. Whereas the addition of α GalCer markedly increased both IL-4 and IFN γ mRNA levels in spleen cells, the addition of RA differentially regulated the production of these cytokines. RA further increased IL-4 mRNA but it decreased the expression of IFN γ mRNA induced by α GalCer (Fig. 6A). Cytokine protein levels, measured by ELISA on cell culture supernatants, provided results consistent with those for cytokine mRNA levels (Fig. 6B). Under similar conditions, ligation of CD38 did not significantly influence the production of these cytokines (data not shown).

Furthermore, intracellular cytokine staining of NKT cells showed that RA augmented the α GalCer-mediated increase of cellular IL-4 protein expression, and at the same time, reduced α GalCer-mediated expression of IFN γ in NKT cells (Fig. 5C), similar to the results from qPCR and ELISA assays in spleen cells and splenic cell culture supernatants. In contrast, ligation of CD38 did not result in any apparent effect on cytokine production (data not shown). Overall, RA significantly rebalanced the ratio of Th2 to Th1 cytokines expressed and produced by α GalCer-stimulated spleen cells and the NKT cell population. Although CD38 played a role in augmenting the cell proliferation and enrichment of spleen NKT cell population, it did not affect the cytokine expression of these cells.

Discussion

As an antigen-presenting molecule, CD1d is closely related to the activity of NKT cells and B cells (Chen et al., 2013; Rossjohn et al., 2012). Thus, the expression and distribution of CD1d may be an important regulator of the downstream immune responses of these cells, such as cytokine and antibody production. CD1d expression is known to be subject to regulation by cytokines and bacterial components (Skold et al., 2005). Previously, we and other investigators have identified transcriptional regulation of the CD1d gene by the Sp1 and Ets families of general transcription factors (Chen and Ross, 2007; Chen and Jackson, 2004; Geng et al., 2005), as well as an RA-dependent specific pathway mediated by a retinoic acid-response element (RARE) that is located in the promoter region of the CD1d gene (Chen and Ross, 2007; Chen and Jackson, 2004; Geng et al., 2005). As CD38 is also a downstream target gene of RA signaling (Mehta and Cheema, 1999), our current studies have focused on these two factors – all-*trans*-RA and the cell-surface coreceptor molecule

CD38 – as each could play a significant yet different role in the regulation of CD1d expression and function.

As our results have shown, both RA and CD38-crosslinking elicited a steady increase of CD1d protein expression, but the combination of RA and CD38 engagement was synergistic and was much more potent, both in human THP-1 cells and mouse splenic DC. Since CD38 is both an ectoenzyme and a signaling molecule that is closely connected to complex intracellular signaling pathways (Flora et al., 2004; Zilber et al., 2005), and since RA also participates in the cell signaling network besides activating its nuclear receptor pathway (Altucci and Gronemeyer, 2004), RA and CD38 engagement may stimulate complex signal transduction pathways that regulate the CD1d expression and protein modification at different steps. For example, CD1d protein is known to go through extensive post-translational modification, such as glycosylation, to become the functional mature protein (Kim et al., 1999; Kunte et al., 2013). The backbone of CD1d is about 38 kDa but the protein size increases to ~50 kDa after modification (Kim et al., 1999). Our western blot analysis illustrated that in the presence of RA, the first CD1d protein to increase, observed at 6 h, migrated as the immature 38-kDa protein band, while a 50-kDa protein became more dominant later. However, CD38 engagement caused a more rapid and dramatic induction of CD1d protein, mainly as the 50-kDa protein, and the combination of RA and CD38 together synergistically increased CD1d protein, both the major 50-kDa and the minor 38-kDa proteins. These results imply that RA and CD38 ligation exert differential effects on the CD1d protein modification, and suggested this may in turn affect the functionality of the protein, which we explored later. However, the results also suggest there is much to learn about the regulation of CD1d protein in terms of modification and function, and therefore future studies at the protein level will be interesting to elucidate the mechanisms of these changes.

It has also been reported that the mature CD1d proteins, similar to MHC class I/II molecules, undergo intensive internalization and intracellular trafficking (Duan and Srivastava, 2012). The recycling of CD1d from the plasma membrane-endosome and the gradual accumulation of CD1d in the late endosome/lysosome compartment are believed to be crucial for immune surveillance and the presentation of its lipid antigens (Jayawardena-Wolf et al., 2001). It has been reported that during the maturation of DC, CD1d expression is not elevated on the cell surface but instead is distributed in the endosome/lysosome compartment (Brigl and Brenner, 2004). We observed that treatment with RA reduced the accumulation of cell surface CD1d protein, which is in contrast to reports for MHC class I molecules (Vertuani et al., 2003), and at the same time, RA significantly increased the amount of intracellular CD1d protein, suggesting RA promoted the redistribution of this protein. RA also synergized with CD38 ligation to markedly increase the levels of both the intracellular and the surface-associated CD1d proteins, suggesting that RA and CD38 regulate CD1d trafficking quite differently but still in an interacting manner. Overall, the increased levels of intracellular and surface CD1d protein may contribute to the elevated antigen presenting activity of CD1d. Further studies are needed to better define the distribution of CD1d protein regulated by RA and CD38 engagement and its role in APC in the process of antigen presentation.

RA and CD38 both are well known to be regulators of cell differentiation, especially the myeloid lineage including DC (Fedele et al., 2004; James et al., 1999). It has been reported that the cell-type specific expression of CD1d greatly affects the functional development of NKT cells (Forestier et al., 2003; Xu et al., 2003; Zimmer et al., 2006), therefore regulated changes in the CD1d expression on APC may be a mechanism that, in turn, regulates NKT cell activation (Skold et al., 2005). As we have shown here, RA significantly increased α GalCer-mediated splenic DC proliferation and NKT cell expansion (Fig. 5D and E). Moreover, RA differentially regulated the α GalCer-induced IL-4 and IFN γ production, consistent with the effect of RA in increasing Th-2 cytokines, and/or increasing the balance of Th2 relative to Th1 cytokines, as noted in previous studies conducted in isolated cells and in vivo (Iwata et al., 2003; Lee et al., 2012; Ma et al., 2005). Since RA could increase CD1d expression in splenic CD11b⁺ cells, as shown previously (Chen and Ross, 2007), these results combined with our findings in THP-1 cells suggest that an increase in CD1d expression, induced by RA, may be important in explaining the recognized effects of RA on NKT cell activities. Although CD38 engagement stimulated a marked increase of CD1d protein, it did not alter the antigen presentation activity in terms of cytokine production. Thus, while the mechanism is not clear at this time, multiple levels of regulation could contribute to this result, such as protein modification and intracellular recycling of CD1d.

A similar regulation was also observed in another antigen presenting system, the B cells. B cells are well known as professional APC which, although having lower affinity for protein antigen than DC, are much more numerous, differ in their tissue distribution from DC, and thus could be very important as APC for certain antigens. Only recently have B cells been studied as carriers of the CD1d molecule, and these studies have demonstrated a close interaction between NKT cells and B cells during humoral immune responses (Allan et al., 2011; Bai et al., 2013). It is known that both RA and CD38 ligation can induce B cell differentiation (Chen and Ross, 2005), and it is especially interesting that RA also increased B cell CD1d expression (Allan et al., 2011; Chen et al., 2013). The increase in B cells having germinal center B cell characteristics by RA and CD38 ligation, especially in the presence of α GalCer, suggests that RA and CD38 signaling are involved in CD1d/ α GalCer-mediated B cell activation and differentiation.

In conclusion, our study has demonstrated that RA and CD38 are potent and distinctive regulators of CD1d protein expression and α GalCer-mediated immune functions, such as NKT cell activity and B cell differentiation. These results provides new information on the regulation of CD1d expression and activity on which to base further studies to examine in detail the processes of protein modification, trafficking, and functionality of CD1d under the regulation of RA and CD38, and to understand the biological effect of RA and CD38 on α GalCer-mediated regulation of innate and adaptive immune responses.

Acknowledgments

Supported by NIH grant DK-41479. The following tetramers were obtained through the NIH Tetramer Facility: Alexa 647-labeled mouse CD1d α GalCer-loaded and unloaded control tetramers.

Abbreviations

APC	antigen-presenting cell
αGalCer	α -galactosylceramide
DC	dendritic cell
LAMP1	lysosomal-associated membrane protein 1
NKT	natural killer T cell

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Fig. 1.

RA and CD38 synergize to increase CD1d protein in THP-1 cells. A. RA time-dependently increased CD1d protein detected by western blot analysis. THP-1 cells were cultured for the different times as indicated with and without RA (20 nM). Fifty μ g of total cellular protein was analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods. B. RA further augmented α -CD38-induced CD1d protein expression. THP-1 cells were treated with α -CD38 antibody (1 μ g/ml) in the presence and absence of 20 nM RA. Images shown represent three independent experiments performed in duplicate.

Fig. 2.

RA and CD38 ligation differentially regulate the cell surface and intracellular expression of CD1d protein. THP-1 cells were treated with RA (20 nM) and/or α-CD38 antibody (1 μg/ml) for either 6 or 48 h. Cells were then stained sequentially with anti-human CD1d antibody and an Alexa 488-conjugated secondary antibody for either surface or intracellular CD1d protein. The stained cells were subjected to flow cytometric analysis. Representative flow histograms show the surface (A) and intracellular (B) expression of CD1d protein in cells treated with RA, α-CD38 antibody, and both combined, for 48 h. Isotype control antibody was used as negative control to set up the gates. C. RA decreased the accumulation of surface CD1d (sCD1d) protein after 48 h treatment. MFI, mean fluorescence intensity,

indicates the relative intensity of the staining on positive cells. D. RA markedly increased intracellular CD1d (inCD1d) protein. E and F. RA synergized with CD38 ligation to increase both surface and intracellular CD1d protein levels at 48 h. Data represent at least three independent experiments performed in triplicates, * RA is different from Medium by Student *t*-test. Different letters denote differences by two-way ANOVA, $a > b > c > d$, $P < 0.01$.

Fig. 3. RA and CD38 engagement up-regulate intracellular CD1d protein, which partially coincides with the plasma membrane-late endosomal/lysosome marker LAMP-1. THP-1 cells were treated for 48 h with RA and/or α -CD38 and then stained for intracellular CD1d protein and visualized using confocal microscopy. A. Representative intracellular staining of CD1d protein is shown for THP-1 cells treated with or without RA (20 nM) and/or α -CD38 (1 μ g/ml), as indicated in the figure. B. Higher magnification (40x) shows the location of CD1d protein in THP-1 cells treated with RA plus α -CD38 for 48 h, after dual staining with anti-CD1d-Alexa 488 (green) and anti-LAMP1-Alexa 568 (red) antibodies.

Fig. 4.

RA and α -CD38 regulated THP-1 cells proliferation. THP-1 cells were cultured in the presence or absence of RA and/or α -CD38 for 18 h. Cells were either incubated with [3 H]-thymidine for another 6 h for the determination of DNA synthesis, or they were harvested for propidium iodide staining to measure the cell cycle activity using flow cytometry. A and B. RA and α -CD38 opposed each other in regulating THP-1 cell proliferation and cell cycle progression as shown by the percentage of cells in the G₂/M phase of the cell cycle. Treatments marked with different letters differ significantly, $P < 0.05$.

Fig. 5.

RA and CD38 ligation increase CD1d/αGalCer-mediated splenocyte proliferation, expand the DC and NKT cell population, and promote B cell differentiation. A and B. Mouse spleen cells were cultured in the presence and absence of βGalCer, αGalCer, RA and/or α-CD38 for up to three days. Cell proliferation was determined each day by [³H]-thymidine incorporation. A. Cell proliferation was induced by αGalCer (100 ng/ml) and further enhanced by RA (20 nM) at 72 h. B. α-CD38 (1 μg/ml) increased αGalCer induced proliferation approximately additively. C. RA and CD38-ligation increased cell surface CD1d and CD38 expression in splenic DC. Splenocytes were isolated from adult mice and cultured for 4 days with treatments as stated above. Cells were then stained for flow cytometry using combinations of the following antibodies: CD1d-FITC/CD11b-PE/CD11c-

PEcy7, or CD38-FITC/CD11b-PE/CD11c-PEcy7. The percentage of CD1d⁺ or CD38⁺ cells were gated on CD11b and CD11c double positive cells. D. RA and CD38-ligation increased α GalCer-mediated DC proliferation. Spleen cells were labeled with CFSE and cultured for 4 days under the same conditions, and then subjected to staining for flow cytometry. The percent of CD11c⁺ cells were evaluated in CFSE diluted cells (CD11b-PE⁺CD11c-PEcy7⁺ in CFSE diluted cells). E. RA and CD38-ligation increased α GalCer-mediated NKT cell proliferation (CD3^{dim}CD49b⁺ cells within the CFSE diluted cell population). The antibody combination was CFSE, CD3-PE and CD49b-APC. F. RA, α -CD38 and α GalCer increased B cells with a germinal center CD138⁺ phenotype. Splenocytes treated as described in D and E were analyzed for CD19⁺CD23⁺CD138⁺ cells. Cells were gated on CD19-PEcy7 and CD23-PE double-positive cells and then counted for CD138-APC⁺ cells. The data represent three independent experiments each performed in triplicate, which were analyzed using one-way ANOVA. Groups with different letters differ significantly, $a > b > c > d > e$, $P < 0.05$.

Fig. 6.

RA regulates CD1d/αGalCer-mediated IL-4 and IFN γ expression. Mouse splenocytes cultured for 4 days with and without RA (20 nM) and/or αGalCer (100 ng/ml) were harvested for RNA isolation and cell staining, and the cell culture supernatants were collected for ELISA. A and B. RA regulated αGalCer-induced IL-4 and IFN γ mRNA expression by qPCR and protein secretion by ELISA measured on cell culture supernatants. C. RA regulated the αGalCer-induced expression of intracellular cytokine (IL-4 and IFN γ) by NKT cells. Cells from the 4-day culture were analyzed for either IL-4-PE or IFN γ -PE⁺ cells, which were gated on the CD1d tetramer-APC⁺ cell population. Letters denote groups that differed from each other, $P < 0.05$.