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Inhibition of acid ceramidase regulates MHC class II antigen presentation and suppression of autoimmune arthritis

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Abstract

The bioactive sphingolipid ceramide affects immune responses although its effect on antigen (Ag) processing and delivery by HLA class II to CD4⁺ T-cells remains unclear. Therefore, we examined the actions of a novel cell-permeable acid ceramidase (AC) inhibitor [(1R,2R) N myristoylamino-(4'-nitrophenyl)-propanediol-1,3] on antigen presentation and inflammatory cytokine production by Ag-presenting cells (APCs) such as B-cells, macrophages, and dendritic cells. We found that AC inhibition in APCs perturbed Ag-processing and presentation via HLA-DR4 (MHC class II) proteins as measured by coculture assay and T-cell production of IL-2. Mass spectral analyses showed that B13 treatment significantly raised levels of four types of ceramides in human B-cells. B13 treatment did not alter Ag internalization and class II protein expression, but significantly inhibited lysosomal cysteinyl cathepsins (B, S and L) and thiol-reductase (GILT),

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Author Contributions

D.Z. performed most of the work, drew the Figures, and wrote the manuscript. L.M.H. also carried out the experiments and analyzed data. X.L. performed transfection assays, quantified protein expression. Z.M.S., A.B. (Aiping Bai), and A.B. (Alicja Bielawska) performed most of the ceramide experiments, provided with the ceramide analogs, and reviewed the manuscript. S.V.R., J.S.N. and Y.A.H. provided critical reagents and constructs for the biochemical studies, and edited the manuscript. A. Haque conceived and designed the experiments, and edited the manuscript. All authors discussed the methods and the results, and reviewed and approved the final content of the manuscript.

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Conflict of Interests

The authors have declared that no conflict of interests exists.

HLA class II Ag-processing, and generation of functional class II-peptide complexes. *Ex vivo* Ag presentation assays showed that inhibition of AC impaired primary and recall CD4⁺ T-cell responses and cytokine production in response against type II collagen. Further, B13 delayed onset and reduced severity of inflamed joints and cytokine production in the collagen-induced arthritis mouse model in vivo. These findings suggest that inhibition of AC in APCs may dysregulate endolysosomal proteases and HLA class II-associated self-antigen presentation to CD4⁺ T-cells, attenuating inflammatory cytokine production and suppressing host autoimmune responses.

Keywords

Acid ceramidase; cytokines; cathepsins; MHC class II; CD4⁺ T-cells; Arthritis

1. Introduction

Ceramide, a bioactive lipid, is now recognized as an important player in multiple cellular signaling pathways implicated in inflammation, differentiation, proliferation, protein trafficking, apoptosis, infection, and cancer [1–4]. Ceramide is known to be generated by three mechanisms: a *de novo* pathway, the salvage pathway, or the sphingomyelin metabolic pathway whereby sphingomyelinases can hydrolyze sphingomyelin in biological membranes to release ceramide [1–3, 5]. The acid, neutral, and alkaline sphingomyelinases exhibit their optimum activity at acidic, neutral, and alkaline pH values, respectively [5–7]. Cellular ceramides are also regulated by ceramide-synthesizing enzymes and ceramide-metabolizing enzymes like acid ceramidase (AC) [4, 6, 8–10]. Ceramide production is increased by many agents such as cytokines [TNF- α , interferon- γ (IFN- γ), etc.], endotoxins, chemotherapeutic agents, vitamin D, heat, radiation, and ceramidase inhibitor [11–15]. While these agents generate ceramides, many of them also are known to be immune regulatory molecules.

Antigen (Ag) processing and presentation can augment or modulate inflammation, differentiation, proliferation, apoptosis, the immune responses to an infectious agent and cancer by its effect on Ag-presenting cells (APCs), monocytes, and T-cells [16–18]. However, little is known about the effect of AC on endolysosomal proteases, Ag-processing in APCs, and T-cell responses. AC is an important regulatory enzyme involved in ceramide metabolism as well as glucocorticoid production and alteration of intracellular sphingosine (SPH) and SPH-1-phosphate (S1P) [6, 15, 19]. AC is known to be synthesized as a 55 kDa precursor polypeptide and often processed into α - and β -subunits within lysosomes [6, 20, 21]. AC may localize both inside and outside of lysosomes and can influence cell growth, differentiation, cytokine production, proliferation, and apoptosis [20, 22, 23]. Thus, the question arises whether AC activities may impact other cellular processes associated with this subcellular compartment, namely the pathway of HLA class II presentation and T-cell recognition. Studies suggest that synthetic ceramide analogs can stimulate antigen presenting cells to increase the expansion of CD8⁺ T cells via the MHC class I pathway [24]. Lipid antigens can also be presented to NK T cells by CD1 molecules [25]. While sphingolipid species regulate a number of cellular events including membrane fluidity and endo/exocytosis [26, 27], very little is known regarding how ceramide analogs affect uptake

of Ags, their processing in the endocytic compartments and presentation via the MHC class II pathway.

Initiation and amplification of an Ag-specific immune response require uptake and cleavage of the Ag by lysosomal thiol-reductase GILT [28, 29] and cathepsins [30] by Ag-processing. The resulting Ag fragments or peptides are presented by HLA-DR molecules via different steps where invariant chain (Ii) and class II-associated Ii peptide (CLIP) [30], HLA-DM [31], lysosome-associated membrane protein-2 (Lamp-2) [32] molecules participate and help transport of the loaded HLA-DR-peptide complex to the surface of the cells. The HLA-DR-Ag complex on the APC triggers the Ag-specific receptor on T-cells and these activated T-cells produce IL-2 cytokine that further elevates T-cell responses. These processes often are explored using test Ags such as IgG κ chain but also are essential for the development of some autoimmune diseases. Autoimmune diseases result from complex interactions among different immune cell types, including both T- and B-cells and professional Ag-presenting cells, resulting in activation of immune cells and production of cytokines. While inflammatory cytokines play important roles in the pathogenesis of autoimmune diseases, it remains unclear how cytokines interact in a complex network of cellular system to sustain inflammation in autoimmune disease. Herein, we used a ceramide analog [(1R,2R) N-myristoylamino-(4'-nitrophenyl)-propandiol-1,3], also called B13 that competitively inhibits intracellular AC [33], to assess its effect on endolysosomal proteases, Ag-processing reactions within APCs, and their ability to trigger T-cell responses. We report that the AC inhibitor B13, but not its inactive analog LCL-618 (B2, [(1R,2R) N-acetyl-(4'-nitrophenyl)-propandiol-1,3]), disrupted self-Ag processing and activation of T-cells in the context of HLA class II proteins. B13 did not affect cell viability but markedly reduced IL-2 cytokine production from T-cells triggered by HLA class II Ag presentation, including the collagen type II (CII) auto-Ag, which is essential for the induction of collagen-induced arthritis (CIA) in rodents. Inhibition of AC by siRNA technology also impaired HLA class II-mediated presentation of Ags to CD4⁺ T-cells, providing a rationale for exploring the modulation of AC to regulate immune responses in autoimmune diseases. Remarkably, B13 treatment inhibited T-cell responses (both primary and recall), and suppressed inflammatory cytokines and the incidence and severity of the disease in the CIA model.

2. Materials and methods

2.1. Ceramide analogs

Ceramide analogs B13, LCL-618 (the inactive B13 analog) and fluorescent NBD-B13 were synthesized at the Medical University of South Carolina (MUSC) Lipidomics Core [34]. AC inhibitor B13 and the control ceramide analog LCL-618 (purity >95%) were first dissolved in ethanol and diluted in Phosphate buffered saline (PBS) so the final ethanol concentration was <0.1% (vol/vol); an analogous ethanol dilution was used as the vehicle control.

2.2. Cell lines

Human B-cell lines which express HLA-DR4 (Frev, Priess.DR4), the human monocytic macrophage cell line (THP-1.DR4), and the murine dendritic cell line (FSDC.DR4) were used as Ag-presenting cells. The IgG κ peptide $\kappa_{188-203}$ -specific T-cell hybridoma 2.18a and

the IgG κ peptide $\kappa_{145-159}$ -specific T-cell hybridoma 1.21 were used as T-cells because of antigenic peptides recognition in the context of HLA-DR4 molecules. [35–37]. These cell lines were obtained from Dr. J.S. Blum (Indiana Univ., Indianapolis, IN). The collagen immunodominant epitope (CII_{261–273})-specific T-cell hybridoma line, 4027/99, was provided by Dr. L. Fugger (Aarhus Univ., Denmark). Cell lines were routinely cultured in complete RPMI and Iscove's modified DMDM media [38, 39].

2.3. Cell viability

Ag-presenting cells were pretreated with various concentrations (0–40 μ M) of B13, LCL-618 (0–20 μ M), or vehicle control for 24 h. After treatment, cells were washed, and checked for viability by using trypan blue dye exclusion method. All subsequent assays used 20 μ M of B13 or LCL-618, unless otherwise indicated.

2.4. Antigens (Ags) and peptides

Self-Ag IgG *kappa* (IgG κ) was obtained from Sigma-Aldrich (St. Louis, MO), and bovine type II cartilage collagen (CII) was obtained from Chondrex, Inc. (Redmond, WA). HLA-DR4-restricted synthetic IgG immunodominant peptide $\kappa_{188-203}$ (sequence KHKVYACEVTHQGLSS), subdominant peptide $\kappa_{145-159}$ (sequence KVQWKVDNALQSGNS), and collagen immunodominant peptide CII_{261–273} (sequence AGFKGEQGPKGEP) were produced by using Fmoc technology and an Applied Biosystems Synthesizer [37, 40, 41]. Peptides with purity greater than 95% were used in the assay.

2.5. siRNA Transfection

The expression of AC was knocked down by employing a specifically designed human AC-siRNA (sequence 5'-AATCAACCTATCCTCCTTCAG-3'). A scramble sequence (5'-AATTCTCCGACGTGTCACGT-3') was used as a control. Both siRNAs were obtained from Qiagen (Valencia, CA). Frev B-cells were transfected with either scrambled siRNA (control) or AC-siRNA (66.7 nM) (test group) using Oligofectamine (Invitrogen, Carlsbad, CA). The assay was performed according to the manufacturer's (Invitrogen) instructions. Functional Ag-processing and presentation assays were carried out after 36h of siRNA transfection. The knockdown efficiency of the transfection in the test sample was monitored by western blotting.

2.6. Ceramide measurements

Analysis of lipid was carried out by the Lipidomics Facility at MUSC. Cellular lipids were extracted from treated and control cells into the organic solvent system, and a qualitative and quantitative analysis of ceramide species was performed by liquid chromatography with tandem mass spectrometry (LC MS) as described [13, 42]. The results of lipid analysis from control and treated groups were presented as lipid level/total cellular protein in pmol/mg.

2.7. Western blot analysis

Cells pretreated with either vehicle alone, or 20 μ M B13, or 20 μ M LCL-618 (control ceramide analog) were analyzed by western blotting for AC (BD Bioscience, Mountain

View, CA), β -galactosidase (B-12), adapter protein 2 (AP2) (H-79), cathepsins B, D, S, L, HLA-DR (L243), invariant chain (Ii) (Pin1.1), GILT, HLA-DM and Lamp-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as described [28, 38, 39]. Relative protein expression was calculated by comparing the band density between test sample and β -actin (Santa Cruz) with a standard Bio-Rad scanning densitometer and was shown as a ratio of specific protein expressed and β -actin for each sample [43].

2.8. Live cell confocal microscopy

Frev B-cells were washed and incubated with NBD (7-nitrobenz-2-oxa-1,3-diazole-4-yl)-labeled B13 and LysoTracker Red for 3 h. Cells were washed and analyzed by a laser scanning confocal microscope plus a Leica TCS SP2 AOBS, 63x water immersion objective as described [44, 45]. Live images of control and treated cells were acquired using excitation lasers 488 and 543. Overlay images were then assembled, zoomed, and cropped for data presentation using Leica software.

2.9. Ag presentation assays

B-cells (Frev, Priess) and the macrophage line (THP-1.DR4) were pretreated with vehicle alone, 20 μ M of B13, or LCL-618 for 6 h, and subsequently incubated with whole Ag (IgG κ) or one of the synthetic peptides ($\kappa_{188-203}$, $\kappa_{145-159}$) for 18 h in media. After washing, cells were co-cultured with the peptide specific T-cell hybridomas (2.18a for $\kappa_{188-203}$, 1.21 for $\kappa_{145-159}$, and 4027/99 for CII₂₆₁₋₂₇₃) for 24 h [38, 39]. For endogenous presentation, Priess B-cells which express endogenous IgG κ were incubated with vehicle alone or B13 for 24 h. Cells were washed, fixed with 1% paraformaldehyde for 5 min, and cocultured with the κ epitope-specific T-cell hybridoma lines for 24 h. The production of IL-2 cytokine by the specific T-cell hybridoma line was measured by ELISA method [38, 39].

2.10. Uptake of Ag and epitope generation

Frev cells were pretreated with 20 μ M B13 or vehicle alone for 6 h and incubated with IgG (50 μ M) for 2–6 h at 37°C in appropriate culture media. Cell lysates were serially diluted and added to the wells of ELISA plate pre-coated with rabbit anti-human κ -Ab (0.8 μ g/ml; Sigma-Aldrich). Biotin-labeled goat F(ab')₂ anti-human κ -Ab (0.1 μ g/ml; Southern Biotechnology Associates, Birmingham, AL), and HRP-streptavidin (0.5 μ g/ml; Pierce, Rockford, IL) were applied to determine the captured IgG κ . HRP activity was recorded using ABTS (BioFX, Randallstown, MD). The absorbance was read at 405 nm.

Cells were also washed, and resuspended in homogenizing buffer (3 mM imidazole, 0.25 M sucrose at pH 7.4, plus 0.1 mM TLCK and 0.5 mM PMSF), fractionated on 17% Percoll in 0.25 M sucrose, and analyzed by western blotting for membrane and early endosomal marker AP2, and lysosomal marker β -galactosidase. These fractions containing class II- κ were then detected with anti-class II antibody (37.1), and cocultured with the $\kappa_{188-203}$ -specific T-cell hybridoma line (2.18a, 2×10^5 /well) at 37°C for 24 h. The culture supernatants obtained were assayed for IL-2 cytokine production by ELISA [38].

2.11. Cathepsin bioassay

Cathepsins B, S, L, and D activities were quantitated according to the BioVision protocol (BioVision, Inc., Milpitas, CA). Briefly, cell lysates containing equal amounts of proteins (50 µg/well) from vehicle/B13 treated Frev cells were mixed with the 50 µl of Reaction Buffer (BioVision, Inc.), and cathepsin activity was determined by using cathepsin specific substrate and FLOUstar OPTIMA flourometer (BMG LabTech, NC, USA).

2.12. Flow cytometry analysis

After treatment with a vehicle or B13 for 24 h, Frev and Priess B-cells were washed, and stained with HLA-DR antibody (L243) for surface class II proteins and CLIP (Cer-CLIP) molecules [43]. Cells were then washed and analyzed on a FACScan utilizing Cell Quest software (BD Bioscience). Background fluorescence was evaluated using an isotype-matched antibody (NN4) as described previously [39, 43].

2.13. Collagen-induced arthritis (CIA) and cellular immune responses

DBA/1 mice (6–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed under SPF (specific pathogen-free) conditions in the animal care facility at MUSC following approval of animal care and use committee. To induce CIA, mice were injected s.c. (subcutaneously) at the base of the tail with 100 µg of CII in 0.05 M acetic acid, which was emulsified with complete Freund's adjuvant (CFA; 4 mg/ml) (Chondrex, VA) at a ratio of 1:1 [46]. Mice received 10 mg/kg of B13 or vehicle alone for five times intraperitoneally at day 15, 18, 21, 24, 27. Mice were monitored every other day for any sign of the disease and graded accordingly using a standardized scale of 0–4. 0 = normal, 1=mild swelling with erythema, 2=significant joint swelling, 3= severe swelling and digit deformity, and 4=maximal swelling with ankylosis. Each joint was blindly scored, with a maximum possible score of 16 per mouse. Mice were sacrificed at peak stages (day 42) of the disease and inguinal lymph node (ILN) cells (3×10^5) were prepared and incubated with CII (20 µg/ml) in the presence or absence of B13 (20 µM) for 72 h. The amount of IL-2 cytokine in the culture supernatants were quantitated by ELISA. The tissue specimens were obtained from the mice on day 33 after immunization. Samples were fixed in 10% formalin, decalcified in formic acid as needed, and then stained with hematoxylin and eosin [47]. All assigned work with mice was approved by the MUSC (Medical University of South Carolina) Animal Protocols (#2012) Review Board. Animal experiments were performed in accordance with the NIH (National Institutes of Health) Guide for Care and Use of Laboratory Animals.

2.14. Analysis of inflammatory cytokines

The cytokine array based on Millipore's MILLIPLEX technology was used to determine inflammatory cytokines from CIA mice sera as described [48]. All samples were tested in triplicate, and cytokine levels were expressed as pg/mL.

2.15. Statistics

The data obtained are expressed as the mean±SEM of triplicate experiments, and analyzed using Student's t-test or one-way ANOVA, with $p < 0.05$ considered statistically significant.

In addition, a nonparametric Wilcoxon Rank Sum test was also used when comparing band intensity obtained from densitometric analysis of distinct protein bands detected in western blot analysis.

3. Results

3.1. B13 elevates cellular ceramides by inhibiting AC protein expression in APCs

Cell-permeable ceramide analogs B13 and LCL-618 were tested in professional APCs to examine their effects on cell viability and Ag presentation to CD4⁺ T cells. Although B13 can reduce cancer cell growth [49], our study here indicated that B13 at 0–20 μM did not show any significant effect on the viability of APCs (B-cells, macrophages and dendritic cells) (Fig. 1A). The structure of the ceramide analog, B13 is shown in the supplemental figure (Fig. S1A). APCs treated with 40 μM of B13 underwent significant cell death (10.3%), and thus 40 μM of B13 was not used in the subsequent experiments. Results showed that B13 (20 μM) treatment significantly reduced AC levels in B-cells as analyzed by western blotting and densitometric analysis, ($p < 0.01$) (Fig. 1, B&C). The structure of the control ceramide analog (LCL-618) was shown in the supplemental figure (Fig. S1B). LCL-618 at 0–20 μM did not show any significant effect on the viability of APCs, as well as Ag presentation (Fig. S1, C&D). Western blot analysis also confirmed that LCL-618 did not significantly alter AC protein expression in B-cells (Fig. 1, B&C). The mass spectrometric analysis showed that B13 treatment elevated several ceramide species particularly C14-Cer, C16-Cer, C18-Cer, C24-Cer in Frev cells (Table I). Taken together, these studies indicated that B13 (20 μM) treatment did not influence cell viability, but instead increased levels of several ceramide species by inhibiting AC protein expression in APCs.

To further examine whether B13 reached endolysosomal compartments of APCs, Frev cells were incubated with NBD-labeled-B13 (NBD-B13) and LysoTracker dye, and viewed with live confocal microscopy. The NBD-B13 localized with the LysoTracker Red in the lysosomal compartments of these B-cells (Fig. 1D), suggesting that inhibition of AC may alter lysosomal proteases crucial for Ag-processing and HLA class II presentation.

3.2. B13 disrupts HLA class II-restricted Ag-processing and activation of T-cells

To examine the capacity of AC in HLA class II-mediated Ag presentation, human B-lymphoblastoid cells (Frev) were pretreated with AC inhibitor (B13) or vehicle (<1% ethanol) for 1h, and subsequently provided self-Ag IgG κ or synthetic κ peptides. After overnight incubation, these Frev cells were washed twice, and cocultured with the $\kappa_{188-203}$ and $\kappa_{145-159}$ peptides specific T-cell hybridomas as described in the methods [50]. The efficiency of the treated Frev cells to activate these T-cell hybridomas was monitored by quantitating the cytokine IL-2 concentrations. B13 pretreatment of B-cells markedly reduced the processing and presentation of class II-restricted epitopes derived from a self-Ag IgG κ up to 65% (Fig. 2A). These data suggested that AC inhibition disrupts class II-restricted exogenous Ag-processing and presentation by B-cells. While Ag-processing and epitope presentation from the whole IgG were diminished by B13, synthetic peptide presentation by HLA class II was not altered (Fig. 2B), suggesting that B13 did not interfere with the

presentation of processed or synthetic peptides by B-cells but altered the efficiency of processing of intact Ag.

In order to determine whether endogenous processing and presentation of intact IgG κ were also disrupted, Priess B-cells (express endogenous IgG κ), were incubated with B13 overnight at 37 C. Cells were washed and cocultured with the peptides ($\kappa_{188-203}$ and $\kappa_{145-159}$) specific T-cell hybridomas as described in the methods. The endogenous presentation of IgG κ chain also was markedly inhibited by up to 74% by B13 (Fig. 2C), suggesting that cell-permeable ceramide analogs may inhibit both exogenous and endogenous Ag-processing and CD4+ T-cell recognition. In contrast, the wild-type B-cells Frev treated with an inactive B13 analog LCL-618 showed no significant changes in Ag-processing and presentation via HLA class II (Fig. 2D). Subsequent assays also showed that this inactive ceramide analog LCL-618 did not influence endogenous Ag-processing and presentation by Priess B-cells to CD4+ T-cells (Fig. S1D).

B13 treatment of macrophages significantly decreased the processing of full-length IgG κ chain and presentation of IgG κ epitopes to CD4+ T-cells; B13 reduced the IL-2 cytokine production up to 69% (Fig. 2E). B13 treatment of dendritic cells also significantly decreased the processing of full-length IgG κ chain and presentation of IgG κ epitopes to CD4+ T-cells; B13 reduced the IL-2 cytokine production up to 76% (Fig. 2F). Like B-cells, B13 treatment of HLA-DR4-expressing THP-1 and FSDC cells did not alter synthetic κ peptide presentation by HLA class II (Fig. S2). Taken together, these data suggest that B13 disrupts HLA class II Ag-processing, and its downstream processes including subsequent CD4+ T-cell responses.

3.3. AC inhibition minimally influences cellular uptake of Ig κ , but markedly reduces Ag-processing and epitope generation for CD4+ T-cell recognition

Ag-processing involves both the uptake of Ag and its cleavage. To determine if AC inhibition by B13 alters internalization of Ig κ by B-cells, Frev cells were pretreated with 20 μ M of B13, and provided 50 μ M of IgG. Cells were then washed, and cell lysates prepared were assayed for internalized κ chain by ELISA. B13 treatment only minimally altered Ig κ uptake by Frev cells (Fig. 3A), which might not be sufficient to affect presentation of class II and stimulation of T-cells observed in Fig 3. In parallel assays, cell viability was also checked by the trypan blue exclusion method, which showed minimal cell death after IgG feeding and treatment with B13 (<4.9%, Fig. 3B).

Percoll density gradient separation of intracellular organelles was employed to fractionate (31 fractions) cellular lysates that may contain processed κ peptides loaded onto class II proteins for stimulating T-cells. Fractions 1–6 contained membrane enriched materials, and fractions 24–30 contained heavier lysosomal organelles, as identified by western blotting for AP2 and β -galactosidase, respectively (data not shown). Class II- $\kappa_{188-203}$ peptide complexes of each fraction were captured by anti-class II antibody (37.1), and tested for IL-2 cytokine production from the activated T-cell hybridomas. The B13-treated Frev cells produced significantly less functional class II- $\kappa_{188-203}$ peptide complexes than vehicle-treated cells (Fig. 3C). These data suggest that B13 treatment did not affect Ig κ uptake, but impaired Ag-

processing reactions for generating HLA class II-peptide complexes for activation of CD4⁺ T-cells.

3.4. B13 modulates AC, GILT, and cysteinyl cathepsins (B, L and S) in professional APCs

Ag-processing and presentation by the HLA class II pathway involves several lysosomal enzymes, including -IFN-inducible-lysosomal thiol reductase (GILT) and cathepsins [28, 40, 51] and can enhance T-cell recognition of HLA-peptide complexes. AC inhibitor B13 significantly reduced the active forms of GILT (30kDa), cysteinyl Cat B (25kDa), Cat L (31kDa), and Cat S (26kDa) proteins in B-cells (Figs. 4, A&B). By contrast, the active form of Cat D was not significantly altered. Further experiments showed that the activity of Cat D was minimally altered by B13 treatment in Frev cells while Cat B, Cat S, and Cat L activities were significantly attenuated (Fig. 4C), suggesting that inhibition of AC differentially affects cysteinyl and aspartyl cathepsin processing and their activities in professional APCs.

Lysosome-associated membrane protein-2 (Lamp-2) may regulate the repertoire of epitopes displayed by HLA class II molecules on B-cells by influencing the ratio of endogenous vs. exogenous Ag presentation [32]. B13 did not influence Lamp-2 expression in Frev cells, but it significantly reduced Lamp-2 in Preiss cells (Figs. 4, A&B). Treatment of Frev B-cells with the inactive ceramide analog LCL-618 did not inhibit cathepsin processing, GILT and Lamp-2 protein expression in B-cells (Fig. S3). Since AC inhibition reduced expression of cathepsin S which cleaves Ii, an important component in the HLA-class II pathway, the effects of B13 on HLA-DR maturation and loading of processed Ag were examined.

3.5. AC inhibition minimally affects class II components in Ag-presenting cells

In general, HLA class II $\alpha\beta$ -Ii complexes are transported to the late endosomal and lysosomal compartments where cathepsin S and other acidic proteases process Ii, trimming Ii so CLIP remains in the groove of class II molecules [51]. HLA-DM molecules edit CLIP proteins from the class II binding groove and help load the processed Ag peptides onto classical HLA class II proteins in the late endosomal and lysosomal compartments [52]. B13 treatment minimally affected the expression levels of class II HLA-DR, Ii, and HLA-DM molecules in B-cells (Fig. S4, A&B).

Ag-presentation is also modulated by reducing the expression of cell surface HLA-DR. AC inhibition did not significantly alter cell surface class II HLA-DR. Staining of Frev cells for HLA-DR showed an MFI of 119.56 and 120.5 for vehicle and B13, respectively, with the L243 antibody (Fig. S4C). Likewise, the HLA-DR MFI in Priess cells was 130.09 and 136.21 after treatment with vehicle and B13, respectively (Fig. S4). These data suggest that AC inhibition may not alter the expression levels of the components of the HLA class II pathway and that Ag-processing reactions rather than class II presentation are modulated by AC inhibition in APCs. However, longer B13 treatment (4 days) elevated cell surface CLIP protein expression in B-cells (Fig. S5), further suggesting that AC inhibition may alter immune recognition via the HLA class II pathway.

3.6. Inhibition of AC by siRNA disrupts HLA class II Ag-processing and presentation to CD4+ T-cells

To ensure that AC inhibition and higher ceramide levels modulated class II Ag-processing and it was not due to an unknown activity of the B13 ceramide analog, a distinct strategy was used to inhibit AC—a small interfering RNA (siRNA) specific for the AC mRNA. Frev cells that were pretreated with AC-specific siRNA for 2 days had significantly lower AC protein than those treated with a scrambled siRNA control (Fig. 5A). Knockdown of AC by siRNA in Frev disrupted class II Ag-processing and presentation in B-cells (Fig. 5B).

3.7. AC inhibition disrupts Ag-processing and presentation to primary T-cells in the mouse CIA model

To test the potential application of AC inhibition to self-Ags, the B-cell line Frev was treated with vehicle alone or B13, incubated with the auto-Ag CII, and tested for CII epitope (261–273) presentation and CD4+ T-cell responses. B13 treatment significantly decreased CII processing and presentation by B-cells, showing reduced IL-2 cytokine production by T-cells (Fig. 5C). To examine whether B13 can inhibit *ex vivo* recall responses to CII, DBA/1 mice were used to be immunized with CII and had developed CIA. Lymph node cells (1×10^5) obtained from arthritic mice were incubated with vehicle alone or B13 in the presence of CII (20 µg/ml) for 72h. The production of IL-2 cytokine in the culture supernatant was measured. B13 treatment in the culture system significantly suppressed recall responses to the arthritic Ag CII (Fig. 5D).

3.8. Inhibition of AC by B13 AC suppresses the development and severity of autoimmune arthritis and decreases inflammatory cytokines in the mouse CIA model.

To test whether AC inhibition during disease development impaired self- or auto-Ag presentation to CD4+ T-cells and altered the severity of joint inflammation, DBA/1 mice received 5 treatments of B13 or vehicle on days 15, 18, 21, 24, 27, and joints were scored based on established clinical criteria. B13 treatment significantly delayed the development of inflamed joints and reduced the joint score (Fig. 6A), which were confirmed by histological analyses (Fig. 6B). B13 treatment also decreased infiltration of polymorphonuclear leukocytes (PMNs) in the joint of CIA mice as compared to those in vehicle controls (Fig. S6). These data suggest that AC inhibition may disrupt auto-Ag-specific T-cell responses *in vivo*, and reduce inflammation and disease symptoms. Proinflammatory cytokines are known to contribute to the initiation and propagation of autoimmune inflammation. To examine whether B13 treatment reduced inflammatory cytokines in CIA mice, serum samples were analyzed by cytokine array as described in the methods. Remarkably, AC inhibition by B13 significantly decreased the levels of inflammatory cytokines TNF-, IL-1, IL-6, IL-12p70, and IL-17 levels in CIA mice (Fig. 6C), suggesting the potential use of ceramide analogs against autoimmune arthritis.

4. Discussion

We here report a new mechanism for modulation of the immune response: inhibition of AC and elevation of four types of ceramide reduce self-Ag processing, its subsequent presentation by HLA class II molecules, and stimulation of CD4+ T-cells. Ag-processing

and presentation by HLA class II play key roles in inflammation, apoptosis of self-reactive T-cells and B-cells that prevent autoimmunity, and induction of an immune responses to infectious agents and cancer cells which involve a number of events including differentiation and proliferation of T-cells and B-cells. Our major finding was that AC can modulate class II-mediated Ag-processing, presentation to T-cells, thereby regulating the initiation and amplification of an Ag-specific immune response. Both strategies of AC inhibition—analogue B13 and an AC-specific siRNA— inhibited class II presentation of intact Ag and reduced IL-2 cytokine production from T-cells.

The multi-step process of Ag-processing and presentation via MHC class II proteins initiate with Ag uptake and its cleavage in the endolysosomal compartments of APCs [53]. GILT and cysteinyl cathepsins S and L are essential for HLA class II Ag-processing and presentation, and reduced processing of HLA class II may be directly linked to inhibition of active cathepsins S and L [28, 51]. We showed that B13 treatment of professional APCs reduced AC expression, augmented levels of several types of ceramide, and disrupted class II-mediated Ag presentation of whole IgG κ Ag but not synthetic κ peptides in the three main types of professional APCs, B-cells, dendritic and monocytic cell lines. Synthetic peptides usually do not require processing by APCs as they are already processed and can directly bind to MHC molecules on the surface of APCs for presentation to CD4+ T cells. Our study suggest that B13 treatment did not significantly alter the uptake of intact IgG κ Ag into Frev B cells; in comparison, C2-ceramide (40–80 μ M) was reported to reduce uptake of HRP into human DCs without affecting cell viability within 48h [54]. This disparity may reflect differences in cell types, Ag structure, and/or ceramide analogs with distinct structure-activity relationships. Additionally, B13 treated Priess B-cell line which expresses endogenous IgG κ chain also showed a similar decrease in Ag presentation. Since presentations of whole IgG κ and endogenous IgG κ were diminished by B13 pre-treatment of cells while the presentation of synthetic κ peptides was unaffected in agreement with previous findings [54], we examined the effects of B13 on several key components of Ag-processing: GILT [28, 29], cathepsins [51, 55], and Lamp-2 proteins [32]. These data suggested that B13 treatment reduced the expression levels of GILT and cathepsins B, S, and L in B-cell lines with minimum alteration of Ii, HLA-DM molecules. Cystenyl and aspartyl cathepsins are involved in Ag processing and generation of antigenic peptides for presentation to CD4+ T cells. Our data suggest that cystenyl cathepsins are perturbed by inhibition of AC. It is not clear at this time why cathepsin D activity is not significantly altered. Our future study may focus on the mechanisms by which AC inhibition differentially affects cystenyl and aspartyl cathepsin in APCs and influences Ag-processing and presentation. Interestingly, B13 treatment reduced Lamp-2 expression, which affects the ratio of exogenous and endogenous peptides loaded onto class II molecules [32], in one of two B cell lines. Protein Ags may be observed in late endosomes to lysosomes, with both of these subcellular locations serving as potential sites of further Ag-processing [56–58]. Interestingly, NBD-B13 colocalized with LysoTracker Red in the endocytic compartment, placing it in close proximity to GILT and cathepsins [28] for modulation of their activity.

Elevation of ceramides is found in Farber disease (FD), which is a rare autosomal recessive disease caused by mutations in the acid ceramidase gene [59]. While quantitation of excess ceramides is used to assist in the diagnosis of FD, it may not provide accurate information

about individual ceramide species in the disease. AC Deficiency in FD is also poorly understood and often misdiagnosed as Juvenile Idiopathic Arthritis (JIA) [60, 61]. Although many different ceramide species and derivatives are involved in FD, the role of specific ones that accumulate in FD remains unclear. Cytokines, chemokines and growth factors such as MCP-1, IL-10, IL-6, IL-12, and VEGF levels are elevated in plasma from Farber patients but not in control or JIA patients [61]. Our study suggests that AC inhibition decreases several cystenyl cathepsins which may dysregulate various physiological and pathological processes, such as maturation of the MHC class II complex, bone remodeling, rheumatoid arthritis and osteoarthritis. Expression of cathepsins B, L, and S in Ag-presenting cells [62] as well as their presence in serum and synovial fluid from arthritic patients are commonly found, and these enzymes contribute to Ag presentation and disease severity [63, 64]. Thus, inhibition of cystenyl cathepsins by AC inhibition may diminish Ag-processing and immune activation, decreasing inflammatory cytokine production and disease severity in the CIA model.

While previous studies have demonstrated a pro-apoptotic function of B13 on tumor cells [49, 65], our results here demonstrated little to no cytotoxicity on professional APCs at 20 μ M concentration. In particular, we showed that AC inhibition reduced the production of inflammatory cytokines and the severity of joint inflammation in the mouse CIA model, a common model for inflammatory arthritis. Indeed self-Ag presentation by class II has been implicated in events like clinically significant autoimmune disorders, such as rheumatoid arthritis, type II hepatitis, multiple sclerosis, pemphigus vulgaris and myasthenia gravis [66–68]. The potential use of optimized AC inhibitors to treat certain autoimmune disorders is supported by our demonstration here that class II-mediated presentation of the auto-Ag CII and inflammatory cytokines are markedly reduced in the presence of B13. Optimized AC inhibitors may also have therapeutic potential for reducing the severity of graft-versus-host disease and inflammation [69], following stem cell or bone marrow transplantation and minimizing the risk of rejection following organ transplantation. However, the effects of other ceramidases or other enzymes involved in ceramide synthesis/metabolism in arthritis remain to be tested.

In conclusion, our results demonstrate that the treatment of cells with the AC inhibitor B13 did not alter Ag uptake but reduced the processing of host-Ags by the HLA class II Ag-processing machinery. The reduced degradation of Ags diminished class II-mediated Ag-specific IL-2 cytokine production by T-cells and suppression of the disease, in part, via downregulation of GILT, cathepsins B and S, important components of the MHC class II Ag-processing pathway. Attenuation of inflammatory cytokines by B13 in the CIA model suggests that inhibition of acid ceramide may have important implications in our understanding of distinct cytokine regulation and development of effective ceramide analogs for treating autoimmune disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Ceramide analogs dysregulate acid ceramidase, cathepsins and thiol reductase (GILT) in the endolysosomal compartments of antigen-presenting cells.

Inhibition of acid ceramidase reduces antigen processing and presentation via the MHC class II pathway.

Ceramide analogs inhibit inflammatory cytokines and T cell responses in vivo.

Inhibition of acid ceramidase suppresses the development and severity of inflamed joints in collagen-induced arthritis in mice.

Fig. 1.

Effects of B13 on cell survival, cellular expression of AC proteins, and intracellular localization in professional APCs. **(A)** Human B-cell lines Frev and Priess, and the human macrophage cell line THP-1.DR4 (10^5) were treated with B13 (0–40 μ M) for 24 h. Viable cells were counted using trypan blue dye exclusion method. **(B)** The B-cell line, Frev, was treated with an inactive ceramide analog LCL-618 (20 μ M) as a control or B13 (20 μ M) for 24 h, followed by western blot analysis for AC protein expression. β -actin was used as a loading control and a reference band to quantitate the relative expression of AC proteins. **(C)** Densitometric analysis of protein bands following treatments with LCL-618 and B13. Significant differences were calculated by student's *t* test; **= $p < 0.01$. **(D)** Live confocal images showing *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] hexanoic acid (NBD)-labeled B13 colocalizes with the LysoTracker Red in human B-cells. The B-cell line, Frev, was incubated with NBD-B13 (20 μ M) and LysoTracker Red for 3 h at 37°C and viewed with live confocal microscopy as described in the methods. NBD-B13 (left panel, green), LysoTracker Red (middle panel, Red), overlay (right panel, yellow). Data are representative of three separate experiments.

Fig. 2.

Effects of B13 on self-Ag processing and presentation by professional APCs such as B-cells, macrophages and dendritic cells. APCs were treated with vehicle, 20 μM B13, or 20 μM LCL-618 for 6 h and loaded with the indicated concentrations of either intact IgG κ or synthetic peptides for 18 h. Their efficiency in stimulating IL-2 cytokine production from the IgG κ peptide κ₁₈₈₋₂₀₃-specific T-cell hybridoma 2.18a (left panel) and from the IgG κ peptide κ₁₄₅₋₁₅₉-specific T-cell hybridoma 1.21 (right panel) was measured by ELISA. **(A)** Effects of B13 on exogenous IgG κ processing in Frev and efficiency of presentation of κ₁₈₈₋₂₀₃ and κ₁₄₅₋₁₅₉ epitopes to CD4⁺ T-cell hybridomas, as measured by IL-2 cytokine production via an ELISA. **(B)** Effects of B13 on exogenous presentation of class II-restricted synthetic κ peptides by Frev cells to CD4⁺ T-cell hybridomas. **(C)** Effects of B13 on endogenous presentation of IgG κ epitopes to T-cells. Priess cells which constitutively express endogenous IgG κ Ag were pretreated with vehicle alone or B13 for 24 h, washed, fixed to prevent further processing of the Ag, and cocultured with the κ peptide specific T-cell hybridomas 2.18a (left panel) and 1.21 (right panel). **(D)** Effect of control ceramide analog, LCL-618, on exogenous processing of intact IgG κ and presentation in Frev cells. **(E)** Effects of B13 on exogenous presentation of IgG κ epitopes by the macrophage cell line THP-1.DR4. **(F)** Effects of B13 on exogenous presentation of IgG κ epitopes by the

dendritic cell line FSDC.DR4. Data are representative of five (A-D) or three (E, F) separate experiments, and expressed as mean IL-2 (pg/ml) $\pm$ SEM of triplicate wells. Significant differences between control and test groups were calculated by student's *t* test; *= $p<0.05$, **= $p<0.01$.

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Fig. 3.

Effects of B13 on quantity and location of internalized κ -chain. After pretreatment with vehicle or 20 μ M B13 for 6 h, Frev cells were incubated with intact 50 μ M IgG for 2 h (**A**) or 18h (**B**) at 37°C in culture media. (**A**) Quantity of detectable internalized κ -chain. Cell lysates were assayed for the presence of internalized κ -chain as described in the methods. (**B**) Treatment and cell viability. Trypan blue exclusion method was used to determine cell viability following B13 treatment and IgG feeding. (**C**) Location of internalized κ -chains. Cell lysates were fractionated on 17% Percoll in 0.25 M sucrose, and the fractions (total=31) were tested for recognition by the Ig $\kappa_{188-203}$ -specific T-cell hybridoma (2.18a) as described. Fractions 1–5 contained lighter materials which were AP2-positive and fractions 26–29 contained lysosomal marker β -galactosidase. Data are representative of three separate experiments. Significant differences were calculated by student's *t* test; ns=not statistically significant, *=*p*<0.05, **=*p*<0.01.

Fig. 4.

Effects of B13 on levels and activities of proteins that may contribute to self-Ag-processing: thiol-reductase GILT and cathepsins B, S, L, and D. **(A)** Expression levels of GILT, cathepsins B, S, L, and D, and Lamp-2 in the B-cell lines Frev and Preiss visualized by Western blot analysis. After cells were treated with vehicle or B13 (20 μ M) for 24 h, cell lysates were analyzed by western blotting for GILT, Lamp-2 and cathepsins B, S, L, D proteins. Blots were reprobbed with β -actin antibody and used as a loading control. Data are representative of at least three separate experiments. **(B)** Quantitative comparison of levels of GILT, Lamp-2, and cathepsins B, S, L, and D in vehicle or B13 treated B-cell lines by densitometric analysis. β -actin was used as a reference protein band to quantitate relative protein expression. Significant differences were calculated by student's *t* test; *= p <0.05, **= p <0.01, and ns = not statistically significant. **(C)** Effect of B13 on cysteinyl and aspartyl cathepsin activities in B-cells. Frev cells grown in the presence or absence of B13 were washed, and cell lysates (50 μ g protein/well) were analyzed by BioVision reagents for cathepsin B, S, L, and D activity as described in the methods. The control value was scaled to 100 for analyses of all cathepsin activity assays. Data are presented as mean SEM of triplicate wells. *= p <0.05, **= p <0.01, and ns = not statistically significant.

Fig. 5.

Inhibition of AC disrupts auto-Ag processing and presentation, as well as ex vivo recall responses to collagen. **(A)** Western blot analysis of AC protein expression in Frev cells treated with either scrambled siRNA or AC siRNA. β -actin was used as a loading control. Densitometric analysis was performed using β -actin as a reference protein band to calculate relative expression via band density. Data are representative of three separate experiments. **(B)** Effects of AC-specific siRNA on IgG κ processing and presentation by B-cells. After treatment with scrambled siRNA control or AC-specific siRNA, Frev cells were incubated with intact IgG κ for 6 h, washed, and cocultured with the $\kappa_{188-203}$ epitope specific T-cell hybridoma line 2.18a for 24 h. T-cell production of cytokine IL-2 was quantitated by ELISA as described. **(C)** Frev cells were treated with vehicle alone or B13 and loaded with CII (0–40 μ g/ml) overnight. Cells were washed and cocultured with the collagen immunodominant epitope CII_{261–273} specific T-cell hybridoma line 4027/99 for 24 h. T-cell production of IL-2 cytokine was quantitated by ELISA as described. **(D)** CIA was induced in DBA/1J mice by immunization with CII as described in the methods. Inguinal lymph node cells (1×10^5) from arthritic mice were incubated with vehicle alone or B13 in the presence of CII (20 μ g/ml) for 72 h. The production of IL-2 cytokine in the culture supernatant was measured by ELISA. Data is representative of at least three separate experiments. * $p < 0.05$, ** $p < 0.01$.

Fig. 6.

Inhibition of AC disrupts the development and severity of autoimmune arthritis in a mouse model of arthritis. **(A)** Effect of B13 on onset and severity of joint inflammation in the CIA model. Mice received 10 mg/kg of B13 or vehicle intraperitoneally on days 15, 18, 21, 24, and 27. Mice were monitored every other day for signs of arthritis, and mean arthritis score was calculated as described in the text. **(B)** Histopathological analysis on day 33 showing signs of proliferation, bone erosion, and synovial hyperplasia in joints from vehicle-treated mice (upper panel). B13 treatment (five times) markedly inhibited those signs of inflammation (lower panel). **(C)** B13 treatment inhibited inflammatory cytokines in a mouse model of CIA. Serum cytokines were analyzed by cytokine array as described in the methods. Experiments were repeated at least three times. Statistical analysis was calculated by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

Table 1.

B13 treatment elevates various ceramide species in human B-cells.

Ceramides	C14-Cer	C16-Cer	C18-Cer	C-18:1-Cer	C20-Cer	C24-Cer	C24:1-Cer	DHC16-Cer	DHSph	DhSph-IP	Sph	Sph-1P
Frev +Vehicle	13.2±1.1	103.2±9.2	10.5±0.8	2.3±0.2	4.3±0.4	33.0±3.1	36.1±3.4	8.7±0.7	0.8±0.1	0.0±0.0	4.2±0.3	0.1±0.0
Frev+B13	21.8±1.5	182.9±15.4	18.0±1.3	4.0±0.4	6.1±0.6	33.5±3.1	55.0±4.1	14.9±1.3	1.2±0.0	0.0±0.0	4.9±0.4	0.1±0.0

Frev B-cells were treated overnight with vehicle alone or B13 (20μM), washed three times with PBS, and cell lysates were analyzed by mass spectrometry for various ceramide species. Ceramide concentrations were shown in picomole (pmole) per mg of protein.