Published in final edited form as:

Mol Genet Metab. 2012 July ; 106(3): 310–322. doi:10.1016/j.ymgme.2012.04.020.

Immunological cell type characterization and Th1–Th17 cytokine production in a mouse model of Gaucher disease

Manoj Kumar Pandey¹, Reena Rani³, Wujuan Zhang^{2,4}, Kenneth Setchell^{2,4}, and Gregory A. Grabowski¹

¹Division of Human Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio

²Division of Pathology, University of Cincinnati College of Medicine, Cincinnati, Ohio

³Division of Immunobiology, University of Cincinnati College of Medicine, Cincinnati, Ohio

⁴Laboratory of Mass Spectroscopy of the Cincinnati Children's Hospital Medical Center and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio

Abstract

Gaucher disease is a lysosomal storage disease resulting from insufficient enzyme acid β -glucosidase (glucocerebrosidase, GCase, EC 4.2.1.25) activity and the resultant accumulation of glucosylceramide. Macrophage (M ϕ) lineage cells are thought to be the major disease effectors because of their secretion of numerous cytokines and chemokines that influence other poorly defined immunological cell populations. Increases in several such populations were identified in a *Gba1* mouse model (D409V/null; 9V/null) of Gaucher disease including antigen presenting cells (APCs), i.e., M ϕ , dendritic cells (DCs), neutrophils (PMNs), and CD4⁺T cells. FACS analyses showed increases in these cell types in 9V/null liver, spleen lung, and bone marrow. T-cells or APCs enhanced activations were evident by positivity of CD40L, CD69, as well as CD40, CD80, CD86, and MHCII on the respective cells. M ϕ , and, unexpectedly, DCs, PMNs, and T cells, from 9V/null mice showed excess glucosylceramides as potential bases for activation of APCs and T cells to induce Th1 (IFN γ , IL12, TNF α ,) and Th17 (IL17A/F) cytokine production. These data imply that excess glucosylceramides in these cells are pivotal for activation of APCs and T cell induction of Th1 and Th17 responses and PMN recruitment in multiple organs of this model of Gaucher disease.

Keywords

Glucosylceramide; antigen presenting cell; lymphocyte; cytokine; stimulatory and co-stimulatory molecules; Gaucher disease

© 2012 Elsevier Inc. All rights reserved.

Correspondence to: Gregory A. Grabowski, M.D., Professor and Director, Division of Human Genetics, Children's Hospital Medical Center, 3333 Burnet Avenue, MLC 4006, Cincinnati, Ohio 45229-3039, Phone: 515-636-7290, greg.grabowski@cchmc.org.

Disclosure/conflict of interest: The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Gaucher disease (GD) is a common lysosomal storage disease that is caused by mutations in the *GBA1* leading to decreased acid β -glucosidase (β -D-glucosyl-N-acylsphingosine glucosylhydrolase (EC 4.2.1.25; GCase) activity [1]. This insufficient activity results from the detrimental effects on GCase's catalytic function, stability, and/or trafficking of more than 300 mutations in the *GBA1* gene [2–6]. The resultant excess lysosomal accumulation of glucosylceramide (GC) and glucosylsphingosine (GS) substrates in macrophage (M ϕ) lineage cells of liver, spleen, lung, and bone marrow are a primary finding in these tissues. The incidence of Gaucher disease worldwide is 1/60,000, but reaches 1/850 in the Ashkenazi Jewish population [7, 8]. Based on the absence or presence and severity of neuronopathic involvement, Gaucher disease has been classified into three clinical phenotypes, non-neuronopathic (type 1), acute neuronopathic (type 2) and chronic neuronopathic (type 3) [9]. A common feature between all three types is the accumulation of GC in the affected tissues. The cell types and mechanisms leading to consequent hepatosplenomegaly, thrombocytopenia, skeletal disease, anemia, and inflammatory signs are poorly understood [10, 11].

GC has a ceramide backbone with a β -D-glucopyranoside bound at the 1-hydroxyl position. The biosynthesis of GC is catalyzed by a UDP-glucose:ceramide glucosyltransferase [glucosylceramide synthase (GCS), EC 2.4.1.80] [12]. GC is the precursor in the synthesis of 300–400 glycosphingolipids in different mammalian cell types [13–15]. These include ceramide and its degradation products that regulate cell proliferation, apoptosis, and modulation of cell signaling pathways [16–18]. These glycosphingolipids also have key roles in diabetes, cancer, kidney, and other common diseases [19–21]. Disruption of the balance between GC synthesis and degradation in Gaucher disease leads to inflammatory conditions and functions in different tissues [11].

Several cytokines have increased levels in Gaucher disease and have been implicated in pathophysiology of the disease including interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), IL-1 receptor antagonist (IL-1Ra), soluble IL2 receptor (sIL-2R), IL-6, IL-8, IL-10, IL-18, hepatocyte growth factor (HGF), macrophage colony-stimulating factor (MCSF), macrophage-inflammatory protein-1 (MIP-1), pulmonary and activation regulated chemokine (PARC), soluble CD14 (sCD14), transforming growth factor-beta1 (TGF β 1), and tumor necrosis factor-alpha (TNF α) [9, 22–24]. However, the associations of Gaucher disease with other immunological phenotypes as well as their state of activation and role in the production of T helper-1 (Th1), T helper-2 (Th2) and T helper-17 (Th17) cytokines are ill defined, but have basic and applied import. Interactions between APCs (i.e., M ϕ , DCs, PMNs) and T lymphocytes seem to be critical in Gaucher disease pathogenesis. These interactions involve several co-stimulatory molecular pairs, including CD28-B7 and, particularly, CD40-CD40L. In APCs, CD40/CD40L activation favors survival, production of cytokines, and expression of enzymes directly involved in the inflammatory response in many diseases [25, 26]. This plethora of effects reinforces the importance of the stimulatory and co-stimulatory molecule mediated signaling pathways in regulating immunity. Thus, activation of APCs and T cells could lead to increased inflammatory responses in Gaucher disease.

Here, the nature, types, and activation states of immunological cells in various organs from a Gaucher disease mouse model, 9V/null, were determined and correlated with GC species contents. These analyses broaden the scope of immunological cell type involvement in Gaucher disease and provide a basis for understanding the complexity of the disease phenotypes.

2. Materials and methods

2.1. Materials

The following reagents were from BD Biosciences (San Jose, CA) or eBiosciences (San Diego, CA): Monoclonal antibodies (mAb) to CD11b-FITC (M1/70), CD11c-APC, F480-APC, F480-PE, Ly6G-APC (RB6-8C5), CD4-FITC, CD8-APC, CD40-PE, CD80-PE, CD86-PE, MHCII-PE, CD69-PE, CD40L-PE, CD44-PE, CD62L-FITC, and their corresponding isotypes antibodies (rat IgG2a-FITC, rat IgG2a-PE, rat IgG2a-APC, rat IgG2b-FITC, rat IgG2b-PE, rat IgG2b-APC, rat IgG2b-PECy5.5, rat IgG2a-Alexa647, Armenian hamster IgG-APC, Armenian hamster IgG2a-PE, Armenian hamster IgG2b-PE, Fc blocking antibodies, and ELISA kits for cytokines (IFN γ , TNF α , TGF β , IL-12p40/70, IL12p70, IL23, IL-4, IL6, and IL-17A/E). Proteome Profiler A was from R&D System (Minneapolis, MN), anti-Profiler A and Bio-Rad Molecular Imager® Gel Doc™ were from Bio-Rad (Hercules, CA). Anti-CD3 and CD28 antibodies were from Bio legend (San Diego, CA), Liberase C1 was from Roche (Indianapolis, IN). DNase and human Gaucher spleen GCs were from Sigma (St. Louis, MO). GMCSF and MCSF were from Peprotech (Rocky Hill, NJ). Anti-CD11c, anti-CD11b, anti-CD4, and anti-CD8 microbeads were from Miltenyi Biotec (Auburn, CA). C12-GC standards were from Matreya, LLC (Pleasant Gap, PA) or Avanti Polar lipids, Inc. (Alabaster, Alabama).

2.2. Mice

The D409V/null mice (9V/null) and WT controls were of the mixed background FVB/C57BL/6J/129SvEvBrd (50:25:25) [1] and were 24–28 weeks of age. Mice were maintained under pathogen-free conditions. Animal care was provided in accordance with National Institute of Health guidelines and was approved by Cincinnati Children's Hospital Medical Center IACUC.

2.3. Cell preparation

Spleens, livers, lungs, bone marrow and blood samples from WT and 9V/null mice were removed aseptically. Single cell suspensions from spleen were obtained by grinding and filtration through a 70-micron cell strainer. Similar suspensions from liver and lung were obtained from minced pieces that were treated with Liberase C1 (0.5mg/mL) and DNase (0.5mg/mL) in RPMI (45min, 37°C). Blood mononuclear cells were obtained after red blood cell (RBCs) lysis. For bone marrow cells, femurs, tibias, and humeri were flushed with sterile phosphate buffered saline (PBS), followed by RBC lysis (155 mM NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA), passage through a strainer, and pelleted by centrifugation at 350 g. Viable cells were counted using a Neubauer chamber and trypan blue exclusion. DCs, M ϕ , CD4⁺ T lymphocytes, and CD8⁺ T lymphocytes were purified from single cell suspensions of liver, spleen, and lung using CD11c, CD11b, CD4 (L3T4), and CD8a (Ly2) microbeads according to the manufacturer's protocol.

2.4. M ϕ generation from bone marrow cells

Bone marrow cells were used to generate M ϕ as previously described [27, 28]. Briefly, fresh bone marrow cells were stimulated with MCSF (10 ng/ml) in complete Dulbecco modified Eagle medium (FBS 10% + 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES and 1 mM sodium pyruvate). Cells were seeded in 6 well tissue culture plate and incubated at 37°C in a 5% CO₂ atmosphere. Five days after seeding the cells, supernatants were discarded and the attached cells were washed with 10 ml of sterile PBS. Ten ml of ice-cold PBS were added to each plate and incubated at 4°C for 10 minutes. The macrophages were detached by gently pipetting the PBS across the dish. The cells were centrifuged at 200 $\times$ g

for 5 minutes and resuspended in 10 ml of above media. The cells were counted, seeded and cultivated in tissue culture plates 12 hours before any further experimental procedure.

2.5. DC generation from bone marrow cells

DC was generated from mice bone marrow cells as previously discussed [29]. Briefly, bone marrow was flushed from the long bones of the limbs and depleted of red cells with ammonium chloride. These bone marrow cells were plated in six-well culture plates (10^6 cells/ml, 3 ml/well) in RPMI 1640 medium supplemented with FBS 10% + 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES and 1 mM sodium pyruvate and 10 ng/ml recombinant murine GM-CSF. At day 0, 2, 4, and 6 of culture, floating cells were gently removed and fresh medium was added. At day 7 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected, counted, seeded and cultivated in tissue culture plates 12 hours before any further experimental procedure.

2.6. Flow Cytometry

For identification of cellular phenotypes in organs, cells were suspended in PBS containing 1% bovine serum albumin. After incubation (15 min, 4°C) with the blocking antibody 2.4G2 (Fc γ RIII/I), cells were stained (45 min, 4°C) with antibodies for different cell types including 1) CD4 for T cells, 2) CD11b and F480 for M ϕ , 3) CD11b and CD11c for DCs, 4) CD11b and Gr1 for PMNs. Cells were also stained with the respective isotype antibodies as controls. Flow cytometric analyses were performed on a FACS Calibur, where M ϕ were gated first by their typical FSC/SSC pattern based on F4/80 positivity and double stained for F4/80 and CD11b. Similarly, DCs were gated for CD11c positivity and double stained for CD11c and CD11b. PMNs were gated for Gr1 high positivity and stained for Gr1 and CD11b. The different APCs were also characterized for stimulatory and co-stimulatory molecules (CD40, CD80, CD86, MHCII). Flow cytometric analyses of T lymphocytes were generated after gating lymphocytes from forward and side scatter and then identifying the CD4⁺ and CD8⁺ T lymphocytes. CD4⁺ T cells were also stained for CD44, CD62L, CD40L, and CD69. A total of 10^6 events were acquired for each cell type of each organ. Absolute numbers of M ϕ , DCs, PMNs and T cells in each organ of WT and 9V/null mice were determined by multiplying the total number of gated cells $\times$ percentage of CD11b⁺ and F480⁺ positive subsets for M ϕ , CD11c^{high} and CD11b⁺ positive subsets for DCs, CD11b⁺ and GR1^{high} positive subsets for PMNs, and CD4⁺ and CD8⁺ positive subsets for T cells. FACS Calibur, LSRII flow cytometer (BD Biosciences, San Jose, CA), and FCS Express software (DeNovo) was used to analyze these data.

2.7. In vivo induction of circulatory PMNs

WT (n=6) and 9V/null (n=6) mice were sacrificed and cardiac blood was collected in EDTA containing tubes. Neutrophil numbers in the circulation were determined using an automated hematology system (Hemavet 850, Drew Scientific, Oxford, CT).

2.8. Cytokines and chemokines in sera and supernatant

Whole blood from WT (n=20) and 9V/null mice (n=20) was obtained by cardiac puncture. Serum was isolated after 1 hr. of incubation at RT followed by the dilution 1:10 with sterile PBS. Serum was used to identify IFN γ , IL-12p40/70, TNF α , TGF β , IL-4, IL-6, and IL-17A/E by commercial ELISA kits and increased concentration of C-X-C chemokines i.e., keratinocyte-derived chemokine (KC) or chemokine (C-X-C motif) ligand 1 (CXCL1) and M ϕ -inflammatory protein-2 (MIP 2) or chemokine (C-X-C motif) ligand 2 (CXCL2) by using proteome profiler A and densitometry according to the manufacturer's instructions. Bone marrow generated M ϕ s were suspended in complete DMEM and DCs were suspended in complete RPMI medium to a concentration of 10×10^6 cells/ml. 200 μ l per well of this

cell suspension was placed in 96 well tissue culture plate and incubated for 48 h at 37° C with 5% CO₂. The medium was then removed and used to measure KC and MIP2 by proteome profiler A and densitometry according to the manufacturer's instructions. Duplicate assays were conducted thrice.

2.9. CD3/CD28 mediated stimulation of T Cells

For T cell activation, anti-CD3 and CD28 antibodies (2 µg/ml) were dissolved in sterile PBS. These antibodies (200 µl/well) were coated onto 96-well plates, incubated overnight at 4°C, and washed twice in sterile PBS. Single cell suspensions (2×10⁵ cells/well) from liver, spleen, and lung of WT and 9V/null mice were plated. Supernatants harvested 96 hrs. after stimulation with anti-CD3/CD28 antibodies were used to determine the concentrations of IFNγ, IL-12p40/70, IL12p70, IL23, TNFα, IL-17A/E, IL-6, and IL-4 and cells were used to measure the expression of CD40L by using FACS staining with antibodies to CD40 L.

2.10. *In vitro* stimulation of Mφ, DCs, and CD4⁺T cells for CD40 and CD40L induction

To evaluate increased expression of CD40 and CD40L on 9V/null Mφ, DCs, and CD4⁺T cells, WT and 9V/null bone marrow generated Mφ and DCs were *in vitro* stimulated with IFNγ (50 ng/ml). Also, purified WT and 9V/null splenic CD4⁺T cells were stimulated with antibodies to CD3/CD28 for 96 hrs. Cells were harvested 96 hrs. after stimulation with IFNγ and anti-CD3/CD28 antibodies and used to measure expression of CD40 and CD40L by FACS staining.

2.11. Dendritic cell and T cell co-culture

A 1:2.5 ratio of dendritic cells (CD11c⁺ CD11b⁺) and T cells (CD4⁺ T cells) purified from WT and 9V/null liver, spleen, and lung were stimulated in the presence and absence of GC (100 ng/ml) for 96 hrs. in complete medium and the cells used for FACS staining with CD11b, CD11c, CD4, CD40, and CD40L markers. The respective supernatants were used to determine the concentrations of IFNγ, IL-12p40/70, IL12p70, IL23, TNFα, IL-17A/E, IL-6, and IL-4.

2.12. Quantification of GCs from immunological cells

GCs were extracted from purified Mφ, DCs, CD4⁺T cells, and CD8⁺T cells from liver, spleen, and lung, as well as PMNs, and bone marrow generated DCs and Mφ of WT and 9V/null mice. GCs were quantified by ESI-LC-MS/MS using a Waters Quattro Micro API triple quadrupole mass spectrometer (Milford, MA) interfaced with Acquity UPLC system. Calibration curves were built for the GC species (C16:0, C18:0, C24:1) using C12-GC as standard. Quantification of GCs with various fatty acid chain lengths was realized by using the curve of each GC species with closest number of chain length. The total GCs in the liver, spleen, and lung were normalized to 1×10⁶ cells.

2.13. Statistical analyses

One-tailed Students t-test (two groups) or analysis of variance (ANOVA) for multiple groups was used to determine significance (Prism Graph Pad™). Non-significant (ns), p>0.05; one asterisk, p<0.05; two asterisks, p<0.01; and three asterisks, p<0.001 are used in the figures.

3. Results

3.1. Identification of Immunological cells and their GC species contents in 9V/null mouse Macrophages (M ϕ)

M ϕ populations from liver, spleen, lung, and BM of WT and 9V/null were compared. Increased numbers of CD11b⁺ F4/80⁺ positive cells were observed in 9V/null liver (2-fold), spleen (6.1-fold), and lung (3.8-fold) ($p < 0.001$) (Fig. 1A and Supplementary Fig. 1A), but not in bone marrow ($p > 0.05$). Matching isotypes (Rat IgG2b and Rat IgG2a) for CD11b⁺ F480⁺ positive cells were negative (Supplementary Fig. 1B). Positivity for stimulatory and co-stimulatory (CD40, CD80, CD86, MHC-II) molecules was assessed with purified M ϕ (1×10^5) from liver, spleen, lung, and bone marrow. Significantly higher ($p < 0.001$) numbers of CD40, CD80, CD86, and MHC-II positive cells were observed with M ϕ from 9V/null liver (Fig. 1B and Supplementary Fig. 1C) and lung (Fig. 1D and Supplementary Fig. 1E). 9V/null splenic M ϕ also had significantly ($p < 0.001$) increased percentages of CD80 and CD86 co-stimulatory molecules, whereas CD40 and MHC II did not reach significance levels (Fig. 1C and Supplementary Fig. 1D). Similarly, 9V/null BM had significantly increased ($p < 0.001$) percentages of CD40 and CD86, whereas CD80 and MHC II did not reach significance levels (Fig. 1E and Supplementary Fig. 1F). M ϕ purified from WT and 9V/null mice organs were analyzed for GC species, i.e., those with different fatty acid acyl chain compositions. Liver and splenic M ϕ from 9V/null mice showed ($p < 0.05$ - 0.001) increases of GCs: 16-0, 22-0, 24-1 and 24-0 (Fig. 1F and 1G). 9V/null lung M ϕ showed similar increases in 16-0, 24-1, and 24-0 ($p < 0.001$) (Fig. 1H). Glycosphingolipids from MCSF generated 9V/null bone marrow M ϕ showed increased accumulation of GC species: 16-0 ($p < 0.05$), 18-0 ($p < 0.05$), 24-1 ($p < 0.001$), and 24-0 ($p < 0.001$) (Fig. 1I).

3.2. Dendritic cells (DCs)

DC populations from the liver, spleen, lung, and BM of 9V/null were compared to WT. CD11c^{high}, CD11b⁺ DCs from 9V/null mice were increased in liver [15.1-fold ($p < 0.001$)], spleen [2.5-fold ($p < 0.001$)], lung [9.3-fold ($p < 0.001$)], and bone marrow [1.7-fold ($p > 0.05$)] (Fig. 2A and Supplementary Fig. 2A). There was no staining of identical isotypes (rat IgGb and rat Armenian hamster IgG) for CD11c⁺ CD11b⁺ positive cells in either organ (Supplementary Fig. 2B). Analyses of 9V/null DCs for stimulatory and co-stimulatory (CD40, CD80, CD86, MHC-II) molecules showed significantly increased positivity ($p < 0.001$) in liver (Fig. 2B and Supplementary Fig. 2C), spleen (Fig. 2C and Supplementary Fig. 2D), lung (Fig. 2D and Supplementary Fig. 2E), and GMCSF generated bone marrow DCs (Fig. 2E and Supplementary Fig. 2F). Purified DCs, from WT and 9V/null mice tissues were also analyzed for GC species. GC species from 9V/null liver and splenic DCs showed increased accumulation: 16-0 ($p < 0.001$) and 24-0 ($p < 0.001$) (Fig. 2F and G). GCs from DCs of 9V/null lungs (Fig. 2H) and bone marrow (Fig. 2I) showed increased accumulation of 16-0, 22-0, 24-1, and 24-0 ($p < 0.001$).

3.3. T cells

CD4⁺ T cell and CD8⁺ T cell phenotypes were evaluated in liver, spleen, lung, and bone marrow. Significantly increased CD4⁺ T cells were found in the liver ($p < 0.001$), spleen ($p < 0.001$), and lung ($p < 0.01$) of 9V/null mice, but not in bone marrow (Fig. 3A and Supplementary Fig. 3A). None of these organs showed such significant ($p > 0.05$), increased CD8⁺ T cells population in 9V/null mice (Fig. 3B). There was no staining of identical isotypes (rat IgG2b and rat IgG2a) for CD4⁺ T cells and CD8⁺ positive T cells in any of these organs (Supplementary Fig. 3B). To determine whether T cells are activated in 9V/null mice, CD4⁺ T cells and CD8⁺ T cells from liver, spleen, and lung were assessed for positivity with CD69 and CD40L. CD69 positive CD4⁺ T cells were increased ($p < 0.001$) in spleen, but not in other organs (Fig. 3C and Supplementary Fig. 3C). CD40L positive CD4⁺

T cell numbers were increased ($p < 0.001$) in liver, spleen, and lung of 9V/null mice (Fig. 3D and Supplementary Fig. 3D). However, CD8⁺ T cells did not show positivity ($p > 0.05$) for CD69 and CD40L antibodies in any of these organs from 9V/null mice (Supplementary Fig. 3E–F). CD4⁺ T cells from these organs were also assessed for the presence of naïve and memory T cells by evaluating their positivity with CD44 and CD62L. None of the 9V/null organs had significant changes ($p > 0.05$) in naïve (CD62L^{high} CD44^{int}) or memory (CD44^{high} CD62L^{int}) CD4⁺ T cell populations (Supplementary Fig. 4A–L).

CD4⁺ T cell GCs were analyzed using purified cells from liver, spleen, and lung of WT and 9V/null mice. In 9V/null liver, CD4⁺ T cells showed increased GC species: 16-0 ($p < 0.001$), 18-0 ($p < 0.05$), 20-0 ($p < 0.001$), 22-0 ($p < 0.001$), 24-1 ($p < 0.001$), and 24-0 ($p < 0.001$); the other species did not show any differences (Fig. 3G). In 9V/null spleen, CD4⁺ T cells showed increased GC species: 16-0, 20-0, 22-0, 24-1, 24-0 ($p < 0.001$), and 18-0 ($p < 0.05$); the other species i.e. 22-1, 26-1, and 26-0 did not show any differences (Fig. 3H). In 9V/null lung, CD4⁺ T cells showed increased GC species: 16-0 and 24-0 ($p < 0.001$) and 24-1 ($p < 0.05$); the other species did not show any differences ($p > 0.05$) (Fig. 3I). Additional analyses showed that the total GCs in liver, spleen, and lung CD8⁺ T cells were 6–29 fold increased compared to WT.

3.4. Serum cytokines

APCs and T cells of 9V/null mice were increased in numbers and had excess GC storage. Also, enhanced positivity was present for stimulatory and co stimulatory molecules. Thus serum levels of cytokines were evaluated in 9V/null mice. The cytokines i.e., IFN γ (Fig. 4A), IL-12p40 (Fig. 4B), TNF α (Fig. 4C), IL-17A/F (Fig. 4D), IL-6 (Fig. 4E), and TGB β (Fig. 4F) were increased ($p < 0.01$ – $p < 0.001$) in 9V/null sera. In contrast, IL-4 was not (Fig. 4G ($p > 0.05$)).

3.5. CD3/CD28 activation specifically enhances the Th1 and Th17 cytokines in 9V/null mice

Increased positivity of 9V/null APCs and CD4⁺ T cells for stimulatory and co-stimulatory molecules suggested their joint cooperation to cause T cell activation and induction of Th1 and Th17 cytokine production in 9V/null mice. To explore this, mononuclear cells from lung, liver, and spleen were stimulated with the CD3/T cell receptor (TCR) complex and the CD28 co-stimulatory molecule. In general, T cell initiation requires two signals from APCs. The first signal comes from ligation of T cell receptor and the major histocompatibility complex (MHC) antigen presented on the surface of APCs. The second signal is via additional co-stimulatory molecules, including the interaction between the CD28 family of T cells and B7 molecules which is CD80 (B7-1) and 86(B7-2) expressed on the APCs responsible for activation of T cells effector functions [27, 29]. T cells incubated in medium alone produced no cytokines (data not shown). Crosslinking 9V/null liver, spleen or lung cells with antibodies to CD28/CD3 led to the increased production of IFN γ , TNF α , IL-12p40, IL12p70, IL23, IL-6, IL-17A/F ($p < 0.001$ – $p < 0.01$) (Figs. 5A–G). In contrast, the Th2 cytokine IL-4 was not increased (Fig. 5H).

3.6. In vitro stimulation of 9V/null APCs and CD4⁺ T cells enhances expression of CD40 and CD40L

Since 9V/null mice have increased CD40 and CD40L on respective APCs and T cells, *in vitro* stimulation of APCs and T cells were assessed for their ability to amplify their CD40 and CD40L positivity. To evaluate this, IFN γ was used to stimulate M ϕ and DCs. Antibodies to CD3 and CD28 were used to stimulate splenic CD4⁺ T cells. Also, GC was used to stimulate DC and T cells in co-culture experiments. Bone marrow generated M ϕ and DCs of 9V/null mice showed increases ($p < 0.010$ – $p < 0.05$) in the CD40 expression, which was maximally stimulated ($p < 0.001$) by IFN γ (Fig. 6A–B). Unstimulated 9V/null CD4⁺ T

cells showed little increase ($p>0.05$) in CD40L expression, but this was markedly enhanced ($p<0.001$) by stimulation with antibodies to CD3 and CD28 (Fig. 6C). Unstimulated WT and 9V/null DC-T cell co-cultures did not show differences ($p>0.05$ - $p<0.05$) in the positivity of CD40 on DCs and CD40L on CD4⁺T cells. Also, the *in vitro* addition of GC to WT and 9V/null DC-T cell co-cultures led to increased percentages ($p<0.001$ - $p<0.001$) of CD40 positive DC (CD11c⁺CD11b⁺) and CD40L positive CD4⁺ T cells from several tissues (Figs. 6D–G).

3.7. CD40-CD40L interaction is needed for Th1 and Th17 cytokines production in 9V/null mice

CD40-CD40L interaction between APCs and T cells causes the development of Th1 and Th17 immune responses [28, 30]. 9V/null mice APCs and T cells showed spontaneous as well as IFN γ , CD3/CD28 and GC induced increased positivity of CD40 and CD40L, as well as increased production of Th1 and Th17 cytokines. The effects of *in vitro* addition of GC on DCs and T cells on the induction of Th1 and Th17 cytokine production in 9V/null mice were evaluated. GC addition led to the production of IFN γ (Fig. 7A), TNF α (Fig. 7B), IL-12p40 (Fig. 7C), IL12p70 (Fig. 7D), IL23 (Fig. 7E), IL-6 (Fig. 7F), and IL17A/F (Fig. 7G), and decreased generation of IL-4 (Fig. 7H) ($p<0.001$ - $p<0.01$) in DC-T cell co-culture supernatants of 9V/null cells.

3.8. IL17 mediated PMNs recruitment

IL-17 has been implicated in induction of mediators that promote granulopoiesis and selective tissue recruitment of PMNs [31–33]. 9V/null CD3 coating as well as DC-T cell co-cultures experiment showed increased concentrations of IL17. Thus, liver, spleen, lung, and bone marrow PMNs were determined: PMNs gated on Gr1^{high} and CD11b positivity showed modest increased percentages in liver [2-fold, ($p<0.01$), spleen (1.5-fold, $p<0.01$), and bone marrow (1.3-fold, $p<0.001$) (Supplementary Fig. 5A–B). This was not the case in the lung ($p>0.05$). Matching isotypes (rat IgG2bFITC and rat IgG2bAPC) for CD11b⁺ + Gr1⁺ PMNs were negative (Supplementary Fig. 5C). Blood counts and FACS staining confirmed increased numbers of PMNs [2–4 fold, ($p<0.001$)] in blood of 9V/null mice (Supplementary Fig. 5D–E). In addition with the IL17, increased concentration of C-X-C chemokines i.e., KC or CXCL1 and MIP 2 or CXCL2 in sera ($p<0.01$ - $p<0.001$) and culture supernatant of both M ϕ ($p<0.01$) and DC ($p<0.001$) implicate the contribution of these elements in the recruitment of PMNs into GCase deficient tissues (Supplementary Figs. 5F–H). GC species from WT and 9V/null mouse bone marrow derived PMNs showed significantly less increases of 16-0, 22-0, 24-1, and 24-0 GCs ($p<0.001$) than those in 9V/null lung M ϕ and DCs. The other GC species showed minor differences ($p>0.05$) (Supplementary Fig. 5I).

4. Discussion

To gain insight into the pathogenesis of Gaucher disease, the present studies characterized the nature and activation states of immune-related cells isolated from several involved visceral organs. Increased frequencies of M ϕ , PMNs, DCs, T cells, and B cells (unpublished data) were shown in 9V/null mice. These immunological cells were associated with excess of GC, increased production of Th1/Th17 cytokines, and differential positivity for stimulatory and co-stimulatory molecules in several visceral tissues of 9V/null mice. This suggests that excess GC induces APCs and T cell stimulatory and co-stimulatory molecules, respectively, with consequent production of Th1, Th17 cytokines and KC/CXCL1, MIP2/CXCL2 chemokines to cause PMN recruitment to propagate the disease state.

M ϕ s have been the major focus of the pathophysiology of Gaucher disease because of their unique histologic appearance and their massive GC contents. This excessive substrate accumulation leads to inflammatory responses and visceral organ abnormalities by

undefined mechanisms [6]. However, other immunological cells and their activation states have been incompletely characterized. Here, the repertoire of immune cells with excess GC accumulation is expanded to also include DCs, PMNs and CD4⁺T cells. Furthermore, the GC species accumulation in Mφ, DCs, and CD4⁺T cells from liver and lung, i.e., tissues with preferential C16 or C24 contents, respectively, and increases in stimulatory and co-stimulatory molecules (CD40, CD80, CD86, MHC-II, CD40L, and CD69) were different compared with such cells from spleen and bone marrow. 9V/null Mφ, DCs, and CD4⁺T cells showed further increased expression of CD40 and CD40L upon activation with IFNγ, antibodies to CD3/CD28, and GC stimulation and similar cytokines profiles sera vis-à-vis increases of IFNγ, IL-12p40, IL12p70, IL23, TNFα, TGFβ, IL6, and IL-17. The enhanced positivity for stimulatory and co-stimulatory molecules of 9V/null APCs and T cells as well as their enhanced production of IL-12/IFN-γ and IL-6/IL-17 support their activation in 9V/null model mice. These results implicate a significant activation of major components of the immune system as a result of defective GCase activity and accumulation of different GC species in many cell types.

CD3 and anti-CD28 antibodies stimulated mononuclear cells from 9V/null liver, spleen, and lung showed increased secretion of APC-derived cytokines, i.e., IL-12 or IL-6, indicating the involvement of CD40-mediated activation of APCs and their interaction with CD40L upregulated T cells. APCs and their involvement in T cell activation and co-stimulation, via CD3/CD28 ligation, were evaluated by *in vitro* analyses of GC effects on induction of DCs and T cell Th1 and Th17 cytokine production. Indeed, GC in DC-T cell co-cultures induced production of T cell and APC-derived cytokines (IFNγ, IL-12p40, IL12p70, IL23, TNFα, TGFβ, IL6, and IL-17) and increased positivity for CD40 and CD40L. These results indicate APC and T cell interactions are important for cytokine-mediated inflammation in 9V/null mice.

In addition, the unexpected increases in Th1 and Th17 effector cells suggest their cooperativity in an activation of the innate immune response as indicated by a few previous studies of WT cells [34, 35]. In particular, the enhanced numbers of GC loaded neutrophils in various visceral tissues of the 9V/null mice was unexpected since this is not a major component of Gaucher disease histology. These results indicate that the autocrine effect of IL-17 on neutrophil recruitment is significant and is differentially expressed in liver, spleen, bone marrow, and blood. Their role in Gaucher disease propagation will require further investigation. These results do support a differentiated cellular metabolism of various GC species in different cell types and organs with potentially differing effects on APCs and T cell interactions and enhancement of the IL-12p40, IL12p70, IL23, IFN-γ as well as TNFα, IL-6, and IL-17 pathways in GCase defective mice.

Increase of damage associated molecular patterns (DAMPs) including endogenous alarm signals as well as pathogen associated molecular patterns (PAMPs) have been recognized in many immunological cells including DCs, Mφ, and PMNs which trigger innate and adaptive immune inflammation in several diseases [36–40]. Our results indicate that presence of excess GC species, particularly C16 and C24 causes the upregulation of stimulatory and co stimulatory molecules (CD40, CD80, CD86, MHC-II, CD40L, CD69) on corresponding APCs (i.e., Mφ, DCs) and T cells of 9V/null mice. This then leads to T cell activation, and Th1/Th17 responses. These immune responses correlate with excess accumulation of C16 and C24 species of GC, thereby potentially implicating these lipids as damage associated molecular patterns that likely trigger an enhanced inflammatory status in Gaucher disease.

This concept is supported by the GC mediated activation of 9V/null CD4⁺T cells by *ex vivo* experiments. This process is mediated by 1) ligation of T cell receptor and the MHC-II present on the APCs, 2) interaction between CD28 family of T cells and B7 molecules, i.e.

CD80 (B7-1) and 86(B7-2), expressed on APCs and more importantly, 3) the ligation of APC CD40 and T cell CD40L. The latter has been found in several animal models of chronic inflammation, e.g. arthritis, graft versus host disease, transplant rejection, lung inflammation, and multiple sclerosis [41–43]. Here, GC added to cultures of 9V/null DC and CD4 T cells led to increased percentages of CD40 positive DCs and CD40L positive CD4⁺T cells, and increased Th1 and Th17 cytokines (IFN γ , IL-12p40, IL12p70, IL23, TNF α , TGF β , IL6, and IL-17) relative to IL-4.

IL-12 and IL-23 are critical for development of naïve and memory T cells, respectively [44–46]. Despite increased secretion of IL12 and IL23 both naïve and memory CD4⁺T cells percentages were similar to WT in all 9V/null tissues. Much higher levels of IL-23, compared to IL-12, were detected in both anti- CD3/CD28 antibodies coated cells and GC stimulated DC- CD4⁺T cells of 9V/null mice. This observation strongly supports the predominant role of IL-23 in mediating Th17 cell response. Notably, the GC-mediated DC-T cell interaction in promoting the Th1 and Th17 responses in 9V/null mice may also relate to its efficiency in inducing the CD40L expression, as CD40-CD40L crosstalk is important to the Th1 and Th17 responses [30, 47–50]. These results support GC as promoting the engagement of CD40 through T cell CD40L; This is a critical step for T cells activation and up-regulation of Th1 and Th17 immune responses [30, 43, 51, 52]. IL-12 cytokine initiates Th1 responses, whereas IL-6 and IL23 lead to Th17 responses [53–57]. The increases in IFN γ , IL12p40, IL12 p70, IL23, IL6, and TGF β , observed in the 9V/null mice implicate excess GC in Th1 and Th17 immune stimulation.

A model is suggested in which excess GC (potentially of particular species) in APCs, i.e., M ϕ , and DCs, and CD4⁺T cells causes their activation and positivity for stimulatory and co-stimulatory molecules (CD40, CD80, CD86, MHCII, CD69, CD40L). These APCs through the CD40 and B7 family molecules and T cells through the CD40L and CD28 molecules (Supplementary Fig. 6A–B) interact and trigger enhanced responses of the Th1 and Th17 family cytokines (Supplementary Fig. 6C). In addition, with the production of IL17 by the combined activity of APCs and T cells, initial M ϕ and DC activation resulting from excess GC directly triggers a vicious cycle for the release of PMN attracting chemokines, i.e. KC/CXCL1 and MIP2/CXCL2, which causes the PMN migration into the different visceral organs (Supplementary Fig. 6D). These results highlight the importance of GC accumulation in APC and CD4⁺T cells in the initiation and propagation of various stimulatory and co-stimulatory molecules as well as enhanced Th1 and Th17 cytokines in 9V/null mice

The present data focuses attention on the here-to-fore unappreciated contributions of many immunological cells, the nature of their excess GC case substrates, and the induced inflammatory state in Gaucher disease. The enhanced inflammatory state in 9V/null mice is linked to excesses of several tissue specific GC, C16-0 and C24-0 species. However, important unresolved questions remain about how these GC species and/or glucosylsphingosine mechanistically affect the different immunological cells in Gaucher disease leading to the induction of Th1 and Th17 cytokine and recruitment of other cell types. These findings should foster the dissection of the essential functions and complex interactions immune perturbations in Gaucher disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Professor Jörg Köhl for helpful discussion, and Stuart Tinch, Venette Inskeep, and Brian Quinn for their excellent technical assistance. This work is supported by a NIH grant (DK 36749) to GAG.

References

1. Xu YH, Quinn B, Witte D, Grabowski GA. Viable mouse models of acid β -glucosidase deficiency: the defect in Gaucher disease. *Am J Pathol.* 2003; 163:2093–2101. [PubMed: 14578207]
2. Zimmer KP, le Coutre P, Aerts HM, Harzer K, Fukuda M, O'Brien JS, Naim HY. Intracellular transport of acid β -glucosidase and lysosome-associated membrane proteins is affected in Gaucher's disease (G202R mutation). *J Pathol.* 1999; 188:407–414. [PubMed: 10440752]
3. Liou B, Kazimierczuk A, Zhang M, Scott CR, Hegde RS, Grabowski GA. Analyses of variant acid β -glucosidases: effects of Gaucher disease mutations. *J Biol Chem.* 2006; 281:4242–4253. [PubMed: 16293621]
4. Sawkar AR, Schmitz M, Zimmer KP, Reczek D, Edmunds T, Balch WE, Kelly JW. Chemical chaperones and permissive temperatures alter localization of Gaucher disease associated glucocerebrosidase variants. *ACS Chem Biol.* 2006; 1:235–251. [PubMed: 17163678]
5. Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. *Lancet.* 2008; 372:1263–1271. [PubMed: 19094956]
6. Grabowski, GA.; Petsko, GA.; Kolodny, EH. Gaucher Disease. In: Valle, D.; Beaudet, AL.; Vogelstein, B.; Kinzler, KW.; Antonarakis, SE.; Ballabio, A., editors. *The Online Metabolic and Molecular Bases of Inherited disease*. New York: Mc Graw Hill; 2010. p. 1-13.
7. Grabowski GA. Gaucher disease: gene frequencies and genotype/phenotype correlations. *Genet Test.* 1997; 1:5–12. [PubMed: 10464619]
8. Charrow J, Andersson HC, Kaplan P, Kolodny EH, Mistry P, Pastores G, Rosenbloom BE, Scott CR, Wappner RS, Weinreb NJ, Zimran A. The Gaucher registry: demographics and disease characteristics of 1698 patients with Gaucher disease. *Arch Intern Med.* 2000; 160:2835–2843. [PubMed: 11025794]
9. Cullen V, Sardi SP, Ng J, Xu YH, Sun Y, Tomlinson JJ, Kolodziej P, Kahn I, Saftig P, Woulfe J, Rochet JC, Glicksman MA, Cheng SH, Grabowski GA, Shihabuddin LS, Schlossmacher MG. Acid β -glucosidase mutants linked to Gaucher disease, Parkinson disease, and Lewy body dementia alter alpha-synuclein processing. *Ann Neurol.* 2011; 69:940–953. [PubMed: 21472771]
10. Hughes D, Cappellini MD, Berger M, Van Droogenbroeck J, de Fost M, Janic D, Marinakis T, Rosenbaum H, Villarubia J, Zhukovskaya E, Hollak C. Recommendations for the management of the haematological and onco-haematological aspects of Gaucher disease. *Br J Haematol.* 2007; 138:676–686. [PubMed: 17655728]
11. Campeau PM, Rafei M, Boivin MN, Sun Y, Grabowski GA, Galipeau J. Characterization of Gaucher disease bone marrow mesenchymal stromal cells reveals an altered inflammatory secretome. *Blood.* 2009; 114:3181–3190. [PubMed: 19587377]
12. Basu S, Kaufman B, Roseman S. Enzymatic synthesis of ceramide-glucose and ceramide-lactose by glycosyltransferases from embryonic chicken brain. *J Biol Chem.* 1968; 243:5802–5804. [PubMed: 5699063]
13. Huwiler A, Kolter T, Pfeilschifter J, Sandhoff K. Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim Biophys Acta.* 2000; 1485:63–99. [PubMed: 10832090]
14. Tepper AD, Diks SH, van Blitterswijk WJ, Borst J. Glucosylceramide synthase does not attenuate the ceramide pool accumulating during apoptosis induced by CD95 or anti-cancer regimens. *J Biol Chem.* 2000; 275:34810–34817. [PubMed: 10945987]
15. Liu YY, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J.* 2001; 15:719–730. [PubMed: 11259390]
16. Shen W, Stone K, Jales A, Leitenberg D, Ladisch S. Inhibition of TLR activation and up-regulation of IL-1R-associated kinase-M expression by exogenous gangliosides. *J Immunol.* 2008; 180:4425–4432. [PubMed: 18354163]
17. Kim TJ, Kang YJ, Lim Y, Lee HW, Bae K, Lee YS, Yoo JM, Yoo HS, Yun YP. Ceramide 1-phosphate induces neointimal formation via cell proliferation and cell cycle progression upstream of ERK1/2 in vascular smooth muscle cells. *Exp Cell Res.* 2011; 317:2041–2051. [PubMed: 21624361]

18. Popa I, Therville N, Carpentier S, Levade T, Cuvillier O, Portoukalian J. Production of multiple brain-like ganglioside species is dispensable for fas-induced apoptosis of lymphoid cells. *PLoS One*. 2011; 6:e19974. [PubMed: 21629700]
19. Chuang YH, Wang TC, Jen HY, Yu AL, Chiang BL. α -Galactosylceramide-induced airway eosinophilia is mediated through the activation of NKT cells. *J Immunol*. 2011; 186:4687–4692. [PubMed: 21383248]
20. Mather AR, Siskind LJ. Glycosphingolipids and kidney disease. *Adv Exp Med Biol*. 2011; 721:121–138. [PubMed: 21910086]
21. Yamashita T. Glycosphingolipid modification: structural diversity, functional and mechanistic integration of diabetes. *Diabetes Metab J*. 2011; 35:309–316. [PubMed: 21977449]
22. van Breemen MJ, de Fost M, Voerman JS, Laman JD, Boot RG, Maas M, Hollak CE, Aerts JM, Rezaee F. Increased plasma macrophage inflammatory protein (MIP)-1 α and MIP-1 β levels in type 1 Gaucher disease. *Biochim Biophys Acta*. 2007; 1772:788–796. [PubMed: 17499484]
23. Yoshino M, Watanabe Y, Tokunaga Y, Harada E, Fujii C, Numata S, Harada M, Tajima A, Ida H. Roles of specific cytokines in bone remodeling and hematopoiesis in Gaucher disease. *Pediatr Int*. 2007; 49:959–965. [PubMed: 18045304]
24. de Fost M, Out TA, de Wilde FA, Tjin EP, Pals ST, van Oers MH, Boot RG, Aerts JF, Maas M, Vom Dahl S, Hollak CE. Immunoglobulin and free light chain abnormalities in Gaucher disease type I: data from an adult cohort of 63 patients and review of the literature. *Ann Hematol*. 2008; 87:439–449. [PubMed: 18274746]
25. Souza HP, Frediani D, Cobra AL, Moretti AI, Jurado MC, Fernandes TR, Cardounel AJ, Zweier JL, Tostes RC. Angiotensin II modulates CD40 expression in vascular smooth muscle cells. *Clin Sci (Lond)*. 2009; 116:423–431. [PubMed: 18785879]
26. Godoy LC, Moretti AI, Jurado MC, Oxer D, Janiszewski M, Ckless K, Velasco IT, Laurindo FR, Souza HP. Loss of CD40 endogenous S-nitrosylation during inflammatory response in endotoxemic mice and patients with sepsis. *Shock*. 2010; 33:626–633. [PubMed: 20473113]
27. Marim FM, Silveira TN, Lima DS Jr, Zamboni DS. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PLoS One*. 2010; 5:e15263. [PubMed: 21179419]
28. Zamboni DS, Rabinovitch M. Nitric oxide partially controls *Coxiella burnetii* phase II infection in mouse primary macrophages. *Infect Immun*. 2003; 71:1225–1233. [PubMed: 12595436]
29. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 1992; 176:1693–1702. [PubMed: 1460426]
30. Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4⁺ T cells. *Proc Natl Acad Sci U S A*. 2009; 106:876–881. [PubMed: 19136631]
31. Witowski J, Pawlaczyk K, Breborowicz A, Scheuren A, Kuzlan-Pawlaczyk M, Wisniewska J, Polubinska A, Friess H, Gahl GM, Frei U, Jorres A. IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO α chemokine from mesothelial cells. *J Immunol*. 2000; 165:5814–5821. [PubMed: 11067941]
32. Forlow SB, Schurr JR, Kolls JK, Bagby GJ, Schwarzenberger PO, Ley K. Increased granulopoiesis through interleukin-17 and granulocyte colony-stimulating factor in leukocyte adhesion molecule-deficient mice. *Blood*. 2001; 98:3309–3314. [PubMed: 11719368]
33. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nature Reviews: Immunology*. 2010; 10:479–489.
34. Momcilovic M, Miljkovic Z, Popadic D, Miljkovic D, Mostarica-Stojkovic M. Kinetics of IFN- γ and IL-17 expression and production in active experimental autoimmune encephalomyelitis in Dark Agouti rats. *Neurosci Lett*. 2008; 447:148–152. [PubMed: 18852028]
35. Li L, Huang L, Vergis AL, Ye H, Bajwa A, Narayan V, Strieter RM, Rosin DL, Okusa MD. IL-17 produced by neutrophils regulates IFN- γ -mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *J Clin Invest*. 2010; 120:331–342. [PubMed: 20038794]

36. Takeuchi O, Akira S. RIG-I-like antiviral protein in flies. *Nature Immunology*. 2008; 9:1327–1328. [PubMed: 19008930]
37. Matzinger P. Friendly and dangerous signals: is the tissue in control? *Nature Immunology*. 2007; 8:11–13. [PubMed: 17179963]
38. Alisi A, Carsetti R, Nobili V. Pathogen- or damage-associated molecular patterns during nonalcoholic fatty liver disease development. *Hepatology*. 2011; 54:1500–1502. [PubMed: 22045668]
39. Pisetsky DS, Gauley J, Ullal AJ. HMGB1 and microparticles as mediators of the immune response to cell death. *Antioxidants and Redox Signaling*. 2011; 15:2209–2219. [PubMed: 21194388]
40. Ochiel DO, Rossoll RM, Schaefer TM, Wira CR. Effect of Estradiol and Pathogen Associated Molecular Patterns (PAMP) on Class II-Mediated Antigen Presentation and Immunomodulatory Molecule Expression in the Mouse Female Reproductive Tract. *Immunology*. 2011
41. Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA, Noelle RJ. Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. *Science*. 1993; 261:1328–1330. [PubMed: 7689748]
42. Adawi A, Zhang Y, Baggs R, Finkelstein J, Phipps RP. Disruption of the CD40-CD40 ligand system prevents an oxygen-induced respiratory distress syndrome. *Am J Pathol*. 1998; 152:651–657. [PubMed: 9502405]
43. van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol*. 2000; 67:2–17. [PubMed: 10647992]
44. Diaz-Montero CM, El Naggar S, Al Khami A, El Naggar R, Montero AJ, Cole DJ, Salem ML. Priming of naive CD8+ T cells in the presence of IL-12 selectively enhances the survival of CD8+CD62Lhi cells and results in superior anti-tumor activity in a tolerogenic murine model. *Cancer Immunol Immunother*. 2008; 57:563–572. [PubMed: 17726606]
45. Henry CJ, Grayson JM, Brzoza-Lewis KL, Mitchell LM, Westcott MM, Cook AS, Hiltbold EM. The roles of IL-12 and IL-23 in CD8+ T cell-mediated immunity against *Listeria monocytogenes*: Insights from a DC vaccination model. *Cell Immunol*. 2010; 264:23–31. [PubMed: 20483409]
46. Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest*. 2006; 116:1310–1316. [PubMed: 16670770]
47. Jiang XF, Cui ZM, Zhu L, Guo DW, Sun WY, Lin L, Wang XF, Tang YF, Liang J. CD40-CD40L costimulation blockade induced the tolerogenic dendritic cells in mouse cardiac transplant. *Int Surg*. 2010; 95:135–141. [PubMed: 20718320]
48. Nyakeriga AM, Ying J, Shire NJ, Fichtenbaum CJ, Chougnet CA. Highly active antiretroviral therapy in patients infected with human immunodeficiency virus increases CD40 ligand expression and IL-12 production in cells *ex vivo*. *Viral Immunol*. 2011; 24:281–289. [PubMed: 21830900]
49. Baker RL, Mallevaey T, Gapin L, Haskins K. T cells interact with T cells via CD40-CD154 to promote autoimmunity in type 1 diabetes. *Eur J Immunol*. 2012; 42:672–680. [PubMed: 22488364]
50. Sakai H, Okafuji I, Nishikomori R, Abe J, Izawa K, Kambe N, Yasumi T, Nakahata T, Heike T. The CD40-CD40L axis and IFN- γ play critical roles in Langhans giant cell formation. *Int Immunol*. 2012; 24:5–15. [PubMed: 22058328]
51. Noelle RJ. CD40 and its ligand in host defense. *Immunity*. 1996; 4:415–419. [PubMed: 8630727]
52. Katzman SD, Gallo E, Hoyer KK, Abbas AK. Differential requirements for Th1 and Th17 responses to a systemic self-antigen. *J Immunol*. 2011; 186:4668–4673. [PubMed: 21402892]
53. Harrington LE, Mangan PR, Weaver CT. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr Opin Immunol*. 2006; 18:349–356. [PubMed: 16616472]
54. Martinez GJ, Nuriyeva RI, Yang XO, Dong C. Regulation and function of proinflammatory TH17 cells. *Ann N Y Acad Sci*. 2008; 1143:188–211. [PubMed: 19076351]
55. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol*. 2010; 28:445–489. [PubMed: 20192806]

56. Hoeve MA, Savage ND, de Boer T, Langenberg DM, de Waal Malefyt R, Ottenhoff TH, Verreck FA. Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. *Eur J Immunol.* 2006; 36:661–670. [PubMed: 16482511]
57. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 2006; 24:179–189. [PubMed: 16473830]

Highlights

- Increased APCs and T cells were observed in 9V/null model of Gaucher disease
- Excess GC in these cell lead to increased stimulatory/costimulatory molecules
- These APCs and T-cells enhanced Th1, Th17, and PMN CXCL1/2 chemokine responses

Fig. 1. Increased Mφ with increased stimulatory and co-stimulatory molecules and excess of GC species in 9V/null mice

Single cell suspensions were prepared from the liver, spleen, lung, and bone marrow of 9V/null and background matched WT mice and analyzed by FACS (See Methods and Materials). (A) Absolute numbers of CD11b⁺ and F4/80⁺ positive Mφ in these tissues (ns, not significant; ***, p<0.001). (B–E) Mφ were purified from a single cell suspensions from the above tissues using CD11b microbeads, gated for positivity with F4/80 and stained for CD40, CD80, CD86, MHC-II and CD11b antibody reactivity (ns, not significant; **, p<0.01; ***, p<0.001). (F–I) GCs from purified Mφ of the indicated tissues were analyzed (ns, not significant; **, p<0.005; ***, p<0.001). WT (filled columns), 9V/null (open columns) and values represent in the mean±SD and group comparison were done by ANOVA in all figures. Three independent experiments were conducted for all Figures.

Fig. 2. Increased DCs with increased stimulatory and co-stimulatory molecules and excess of GC species in 9V/null mice

Single cell suspensions were prepared from 9V/null and WT mice, and DCs were gated on CD11c high cells on FSC/SSC compartment and stained for CD11b and CD11c markers. A) Absolute numbers of CD11c⁺ CD11b⁺ positive DCs from various tissues (ns, not significant; ***, p<0.001). (B–E) DCs were purified using CD11c Microbeads. These DCs were gated for CD11c^{high} positivity and stained for CD40, CD80, CD86, MHC-II and CD11b positivity (**, p<0.01; ***, p<0.001). (F–I) GC species were analyzed (see Methods and Materials) from purified DCs as in Figure 1 (ns, not significant; two asterisks, p<0.005; three asterisks, p<0.001). WT (filled columns), 9V/null (open columns) and values represent in the mean±SD and group comparison were done by ANOVA in all figures. Three independent experiments were conducted for all Figures.

Fig. 3. Increased CD4⁺ T cells with increased positivity for stimulatory molecules and excess of GC species in 9V/null mice

Single cell suspensions were from liver, spleen, lung, and bone marrow of WT and 9V/null mice. T cells were isolated by lymphocyte gating and stained with CD4 and CD8 antibodies. (A–B) Absolute numbers of CD4⁺ CD8⁺ T cells and CD8⁺ CD4⁺ T cells are shown from various tissues (ns, not significant; two asterisks, $p < 0.01$; three asterisks, $p < 0.001$). (C–D) CD4⁺ T cells were purified from single cell suspensions using a CD4 (L3T4) T cell isolation kit and counter stained with CD4/CD69, and CD4 CD40L antibodies. WT (filled column) and 9V/null (open column) FACS analyses from the different tissues are shown (ns, not significant; ***, $p < 0.001$). (E–G) GC species were analyzed from purified CD4⁺ T cells (ns, not significant; two asterisks, $p < 0.005$; three asterisks, $p < 0.001$).

Fig. 4. Serum Cytokines in 9V/null mice
IFN- γ (A), IL12p40 (B), TNF- α (C), Th17A/F (D), IL-6(E), TGF β (F), and IL4(G) concentrations in serum of WT (n=20, filled column) and 9 V/null mice (n=20, open column) were determined by ELISA (mean $\pm$ SD). Group comparisons were with the student's t test (**, p<0.01; ***, p<0.001).

Fig. 5. CD3/CD28 mediated activation of T cells and cytokine production in 9V/null mice Liver, spleen, and lung cells from WT (filled column) and 9V/null (open column) mice were treated with rat-antimouse CD3 and CD28 antibodies. Culture supernatants were harvested 96 hrs. after T cell stimulation and assayed for IFN- γ (A), TNF α (B), IL12p40 (C), IL12p70 (D), IL23 (E), IL6 (F), IL17A/F (G), and IL4 (H) cytokines. Data are representative of three independent experiments. Values (mean $\pm$ SD) and group comparisons were by ANOVA (ns, not significant, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig. 6. Activated 9V/null APCs and CD4⁺T cells show increased mean fluorescence intensity for CD40 and CD40L

Bone marrow generated Mφ and DCs and splenic CD4⁺T cells from WT (filled column) and 9V/null (open column) mice were activated in the presence and absence of IFN γ and rat-antimouse CD3 and CD28 antibodies. After incubation, CD40 positivity on Mφ (A) and DCs (B) and CD40L positivity on CD4⁺T cells (C) were measured by FACS. In a separate experiment, dendritic cells and CD4⁺T cells purified from WT and 9V/null liver, spleen, and lung were co cultured in the presence and absence of GC. After incubation, the frequencies of CD40 positive DCs (D–E) and CD40L positive CD4⁺T cells (F–G) were determined by FACS. Data are representative of three independent experiments (mean $\pm$ SD), and group comparisons were done by ANOVA (ns, not significant, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig. 7. CD40-CD40L ligation between dendritic cells and CD4⁺T is critical for Th1 and Th17 cytokines production

Dendritic cells and CD4⁺T cells purified from WT (filled column) and 9V/null (open column) liver, spleen, and lung were stimulated by adding GC. After incubation, concentrations of IFN γ (A), TNF α (B), IL-12p40 (C), IL-12p70 (D), IL-23(E), IL-6(F), IL-17A/F (G), and IL-4 (H) cytokines in culture supernatants were determined by ELISA. Data are representative of three independent experiments (mean $\pm$ SD), and group comparisons were done by ANOVA (ns, not significant, p>0.05; **, p<0.01; ***, p<0.001).