AWARD NUMBER: W81XWH-16-1-0384
PR152294

TITLE:

Bioactive Sphingolipids and Wound Healing

PRINCIPAL INVESTIGATOR: Mariana Nikolova-Karakashian

CONTRACTING ORGANIZATION: University of Kentucky
Lexington, KY 40526

REPORT DATE: September 2018

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
SEPT 2018	Final	1AUG2016 - 31MAY2018

4. TITLE AND SUBTITLE
Bioactive Sphingolipids and Wound Healing

5a. CONTRACT NUMBER
W81XWH-16-1-0384

5b. GRANT NUMBER
PR152294

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Mariana Nikolova-Karakashian

E-Mail: mnikolo@uky.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Kentucky
Department of Physiology
Chandler Medical Center, MS 508
800 Rose Street
Lexington, KY 40536-0298

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Difficult to heal wounds are of significant health concern for military personnel and their family, especially those linked to diabetes, since diabetes has reached epidemic proportions in the Western Society. Despite being linked to high mortality, low quality of life and other health complications, wounds that are difficult to heal are still a mystery, and most pharmacologic regimens are ineffective. One of the key finding of this this proposal is that sphingomyelinase, a protein that exhibit regulatory function and determines the extent of inflammation and proliferation at the wound site is defective during obesity and this insufficiency leads to exacerbation of TNFalpha production by macrophages. . More importantly we show that addition of the product of this protein, termed ceramide or its metabolites to the wounds helps wound repair and decreases pain-associated with these wounds in the mouse. Someone unexpectedly we also found that the mechanisms by which ceramide and its key metabolite, Sphingosine-1-phosphate facilitate wound repair are distinct, evident by a distinct temporal pattern of healing of wounds following the treatment with ceramide and with Sphingosine-1-phosphate. Overall, our experiments confirm the main hypothesis and indicate that ceramide, and sphingosine-1-phosphate are likely therapeutic tools to help the wound healing process in diabetic patients.

15. SUBJECT TERMS
Diabetes, macrophages, ceramide, Sphingosine-1-phospate

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	14	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

The subject of this research was the mechanism of delayed wound healing. Chronic wounds present a major morbidity and life-treating complication in diabetic patients. Normal wound healing involves three stages. First comes inflammation, then new tissue formation, and finally tissue remodeling. These are regulated in a temporal, spatial, and cell-type specific manner. Wounds that fail to repair, however, often show signs of chronic inflammation without progression to latter stages of wound healing. The purpose of this research was to test an innovative hypothesis that an enzyme called Lysosomal Sphingomyelinase (L-SMase) and its metabolic products ceramide and sphingosine-1-phosphate (S1P) coordinately regulate inflammation, angiogenesis, and proliferation but which function is impaired in diabetes. The scope of the research involved experimentation using mice to test first the effects of diabetes on sphingomyelinase and second whether substitution of sphingomyelinase products, ceramide and Sphingosine-1-phosphate will accelerate the wound healing process.

KEYWORDS:

Diabetes type II
Obesity
Impaired wound healing
Tumor necrosis factor alpha
Sphingomyelinase
Ceramide
Sphingosine-1-phosphate
Serum markers

1. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Major Task 1: Decipher the role of L-SMase in the wound healing process
Major Task 2: Test whether serum S-SMase activity is a diagnostic and treatment biomarker of delayed wound healing.
Both tasks were accomplished by 80%

- **What was accomplished under these goals?**
Accomplishment 1: According to the hypothesis proposed in Major Task 1, obesity leads to a decline in the activity of lysosomal sphingomyelinase in macrophages which is associated with elevated secretion of pro-inflammatory cytokine, TNFa which serves to stall the wound healing process in the first, inflammatory stage and delay the progression into proliferative phase. To test this we perform studies in three different model of obesity. Mice fed Western diet (containing 35% saturated fat and 1.5% cholesterol), Mice fed High fat diet (65% of calories from fat, no cholesterol) and ob/ob mice, which is a genetic model of obesity. Following 12 weeks on diet, mice were sacrificed peritoneal macrophages were isolated and cultured, serum was

collected and the activity of the lysosomal (in the macrophages) and secretory (in the serum or in the conditioned medium) form of sphingomyelinase were measured by HPLC. In both cases of diet induced-obesity, but not in the genetic model, the ob/ob mice) there feeding high fat diet was associated with significantly lower Lysosomal Sphingomyelinase activity. In all three models of obesity the activity of S-SMase in serum was substantially elevated in obese animals. These effects were seen in both, male and female mice and were not dependent on the age of the animals. A representative datum is shown in Fig. 1.

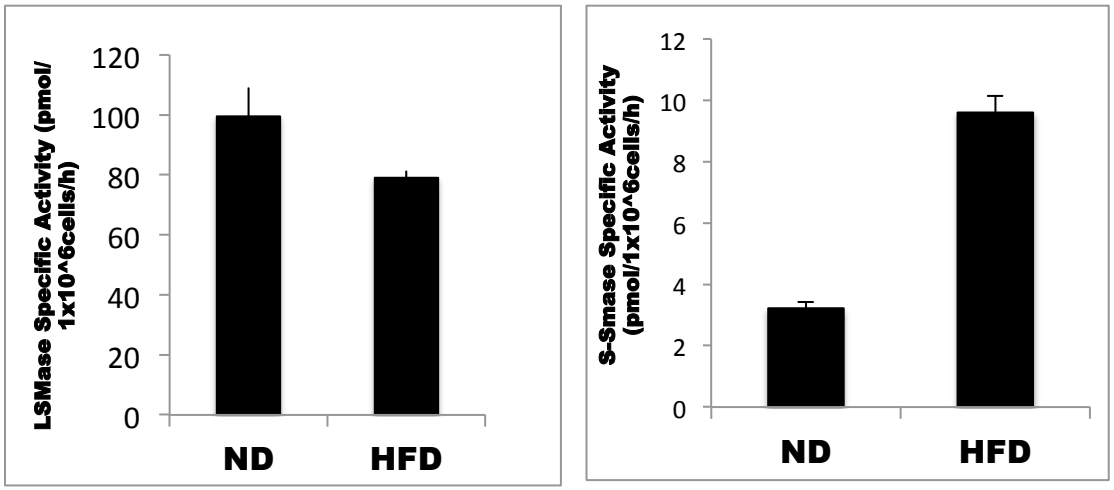

Fig.1. Suppression of macrophage lysosomal sphingomyelinase and stimulation of serum secretory sphingomyelinase in obese rodents. C57Bl6 mice (6 per group) fed standard rodent show (ND) or high fat diet (HFD) for 12 weeks. Lysosomal sphingomyelinase measured in isolated peritoneal macrophages and in serum by HPLC and fluorescence

Since peritoneal macrophages could be different then macrophage isolated from wound, we performed a studied where obese and lean mice were wounded. The wounds were allowed to heal for 5 days and in collaboration with Dr. Ashley Seifert at the College of art and Science at the University of Kentucky, we isolated macrophages from the wounds. These wound macrophages exhibited trends consistent with the changes in peritoneal macrophages and macrophages from wounds of obese animals had lower S-SMase activity. More importantly using flowcytometry, we assessed the levels of TNFa produced in the wound macrophages and they were significantly elevated (by almost 30% higher) in the wound macrophages form obese mice as compared to those of lean animals.

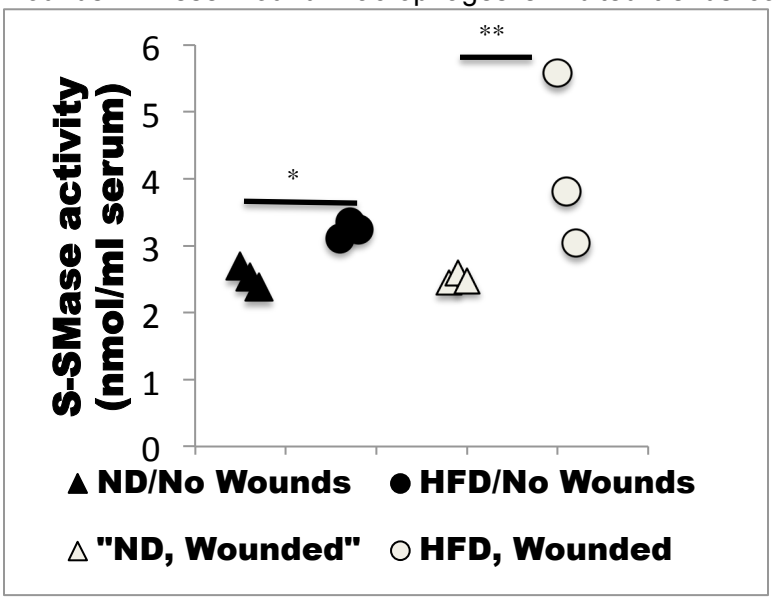

Fig. 2. Changes in serum S-SMase during obesity/wounding.

***Conclusion**: These results showed that indeed diet-induces obesity is*

associated with lower macrophage sphingomyelinase activity, which leads to elevated TNFa production. These observations completely support the hypothesis in the grant application.

Accomplishment 2: According to our hypothesis, serum S-SMase activity could be a diagnostic biomarker of delayed wound healing. All animal experiments consistently showed that serum S-SMase activity is elevated during obesity. In the case of diet-induced obesity but not in ob/ob mice, these increases negatively correlate with the rate of Lysosomal sphingomyelinase. These results suggest that for diet-induced obesity, S-SMase could be a marker of the rate of wound healing.

Somewhat surprisingly, wounding of obese mice alone led to further stimulation of S-SMase activity (Fig. 2), an observation, which significance we are still investigating.

Conclusions and further studies. These animal studies consistently show that elevation in S-SMase activity is linked to obesity, but also to the wounding process. Ongoing studies aimed to test this hypothesis in humans.

Accomplishment 3: To directly test the role of decreased lysosomal sphingomyelinase and its products, ceramide and sphingosine-1-phosphate as a causative factor in delaying wound healing, we treated split wound for up to 6 days with formulations containing ceramide , sphingosine-1-phosphate of vehicle. These experiments were done in collaboration with Dr. Brad Taylor, a leading expert in pain and inflammation. As shown in Fig. 3 the ectopic application of these lipid metabolites, but not dihydroceramide, a biologically inactive ceramide analogue were highly effective in promoting wound healing process.

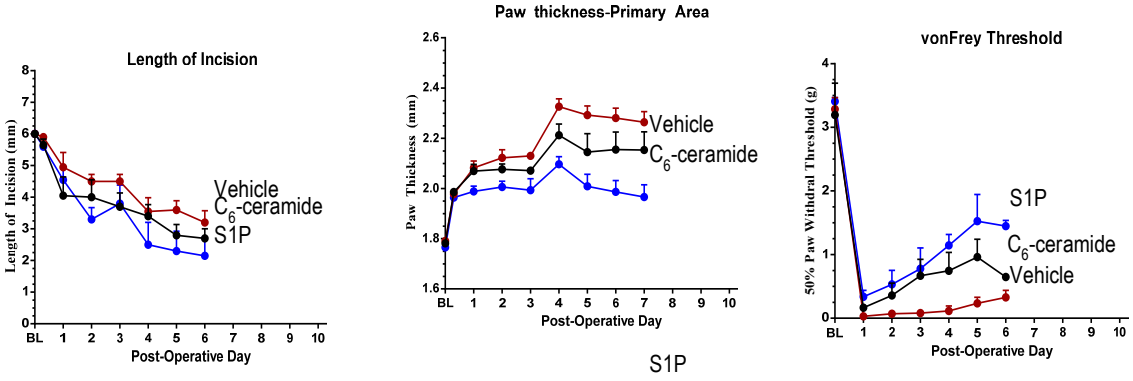

Fig. 3. Ceramide and sphingosine-1-phosphate (S1P) both facilitate wound healing, reduce inflammation and pain in mouse model.

Conclusion: *Exogenous delivery of ceramide and sphingosine-1-phosphate promotes wound healing, decreases inflammation and reduces pain.*

- **What opportunities for training and professional development has the project provided?**

Nothing to Report.

- **How were the results disseminated to communities of interest?**

The results were present at 2 conferences as oral presentations:

South Eastern Regional Lipid Conference, Cashiers, NC, November 2017

The Sphingolipid Club, Italy, October, 2017

A publication is being prepared for submission to the Journal of Lipid Reports

- **What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to Report

2. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

Our studies provided very intriguing data in regards to negative regulation of the enzymatic activity of lysosomal Sphingomyelinase and offered the first evidence that by affecting the levels of TNFa, this enzyme is potential new target for the treatment of impaired wound healing in diabetic patients.

- **What was the impact on other disciplines?**

Nothing to Report

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

3. **CHANGES/PROBLEMS:**

Nothing to Report

4. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  Report only the major publication(s) resulting from the work under this award.

  - **Journal publications.**

    Manuscript in preparation:

    "Secretory sphingomyelinase and macrophage functions during obesity: implication of ceramide in wound healing". Deevska, G, Taylor, B.K, Donahue, R.R., Simkin, J., Seifert, A., Karakashian, A., Nikolova-Karakashian, M.N. For submission to J. Lipid Research

  - **Other publications, conference papers, and presentations.**

1. *"Secretory Sphingomyelinase and macrophage functions during obesity" Deevska, G and Nikolova-Karakashian, , oral presentation for the 52nd Southeastern Regional Lipid Conference, High Hampton inn, Cashiers, NC, Nov. 8010, 2017*

See appendix for abstract

2. *"Secretory Sphingomyelinase and macrophage functions during obesity" Deevska, G and Nikolova-Karakashian, oral presentation for the XII Sphingolipid Club Meeting and SLC Advanced Course, Trabia, Italy, Sept. 6-10, 2017*

See appendix for abstract.

- **Website(s) or other Internet site(s)**

  Nothing to report

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

  Nothing to report

5. **Other Products**

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Mariana Nikolova-Karakashian, no change

Liang, Ying, no change.

Alex Karakashian, no change

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

- **What other organizations were involved as partners?**

Nothing to Report

6. **SPECIAL REPORTING REQUIREMENTS**

- **N/A**

7. **APPENDICES:**

XII Sphingolipid Club Meeting

And

SLC Advanced course

Meeting Proceedings

Trabia (Sicily, Italy)

Hotel Torre Artale

Sept. 6-10, 2017

T11

SECRETORY SPHINGOMYELINASE AND MACROPHAGE FUNCTIONS DURING OBESITY

Deevska, G, and Mariana Nikolova-Karakashian

University of Kentucky College of Medicine, Department of Physiology, Lexington KY.

Acid sphingomyelinase gene encodes two forms of acid sphingomyelinase, the lysosomal (L-SMase) and the secretory (S-SMase). Elevated S-SMase activity has been found in patients with heart failure, sepsis, Hepatitis C, and fatty liver disease, as well as in many animal models. Our earlier studies have shown that obesity is also associated with elevated serum S-SMase. The goal of the current study is to evaluate the mechanism and consequences of these obesity-associated increases in S-SMase.

C57Bl6 mice fed high fat (12 weeks) and ob/ob mice were used in these experiments. Diet-induced obesity was associated with elevated serum S-SMase activity. *Ex vivo* studies with peritoneal macrophages and aortic discs from obese and lean mice suggested that increased macrophage secretion is likely responsible for the elevated serum S-SMase activity. The increased in S-SMase release was accompanied by a reciprocal decline in the activity of L-SMase, suggesting a shift in the post-translational processing of the ASMase precursor. To test whether this shift has an impact on macrophage function, wound-healing model was used. Obese and slim C57Bl6 mice received 4 dorsal wounds. Activated macrophages, isolated from the wounds, exhibited 6-8 fold higher-S-SMase activities as compared to peritoneal macrophages. Obesity was associated with even greater increases in S-SMase and decline in L-SMase. Wound macrophages isolated from obese mice also exhibited higher macrophage TNFα release. Loss- and gain- of function studies indicated that both, the decline in L-SMase and the increase in S-SMase seen in obese animals, influence TNFα secretion in response to wounding or direct LPS stimulation.

These results suggest that obesity is associated with a shift in the processing of acid SMase in activated macrophages, which impacts the capacity to secrete TNFα. Earlier studies have indicated that the underlying mechanism likely involves regulation of the activity of TNFα converting enzyme, TACE.

Acknowledgements. This work was supported by CDMRP Discovery Award PR152294

10:30-12:05 : *Session 3 - SL Enzymology and Metabolism*
Chairs: ***A. Gomez-Muñoz, E. Meacci***

10:30-10:50: **J. Fernandez-Checa** Role of acid sphingomyelinase in drug-induced liver injury and steatohepatitis

10:50-11:05: **H. Le Stunff** Ceramide metabolism in hypothalamus causes insulin central resistance and dysregulation of glucose homeostasis during obesity

11:05-11:20: **M. Nikolova-Karakashian** Secretory sphingomyelinase and macrophage functions during obesity

11:20-11:35: **C. Bandet** The ceramide transporter CERT is a regulator of lipid-induced insulin resistance in muscles

11:35-11:50: **F. Bonezzi** Myriocin as post-conditioning therapeutic in myocardial ischemia/ reperfusion damage: regulation of sphingolipid metabolism for the recovery from infarction

11:50-12:05: **N. Krupenko** CERS6 is a key mediator of the ceramide response to dietary folate in mouse liver

12:05-12:20: **A. Huwiler** Sphingosine kinase-2 deficiency ameliorates kidney fibrosis in a mouse model of unilateral ureteral obstruction by upregulating Smad7

12:20-13:00 : *Session 4 - SLs in Sepsis and Infection*
Chairs: ***P. Signorelli, M. Machala***

12:20-12:50: **M. Gräler** The role of sphingosine-1-phosphate in sepsis

12:50-13:00: **M. Del Poeta** Exploiting sphingolipids against fungal infections

13:00 : Lunch

14:15-15:15 : Poster flash presentations (2 min/2 slides)
Chairs: ***A. Caretti, A. Huwiler***

15:15-16:30 : *Session 5 - SLs in Cell Death, Survival and Differentiation*
Chairs: ***M. Krönke, E. Albi***

15:15-15:30: **D. Leonetti** Circulating acid sphingomyelinase and ceramide contribute to radiation-induced intestinal toxicity

15:30-15:45: **F. Bilal** Sphingomyelin synthase 1 (SMS1) downregulation contributes to CD95L and TRAIL resistance in melanoma

15:45-16:00: **L. Anastasia** The physiological role of sialidase NEU3

16:00-16:15: **F. Peters** Ceramide synthase 4 coordinates stem cell maintenance and cell fate decision

16:15-16:30: **F. Paris** Change in sphingolipids profiles in endoplasmic reticulum, mitochondria-associated membranes and mitochondrion upon induction of apoptosis

16:30 : Free time

20:30 : Dinner

52nd Annual

Southeastern Regional Lipid Conference

High Hampton Inn, Cashiers, NC

November 8-10, 2017

9:30 AM	**Mapping the transmembrane topology of mammalian ORMDL proteins in the endoplasmic reticulum using the Substituted Cysteine Accessibility Method (SCAM™)** *Davis, D., Suemitsu, J., Wattenberg, B.*	Pavilion
9:45 AM	**Alterations of dietary folate induce sphingolipid response in mouse liver** *Barron, K., Krupenko, N.*	
10:00 AM - 10:30 AM	Coffee Break	Pavilion
10:30 AM - 12:00 PM	**Oral Session IV** **Lipids in Pathobiology and Regenerative Medicine** **Chair: David Montefisco**	Pavilion
10:30 AM	**Glycosphingolipid Catabolism Mediates Mesangial Cell IL-6 Production** *Rodgers, J., Sundararaj, K., Drake, R., Janech, M., Oates, J., Nowling, T.*	Pavilion
10:45 AM	**Non-Canonical d16-Base Sphingolipids May Protect Hepatocytes from Alcohol Toxicity by Activating Autophagy** *Cowart, L.A., Roddy, P., Montefusco, D.*	Pavilion
11:00 AM	**Sphingolipids and Brain White Matter in Aging and Mild Cognitive Impairment** *Benitez, E., Hammad, S.*	Pavilion
11:15 AM	**Secretory Sphingomyelinase And Macrophage Functions During Obesity** *Deevska, G, Nikolova-Karakashian, M.*	Pavilion
11:30 AM	**Engineered delivery of sphingosine 1-phosphate receptor compounds improves volumetric muscle defect healing** *San Emeterio, C., Ogle, M., Botchwey, E.*	Pavilion
11:45 AM	**Cream-based Phosphatidylglycerol Inhibited Epidermal Hyperplasia in an Imiquimod-induced Mouse Model of Psoriasis** *Choudhary, V., Cohen, E., Uaratanawong, R., Zhang, C., Bollag, W.*	Pavilion
12:00 PM - 12:30 PM	Business Meeting Co-Chair: Guanghu Wang, PhD Selection of 2018 Co-Chair	Pavilion
12:30 PM - 1:30 PM	Lunch	Dining Room
1:30 PM	Conference Adjournment	

Abstract #22

Secretory Sphingomyelinase and Macrophage Functions During Obesity

Deevska, G, and Mariana Nikolova-Karakashian

University of Kentucky College of Medicine, Department of Physiology, Lexington, KY

Acid sphingomyelinase gene encodes two forms of acid sphingomyelinase, the lysosomal (L-SMase) and the secretory (S-SMase). Our earlier studies have shown that obesity is associated with elevated serum S-SMase. The goal of the current study is to evaluate the mechanism and consequences of these obesity-associated increases in S-SMase. C57Bl6 mice fed high fat and ob/ob mice were used in these experiments. *Ex vivo* studies with peritoneal macrophages and aortic discs from obese and lean mice suggested that increased macrophage secretion is likely responsible for the elevated serum S-SMase activity. The increased in S-SMase release was accompanied by a reciprocal decline in the activity of L-SMase, suggesting a shift in the post-translational processing of the ASMase precursor. To test whether this shift has an impact on macrophage function, wound-healing model was used. Obese and slim C57Bl6 mice received 4 dorsal wounds. Activated macrophages, isolated from the wounds, exhibited 6-8 fold higher-S-SMase activities as compared to peritoneal macrophages. Obesity was associated with even greater increases in S-SMase and decline in L-SMase. Wound macrophages isolated from obese mice also exhibited higher macrophage TNFα release. Loss- and gain- of function studies indicated that both, the decline in L-SMase and the increase in S-SMase seen in obese animals, influence TNFα secretion in response to wounding or direct LPS stimulation. These results suggest that obesity is associated with a shift in the processing of acid SMase in activated macrophages, which impacts the capacity to secrete TNFα. Earlier studies have indicated that the underlying mechanism likely involves regulation of the activity of TNFα converting enzyme, TACE. This work was supported by CDMRP Discovery Award PR152294