Regulation of Fluid-phase Uptake in Podocytes by Albumin-associated Lipids

Jun-Jae Chung
Washington University in St. Louis

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Regulation of Fluid-phase Uptake in Podocytes by Albumin-associated Lipids

and

Role of NKG2D in Obesity-induced Adipose Tissue Inflammation and Insulin Resistance

By

Jun-Jae Chung

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2014

St. Louis, Missouri
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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>7AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>8-bromo-cAMP</td>
<td>8-Bromoadenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>8-CPT-2Me-cAMP</td>
<td>8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acetyl-coenzyme A acetyltransferase</td>
</tr>
<tr>
<td>acetyl-LDL</td>
<td>Acetylated-low density lipoprotein</td>
</tr>
<tr>
<td>Angptl4</td>
<td>Angiopoietin-like 4</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>3'-5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD2AP</td>
<td>CD2-associated protein</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Macrophage colony-stimulating factor-1</td>
</tr>
<tr>
<td>CTLs</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet-induced obese</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>EPA</td>
<td>Dicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETA</td>
<td>Eicosatrienoic acid</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FFAR</td>
<td>Free fatty acid receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fLuc</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FSGS</td>
<td>Focal segmental glomerular sclerosis</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance tests and</td>
</tr>
<tr>
<td>H60</td>
<td>Histocompatibility 60</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>H-ESRD</td>
<td>Hypertension-associated ESRD</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HIVAN</td>
<td>HIV-associated nephropathy</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
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viii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance tests</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MALD</td>
<td>Mapping by admixture linkage disequilibrium</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MIC</td>
<td>MHC class-I polypeptide-related chain</td>
</tr>
<tr>
<td>MULT1</td>
<td>ULBP-like transcript 1</td>
</tr>
<tr>
<td>ND</td>
<td>Normal diet</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural-killer group 2, member D</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood nononuclear cells</td>
</tr>
<tr>
<td>pCMB</td>
<td>p-Chloromercuriobenzoate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>PI-3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RAE</td>
<td>Retinoic acid early transcript</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Cell Expressed and Secreted</td>
</tr>
<tr>
<td>rGM-CSF</td>
<td>Recombinant granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific Pathogen Free</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>ULBP</td>
<td>Cytomegalovirus UL16-binding proteins</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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</tbody>
</table>
ACKNOWLEDGMENTS

The past several years spent as a graduate student has been a quite the rollercoaster ride. It was a complicated collage of hope, disappointment, joy, despair, excitement, and irritation, all on a canvas of constant low-level angst. As I am not far removed enough from my doctoral candidacy, it’s still unclear to me what this piece of work looks like. I suppose it will become clearer in time. One thing for certain is that many pieces of the mosaic were influenced by the many people I had the pleasure of interacting with on a daily basis. To these people I would like to express my deep gratitude.

All of the work done would not have been possible without the support and guidance of my advisor Dr. Andrey Shaw. He provided an environment for me to find and explore topics that caught my interests while somehow managing to keep it integrated into the many other research projects going on in the lab. The brilliance and perseverance he demonstrates in asking interesting questions and pursuing them has been a true inspiration.

I am grateful to the past and present members of the Shaw lab. Not only did they provide me with great ideas and comments with regard to my research, the intelligence and diligence of many spurred me to become a better researcher. But most of all, I want to thank them for all the memorable quotes and moments. I want to specially thank Shuba for being a good a benchmate as one could hope for, and Al for being such a great friend and big brother.

I would like to thank my thesis committee members. I was fortunate to have a committee that included members from a diverse research background to guide me through my somewhat disjointed research projects.
Last but not least, I want to mention my friends and family. I want to thank my friends John and Jinho for all the great times. My parents have always been my inspiration and role models. I want to thank them for their unwavering love and support. I cannot express the depth of my appreciation and gratitude for Jiyeon, my wife, who has been with me every step of the way. Finally, the saddest moment during my time here was when I lost my older brother, Won-Jae. I will always remember his great passion and dedication for any and all of his endeavors and his amazing sense of wit and humor.
Podocytes are specialized epithelial cells in the kidney glomerulus that play important structural and functional roles in maintaining the filtration barrier. In nephrotic syndrome, a major breakdown of the kidney filtration barrier associated with proteinuria, hyperlipidemia, and edema, podocytes undergo changes in morphology and appear to internalize serum proteins. We postulated that fluid-phase uptake by podocytes might play a role in maintaining the integrity of the filtration barrier. Using fluid-phase tracers, we show that podocytes *in vivo* actively internalize fluid from the serum and that the rate of internalization is enhanced when the filtration barrier is disrupted. *In vitro* experiments demonstrated that lipids associated with serum albumin stimulate macropinocytosis in podocytes. This process was specific to podocytes as known stimuli that induce macropinocytosis in other cells had no effect on podocytes, while
serum lipids did not stimulate macropinocytosis in other cells. A candidate lipid approach showed that certain unsaturated free fatty acids stimulate macropinocytosis through G protein coupled receptors, FFAR1, FFAR2 and FFAR3 and the Gβ/Gγ complex. Meanwhile, long-chain fatty acids were found to induce apoptosis in podocytes. These results suggest that podocytes sense the disruption of the filtration barrier via free fatty acids carried by albumin and respond to the increased protein by enhancing fluid-phase uptake. However, alterations in free fatty acids to albumin ratio during nephrotic syndrome may lead to dysregulation of macropinocytosis and podocyte toxicity and play an important role in the development of podocyte diseases.

II

The early events that initiate inflammation in the adipose tissue during obesity are not well defined. It is unclear whether the recruitment of CD8 T cells to the adipose tissue during onset of obesity occurs through antigen-dependent or –independent processes. We have previously shown that interaction between NKG2D (natural-killer group 2, member D) and its ligand Rae-1ε is sufficient to recruit cytotoxic T lymphocytes to the pancreas and induce insulitis. Here, we tested to see whether NKG2D-NKG2D ligand interaction is also involved in obesity-induced adipose tissue inflammation and insulin resistance. We observed a significant induction of NKG2D ligand expression in the adipose tissue of obese mice, especially during the early stages of obesity. However, mice lacking NKG2D developed similar levels of insulin resistance and adipose tissue inflammation compared to control mice when placed on a high fat diet. Moreover, overexpression of Rae-1ε in the adipose tissue did not increase immune cell infiltration to the adipose tissue neither in the setting of a normal or high fat diet. These results
indicate that, unlike in the pancreas, NKG2D-NKG2D ligand interaction does not play a critical role in obesity-induced inflammation in the adipose tissue.
CHAPTER I

Regulation of Fluid-phase Uptake in Podocytes by Albumin-associated Lipids
INTRODUCTION

The kidney glomerular filter is a size- and charge-selective barrier that allows the filtration of blood without the loss of large serum proteins into the urine. The filter is composed of three layers - fenestrated endothelial cells, the glomerular basement membrane (GBM), and podocytes (also known as visceral epithelial cells) (Haraldsson et al. 2008). Although the fenestrations in the endothelial layer are relatively large (approximately 70 nm to 100 nm in diameter) and would allow free filtration of fluid and proteins, the fenestrations are covered by a glycocalyx-like layer that can provide partial barrier functions. The GBM, which consists mainly of laminin, type IV collagen, nidogen, and heparan sulfate proteoglycans, is thought to act as a size- and charge-selective filter or gel. On the outer aspect of the GBM, podocytes spread out across capillary loops and form a tight interdigitating network of cytoskeletal extensions known as foot processes. The space between adjacent foot processes is covered by a slit diaphragm, which is composed of cell surface proteins such as nephrin, Neph1, and P-cadherin.

Despite significant progress, the exact mechanism of glomerular filtration is yet to be completely understood and has been the subject of modeling studies (Edwards et al. 1999; Deen et al. 2001; Lazzara and Deen 2001; Smithies 2003). These current models predict that the size-selectivity of the GBM is not 100% effective and that it is, at least to a certain extent, permeable to serum proteins. Typically, in humans, approximately 180 liters of blood is filtered by the kidney daily. Even if 99.9% of albumin is prevented from crossing the GBM, as predicted by the modeling studies, the high volume of renal blood flow would still result in a significant amount of serum protein crossing the GBM. 0.1% of this blood flow would mean that nearly 9 grams of albumin and 180 mg of immunoglobulin G (IgG) cross the GBM every 24 hours. Since the
slit diaphragm is considered to be impermeable to large protein molecules such as albumin, we considered the possibility that podocytes have a mechanism to actively transport these proteins from the GBM into the primary urinary space.

The notion that podocytes are capable of internalizing and removing proteins from the GBM was proposed over 40 years ago (Farquhar et al. 1957; Farquhar et al. 1957). Numerous studies using electron microscopy have shown protein-containing vesicles in podocytes under normal and, to an enhanced degree, under nephrotic conditions (Farquhar and Palade 1960; Farquhar and Palade 1961; Farquhar et al. 1961; Vogt et al. 1968; Venkatachalam et al. 1970; Caulfield and Farquhar 1974). The constitutive expression of the neonatal Fc receptor, a transcytosis receptor for immunoglobulin and albumin, as well as other receptors for immunoglobulin and complement, suggests that podocytes may handle this protein by internalization and transcytosis (Akilesh et al. 2008).

Fluid-phase uptake in cells occurs through two distinct processes (Doherty and McMahon 2009). Micropinocytosis occurs either during the receptor-mediated endocytosis in a clathrin-dependent manner or as a constitutive process that internalizes fluids in clathrin-independent vesicles. Micropinocytosis results in small vesicles less than 0.1 μm in diameter. The second process, macropinocytosis, involves the formation of large, 0.2 - 5 μm vesicles that form at sites of membrane ruffling (Kerr and Teasdale 2009). Macropinocytosis is clathrin-independent but requires actin, Rac and PI-3 kinase (PI3K). In certain cells like immature dendritic cells and macrophages, macropinocytosis occurs as a constitutive process while in other cells, macropinocytosis can be induced by extracellular stimuli like growth factors. It is a highly efficient process allowing internalization of up to one cell volume of fluid/hour. Because of the
larger size of the vesicles, macropinocytosis is also more efficient for the uptake of molecules that are larger than 100 kDa in size.

Here we investigated the endocytic properties of podocytes and discovered an unexpected high capacity of podocytes to carry out macropinocytosis. We found that macropinocytosis in podocytes is regulated as it could be induced in response to lipids that were carried by albumin. Unlike previously identified regulators of macropinocytosis, we found that lipid-binding G protein-coupled receptors (GPCRs) and Gβ/Gγ subunits were involved in stimulating macropinocytosis in podocytes. We propose that exposure of podocytes to albumin-associated lipids may act as a signal to trigger macropinocytosis and potentially allow for transcytosis of proteins from the GBM into the primary urinary space.
MATERIALS AND METHODS

Reagents

FITC-Ficoll (70 kDa), FITC-dextran (10, 70, 150, 500, 2000 kDa), bovine serum albumin, mouse serum IgG, mannan, p-Chloromercuriobenzoate (pCMB), wortmannin, cytochalasin D, recombinant EGF, ovalbumin, 8-Br-cAMP, 8-pCPT-2′-O-Me-cAMP, forskolin, 3-isobutyl-1-methylxanthine (IBMX), isoproterenol, fluorescein, anti-podocin Ab, and anti-β-actin Ab were obtained from Sigma (St. Louis, MO). Fatty acid-free bovine serum albumin was purchased from SeraCare Life Sciences (Milford, MA). Human serum albumin was purchased from Williams Medical Company (Placentia, CA). Recombinant PDGF was purchased from Life Technologies (Carlsbad, CA). Anti-phospho-ERK Ab was obtained from Cell Signaling Technology (Danvers, MA). Rhodamine B-dextran (70 kDa) was purchased from Nanocs (New York, NY). Oleic acid, eicosatetraenoic acid, eicosapentaenoic acid, and docosahexaenoic acid were obtained from Nu-Chek Prep (Elysian, MN). Gallein was purchased from Tocris Bioscience (Bristol, UK). Anti-Gβ1 and anti-Gβ2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- laminin α5 (Miner et al. 1997) and anti-CD2AP (Dustin et al. 1998) antibodies have been previously described.

Mice

All mice were housed in a specific pathogen-free facility at Washington University School of Medicine. C57BL/6J and 129/SvJ mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Laminin β2 knockout (lamb2–/–) mice with muscle-specific rescue transgene
has been previously described (Noakes et al. 1995; Jarad et al. 2006; Miner et al. 2006). For
BSA injection experiments, mice (C57BL/6J and 129/SvJ mixed background) were given daily
intraperitoneal injection of endotoxin-free bovine serum albumin (15 mg/g body weight in PBS)
for 4 days. Care and use of mice were conducted in accordance with protocols approved by the
Animal Studies Committee at Washington University in St. Louis (Protocol Number: 20120013),
in compliance with the Animal Welfare Act.

**Cell culture**

Conditionally immortalized podocytes were generated as described previously (Mundel
et al. 1997) and maintained at 33°C in RPMI1640 supplemented with 10% fetal bovine serum, 1
mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM β-
mercaptoethanol, and 100 U/ml recombinant mouse IFNγ (a gift from Robert Schreiber,
Washington University, St. Louis, MO).

Bone marrow-derived dendritic cells were cultured as previously described (Lutz et al.
1999). Briefly, bone marrow cells were cultured in IMDM supplemented with 10% fetal bovine
serum (Hyclone), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml
streptomycin, 0.1 mM β-mercaptoethanol, and 100 ng/ml rGM-CSF (R&D System, Wiesbaden-
Nordenstadt, Germany) for eight days.

**Electron microscopy**

Slices of kidney cortex or podocytes cultured on coverslips were fixed overnight in 4%
paraformaldehyde and 4% glutaraldehyde in 0.1 M sodium cacodylate buffer followed by
repeated washes in PBS. After postfixation in 1% osmium tetraoxide, the samples were reduced with 1.5% potassium ferrocyanide for 1 hr at 4°C. Subsequently, the samples were washed, dehydrated in graded alcohol, and embedded in resin according to standard protocols.

**In vivo endocytosis**

Mice were injected intravenously with a bolus of FITC-Ficoll (70 kDa) or FITC-dextran (10 kDa) in 0.9% NaCl at a dose of 250 µg/g of body weight. 24 hrs after injection, kidney was harvested from mice, embedded in OCT (VWR, Radnor, PA), and snap-frozen in methylbutane cooled in a dry ice-ethanol bath. Cryosections (8 µm) were applied to charged slides using a cryostat.

For immunofluorescence assays, the kidney sections were fixed with 1% paraformaldehyde (PFA) in PBS for 5 min, followed by blocking and permeabilization with 2% FBS in PBS with 0.1% saponin. The slides were incubated with antibodies against laminin α5 or podocin for 1 hour at room temperature followed by extensive washing. Fluorescently conjugated secondary antibodies were then applied for 1 h at room temperature. After several washes, the slides were imaged on an Olympus FV1000 confocal microscope (Center Valley, PA).

**In vitro macropinocytosis assay**

Mouse podocytes in 6 well plates were serum starved with serum-free RPMI for 2 hours. The cells were then incubated in RPMI supplemented with 0.5 mg/ml FITC- or Rhodamine B-dextran for 30 min on ice or at 37°C. Subsequently the cells were washed three times with ice-
cold PBS and fixed with 4% paraformaldehyde for confocal microscopy or trypsinized and resuspended in FACS buffer (2% FBS, 2mM EDTA in 1× PBS) for flow cytometry. FACS data was collected on FACSCantoII or LSRII flow cytometers (BD Biosciences, San Jose, CA) and analyzed with FlowJo (Treestar, Ashland, OR).

**Western Blots**

Podocytes were lysed in a modified RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM Na3VO4, 1 mM NaF) for 30 min on ice. The protein content of the lysates were quantified with a protein assay kit (Bio-Rad). Equivalent amounts of each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted for phospho-ERK, CD2AP, Gβ1, Gβ2, or β-actin. The blots were visualized by ECL or infrared imaging on a LI-COR Odyssey system (LI-COR, Lincoln, NE).

**Lipid extraction**

Lipids were extracted from BSA or HSA using the Bligh-Dyer method with slight modifications. 3 ml of 20% BSA in ddH2O was added to a mixture of 6 ml chloroform and 12 ml methanol in a glass tube and mixed on an orbital shaker for 15 min at room temperature. 6 ml of chloroform was added to the mixture while vortexing at low speeds followed by 6 ml of 1 M NaCl. The samples were centrifuged at 1000 RPM for 5 minutes to separate the aqueous and organic phases. The lower organic phase was carefully collected, aliquoted into 1 ml tubes, evaporated under vacuum, and stored at -80°C.
For endocytosis assays, the lipids were resuspended in 30 µl of DMSO and the cells were treated with 1-5 µl/ml. Assuming recovery of 80% of the lipids during extraction, this is estimated to be equivalent to the amount of lipids bound to BSA in a 0.1 - 0.5 % solution.

**Generation of stable podocytes**

Lentiviral constructs containing small hairpin RNAs (shRNAs) targeting FFAR isoforms, Gβ isoforms, and firefly luciferase (control) were generated using the pFLRu-YFP lentiviral vector (provided by Y. Feng and G. D. Longmore, Washington University, St. Louis, MO) (Feng et al. 2010). The targeting sequence were: fLuc, CCA ACC CTA TTC TCC TTC T; FFAR1, ATC TTT GCC TTG GCC CAC TTT G; FFAR2, CGG TTT GCT ACT GAT CCG CAA T; FFAR3, ACT GGA ACT GAA GGT AAA GAA T; GNB1, CAT TAT CTG TGG TAT CAC A; GNB2, CAT CTG CTC CAT CTA TAG T. PCR products containing the U6 promoter followed by the shRNA sequences were cloned into the pFLRu-YFP vector using the XbaI/XhoI site.

For lentivirus production, 293T cells were transfected with the lentiviral constructs along with packaging plasmids. Proliferating podocytes were infected with supernatants containing lentivirus and 8 µg/ml protamine sulfate for 24 hours. 7 days after infection, cells expressing high levels of YFP were sorted on a BD FACSAria II flow cytometer (BD Biosciences).

To generate podocytes stably expressing constitutively active Gαs, cells were transfected with a pcDNA3.1 vector containing Gαs (Q227L) (a gift from Kun-Liang Guan, UCSD). Stably transfected cells were selected by serial passage in 400 µg/ml G418 for 1 week.

**cAMP FRET assays**
A pcDNA3 plasmid containing the FRET-based cAMP biosensor $\text{Epac}^{VV}$ was a gift from Kees Jalink (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Cultured podocytes were transfected with $\text{Epac}^{VV}$ using the Nucleofector system (Lonza, Basel, Switzerland) and seeded on glass bottom dishes (MatTek, Ashland, MA). Images were obtained using an Olympus FV1000 confocal microscope in a temperature controlled chamber (37°C). The cells were excited at 440 nm and donor and acceptor emission was detected simultaneously using a 510 nm beamsplitter and two photomultipliers with optical filters: 465 - 495 nm (CFP) and 535 - 565 nm (YFP).

FRET measurements were quantified using ImageJ (NIH) and the ‘Ratio Plus’ plugin. After regions were drawn around individual cells, emission ratios were obtained by calculating FRET intensities divided by donor intensities. Time-course ratio measurements were normalized to baseline values before stimulation. The FRET value was set at 1 at the onset of the experiment.

**Statistical analyses**

Data were routinely presented as mean +/- standard deviation (SD). We determined significance by the Student's t test or ANOVA. We considered a P value of <0.05 as statistically significant.
RESULTS

Podocytes are highly endocytotic in vivo

Electron micrographs of the kidney often show intracellular vesicles inside podocytes (Fig 1a) (Farquhar and Palade 1960; Farquhar and Palade 1961; Farquhar et al. 1961; Vogt et al. 1968; Venkatachalam et al. 1970; Venkatachalam et al. 1970; Caulfield and Farquhar 1974). To determine whether these are endocytic vesicles, we injected mice intravenously with two different fluorescent fluid-phase markers (70kDa FITC-ficoll or 10kDa FITC-dextran). 24 hours after injection, imaging of the kidney glomerulus showed fluorescence labeling in distinct vesicles suggesting endocytic uptake (Fig 1b, 2b).

To determine whether fluid-phase uptake is increased during proteinuria, we performed similar experiments with a mouse model of nephrotic syndrome, the laminin β2 knockout (lamb2−/−) mice (Noakes et al. 1995; Jarad et al. 2006; Miner et al. 2006). Lamb2−/− mice have a defect in the glomerular filtration barrier, allowing serum proteins to pass into the urine. Injection of fluorescent tracers into 3 week-old lamb2−/− mice resulted in significantly increased numbers of fluorescently labeled intracellular vesicles that co-localized in cells that labeled with podocin, a podocyte-specific marker (Fig 1c and d, 2b and c).

Albumin stimulates macropinocytosis in podocytes

To test whether fluid-phase endocytosis in podocytes could be studied in vitro, we incubated immortalized podocytes in culture with fluid-phase fluorescent markers. In the absence of any stimulus, moderate amounts of uptake were detected by both immunofluorescence and flow cytometry when podocytes were incubated with 70 kDa FITC-
dextran at 37 degrees, but not at 4 degrees (Fig 3a and b). To mimic proteinuria, when podocytes are exposed to increased amounts of serum proteins, we incubated podocytes with 5 - 15% bovine serum albumin (BSA). The addition of BSA to the media dramatically increased the uptake of the fluid-phase tracers in a dose-dependent manner (Figure 3a-c). This effect was specific to albumin as the addition of immunoglobulin, another abundant protein in the serum, did not have any effect (Figure 3d). Importantly, stimulation of endocytosis by BSA was also observed in mice injected with BSA (Fig 4).

Fluid-phase uptake in cells is carried out by micropinocytosis and macropinocytosis. These processes can be distinguished by the size of the endocytic vesicles and their differential ability to internalize fluorescent tracers of larger sizes. BSA stimulation induced the uptake of high molecular weight fluorescence tracers (500 kDa and 2000 kDa), suggesting that macropinocytosis was the major endocytic process stimulated by BSA (Fig 5a). Immunofluorescence imaging confirmed that the size of the fluorescent vesicles ranged from 0.2 μm to 0.6 μm, consistent with macropinosomes (Fig 3a). Macropinocytosis is dependent on PI3K activation and actin remodeling and requires aquaporins to expel excess water from the vesicles. Incubation with inhibitors of aquaporin (pCMB), PI3K (wortmannin), or actin polymerization (cytochalasin D) blocked dextran uptake in podocytes (Fig 5b). This confirmed that the increased fluid-phase uptake stimulated by BSA was mediated by macropinocytosis.

A well-known stimulus of macropinocytosis is growth factors. It has been shown that macrophage colony-stimulating factor-1 (CSF-1), epidermal growth factor (EGF), and platelet-derived growth factor are able to activate macropinocytosis in some cell types (Haigler et al. 1979; Racoosin and Swanson 1989). Incubation of podocytes with EGF or PDGF did not increase macropinocytosis in podocytes even though downstream targets like ERK were
activated (Fig 5c). The mechanism involved in regulation of macropinocytosis in podocytes is therefore distinct from that manifest in dendritic cells.

Macropinocytosis is thought to be a specialized process specific to certain cell types such as dendritic cells and macrophages. To assess the level of macropinocytosis induced by BSA in podocytes, we compared it to the rate of macropinocytosis in immature dendritic cells, a cell considered to have a high rate of macropinocytosis (Norbury 2006). As expected, dendritic cells exhibited significantly higher levels of constitutive dextran uptake compared to untreated podocytes (Fig 5d). However, stimulation of podocytes with 7.5% BSA induced a rate of macropinocytosis that was comparable to dendritic cells, and at 15% BSA, podocytes surpassed dendritic cells in their ability to endocytose dextran (Fig 5d). BSA did not stimulate fluid-phase uptake in dendritic cells, suggesting that the regulatory mechanism of macropinocytosis in podocytes is distinct.

Morphologically, exposure to BSA induced the formation of numerous filopodia and actin ring structures (Fig 6). There was significant activation of actin structures as indicated by the enrichment of actin at the cell periphery (Fig 6). These changes are consistent with activation of the small GTPase Cdc42, which is known to be associated with induction of macropinocytosis (Hall 1998; Garrett et al. 2000).

**Lipids bound to albumin are the stimuli for podocyte macropinocytosis**

A major function of albumin is to bind hydrophobic molecules suggesting that macropinocytosis in podocytes might be stimulated by the serum lipids present in the commercial albumin preparations (van der Vusse 2009). Delipidated albumin did not induce macropinocytosis, suggesting that the albumin protein itself is not the stimulus (Fig 7a).
Meanwhile, lipids extracted from the BSA were able to induce fluid-phase uptake (Fig 7b).

Importantly, lipids extracted from clinical grade human serum albumin (HSA) could also stimulate macropinocytosis (Fig 7b), indicating that the lipid activity was not a contaminant but a normal physiological lipid present in serum.

The activity in the lipid extracts was resistant to boiling or hydrolysis in acid or base (data not shown). Incubating the lipids with neutral hydroxylamine (cleavage of thioester bonds), methanolic HCl (cleavage of ester, vinyl ether, glycosidic, and amide bonds), or periodate (oxidation of diols in sugar moieties) also did not significantly reduce activity (data not shown).

To purify the active compound, the extracted lipids were separated using silica-based HPLC (Fig 8). The fractions that displayed the highest macropinocytosis-stimulating activity were pooled together and separated by reverse-phase HPLC (Fig 8). Mass spectrometric analysis showed that these fractions were a complex mixture of hundreds of different lipid molecules (data not shown).

An analysis of the lipids extracted from HSA showed that the active compound migrated in the same fractions on straight and reverse phase HPLC as the active components from BSA (data not shown). This further supports the idea that the bioactive lipid is a physiological lipid present in serum.

In a parallel effort to identify the active lipid compound, we tested a variety of candidate lipid molecules. Initial assays using bioactive lipids (lysophosphatidic acid, prostaglandins, lipopolysaccharide, sphingosylphosphorylcholine, etc.) or lipids with various polar head groups (choline, ethanolamine, n-dansyl-ethanolamine, n-pyrenesulfonyl-ethanolamine) did not result in stimulation of macropinocytosis (data not shown). We therefore next tested several saturated and unsaturated fatty acids of various lengths. Surprisingly, several different fatty acids including oleic acid, eicosatrienoic acid (ETA), eicosapentaenoic acid (EPA), and
Docosahexaenoic acid (DHA) stimulated macropinocytosis in a dose-dependent manner (Fig 9). When added together, the different lipid species had an additive effect on macropinocytosis and induced dextran uptake to levels comparable to BSA lipid extracts (Fig 9). This suggested that more than one lipid, including several different fatty acids, might be responsible for inducing macropinocytosis in podocytes.

Stimulation of macropinocytosis by albumin-associated lipids is mediated by lipid-binding GPCRs and Gβ

Since many lipids are known to induce signaling in cells using specific receptors, we analyzed podocyte microarrays (Akilesh et al. 2011) to identify lipid receptors expressed in podocytes focusing on G protein-coupled receptors (GPCRs), the major lipid binding receptors. The results showed that podocytes express a variety of fatty acid receptors including FFAR1, FFAR2 and FFAR3 (data not shown). The ligands of FFAR1 are medium- and long-chain fatty acids while FFAR2 and FFAR3 bind to short-chain fatty acids. To determine their roles in podocyte macropinocytosis, we used shRNA to knock down all three free fatty acid receptors (Fig 10). Lipid-induced macropinocytosis was significantly decreased when any one of the FFAR isoforms were knocked down (Fig 10).

GPCRs stimulate the disassembly of heterotrimeric G proteins composed of the Gα, Gβ, and Gγ subunits. Upon activation of the receptor, the G protein subunits dissociate from the receptor and activate downstream effectors through the free Gα subunit or by the Gβ/Gγ subunit complex. Distinct α subunits activate specific downstream effects including modulation of intracellular cAMP concentration. To determine whether cAMP levels are altered in podocytes in response to albumin-associated lipids, podocytes were transfected with a cAMP fluorescence
resonance energy transfer (FRET) biosensor and analyzed using time-lapse confocal microscopy. Activation with known stimulators of cAMP (Forskolin + IBMX or isoproterenol) induced a rapid increase in FRET that was maintained for over 20 minutes or plateaued at a reduced level, respectively (Fig 11). Stimulation with the albumin-associated lipids induced a response that was slightly delayed but induced a level of FRET activity similar to isoproterenol within 10 minutes after exposure (Fig 11). This suggested that albumin-associated lipids activate GPCRs that stimulate cAMP production.

We next tested whether cAMP could induce macropinocytosis in podocytes. Podocytes were treated with forskolin (an activator of adenylate cyclase), 8-bromo-cAMP (a non-selective cAMP analog), or 8-CPT-2Me-cAMP (a cAMP analog that activates Epac, but not PKA). Contrary to our expectations, none of these manipulations affected dextran uptake (Fig 12a). Furthermore, macropinocytosis was not induced when a constitutively active form of $\text{G}_\alpha_S$ (Q227L) was overexpressed (Fig 12b). These results indicate that although cAMP levels change in response to lipids, it is not mediating activation of macropinocytosis.

In addition to triggering the activation of $\text{G}_\alpha$ subunit, GPCR signaling induces the release and activation of the $\text{G}_\beta/\text{G}_\gamma$ complex which is known to activate its own set of downstream effectors including PI3K and the small GTPases Rac1 and Cdc42. To determine whether the $\text{G}_\beta/\text{G}_\gamma$ complex was mediating lipid-induced macropinocytosis, we incubated podocytes with gallein, an inhibitor of $\text{G}_\beta$. Fluorescein, a compound similar in structure to gallein but does not inhibit $\text{G}_\beta$, was used as control. In contrast to fluorescein, gallein reduced basal and lipid-induced dextran uptake in podocytes (Fig 13a). In addition, high concentrations of gallein almost completely blocked lipid-induced macropinocytosis, suggesting that $\text{G}_\beta/\text{G}_\gamma$ activity was mediating this pathway.
These results were confirmed by using shRNA to inhibit expression of Gβ in podocytes. Our expression analysis of podocytes showed that of the four Gβ isoforms, only Gβ1 and Gβ2 are expressed in cultured podocytes (data not shown). Lentiviral constructs expressing shRNAs that target Gβ1 and Gβ2 were generated. We also generated a construct that contains the two shRNAs in tandem to target both isoforms. YFP co-expressed by the lentivirus allowed us to purify transduced cells. After lentiviral infection, podocytes expressing high levels of YFP were sorted using flow cytometry (Fig 14). Compared to the control (firefly luciferase), the GNB1, or the GNB2 knockdown cells, the fraction of YFP\textsuperscript{hi} cells was significantly lower in the GNB1/GNB2 double knockdown podocytes. This was likely due to the deleterious effect of Gβ depletion on cell survival and/or proliferation. Gβ knockdown was confirmed by immunoblotting with isoform-specific antibodies that showed nearly complete reduction of specific Gβ isoforms in GNB1 and GNB2 single knockdown podocytes (Fig 13b). Knockdown was less efficient in the GNB1/GNB2 double knockdown cells, consistent with the idea that the complete absence of Gβ is toxic to the cells. As expected, knockdown of either Gβ1 or Gβ2 resulted in an impaired macropinocytotic response to lipid treatment (Fig 13c). The compensatory increase in the expression of the other Gβ isoform in the single knockdown cells may have ameliorated the magnitude of the effect on macropinocytosis (Fig 13b). Indeed, knockdown of both Gβ isoforms resulted in a further reduction in macropinocytosis (Fig 13c).
DISCUSSION

The presence of large endocytotic vesicles within podocytes is one of the first features seen in podocytes using electron microscopy (Farquhar et al. 1957). These vesicles have been speculated to be transcytotic vesicles of primary urine (Vernier et al. 1958) or protein reabsorption droplets (Feldman and Fisher 1959; Venkatachalam et al. 1969), phagosomes removing subepithelial immune deposits (Rantala 1981) and giant protamine heparin aggregates (Sharon et al. 1978). In the last 10 years, evidence has been accumulating implicating the important role of the endocytic pathway in normal and diseased podocytes. We showed that CD2AP hapoinsufficient mice had defects in multivesicular body formation and this was associated with subepithelial immunoglobulin deposits and an increased susceptibility to glomerular disease (Kim et al. 2003). More recently Ishibe and colleagues have shown the importance of the endocytic proteins, synaptojanin, endophilin and dynamin for the normal function of the podocyte (Soda et al. 2012). The functional significance of these vesicles has remained unresolved.

Our results reveal that these vesicles likely represent macropinosomes and that they function to internalize proteins that cross the glomerular basement membrane. Our studies in mice using fluorescent tracers demonstrated that podocytes are capable of significant fluid-phase uptake, and that the level of fluid-phase uptake is dramatically increased in proteinuric mice. As the primary defect in the model of nephrotic syndrome that we used (lamb2-/-) mice is in the GBM (Noakes et al. 1995; Jarad et al. 2006; Miner et al. 2006), this suggested that the increased fluid-phase uptake was not due to pathological changes in podocytes, but rather due potentially to stimulating factors in the serum that were traversing the compromised GBM.
Our experiments suggest that macropinocytosis is stimulated by fatty acids that are associated with albumin. While most of our experiments were carried out using BSA, the evidence supports the idea that the results we obtained were not due to a contaminant in the commercially available BSA preparations. Lipids isolated from clinical grade HSA behaved by HPLC in a manner that was similar to lipids isolated from BSA. Furthermore, the fatty acids that stimulated macropinocytosis are fatty acids present in substantive concentrations in normal serum. The potential effects of endotoxin on our experiments were excluded by multiple assays of endotoxin in BSA, which demonstrated insignificant amounts of contamination in the BSA. Moreover, LPS treatment had no effect on macropinocytosis (data not shown).

We found that macropinocytosis in podocytes was stimulated by multiple lipid species and was mediated by multiple lipid-binding GPCRs. Each lipid had a dose-dependent effect on endocytosis and we saw an additive effect when we combined the lipids together. This was shown to be mediated by the Gβ/Gγ subunits which are, unlike the Gα subunits, shared by the all of the fatty acid receptors and are known to activate key mediators of macropinocytosis (e.g., PI3K, Rac1, and Cdc42). Indeed, macropinocytosis was not affected by manipulating intracellular levels of cAMP, a downstream mediator of GαS, but was inhibited by chemical inhibitors or shRNA-mediated knockdown of Gβ.

The role of macropinocytosis has been shown to vary depending on cell type. Certain cell types exhibit constitutive macropinocytosis. For example, immature dendritic cells use macropinocytosis as a means of surveying the surrounding environment for foreign pathogens (Sallusto et al. 1995). For axenic strains of Dictyostelium and Ras-transformed cells, macropinocytosis serves as a major supply pathway for nutrients (Hacker et al. 1997; Commissio et al. 2013). In other situations, macropinocytosis is transiently induced by certain stimuli such
as phorbol esters or growth factors such as EGF or CSF-1 (Haigler et al. 1979; Racoosin and Swanson 1989; Swanson 1989).

In the case of podocytes, we propose that macropinocytosis functions to remove large serum proteins that are able to enter and cross the GBM. In the sieving model of the glomerular filtration barrier, the endothelial fenestrations, the collagen and laminin matrix of the GBM, and the pores of the slit-diaphragm function together to form an efficient size-selective filter, preventing the passage of molecules larger than 60kD. It has been argued that such models would require an active clearance mechanism to prevent the filter from clogging. This has led to alternative models where the filtration barrier is thought to function more as a gel, allowing the passage of all molecules but limiting the molecular transit in a manner that is inversely proportional to the size of the molecule. In either case, both models would require an active mechanism to handle large molecules that cross the GBM. The high expression of receptors for albumin, IgG, and complement on the podocyte support the idea that the podocyte plays a specialized role in recycling of serum proteins that cross the GBM.

Since macropinocytosis is an energy consuming process, it seems reasonable that podocytes would regulate their level of macropinocytosis with the amount of serum proteins that have crossed the barrier. Our data suggest that podocytes respond to fatty acids that are bound to albumin and the recognition of fatty acids serves as a proxy for albumin. It is interesting that multiple fatty acid species were able to stimulate macropinocytosis through several different receptors. We speculate that this redundancy allows podocytes to detect a broader spectrum of ligands and ensures induction of a response regardless of fluctuations in the serum levels of individual lipid molecular species. It also suggests that metabolic or inflammatory conditions
that alter the type and amount of fatty acid molecular species could also have an effect on podocyte function and the glomerular filtration barrier.

Macropinocytosis could also be important in podocytes as a source of energy and nutrition. Podocytes are unique in that they are located wholly in the urinary space and are separated from the blood supply by the GBM. The filtration barrier might function to restrict access to certain types of nutrients, partially explaining why podocytes exhibit constitutive autophagy and why impairing autophagy is so detrimental to podocytes (Burgess et al. 2008; Bour et al. 2009). It is plausible to speculate that, as in amoeba and Ras-transformed cells (Hacker et al. 1997; Commisso et al. 2013), serum proteins internalized via macropinocytosis are degraded and used as a source of energy and anabolic substrates. Time-lapse experiments revealed that macropinocytic vesicles indeed merged with lysosomal compartments over time (data not shown).

The fact that macropinocytosis at basal states is low and only induced transiently suggest that sustained activation of macropinocytosis could be detrimental. The level of macropinocytosis induced during proteinuric states is likely maladaptive. In support of this notion, we observed swollen podocytes with numerous protein-filled vesicles and partially fused foot processes in mice injected with BSA for up to 8 days (data not shown). Chronic stimulation of macropinocytosis may lead to excessive uptake of fluids and serum proteins that overload the degradative pathway leading to cell dysfunction. Accumulation of albumin in podocytes has been shown to be toxic and lead to podocyte loss as well as glomerulosclerosis (Barnes and Karin 1997; Cai et al. 2005; Bastarrachea et al. 2007; Chen et al. 2011). In addition, it has been demonstrated recently that hyperstimulation of macropinocytosis leads to lysosomal dysfunction and cell death (Overmeyer et al. 2008; Li et al. 2010; Nara et al. 2012). Chronic activation of
Rac1 and/or Cdc42 stimulated by the fatty acids could also lead to exacerbation of proteinuria. We and others have demonstrated that dysregulation of Rac1 or Cdc42 in podocytes leads to podocyte foot process effacement and proteinuria in mice (Carr et al. 2004; Carr et al. 2005; Scott et al. 2012; Blattner et al. 2013). Thus, we suspect that low level exposure of podocytes to fatty acids allows for macropinocytosis-mediated maintenance of the glomerular filter, but sustained stimulation by fatty acids could lead to foot process effacement and persistent proteinuria.

Hyperlipidemia is one of the defining characteristics of nephrotic syndrome, a severe proteinuric condition caused by podocyte dysfunction. Our work suggests that exposure to elevated serum lipid concentrations in nephrotic states could be a cause of podocyte dysfunction. In support of this idea, numerous studies have reported reductions in proteinuria and improvements in renal function in nephrotic patients treated with lipoprotein apheresis to reduce hyperlipidemia (Hattori et al. 1993; Yorioka et al. 1997; Yokoyama et al. 1998; Muso et al. 1999; Daimon et al. 2000; Stenvinkel et al. 2000; Muso et al. 2001; Hattori et al. 2003; Nakamura et al. 2005; Carr et al. 2006; Caspar-Bauguil et al. 2006). This implies that free fatty acids may function as a key player in a vicious cycle that sustains proteinuria. An initial trigger for proteinuria (i.e., infection, toxins, inflammation) would lead to loss of albumin and decreased lipid-buffering capacity of serum, resulting in an increase of uncomplexed lipids. This would result in increased exposure of podocytes to free fatty acids, causing further podocyte damage and foot process effacement through activation of Rac/Cdc42 and/or lysosomal dysfunction, thereby perpetuating the cycle of macropinocytosis and proteinuria. Thus it will be interesting to see whether chronic stimulation of the macropinocytic pathway by serum lipids is a mechanism driving the pathogenesis of nephrotic syndrome.
In conclusion, we have described an inducible system of macropinocytosis in podocytes. A number of different lipid species bound to albumin in serum can stimulate macropinocytosis in podocytes though lipid-binding receptors and signaling through Gβ/Gγ. We propose a model in which podocytes detect accumulation of proteins in the subepithelial space via lipids and subsequently remove them by engaging a highly efficient mechanism of endocytosis. This has the role of preventing the glomerular filtration barrier from clogging and/or providing a source of energy for the podocytes, but may have maladaptive consequences when sustained chronically.
Figure 1. Podocytes are highly endocytotic cells in vivo and endocytosis is stimulated under proteinuric conditions

(A) Cross-section of the glomerular filter of a wild-type mouse obtained by electron microscopy. Arrows indicate vesicles located inside podocytes. (B-D) Wild-type (B) or lamb2/- mice (C, D) were intravenously injected with 70 kDa FITC-ficoll (green). 24 hrs after injection, glomerular sections were stained for laminin α5 (B, C) or podocin (D) (red) and counterstained with DAPI (blue). Insets are enlarged images of the indicated area.
Figure 2. Podocytes are highly endocytotic cells in vivo and endocytosis is stimulated under proteinuric conditions

(A-C) Wild-type (A) or lamb2−/− mice (B, C) were intravenously injected with 10 kDa FITC-dextran (green). 24 hrs after injection, glomerular sections were stained for laminin α5 (A, B) or podocin (C) (red) and counterstained with DAPI (blue). Insets are enlarged images of the indicated area.
Figure 3. Albumin stimulates fluid-phase uptake in cultured podocytes

(A) Cultured podocytes were incubated with or without 10% BSA at 4°C or 37°C in the presence of 70 kDa FITC-dextran for 30 minutes and analyzed by fluorescence microscopy. Upper inset is an enlarged image of the indicated area. Lower inset is an electron microscopy image of a cultured podocyte incubated with 10% BSA. Scale bars are 0.5 µm. (B) Cells treated identical to (A) were analyzed by flow cytometry. (C, D) Podocytes were incubated with indicated concentrations of BSA or IgG in the presence of 70 kDa FITC-dextran for 30 minutes and analyzed by flow cytometry. Results are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01
**Figure 4. Albumin stimulates fluid-phase uptake in podocytes in mice**

(A-D) Control mice (A) or mice given daily injections of BSA for 4 days (B, C) were intravenously injected with 70 kDa FITC-ficoll (green). 24 hrs after injection, glomerular sections were stained for laminin $\alpha_5$ (A, B) or podocin (C) (red) and counterstained with DAPI (blue). Insets are enlarged images of the indicated area. (D) The glomerulus of mice injected daily with BSA for 4 days was analyzed by electron microscopy. Arrows indicate protein-filled vesicles located inside podocytes.
Figure 5. *Albumin stimulated fluid-phase uptake in podocytes occurs by macropinocytosis*

(A) Podocytes were incubated with 70 kDa, 500 kDa, or 2000 kDa FITC-dextran in the presence of 7.5% BSA and mannan (1 mg/ml) as indicated for 30 minutes and analyzed by flow cytometry. (B) Podocytes were pretreated with pCMB (100 µM), wortmannin (1 µM), or cytochalasin D (10 µM) for 30 min. Subsequently, the cells were incubated with 15% BSA in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. (C) Podocytes were incubated with EGF (100 nM) and PDGF (25 nM) in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. Inset is an immunoblot of cells for ERK phosphorylation and CD2AP as loading control. (D) Bone-marrow derived dendritic cells and podocytes were incubated with BSA as indicated in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. All results are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01
Figure 6. Albumin stimulates formation of actin structures in podocytes

Podocytes were fixed after incubating with 5% BSA for 5 minutes, stained with phalloidin, and analyzed with fluorescence microscopy. Insets are enlarged images of the indicated area displaying formation of filopodia (upper) and actin ring structures (lower).
Figure 7. Podocyte macropinocytosis is stimulated by albumin-associated lipids

(A) Podocytes were incubated with 7.5% BSA or fatty acid-free BSA in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. (B) Podocytes were incubated with 7.5% BSA, BSA lipid extract, or HSA lipid extract in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. All results are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01
Figure 8. Fractionation of albumin-associated lipids by HPLC

(A) Lipids extracted from BSA were fractionated by silica-based HPLC and each fraction was tested for ability to induce macropinocytosis in podocytes. (B) Fractions 20 and 21 from (a) were combined and fractionated by reversed-phase HPLC. Each fraction was again tested for macropinocytosis induction. Fraction 52 was further analyzed by mass spectrometry. All results are shown as fold change in mean fluorescence intensity.
Figure 9. Podocyte macropinocytosis is stimulated by free fatty acids

(A) Podocytes were incubated with increasing concentrations (1 or 10 µM) of lipids as indicated in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. (D) Podocytes were incubated with individual lipids (2 µM) or a mixture of the lipids (2 µM each) in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. All results are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01 vs DMSO.
Figure 10. **Stimulation of macropinocytosis by albumin-associated lipids is mediated by free fatty acid receptors**

(A) FFAR knockdown podocytes were analyzed by real time quantitative PCR. Results are shown as fold change of mRNA levels compared to control (firefly luciferase) knockdown cells. * p<0.05, ** p<0.01 vs. fLuc control. (B) Control and FFAR knockdown podocytes were incubated with Rhodamine B-dextran and BSA lipid extracts for 30 min and analyzed by flow cytometry. Results are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05
Figure 11. Albumin-associated lipids induce GaS activation in podocytes

(A) Podocytes expressing the cAMP FRET sensor $\text{Epac}^{\text{VV}}$ were treated with Forskolin (25 µM) + IBMX (100 µM), Isoproterenol (10µM), or 3 µl/ml BSA lipid extract and analyzed by real time confocal microscopy. Compounds were added at 4 min. Brighter intensity indicates higher CFP/YFP ratios. (B) Changes in CFP/YFP ratios are plotted against time. Arrow indicates time of treatment. (n= 4, Forskolin + IBMX; 4, Isoproterenol; 10, BSA lipid extract)
Figure 12. Stimulation of macropinocytosis by albumin-associated lipids is not mediated by Ga activation

(A) Podocytes were incubated with 3 μl/ml BSA lipid extract, forskolin (10 μM), 8-Br-cAMP (10 μM), or 8-CPT-2Me-cAMP (10 μM) in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. (B) Control or podocytes expressing constitutively active GaS (Q227L) were incubated with BSA lipid extract in the presence of FITC-dextran for 30 minutes and analyzed by flow cytometry. Results are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05
Figure 13. Stimulation of macropinocytosis by albumin-associated lipids is mediated by Gβ activation

(A) Podocytes were pretreated with 2 or 10 µM fluorescein or gallein for 30 minutes. Subsequently, the cells were incubated with BSA lipid extracts in the presence of Rhodamine B-dextran for 30 min and analyzed by flow cytometry. (B) Podocytes expressing shRNAs targeting firefly luciferase (fLuc), GNB1, GNB2, or GNB1 and 2 (GNB1/2) were immunoblotted for Gβ1 and Gβ2 expression. β-actin was used as loading control. (C) GNB knockdown podocytes were incubated with BSA lipid extracts in the presence of Rhodamine B-dextran for 30 min and analyzed by flow cytometry. Results in (A) and (C) are shown as fold change in mean fluorescence intensity. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01
Figure 14. Flow cytometry analysis of podocytes infected with GNB knockdown lentivirus

(A-E) Podocytes were infected with lentiviral vectors expressing YFP and shRNAs targeting firefly luciferase (fLuc) or different GNB isoforms. Cells expressing high levels of YFP (gated region) were sorted and used for further analysis. Note the lower number of YFP-positive cells, especially the significant reduction of the YFP$^{hi}$ populations in podocytes infected with GNB1/GNB2 shRNA lentivirus (E).
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CHAPTER II

ApoL1 Protects Podocytes from Fatty Acid-Induced Cell Death
INTRODUCTION

Chronic kidney disease (CKD), which refers to the progressive loss of renal function, affects millions of people in the United States (NIDDK 2009). CKD is classified into 5 stages based on the extent of kidney damage and pathology including glomerular filtration rate, plasma creatinine, and proteinuria levels. The final stage, also known as end stage renal disease (ESRD), is the complete loss of kidney function, when the patient cannot continue daily life without dialysis or a transplant. Diabetes and hypertension the leading causes of ESRD. In addition, mutations in several genes involved in maintaining the structure and function of the kidney have been linked to CKD (Friedman and Pollak 2011).

African Americans develop ESRD at four to five times higher rates than European Americans. In 2008, two studies utilizing mapping by admixture linkage disequilibrium (MALD) identified a locus in chromosome 22 (which included the MYH9 gene) that conferred the increased risk for nondiabetic kidney diseases in African Americans (Kao et al. 2008; Kopp et al. 2008). However, subsequent studies failed to provide convincing evidence to support the etiological role of Myh9 in CKD. Later, two groups incorporated data from the 1000 genomes project and identified two coding sequence variants in APOL1, a gene immediately downstream of MYH9, that had the strongest associations with CKD in African Americans (Genovese et al. 2010; Tzur et al. 2010). The two variants are referred to as G1 (S342G and I384M substitutions) and G2 (deletion of N388 and Y389). Approximately 50% of the African American population have either one or two risk alleles, and individuals carrying both G1 and G2 alleles have a significantly increased risk of focal segmental glomerular sclerosis (FSGS), hypertension-associated ESRD (H-ESRD), and HIV-associated nephropathy (HIVAN).
ApoL1 was originally identified as a minor component of high-density lipoprotein (HDL) (Duchateau et al. 1997). Five additional ApoL isoforms have since been identified and are located in a tight cluster from chromosome 22q12.3 to 13.1 (Duchateau et al. 2001; Page et al. 2001). It is expressed only in humans and certain primates, but not in rodents. ApoL1 has been shown to be able to bind various lipid species including phosphatidic acids, cardiolipin, and phosphoinositides (Wan et al. 2008; Zhaorigetu et al. 2008). In addition, clinical data reveal an association of ApoL1 with hyperlipidemia (Duchateau et al. 2000; Albert et al. 2005; Li et al. 2009). These results indicate that ApoL1 is involved in lipid handling and transport.

ApoL1 is also involved in trypanosome immunity, apoptosis, and autophagy. When circulating ApoL1 is endocytosed by trypanosomes and trafficked to the lysosome, it undergoes a conformational change to form an anion channel. This causes swelling of the lysosome and eventually kills the trypanosome (Vanhamme et al. 2003; Perez-Morga et al. 2005). In addition, ApoL1 was shown to be a novel BH3-only protein that induces autophagic cell death when overexpressed in various cancer cell lines (Wan et al. 2008; Zhaorigetu et al. 2008).

Despite mounting evidence of its genetic association, it is still unclear how mutations in ApoL1 predispose carriers to CKD. In a study by Reeves-Daniel et al., donor kidneys with ApoL1 risk alleles had a higher incidence of early failure when transplanted (Reeves-Daniel et al. 2011). This indicates that alteration of ApoL1 function in the kidney is, at least in part, responsible for increased susceptibility to CKD. In normal kidney, ApoL1 is expressed exclusively in podocytes (Madhavan et al. 2011). Podocytes are epithelial cells that form interdigitating foot processes which wrap around the capillaries in the glomerulus (Greka and Mundel 2012). The space between the foot processes is connected by a network of proteins known as the slit diaphragm, which along with the glomerular basement membrane, form the
sieve through which blood is filtered to make urine. Disruption of the foot processes or loss of podocytes leads to massive proteinuria, a hallmark of renal failure. Thus, it is reasonable to speculate that ApoL1 may be required for normal function and/or survival of podocytes.

It has long been suspected that abnormal lipid metabolism can lead to kidney dysfunction. Obesity increases the risk of CKD independent of hypertension and diabetes (Wahba and Mak 2007). In addition, hypertriglyceridemia and hypercholesterolemia are associated with podocyte damage and mesangial sclerosis (Joles et al. 2000). Interestingly, lipid accumulation in the glomeruli and tubules of kidney has been observed in various models of kidney dysfunction in mice and human (Bobulescu 2010). Lipid accumulation in cells not equipped to handle excess lipids is known to cause cellular dysfunction and apoptosis in a process termed lipotoxicity (Schaffer 2003).

Based on these observations, we investigated whether podocytes are sensitive to lipid-induced cell toxicity. The possible role of ApoL1 in protecting podocytes from hyperlipidemic conditions is also examined.
MATERIALS AND METHODS

Cell culture

Conditionally immortalized mouse podocytes were generated as described previously (Mundel et al. 1997) and maintained at 33°C in RPMI1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM β-mercaptoethanol, and 100 U/ml recombinant mouse IFNγ (a gift from Robert Schreiber, Washington University, St. Louis, MO).

Conditionally immortalized human podocytes were a gift from Moin Saleem (University of Bristol, UK). The cells were maintained at 33°C in RPMI1640 supplemented with 10% fetal bovine serum, 10 µg/ml insulin (Sigma), 5.5 µg/ml transferrin (Sigma), 5 ng/ml sodium selenite (Sigma), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM β-mercaptoethanol as previously described (Saleem et al. 2002).

Lipotoxicity assays

For palmitate-induced cell death assays, palmitic acid or oleic acid (Nu-Chek Prep) was conjugated to BSA at a 2:1 molar ratio. Podocytes at 40 - 50% confluency were incubated in complete growth medium and BSA-fatty acid conjugates for 24 or 48 hours.

For cholesterol-induced cell death assays, podocytes were loaded with free cholesterol by incubating the cells with 100 µg/ml acetyl-LDL (Alfa Aesar, Ward Hill, MA) for 24 hours in the presence of 10 µg/ml Sandoz 58035 (Sigma), which inhibits ACAT-mediated cholesterol esterification (Yao and Tabas 2000).
At the end of the incubation period, the cells were collected, stained with Annexin V antibodies and PI or 7AAD using an apoptosis detection kit (BD biosciences), and analyzed on a BD FACSCantoII flow cytometer (BD biosciences) and FlowJo (Treestar).

**Podocyte transfection**

Mammalian expression vectors (pCMV6-AC-GFP) containing the cDNAs of ApoL1 – 4 and a C-terminal GFP tag were purchased from Origene (Rockville, MD). G1 (S342G, I384M) and G2 (deletion of N388 and Y389) variants of ApoL1 were generated by site-directed mutagenesis from the pCMV-ApoL1-GFP construct using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA).

Immortalized mouse podocytes were transfected with the Nucleofector Kits for Mammalian Epithelial Cells (Lonza, Basel, Switzerland) using the protocol number T-020. The cells were maintained for 24 hours after nucleofection before treatment of fatty acids for lipotoxicity assays.

**Generation of ApoL1 knockdown podocytes**

Lentiviral constructs containing small hairpin RNAs (shRNAs) targeting ApoL1 and firefly luciferase (control) were generated using the pFLRu-YFP lentiviral vector (provided by Y. Feng and G. D. Longmore, Washington University, St. Louis, MO) (Feng et al. 2010). The targeting sequences were: fLuc, CCA ACC CTA TTC TCC TTC T; ApoL1, GGA CAA CCT TGC AAG ACA A. PCR products containing the U6 promoter followed by the shRNA sequences were cloned into the pFLRu-YFP vector using the XbaI/XhoI site.
For lentivirus production, 293T cells were transfected with the lentiviral constructs along with packaging plasmids. Proliferating podocytes were infected with supernatants containing lentivirus and 8 µg/ml protamine sulfate for 24 hours. 7 days after infection, cells expressing high levels of YFP were sorted on a BD FACSARia II flow cytometer (BD Biosciences).

**Western Blots**

Podocytes were lysed in a modified RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM Na3VO4, 1 mM NaF) for 30 min on ice. The protein content of the lysates were quantified with a protein assay kit (Bio-Rad). Equivalent amounts of each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against ApoL1 (Sigma) or β-actin (Sigma). The blots were visualized by ECL.
RESULTS

Podocytes are sensitive to palmitate-induced cell death, but not cholesterol-induced cell death

Numerous studies have reported that long-chain fatty acids but not unsaturated fatty acids induce apoptosis in cells such as pancreatic β-cells, cardiac myocytes, and skin fibroblasts (Rosenthal 1981; Zhang et al. 1992; de Vries et al. 1997; Maedler et al. 2001). In order to determine whether podocytes are sensitive to lipotoxicity, immortalized mouse podocytes were incubated with increasing concentrations of BSA-conjugated palmitic acid or oleic acid. Results showed that palmitic acid induced podocyte apoptosis in a dose-dependent manner while oleic acid did not induce cell death even at the highest concentrations tested (Fig. 1a and b). Cell death was detectable at after 24 hours and was significantly increased after 48 hours (Fig 1c). Palmitic acid was similarly induced apoptosis in human podocytes (data not shown).

Intracellular accumulation of unesterified cholesterol has also been shown to induce cell toxicity (Warner et al. 1995). Mouse podocytes were loaded with free cholesterol by incubating in acetylated-LDL in the presence of Sandoz 58-035, which inhibits acetyl-coenzyme A acetyltransferase (ACAT)-mediated cholesterol esterification. In contrast to palmitic acid, there was no increase in podocyte cell death after cholesterol overload (Fig 2).

ApoL1 protects podocytes from palmitate-induced cell death

ApoL1 is only expressed in higher primates and is therefore absent in mouse podocytes. To determine whether ApoL1 could protect podocytes from palmitate-induced cell death, cells
were transfected with GFP-tagged ApoL1 and treated with palmitic acid. Analyses using flow cytometry revealed that ApoL1-expressing GFP⁺ cells were more resistant to palmitate-induced cell death than GFP⁻ cells (Fig 3a and b).

Isoforms of ApoL1 appear to have risen from recent gene duplication and have a high level of homology at the level of amino acid sequence (Page et al. 2001). Assays using cells overexpressing GFP-tagged ApoL2, 3, and 4 demonstrated that ApoL3 can also protect podocytes from lipotoxicity, albeit to a lesser degree than ApoL1 (Fig 3b).

To confirm these findings, shRNA-mediated gene silencing was used to knockdown the expression of ApoL1 in immortalized human podocytes (Fig 4a). Reduction of ApoL1 did not affect cell viability in cells cultured with BSA or BSA-conjugated oleic acid (Fig 4b). In contrast, when incubated in palmitic acid, podocytes expressing decreased levels of ApoL1 displayed significantly higher rates of apoptosis as indicated by Annexin V and 7AAD staining (Fig 4b).

**ApoL1 G1 and G2 variants have similar effects in reducing lipotoxicity compared to wild-type ApoL1**

The increased risk of CKD in populations of African descent has been attributed to the G1 and G2 sequence variants in the ApoL1 gene (Genovese et al. 2010). Mouse podocytes were transfected with either wild-type or the G1 or G2 variants of ApoL1-GFP and treated with BSA-conjugated palmitic acid for 24 hours. Analysis of the ratio of GFP⁺:GFP⁻ cells in the viable fraction revealed that the G1 and G2 mutations do not have an effect on the ability of ApoL1 in modulating lipotoxicity (Fig 5).
DISCUSSION

One of the hallmarks of nephrotic syndrome is hyperlipidemia, or abnormal elevation of serum lipid levels. It was recently demonstrated that the elevated serum ratio of free fatty acids to albumin caused by proteinuria stimulates secretion of angiopoietin-like 4 (Angptl4) from heart, muscle, and adipose tissues (Clement et al. 2014). The circulating Angptl4 reduces uptake of free fatty acids in the peripheral organs by inhibiting lipoprotein lipase (LPL) and thereby causes hyperlipidemia. This indicates that dysregulation of serum lipid levels is a result of protein loss during nephrotic states.

However, there several lines of evidence suggest that lipids could directly cause renal injury. Hyperlipidemia has been shown to be an independent risk factor for developing proteinuria and chronic kidney diseases (Samuelsson et al. 1998; Tozawa et al. 2002; Schaeffner et al. 2003; Astor et al. 2006; Lee et al. 2009). In addition, numerous studies have demonstrated that lipoprotein apheresis to reduce hyperlipidemia effectively reduces proteinuria and improves renal function in nephrotic patients (Hattori et al. 1993; Yorioka et al. 1997; Yokoyama et al. 1998; Muso et al. 1999; Daimon et al. 2000; Stenvinkel et al. 2000; Muso et al. 2001; Hattori et al. 2003; Nakamura et al. 2005; Carr et al. 2006; Caspar-Bauguil et al. 2006). Furthermore, rodent models with genetically- or diet-induced hyperlipidemia develop progressive glomerular damage (Koletsky 1975; Imai et al. 1977; Kasiske et al. 1985; Keane et al. 1991; Grone et al. 1993; Ruan et al. 2003). The pathological effects of lipids on renal function have been attributed to the induction of inflammatory stress, oxidative stress, and endoplasmic reticulum stress (Ruan et al. 2009).
Our results demonstrate that saturated fatty acids can directly induce apoptosis in podocytes. These results have been confirmed by recent reports of similar findings (Sieber et al. 2010; Yasuda et al. 2014). This suggests that free fatty acids may play a prominent role in the development of podocyte diseases. In healthy subjects, the vast majority (more than 99.9%) of free fatty acids in the serum is bound to albumin (Richieri and Kleinfeld 1995), and the amount of saturated fatty acids that podocytes are exposed to would be insufficient to cause injury. However, increased serum lipid concentrations manifest in obese and diabetic subjects would result in elevated levels of unbound fatty acids. This could trigger podocyte injury and induce proteinuria. Loss of albumin due to proteinuria, and the subsequent decrease in the fatty acid buffering capacity of the serum, would then further increase in the levels of unbound fatty acids. Saturated fatty acids could therefore act as both an initial trigger of podocyte injury as well as a mediator of a vicious cycle. Interestingly, it has been shown that free fatty acids can also cause tubulointerstitial injury, demonstrating that the lipotoxic effects of fatty acids are not limited to the glomerulus (Kamijo et al. 2002; Thomas et al. 2002).

Genetic association studies have provided very compelling data implicating the role of ApoL1 in the development of chronic kidney diseases (Genovese et al. 2010; Tzur et al. 2010; Genovese et al. 2013). However, the function of ApoL1 and the mechanisms underlying the association of ApoL1 sequence variants with increased risk of developing chronic diseases have not been elucidated. We provide evidence that ApoL1 is able to dampen the lipotoxic effects of palmitic acid in podocytes. This was demonstrated by overexpressing ApoL1 in mouse podocytes, which do not express ApoL1, or by knocking down ApoL1 expression in human podocytes.

ApoL1 was initially identified as a component of HDL (Duchateau et al. 1997), and has thus been predicted to have functions related to lipid transport. In support of these speculations,
ApoL1 is capable of binding to several lipid species, such as phosphatidic acid, cardiolipin, and phosphoinositides (Wan et al. 2008). Our results suggest that ApoL1 may be involved in the binding and sequestering of intracellular fatty acids or the export of fatty acids. Disruption of ApoL1 function in podocytes may result in the loss of the ability to cope with increased influx of fatty acids. Notably, ApoL3, another member of the ApoL family with a high degree of homology to ApoL1, also decreased palmitate-induced cell death in podocytes suggesting that it could have similar functions with ApoL1.

The G1 (S342G and I384M) and G2 (N388, Y389 deletion) mutations did not affect the ‘lipo-protective’ properties of ApoL1. One explanation is that the residues mutated in the G1, G2 variants and the associated increase in susceptibility to renal injury are not related to the lipid-handling aspects of ApoL1 function. On the other hand, it is possible that the lipid-related functions of ApoL1 are indeed important for podocyte function and survival, and the G1 and G2 residues are merely residues that are in close linkage to the actual amino acids critical for ApoL1 function in lipid handling. Further association studies with larger samples sizes may reveal additional sequence variations in the ApoL1 gene to be investigated.

In conclusion, these results demonstrate the potential role of saturated free fatty acids in the pathogenesis of podocyte diseases and suggest a possible mechanism to explain the association of ApoL1 with chronic kidney diseases.
Figure 1. Podocytes are sensitive to palmitate-induced cell death

(A, B) Mouse podocytes were incubated with 250 µM BSA or fatty acids conjugated to BSA (FFA:BSA ratio 2:1) at indicated concentrations for 48 hours. (A) Cells were stained with α-Annexin V Ab and PI and analyzed by flow cytometry. Percent of PI⁺ cells are depicted in (B).

(C) Mouse podocytes were incubated with BSA or fatty acids conjugated to BSA as indicated for 24 or 48 hours. Percent of Annexin V⁺ cells are shown.
A

BSA

$H_2O_2$

oleic acid
250 $\mu$M

oleic acid
500 $\mu$M

palmitic acid
62.5 $\mu$M

palmitic acid
125 $\mu$M

palmitic acid
250 $\mu$M

palmitic acid
500 $\mu$M

Annexin V

B

C

% $PI^+$ cells

% Annexin V cells

BSA $H_2O_2$

oleic acid

palmitic acid

24 hrs

48 hrs
**Figure 2. Podocytes are resistant to cholesterol-induced cell death**

Mouse podocytes were pretreated with 10 µg/ml Sandoz 58-035 or DMSO for 24 hours. Subsequently, cells were incubated with 100 µg/ml acetyl-LDL for 24 hours, stained with 7AAD and analyzed by flow cytometry.
Figure 3. Overexpression of ApoL1 in podocytes reduces palmitate-induced cell death

(A) Mouse podocytes were transfected with ApoL1-GFP. 24 hours after transfection, the cells were treated with 500 µM palmitate for 24 hours, stained with PI, and analyzed by flow cytometry. (B) Mouse podocytes were transfected with different ApoL isoforms or GFP and treated as in (A). The graph represents the fold change in the ratio of GFP+/GFP- cells in the live cell gate (PI−) after palmitate treatment. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01 vs. cells transfected with GFP only.
Figure 4. Knockdown of ApoL1 in podocytes increases palmitate-induced cell death

(A) Western blot of control and ApoL1 knockdown human podocytes. β-actin was used as loading control. (B) Control and ApoL1 knockdown podocytes were treated with BSA or 500 µM fatty acids as indicated for 24 hours. Graph represents percent of Annexin V⁺/7AAD⁺ cells. The data represent mean ± SD of multiple experiments. ** p<0.01
A

control
ApoL1 shRNA

ApoL1
β-actin

B

% Annexin V+/7AAD^+ cells

BSA oleic acid palmitic acid

control ApoL1 shRNA

**
Figure 5. ApoL1 G1 and G2 variants reduce lipotoxicity in podocyte to a similar degree compared to wild-type ApoL1

Mouse podocytes were transfected with wild-type or G1/G2 variants of ApoL1. 24 hours after transfection, the cells were treated with 250 µM palmitate for 24 hours and stained with PI. The graph represents the fold change in the ratio of GFP⁺/GFP⁻ cells in the live cell gate (PI⁻) after palmitate treatment.
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CHAPTER III

Role of NKG2D in Obesity-induced Adipose Tissue Inflammation

and Insulin Resistance
INTRODUCTION

Obesity has become a serious health issue worldwide. A key metabolic feature that commonly accompanies obesity is insulin resistance, which refers to an inadequate response by insulin target tissues to the physiologic effects of circulating insulin. The major functions of insulin include stimulation of glucose uptake in skeletal muscle, inhibition of hepatic glucose production, and suppression of lipolysis in adipose tissue. Insulin resistance is a serious health concern because it increases the likelihood of developing type 2 diabetes and other metabolic disorders, including hypertension, nonalcoholic fatty liver disease, and cardiovascular diseases (Kahn et al. 2006).

Recent studies have pointed to chronic inflammation in insulin target tissues as one of the causal links between obesity and insulin resistance (Gregor and Hotamisligil 2011; Osborn and Olefsky 2012; Odegaard and Chawla 2013). Multiple inflammatory cytokines (TNF-α, IL-6, etc.) and signaling pathways (JNK, NF-κB) have been implicated in obesity-induced insulin resistance (Hotamisligil et al. 1993; Barnes and Karin 1997; Karin et al. 1997; Yuan et al. 2001; Hirosumi et al. 2002; Cai et al. 2005; pArkan et al. 2005).

The adipose tissue plays a key role in regulating systemic metabolism. In addition to being a storage depot for lipids, the adipose tissue secretes a number of paracrine and endocrine factors, known as adipocytokines, that modulate metabolism and inflammation in the liver and muscle and pancreatic islets (Ouchi et al. 2011). In obese mice, the secretion profile of the adipose tissue is altered by the pro-inflammatory milieu. Secretion of pro-inflammatory adipocytokines, such as resistin, IL-6, and TNF-α, is increased while anti-inflammatory and insulin-sensitizing adipocytokines like adiponectin are down-regulated. Therefore it is thought
that dysregulation of the adipose tissue induces insulin-resistance and inflammation in major metabolic tissues during obesity.

Despite much effort, the initiating events that cause inflammation in obese adipose tissue are yet to be clearly understood. Hypoxia and ER stress have been shown to activate inflammatory signaling pathways in obese adipose tissue, but it is unclear whether they are the primary switch that triggers inflammation (Ozcan et al. 2004; Ozcan et al. 2006; Hosogai et al. 2007; Ye et al. 2007; Yoshiuchi et al. 2008).

Various immune cell types have been implicated in obesity-induced insulin resistance (Mathis 2013). Among them, macrophages are considered to be the major mediators of adipose tissue inflammation (Olefsky and Glass 2010; Chawla et al. 2011). Macrophages can constitute up to ∼40% of the cell number in the adipose tissue during obesity are responsible for the majority of \( \text{Tnfa} \) expression in the adipose tissue (Weisberg et al. 2003; Xu et al. 2003). Additionally, in contrast to the resident macrophages found in the adipose tissue of lean mice, the macrophages recruited to the adipose tissue during obesity are pro-inflammatory (Lumeng et al. 2007). The resident macrophages display characteristics of “alternatively activated” or M2 macrophages and express anti-inflammatory cytokines such as IL-10, while the newly infiltrating macrophages are of the “classically activated” or M1 category and express high levels of TNF-\( \alpha \) and iNOS (Lumeng et al. 2007; Martinez et al. 2008).

More recently, T cells have emerged as a key component in obesity-induced adipose tissue inflammation. Multiple studies have reported increased numbers of CD8 T cells in the adipose tissue of obese rodents and humans along with elevated levels of IFN-\( \gamma \) and RANTES, which are important for T cell function and recruitment (Caspar-Bauguil et al. 2005; Wu et al. 2007; Kintscher et al. 2008; Rausch et al. 2008; Rocha et al. 2008; Duffaut et al. 2009).
Interestingly, accumulation of T cells prior to infiltration of macrophages and the onset of insulin resistance, giving rise to the idea that recruitment of pro-inflammatory T cells could be a primary event that initiates adipose tissue inflammation (Kintscher et al. 2008; Duffaut et al. 2009). More detailed studies of the different T cell subsets have shown that the number of regulatory T cells (T\textsubscript{reg} cells), which occupy the adipose tissue of lean mice and restrict inflammation, decreases during the progression of obesity (Feuerer et al. 2009; Winer et al. 2009; Deiuliis et al. 2011). Meanwhile, CD8 and T\textsubscript{H}1 CD4 cell numbers are increased in the obese adipose tissue, and depletion of CD8\textsuperscript{+} cells alleviated adipose tissue inflammation and insulin resistance in obese mice (Nishimura et al. 2009; Winer et al. 2009; Yang et al. 2010). Together, these studies describe a situation during the onset of obesity where CD8\textsuperscript{+} and T\textsubscript{H}1 CD4\textsuperscript{+} cells overwhelm the immune-regulatory function of T\textsubscript{reg} cells, leading to subsequent infiltration of macrophages and chronic inflammation in the adipose tissue.

Still, the question of what initiates the infiltration and activation of adipose tissue T cells remains to be answered. It has been suggested that T cells are responding to chemokines and cytokines produced in the adipose tissue in response to cell death or hypoxia (Cinti et al. 2005; Rausch et al. 2008; Feuerer et al. 2009). A more provocative idea is that certain antigens produced in the adipose tissue under obese conditions could direct immune cell responses. This hypothesis is supported by the observation that adipose tissue T cells express a restricted repertoire of T cell receptors (TCRs), whose profile is unique from that of T cells in the spleen or lymph nodes (Feuerer et al. 2009; Winer et al. 2009; Yang et al. 2010). Though intriguing and novel, these models require further investigation to be validated.

An alternative way of T cell recruitment and accumulation in tissues is through the interaction between certain high affinity receptors and their ligands independent of antigen. Our
lab has previously shown that NKG2D (natural-killer group 2, member D), a cell surface receptor expressed in cytotoxic T lymphocytes (CTLs), is sufficient to induce recruitment of CTLs to pancreatic islets over-expressing its ligand and cause insulitis (Markiewicz et al. 2012). NKG2D is a stimulatory receptor expressed in NK cells, CD8+ T cells, γδ T cells, and macrophages (Raulet 2003). Engagement of murine NKG2D by one of its various ligands is sufficient to fully activate NK cells and macrophages, while in CD8+ T cells, it functions primarily as a co-stimulatory receptor that amplifies TCR signaling (Groh et al. 2001; Jamieson et al. 2002; Markiewicz et al. 2005). NKG2D has been shown to be involved in various immune responses, such as viral immunity, tumor surveillance, and autoimmune diseases, and its importance is underlined by the various ways viruses and tumors have evolved to evade recognition by NKG2D (Burgess et al. 2008; Van Belle and von Herrath 2009).

NKG2D binds to a number of distinct ligands that share structural homology with MHC class-I proteins with high affinities (nanomolar to low micromolar range) (Raulet 2003). The list of NKG2D ligands include two MHC class-I polypeptide-related chain (MIC) proteins and six cytomegalovirus UL16-binding proteins (ULBP) in human and five retinoic acid early transcript (RAE) proteins, three histocompatibility 60 (H60) proteins, and ULBP-like transcript 1 (MULT1) in mice. The NKG2D ligands are usually not expressed in normal adult tissues, but are up-regulated in response to various ‘cellular stress conditions’, such as oxidative stress, viral infection, and DNA damage (Samarakoon et al. 2009). The induced ligands are thought to act as a signal to the immune system for the removal of damaged cells.

The adipose tissue and other major metabolic tissues of obese mice and humans are characterized by numerous stress conditions, such as nutrient stress (from elevated glucose and lipid levels), ER stress, oxidative stress, hypoxia, and chronic inflammation (Hotamisligil 2006; Ye 2009).
Some of these stress stimuli overlap with conditions previously shown to up-regulate NKG2D ligand expression. In fact, the human NKG2D ligands MICA and MICB have been shown to be induced in the liver of obese patients (Kahraman et al. 2010). This leads to an interesting hypothetical model in which the stress-inducing environment of the adipose tissue during obesity could cause the induction of NKG2D ligand expression and recruitment of CD8^+ T cells. This study was designed to test the validity of this model and determine the possible role of NKG2D in obesity-induced insulin resistance.
MATERIALS AND METHODS

Cell Culture

3T3-L1 fibroblasts (from American Tissue Type Culture Collection) were maintained and differentiated as previously described (Song et al. 2008). For NKG2D ligand expression experiments, differentiated 3T3-L1 cells (day 7, ~95% differentiated) were treated with 5 µg/ml insulin (Sigma), 50 ng/ml TNF-α (Peprotech, Rocky Hill, NJ), 3 µg/ml tunicamycin (EMD Millipore, Darmstadt, Germany), 50mU/ml glucose oxidase (Sigma), 100 ng/ml LPS (Invivogen, San Diego, CA), and 500 µM palmitate (Nu-Chek Prep) conjugated to 250 µM fatty acid-free BSA (SeraCare Life Sciences) for 24 hrs.

Mice

All mice were housed in a specific pathogen-free facility at Washington University School of Medicine. C57BL/6J and ob/ob (B6.V-Lepob/J) mice were purchased from The Jackson Laboratory. NKG2D-deficient (Klrk1−/−) mice have been previously described (Zafirova et al. 2009). The PCCALL-Rae1ε mice have been previously described (Markiewicz et al. 2012). Adiponectin-Cre transgenic mice were provided by Dr. P. Scherer (University of Texas Southwestern Medical Center) (Wang et al. 2010). High-fat diet (HFD) mice were fed a 60 kcal% fat diet from Research Diets, Inc. (New Brunswick, NJ). Normal chow control mice were fed a diet of 13 kcal% fat from LabDiet (St. Louis, MO). Care and use of mice were conducted in accordance with protocols approved by the Animal Studies Committee at Washington University in St. Louis (Protocol Number: 20120013), in compliance with the Animal Welfare Act.
Real-time Quantitative PCR Analysis

RNA was extracted from 3T3-L1 cells, epidydymal fat tissue, or fractionated adipocytes and stromal vascular cells with TRIzol (Life Technologies). RNA was reverse transcribed using random primers with the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time quantitative PCR was performed using the SYBR Green method on the 7500 Fast Real-Time PCR system (Life Technologies). Transcript levels were normalized to HPRT or β-actin.

The sequence of primers were: Rae-1 pan F - CCA CCT GGG AAT TCA ACA TC; Rae-1 pan R - TGA TCT TGG CTT TTC CTT GG; Mult-1 F - CAA AGG TCT GCT GCT TCA CA; Mult-1 R - TGC TTG TGT CAA CAC GGA AT; H60b F - TGC CTC AAC AAA TCG TCA TC; H60b R - CAC TCA GAC CCT GGT TGT CA; CD68 F - TAC CCA ATT CAG GGT GGA AG; CD68 R - ATG GGT ACC GTC ACA ACC TC; TNF-α F - ACG GCA TGG ATC TCA AAG AC, TNF-α R - AGA TAG CAA ATC GGC TGA CG; MCP-1 F - TCC CAA TGA GTA GGC TGG AG; MCP-1 R - TCT GGA CCC ATT CCT TCT TG; adiponectin F - GAC AAG GCC GTT CTC TTC AC; adiponectin R - CAG ACT TGG TCT CCC ACC TC; adipin F - AGC GAT GGT ATG ATG TGC AG; adipin R - ATT GCA AGG GTA GGG GTC TC; β-actin F - GAA GAG CTA TGA GCT GCC TGA; β-actin R - GCA CTG TGT TGG CAT AGA GGT, HPRT F - ATC AGT CAA CGG GGG ACA TA; HPRT R - AGA GGT CCT TTT CAC CAG CA

Fat Tissue Fractionation
The epidydmal fat tissue was isolated from euthanized mice, rinsed in PBS, weighed, and minced into small pieces (~1 mm). The minced tissue was digested in KRH buffer (4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 118 mM NaCl, 20 mM HEPES [pH 7.5]) supplemented with 1.5% bovine serum albumin (BSA) (SeraCare Life Sciences), 30 μg/ml Liberase TM (Roche Applied Science, Indianapolis, IN), and 1 μg/ml DNaseI (Sigma) at 37°C for 25~40 minutes with vigorous shaking. The digested samples were strained through a sterile 250 μm nylon mesh (Sefar, Buffalo, NY) and centrifuged at 200g for 10 min. The floating cells were collected as the adipocyte fraction and the pelleted cells were collected as the stromal vascular fraction (SVF). The adipocytes were washed twice with KRH buffer + 1.5% BSA before being processed for RNA extraction. The SVF was resuspended in ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) and incubated at room temperature for 2 minutes to deplete erythrocytes, washed twice with KRH buffer + 1.5% BSA, and finally resuspended in FACS buffer (1% BSA, 2 mM EDTA in 1×PBS) for flow cytometry or processed for RNA extraction.

Isolation of Splenocytes and Peripheral Blood Mononuclear Cells (PBMCs)

Spleen harvested from euthanized mice were homogenized and filtered through a 40-micron cell strainer. The cells were then washed with PBS, depleted of erythrocytes with ACK lysis buffer, washed twice with PBS, and resuspended in FACS buffer.

For PBMCs, whole blood was collected from mice in EDTA-coated vacutainers (BD Biosciences). The blood was mixed with PBS and centrifuged at 300g for 10 minutes. The cells were then resuspended in ACK lysis buffer, washed twice with PBS, and resuspended in FACS buffer.
** Immunofluorescent Staining and Flow Cytometry 

Single-cell suspensions from the SVF of fat tissue, spleen, and blood samples were incubated in FcBlock (BD Biosciences) for 15 min at 4°C. The cells were then stained with fluorophore-conjugated antibodies for 20 min at 4°C in the dark. The antibodies were purchased from BD Biosciences (CD8-FITC, CD11c-FITC, NK1.1-FITC, CD4-PE/Cy7, CD45.2-APC), eBioscience (San Diego, CA) (NKG2D-PE, F4/80-PE/Cy7, CD11b-APC/eFluor780), or BioLegend (San Diego, CA) (CD3ε-Pacific Blue). Dead cells were excluded with 7-AAD staining (BD Biosciences). Flow cytometry data was acquired with FACSCantoII or LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo (Treestar).

** Metabolic Studies 

Male mice were placed on a HFD beginning at 8 weeks of age, and body weight was monitored weekly. Mice were fasted overnight (12 h) prior to measuring fasting blood glucose or performing glucose tolerance tests (GTT) and insulin tolerance tests (ITT). For GTT, glucose (1 g/kg in saline) was administered by intraperitoneal (i.p.) injection. Blood glucose levels were measured before and 15, 30, 60, 120 min after glucose injection with a handheld ContourTS glucometer (Bayer, Tarrytown, NY). For ITT, insulin (0.75 U/kg, Humulin R, Lilly, Indianapolis, IN) was administered by i.p. injection and blood glucose levels were measured before and 15, 30, 60, 120 min after insulin injection.

** CTL Adoptive Transfer 


CTL adoptive transfer experiments were performed as described previously (Markiewicz et al. 2012). Briefly, CTLs were generated \textit{in vitro} by culturing splenocytes and lymph node cells from OT-1 T cell receptor transgenic mice in IMDM + 10% FCS with 1 μM OVA peptide (SIINFEKL) (provided by Dr. P. Allen, Washington University School of Medicine) for 5 days. Live cells were harvested using Ficoll-hypaque (GE Healthcare, Piscataway, NJ) and labeled with 1 μM CFSE (Invitrogen) and injected intravenously (10^7 cells/mouse). Epididymal adipose tissue and spleen were harvested 24 hours after injection and processed for flow cytometry.

\textit{Statistical analyses}

Data were routinely presented as mean +/- standard deviation (SD). We determined significance by the Student's t test or ANOVA. We considered a P value of < 0.05 as statistically significant.
RESULTS

Induction of NKG2D ligands in fat tissue of obese mice

NKG2D binds to a number of distinct ligands which are usually not expressed in normal adult tissues, but are up-regulated in response to various ‘cellular stress conditions’, such as oxidative stress, viral infection, and DNA damage (Samarakoon et al. 2009). The induced ligands are thought to act as a signal to the immune system for the removal of damaged cells. In order to identify factors that could potentially regulate NKG2D ligands in adipocytes, we incubated differentiated 3T3-L1 cells with factors that induce various cell responses. Insulin and TNF-α treatment caused a significant increase in the mRNA levels of different NKG2D ligands at varying concentrations. Insulin had the greatest effect on Rae-1 mRNA level among the NKG2D ligands (Fig. 1a) while TNF-α treatment increased both Rae-1 and Mult-1 (Fig. 1a and b). H60b mRNA levels were only moderately affected by insulin and TNF-α (Fig. 1c). Meanwhile, incubation in tunicamycin, glucose oxidase, LPS, and palmitate did not have an effect (Fig. 1d).

Insulin and TNF-α levels are known to be elevated during obesity in humans and rodents. To address whether NKG2D ligands are induced by obesity, we analyzed the adipose tissue of diet-induced obese (DIO) mice. RNA was isolated from epididymal adipose tissue obtained from mice fed either a normal diet (ND) or a high-fat diet (HFD) and analyzed by real-time qPCR. Rae-1 and Mult-1 mRNA levels were increased approximately 5-fold and 17-fold respectively in the DIO mice compared to lean control mice (Fig. 2a). H60b expression level was not altered in DIO mice. As control, we confirmed increased expression of CD68, TNF-α, and MCP-1 and decreased expression of adiponectin and adipins in DIO mice (Fig. 2b).
To confirm these results, we also analyzed the adipose tissue of \textit{ob/ob} mice, a genetic model of obesity (Fig. 2c, d). Similar to the DIO mice, Rae-1 and Mult-1 transcript levels were increased in the \textit{ob/ob} mice compared to lean \textit{ob/+} controls (Fig. 2c). In addition, H60b mRNA level was also slightly increased in the \textit{ob/ob} mice (Fig. 2c).

The adipose tissue is composed of adipocytes, immune cells, vascular cells, and undifferentiated preadipocytes. In order to determine which cell types express NKG2D ligands, the adipose tissue was fractionated into adipocytes and the stromal vascular fraction (SVF) via collagenase digestion. Rae-1 was expressed predominantly in the adipocyte fraction while Mult-1 had similar expression levels in the adipocytes and the SVF (Fig. 3a). H60b expression was higher in the adipocyte fraction (Fig. 3a).

Induction of NKG2D ligands in the adipose tissue of obese mice led us to hypothesize that this could be a mechanism by which CD8 T cells are recruited to the adipose tissue to initiate inflammation during obesity. Infiltration of CD8 T cells to the adipose tissue of DIO mice has been shown to occur relatively soon after starting the HFD regimen (4~5 weeks after initiation of diet). In order to see whether NKG2D ligand induction occurs within a similar time frame, we measured the time-course kinetics of NKG2D ligand expression in DIO mice (Fig. 3b). The mRNA levels of Rae-1 and Mult-1 were highest at the earliest time-point measured (3 weeks post-initiation of diet) and gradually decreased during the progression of obesity. These results suggest that early changes that occur in the adipose tissue of mice on a HFD induce expression of NKG2D ligands.

\textit{Obesity-induced insulin resistance and adipose tissue inflammation in NKG2D-deficient mice}
The NKG2D–deficient (klrk−/−) mice lack expression of NKG2D in all immune cells including CD8 T cells. To directly test whether NKG2D ligands are involved in the development of obesity-induced insulin resistance, we placed wild-type and klrk−/− mice on a HFD. Age-matched male mice were divided into two groups and placed on either ND or HFD. We measured the changes in body weight and fasting glucose levels for 18 weeks. In order to measure insulin sensitivity, we performed glucose tolerance test (GTT) and insulin tolerance test (ITT) at 6, 12, and 18 weeks after starting the diet regimen.

At baseline (8-weeks old), there was no difference between WT and klrk−/− mice in body weight (Fig. 4a) and fasting blood glucose levels (Fig. 4b). Rapidly within a few weeks, mice on the HFD began to outweigh mice on the normal diet, and the gap between the two groups continued to grow until the end of the experiment. There was no difference in body weight change between WT and klrk−/− mice in either the ND or HFD group throughout the experiment.

In GTT experiments (Fig. 5a), obese mice were significantly less tolerant compared to lean mice in both WT and klrk−/− mice. However, there was no difference between WT and klrk−/− mice in any of the time points. Similar results were obtained in ITT assays (Fig. 5b) with WT and klrk−/− mice displaying similar degrees of insulin sensitivity. Fasting glucose, which is also indicative of insulin sensitivity, was not altered in klrk−/− mice (Fig. 4b).

At the end of 18 weeks on the diet regimen, the epididymal adipose tissue was harvested to analyze immune cell infiltration. The SVF was isolated by collagenase digestion, stained for cell surface markers, and analyzed by flow cytometry. Consistent with previous reports, there was a significant increase in the number of F4/80+CD11c+ M1 macrophages and CD8 T cells in the adipose tissue of HFD-fed mice compared to lean counterparts (Fig. 6b). However, the number of M1 macrophages and CD8 T cells in the adipose tissue were similar in WT and klrk−/−
mice (Fig. 6a, d). We also confirmed that there was no difference in CD8 T cell infiltration after 3 weeks of HFD, when NKG2D ligand expression was highest (data not shown). These results indicate that NKG2D does not affect CD8 T cell recruitment to the adipose tissue during obesity.

**Obesity-induced adipose tissue inflammation in fat-specific Rae-1ε transgenic mice**

In order to confirm these results, we generated a transgenic mice specifically over-expressing Rae-1ε in the adipose tissue (AdpnCre⁺ Rae⁺) by crossing adiponectin-Cre mice (Wang et al. 2010) with PCCALL-Rae1ε mice (Markiewicz et al. 2012). Real-time qPCR was used to confirm Rae-1ε over-expression in the adipose tissue (Fig. 7a). Rae-1 mRNA levels were not altered in liver and muscle (data not shown), indicating specific overexpression of Rae-1ε in the adipose tissue. It is known that NKG2D expression in NK cells and T cells is down-regulated in transgenic mice constitutively overexpressing NKG2D ligands (Oppenheim et al. 2005; Wiemann et al. 2005). Indeed, we observed loss of NKG2D expression in NK cells isolated from the adipose tissue, but not spleen or blood, again confirming fat-specific overexpression of Rae-1 in the Adpn⁺ Rae⁺ mice (Fig. 7b).

To examine the early events of obesity-induced immune cell infiltration, these transgenic mice (AdpnCre⁺ Rae⁺) were placed on a high-fat diet for 5 weeks along with their littermate controls (AdpnCre⁺ Rae⁺). At the end of 5 weeks, the SVF from the adipose tissue were analyzed by flow cytometry. Adipose tissue from obese mice contained approximately twice the number of M1 macrophages and CD8 T cells compared to lean mice (Fig. 8a, d). However, the number of CD8 T cells and M1 macrophages in the adipose tissue was not altered by fat-specific overexpression of Rae-1ε (Fig. 8a, d). These results are consistent with the observations
made in $k_{lrk}^{-/-}$ mice and confirm that NKG2D is not involved in the recruitment of CD8 T cells to the adipose tissue during obesity.

The $k_{lrk}^{-/-}$ mice and AdpnCre$^+$ Rae$^+$ mice used in the previous experiments were housed in specific pathogen-free (SPF) conditions, and therefore the number of circulating CTLs is minimal. In mice, NKG2D is expressed in activated CTLs, but not in naïve CD8 T cells. We therefore speculated that the lack of a phenotype could be due to low numbers of circulating CTLs. In addition, it was possible that the constitutive overexpression of Rae-1$\epsilon$ was down-regulating NKG2D expression in circulating T cells. To circumvent these issues, and to directly test whether up-regulation of NKG2D ligands can induce recruitment and accumulation of cytotoxic CD8 T cells in the adipose tissue, we intravenously injected CFSE-labeled CTLs into control and AdpnCre$^+$ Rae$^+$ mice. 24 hours after injection, we measured the number of CFSE$^+$ CTLs recruited to the spleen and adipose tissue (Fig. 9). Similar numbers of CFSE$^+$ CTLs were detected in the spleen of AdpnCre$^+$ Rae$^-$ and AdpnCre$^+$ Rae$^+$ mice, indicating that equal number of CTLs was successfully transferred to each mice. In the adipose tissue, overexpression of Rae-1$\epsilon$ did not result in increased recruitment of CTLs. These results demonstrate that, in contrast to pancreatic islets, NKG2D-NKG2D ligand interaction is not sufficient to drive accumulation of NKG2D-expressing CTLs in the adipose tissue.


DISCUSSION

In this study, we tested a model of antigen-independent recruitment of CD8$^+$ T cells to the adipose tissue during obesity via NKG2D-NKG2D ligand engagement. Results from our experiments suggest that interaction between the immunoreceptor NKG2D and its cognate ligands is not critically involved in the development of adipose tissue inflammation during diet-induced obesity. Neither deletion of NKG2D nor fat-specific overexpression of an NKG2D ligand had significant effects on insulin sensitivity or infiltration of immune cells to the adipose tissue in the setting of a high-fat diet.

This lack of phenotype does not seem to be due to strain-specificity of the mice used in our experiments. The composition of immune cells is known to vary among different strains of mice (Petkova et al. 2008). In addition, the immune system is compromised in certain genetic obese mice models, such as the ob/ob mice or the db/db mice, because leptin is required for immune cell homeostasis, especially T cells (Lam and Lu 2007). In order to avoid such variables, we chose to utilize the diet-induced obese (DIO) mouse model using C57BL/6 mice. The C57BL/6 strain is the predominant strain used to study nutrient-dependent metabolic disorders in the literature and was the strain used to demonstrate the involvement of various immune cells in obesity-induced inflammation.

The NKG2D-deficient mice display a NK cell hyperproliferation phenotype (Zafirova et al. 2009), which could be a confounding factor in the case that NK cells are also involved in adipose tissue inflammation. However, as of date, NK cells have not been clearly implicated in obesity-induced insulin resistance. There have been conflicting reports of changes in the number
of NK cells in the adipose tissue during obesity. In our experiments, we did not observe a clear reproducible pattern of NK cell recruitment.

Similar to the NKG2D-deficient mice, we did not observe any difference in immune cell infiltration in the fat-specific Rae-1ɛ mice. We could not directly determine the protein level of Rae-1ɛ in the adipocytes of the fat-specific Rae-1ɛ mice due to the lack of antibodies suitable for immunohistochemistry or Western blots and the technical difficulties in performing flow cytometry with adipocytes. However, we did observe a nearly complete down-regulation of NKG2D specifically in NK cells isolated from the adipose tissue, but not in cells isolated from spleen or blood. It is well known that engagement of Rae1ɛ by NKG2D results in NKG2D down-regulation (Groh et al. 2002; Ogasawara et al. 2003; Oppenheim et al. 2005). Therefore, these results show that the level of transgene expression was sufficient to completely quench NKG2D expression on NK cells in the adipose tissue, suggesting that Rae-1ɛ is being expressed at biologically relevant levels.

Although we observed induction of both Rae-1 and Mult-1 in obese mice, only Rae-1 was expressed specifically in fat cells. As adiponectin-Cre mice induce transgene expression only in fat cells and not in stromal vascular cells, we speculated that overexpressing Rae-1ɛ would closely resemble the expression pattern of NKG2D ligands during obesity. However, it is possible that overexpressing Mult-1 instead could have yielded different results.

It has been shown that circulating levels of CD8+ T cells in male C57BL/6 mice are relatively low compared to other mouse strains. In addition, NKG2D is only expressed in activated CD8+ T cells, the number of which may not be sufficient in mice raised in a SPF facility. In order to circumvent these issues, we adoptively transferred activated CTLs into the fat-specific Rae-1ɛ transgenic mice. Contrary to our expectations, overexpression of Rae-1ɛ in
adipocytes did not lead to accumulation of CTLs in the adipose tissue. This is in contrast to previous results from similar experiments performed in the pancreatic islets (Markiewicz et al. 2012). This may be due to differences in the architecture of the vasculature between the pancreas and the adipose tissue, which could affect how surface proteins are detected by lymphocytes in circulation.

Despite the lack of evidence for the involvement of NKG2D in obesity-induced inflammation and insulin resistance, it is still interesting that NKG2D ligands are up-regulated in the adipose tissue in response to a high fat diet. In fact, the human NKG2D ligands MICA and MICB have been shown to be induced in the liver of obese patients (Kahraman et al. 2010). In addition, NKG2D ligands are induced in the aorta of ApoE−/− mice fed a western diet and are involved in atherosclerotic plaque formation (Xia et al.). The nature of the response of the immune system to NKG2D ligand induction necessitates a tight regulation of its expression. Previous studies have shown that various ‘cell stress’ conditions, including DNA damage and viral infection, can induce NKG2D ligand expression. However, it seems that such cues are not sufficient to up-regulate NKG2D ligands in all cell types. It is more likely that different signals trigger the expression of the multiple NKG2D ligands in different cell types. Indeed, insulin and TNF-α was able to significantly increase transcript levels of Rae-1 and Mult-1, but not H60b, in differentiated 3T3-L1 adipocytes. Notably, high-fat diet resulted in the up-regulation of the same subgroup of NKG2D ligands, namely Rae-1 and Mult-1. Therefore, it is tempting to speculate that these signals are also responsible for the induction of NKG2D ligands in the adipose tissue in vivo given that insulin and TNF-α levels are known to be elevated in obesity.

Interestingly, multiple NKG2D ligands were expressed in undifferentiated 3T3-L1 cells and the expression levels decreased after differentiation (data not shown). This could be
attributed to the cease of proliferation that occurs when 3T3-L1 cells differentiate. It was recently demonstrated that Rae-1 expression is regulated by the transcription factor E2F, which is associated with cell cycle. On the other hand, it is also possible that NKG2D ligand expression is actively repressed by the differentiation process. Identification of such factors would shed light on how the strict regulation of NKG2D ligands is achieved in various cell types.

The biological significance of NKG2D ligand induction in the adipose tissue during obesity is unclear. Adipocytes in the adipose tissue of HFD-fed mice are subjected to various cell stress conditions due to increased metabolic loads, hypoxic conditions, and proinflammatory environment. Hence, the most obvious explanation would be that NKG2D ligands are being expressed by damaged adipocytes. It should be noted that the different types of NKG2D ligands do not seem to respond in the same manner. Only Rae-1 and Mult-1 were induced by the introduction of the high-fat diet while H60b levels remained the same. Furthermore, Rae-1 was predominantly found in adipocytes whereas Mult-1 expression levels were similar in adipocytes and stromal vascular cells. How these differences affect adipose tissue homeostasis and/or pathology during obesity remain to be determined.

In summary, our results rule out the interaction between NKG2D and its ligands as a possible mechanism of CD8$^+$ T cell recruitment to the adipose tissue during obesity. Our findings regarding the induction of NKG2D ligands in the adipose tissue during obesity provide a new model to investigate the mechanisms involved in the regulation of NKG2D ligand expression.
Figure 1. Insulin and TNF-α increases NKG2D ligand expression in 3T3-L1 adipocytes.

(A-C) Differentiated 3T3-L1 cells were treated with insulin (0.5 - 5 μg/ml) or TNF-α (10 - 100 ng/ml) for 24 hours. The cells were then analyzed by quantitative PCR to determine the expression levels of (A) Rae-1, (B) Mult-1, and (C) H60b. (D) Differentiated 3T3-L1 cells were treated with insulin, TNF-α, tunicamycin, glucose oxidase, LPS, and palmitate. The cells were then analyzed by quantitative PCR for NKG2D ligand expression. The results were normalized to HPRT levels and are shown as fold-increase over control. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01 vs. control
Figure 2. *NKG2D ligands are increased in the adipose tissue of obese mice.*

Expression of NKG2D ligands in the epididymal adipose tissue from (A) DIO mice (8 weeks on HFD) and (C) *ob/ob* mice was determined by quantitative PCR. mRNA levels of CD68, TNF-α, MCP-1, adiponectin, and adipsin were measured in (B) DIO mice and (D) *ob/ob* mice for control. The results were normalized to HPRT levels and are shown as fold-increase over lean control mice. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01 vs. lean control mice.
Figure 3. NKG2D ligands are increased in the adipocytes of obese mice.

(A) The epididymal adipose tissue of DIO mice was fractionated into the stromal vascular fraction and adipocytes and analyzed by quantitative PCR for expression of NKG2D ligands. (B) Adipocytes were obtained from the epididymal adipose tissue of DIO mice at different time points after starting high-fat diet. The adipocytes were then analyzed by quantitative PCR for expression of NKG2D ligands. The results were normalized to β-actin levels and are shown as fold over (A) stromal vascular fraction or (B) lean control mice. The data represent mean ± SD of 3 independent experiments. * p<0.05, ** p<0.01 vs. (A) stromal vascular fraction or (B) lean control mice.
Figure 4. Body weight gain and fasting glucose levels are not altered in klrk<sup>−/−</sup> mice.

(A) Body weight of WT and klrk<sup>−/−</sup> mice placed on normal diet or high-fat diet for 18 weeks. (B) Fasting blood glucose levels measured after 0, 6, 12, and 18 weeks on high-fat diet.
Figure 5. Susceptibility to obesity-induced insulin resistance is not altered in klrk<sup>−/−</sup> mice.

(A) Glucose tolerance test and (B) insulin tolerance tests were performed after 0, 6, 12, and 18 weeks on high-fat diet. The numbers of mice used for each experiment were at least 10 for WT normal diet, at least 9 for WT high-fat diet, at least 16 for klrk<sup>−/−</sup> normal diet, and at least 18 for klrk<sup>−/−</sup> high-fat diet.
A

- WT normal diet
- KO normal diet
- WT high-fat diet
- KO high-fat diet

blood glucose (mg/dL)

week 0

week 6

week 12

week 18

B

blood glucose (mg/dL)

week 0

week 6

week 12

week 18
Figure 6. Immune cell infiltration of adipose tissue during obesity is not altered in klrk^−/− mice.

The stromal vascular fraction was isolated from the epididymal adipose tissue of DIO and lean mice after 18 weeks on high-fat diet regimen. The cells were stained with antibodies to identify (A) M2 macrophages (F4/80^+, CD11c^-), (B) M1 macrophages (F4/80^+, CD11c^+), (C) CD4 T cells (CD3ε^+, CD4^+), (D) CD8 T cells (CD3ε^+, CD8^+), and (E) NK cells (CD3ε^-, NK1.1^+) and analyzed by flow cytometry. The cells were gated on 7AAD^-, CD45^+ populations. The data represent mean ± SD of 3 independent experiments.
Figure 7. Rae-1ε is overexpressed specifically in the adipose tissue of AdpnCre⁺ Rae⁺ mice.

(A) The epididymal adipose tissue from AdpnCre⁺ Rae⁺ and control mice were analyzed by quantitative PCR to determine Rae-1 expression. The results were normalized to β-actin levels and are shown as fold-increase over AdpnCre⁻ Rae⁻ mice. The data represent mean ± SD of 3 independent experiments. ** p<0.01 vs. AdpnCre⁻ Rae⁻ mice. (B) Splenocytes, peripheral blood mononuclear cells (PBMC), and stromal vascular fractions isolated from the epididymal adipose tissue of AdpnCre⁻ Rae⁺ and AdpnCre⁺ Rae⁺ mice were stained with NKG2D antibodies and analyzed by flow cytometry. Cells were gated on 7AAD⁻, CD45⁺, CD3ε⁻, NK1.1⁺ populations. NKG2D is down-regulated only in NK cells isolated from the adipose tissue.
A

![Graph showing relative mRNA level normalized to β-actin for AdpnCre- Rae- and AdpnCre+ Rae+ conditions.](image)

B

![Flow cytometry plots for NKG2D and FSC for splenocytes, PBMC, and SVF.](image)
Figure 8. Immune cell infiltration of adipose tissue during obesity is not altered in AdpnCre$^+$ Rae$^+$ mice.

The stromal vascular fraction isolated from the epididymal adipose tissue of AdpnCre$^+$ Rae$^-$ and AdpnCre$^+$ Rae$^+$ mice after 5 weeks on high-fat diet regimen. The cells were stained with antibodies to identify (A) M2 macrophages (F4/80$^+$, CD11c$^-$), (B) M1 macrophages (F4/80$^+$, CD11c$^+$), (C) CD4 T cells (CD3ε$^+$, CD4$^+$), (D) CD8 T cells (CD3ε$^+$, CD8$^+$), and (E) NK cells (CD3ε$^-$, NK1.1$^+$) and analyzed by flow cytometry. The cells were gated on 7AAD$^-$, CD45$^+$ populations. The data represent mean ± SD of 3 independent experiments.
Figure 9. Rae-1ε overexpression does not induce CTL accumulation in the adipose tissue.

CTLs generated from OT-1 mice were labeled with CFSE and injected intravenously. A group of mice were injected with PBS for control. Spleen and epididymal adipose tissue were isolated 24 hours after injection and analyzed by flow cytometry. Cells were gated on 7AAD⁻, CD45⁺, CD3ε⁺ populations. The data are representative of at least 3 individual mice per group.
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