METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

Epidermis-Activated Gasdermin-A3 Enhances Thermogenesis of Brown Adipose Tissue through IL-6/Stat3 Signaling

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Missense mutations in the gasdermin-A3 (*Gsdma3*) gene are associated with skin inflammation and hair loss in mice. However, the physiological function of *Gsdma3* remains unclear. Herein, we reported that mice carrying the *Gsdma3* Y344H mutation that encodes a presumptive activated form of Gsdma3 show increased heat production along with lower body fat percentages. Detailed analysis indicated that this metabolic phenotype is mediated by serum IL-6—induced up-regulation of thermogenesis in brown adipose tissue. The mutant form of Gsdma3 promotes the expression of IL-6 in the epidermis in a c-Jun N-terminal kinase (JNK) signaling—dependent manner. The higher whole-body heat production in alopecia and excoriation mice could be suppressed by an IL-6 receptor/GP130 inhibitor. Our results uncovered Gsdma3/IL-6—dependent cross talk between the skin and brown adipose tissue. (*Am J Pathol* 2019, 189: 1041–1052; <https://doi.org/10.1016/j.ajpath.2019.01.012>)

Skin is traditionally defined as a barrier for the human body to resist mechanical damage and invasion by pathogenic microorganisms. Recently, several studies found that epidermal lipid metabolism plays a crucial role in maintaining whole-body metabolic homeostasis.¹ For instance, skin-specific stearyl-CoA desaturase-1 (*Scd1*) knockout mice showed an increase in energy expenditure and resistance to high-fat diet—induced obesity.^{2,3} Skin also mediates adaptive thermogenesis under cold conditions. Thermoreceptors in the skin can activate the neural circuits that connect with the hypothalamus under cold exposure. The hypothalamus further releases norepinephrine into the blood and activates brown adipose tissue (BAT) heat production.^{4,5}

Inflammatory skin diseases, such as psoriasis, are also associated with metabolic syndrome. Psoriasis has been identified as a risk factor for metabolic syndromes, such as obesity and type 2 diabetes, by several clinical studies.^{6–8} Another study reports that after UV irradiation, the epidermis secretes several cytokines to regulate s.c. fat

metabolism.⁹ Increase of metabolic rate and heat production is also common after burn injury, along with up-regulation of uncoupling protein 1 (UCP1) in BAT.^{10–13} Even for 9 to 12 months after severe burn, the patients are still in a hyper-metabolic situation.¹⁴ These reports show a close relevance between skin and whole-body metabolism in responding to inflammation. However, the mechanistic relationship between inflammatory skin diseases and metabolic syndrome is still unknown.

The gasdermin-A3 (*Gsdma3*) gene, a member of the *Gasdermin* family, is expressed specifically at a high level in the epidermis.¹⁵ Several mouse strains carrying *Gsdma3*

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missense mutations have been reported to associate with cicatricial alopecia, hyperkeratosis, sebaceous gland atrophy, and corneal opacity.^{16–19} Nevertheless, the physiological function of Gsdma3/GSDMA is still unclear.²⁰

The alopecia and excoriation (AE) phenotype in *Gsdma3* mutant mice (without additional mutations) is mediated by skin inflammation and can be rescued by immunosuppressant.²¹ We also found that the N terminus of Gsdma3 is the active domain for inducing cell death, and the C terminus of Gsdma3 suppresses the N-terminus function in a self-inhibitory manner.²⁰ Interestingly, all mutations reported so far are missense point mutations in the C terminus, which abolish its self-inhibition function. Therefore, the mutant forms of Gsdma3 actually represent the active state of Gsdma3. Our finding was confirmed by studies of gasdermin-D (*GSDMD*), another member of the *Gasdermin* family.^{16–19} The activated GSDMD is activated by caspase-1/4/5/11-mediated cleavage in the linker region between the N terminus and the C terminus. However, the potential protease for Gsdma3 activation remains unknown.

In this study, it was found that Gsdma3 activation in skin showed decreased body fat and increased whole-body heat production. Further study showed that the metabolic change is dependent on the up-regulation of IL-6 expression, which further activates BAT thermogenesis.

Materials and Methods

Mice

All animal welfare and experimental procedures were approved by the Animal Care and Use Committee of the Model Animal Research Center, Nanjing University (Nanjing, China). The wild-type (WT) C57BL/6J mice were obtained from the National Database of Mouse Genetic Resources (Nanjing, China). The AE strain was generated in an ethyl nitrosourea mutagenesis program, which was reported previously, that had been backcrossed to the C57BL/6J strain for >10 generations.²¹ All mice were maintained in a specific pathogen-free animal facility under a 12-hour light-dark cycle at constant temperature (23°C), fed a standard chow diet ad libitum, and hydrated with acidified water, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The WT littermates were used as controls.

Cell Culture and Treatment

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS; Invitrogen, San Diego, CA), with 5% CO₂ at 37°C. HaCaT-N and HaCaT-CW cell lines infected by TRIPZ–Gsdma3–N-terminus and TRIPZ–Gsdma3–C-terminus lentivirus were reported previously.²⁰ JNK inhibitor SP600125 and extracellular signal-regulated kinase inhibitor U1026, used to treat HaCaT-N and HaCaT-CW cell lines, respectively, were purchased from Selleck Chem (Houston, TX).

Body Composition Measurement

Mice body composition parameters, including fat mass and lean mass values, were assessed with dual-energy X-ray absorptiometry using a Lunar PIXImus II densitometer (PIXImus 2; GE Lunar, Chicago, IL), according to the instructions of the manufacturer.

Primary Brown Adipocyte Isolation and Culture

Primary brown adipocytes were isolated and cultured, as previously described.²² Briefly, 2-week-old C57BL/6J mice were sacrificed, and their interscapular brown fat tissues were harvested and digested by collagenase A (CLS004196; Worthington, Lakewood, NJ). Primary brown adipocytes were filtered through 70-μm filters and then cultured in DMEM containing 10% FBS and 2 nmol/L 3,3',5'-Triiodo-L-tyrosine (T3; Sigma-Aldrich, St. Louis, MO). After confluence, primary brown adipocytes were stimulated with DMEM containing 10% FBS, 850 nmol/L insulin (Sigma-Aldrich), 0.5 μmol/L dexamethasone (Sigma-Aldrich), 250 μmol/L 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 1 μmol/L rosiglitazone (Sigma-Aldrich), 0.125 nmol/L indomethacin (Sigma-Aldrich), and 2 nmol/L T3 for 2 days. The induction medium was switched to DMEM containing 10% FBS, 160 nmol/L insulin, and 2 nmol/L T3 for 2 days. Then, cells were incubated in DMEM with 10% FBS and 2 nmol/L T3. The primary brown adipocytes were treated with serum from AE or WT mice for 4 hours to harvest.

Administration of Anti-IL-6

The IL-6-neutralizing monoclonal antibody and the control IgG were produced by Bio X Cell (West Lebanon, NH). For specific blocking of IL-6 signaling, mice were intraperitoneally injected with 500 μg neutralizing anti-IL-6 monoclonal antibody, which had been shown to be effective in blocking IL-6 signaling in previous work.²³ In the control group, mice received 500 μg IgG antibody. Antibodies were injected in a volume of 200 μL phosphate-buffered saline (PBS) 24 hours before metabolic cage analysis.

Protein Extraction and Western Blot Analysis

Samples were rinsed quickly in 2 mL of modified radioimmunoprecipitation assay buffer (radioimmunoprecipitation assay base with 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, and cocktail protease inhibitors; Sigma-Aldrich). The lysate was incubated at 4°C for 2 hours while rotating and then centrifuged at 12,000 × g for 15 minutes at 4°C. The supernatant protein concentrations were determined with Bradford reagent (Sangon Biotech, Shanghai, China). Protein samples were electrophoresed using SDS-PAGE with the appropriate percentage of acrylamide (10% to 15%), transferred to an Immobio-P^{SQ} transfer membrane (Merck Millipore, Tullagreen, Carrigtwohill, Ireland), and

incubated with primary antibodies. Antibodies used are given in [Table 1](#).

Histology

Adipose tissues were isolated and fixed in 4% para-formaldehyde and divided into sections. Hematoxylin and eosin staining was performed routinely. Frozen sections for immunofluorescence were washed in PBS three times, 5 minutes each time, and then blocked in blocking buffer (5% bovine serum albumin and 0.1% Tween-20 in PBS) at room temperature for 1 hour. Then, sections were incubated with primary antibody, which was diluted in blocking buffer overnight at 4°C. On the following day, sections were washed in PBS and incubated with secondary antibody diluted in PBS at room temperature for 1 hour. Finally, after washing with PBS three times, sections were mounted in 50% glycerol. Sections were imaged using confocal microscopy (Leica, Buffalo Grove, IL).

RNA Extraction and Quantitative Real-Time PCR

RNA was extracted using RNAiso reagent (Takara, Dalian, China), according to the manufacturer's instructions. The cDNA was reverse transcribed from 1 µg total RNA using a first-strand cDNA synthesis kit (Takara). An ABI 7300 and SYBR Premix Ex Taq (Takara) were used for quantitative real-time PCR. Primer sequences are summarized in [Table 2](#).

Indirect Calorimetry and Metabolic Assays

Mice were housed individually in a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH) with free access to food and water and the same dark and light periods of 12:12-hour cycles. O₂ consumption, CO₂ production, and activity were measured continuously for 2 days after 1 day to allow for adaptation, and respiratory exchange ratio and heat production were computed automatically. Core body temperatures were detected rectally using a thermocouple (Physitemp, Clifton, NJ).

GTT and Insulin Tolerance Test

For the glucose tolerance test (GTT) experiment, WT and AE mice, fasted for 16 hours, were injected intraperitoneally with D (+) glucose (Sigma-Aldrich; 2 g/kg body weight). For the insulin tolerance test experiment, WT and AE mice, fasted for 6 hours, were injected intraperitoneally with insulin (Novo Nordisk Pharmaceutical Industries, Malov, Denmark; 0.75 U/kg body weight). Blood glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 minutes after glucose or insulin administration. Blood glucose concentrations were measured using a Breeze 2 Blood Glucose Meter (Bayer HealthCare LLC, Mishawaka, IN).

Statistical Analysis

The results were performed by using GraphPad Prism 6 (GraphPad Software, La Jolla, CA) and Excel (Microsoft, Redmond, WA). Data were analyzed with unpaired *t*-test and presented as the means ± SEM. *P* < 0.05 was considered statistically significant.

Results

Gsdma3-Mutant AE Mice Have Less Body Fat and Higher Heat Production

There was no significant difference in body weight between WT and AE male mice (*P* = 0.1245) ([Figure 1A](#)). However, the AE mice showed significantly decreased fat mass compared with WT mice when the fat mass and lean mass composition was measured by bone densitometry (dual-energy X-ray absorptiometry or double-energy X-ray absorptiometry; *P* = 0.0002) ([Figure 1A](#)). The percentage of body fat was also decreased in AE mice (*P* < 0.0001) ([Figure 1B](#)). By weighing the dissected BAT (*P* = 0.0002), s.c. fat (*P* < 0.0001), and visceral fat (*P* < 0.0001) tissues, all of the fat tissues were found to be significantly decreased in AE mice ([Figure 1, C and D](#)).

The energy metabolism was compared in WT and AE mice by using metabolic cages. All metabolic cage data

Table 1 List of Primary Antibodies Used in This Study

Target	Catalog number	Company
Phosphorylated Stat3 (Tyr705)	9145	Cell Signaling Technology (Danvers, MA)
Stat3	12640	Cell Signaling Technology
Phosphorylated SAPK/JNK (Thr183/Tyr185)	4668	Cell Signaling Technology
SAPK/JNK	9252	Cell Signaling Technology
Phosphorylated Erk1/2 (Y204)	BS4621	Bioworld (Minneapolis, MN)
Erk1/2	BS1112	Bioworld
Phosphorylated c-Jun (Ser73)	3270	Cell Signaling Technology
InVivoMAb rat IgG1 isotype control	BE0088	Bio X Cell
InVivoMAb anti-mouse IL-6	BE0046	Bio X Cell

Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase.

Table 2 Sequences of qPCR Primers Used in This Study

Primer name	Forward primer	Reverse primer
Human-specific primers		
IL-1 β	5'-ATGATGGCTTATTACAGTGGCAA-3'	5'-GTCGGAGATTTCGTAGCTGGA-3'
IL-2	5'-AACTCCTGTCTTGCATTGCAC-3'	5'-GCTCCAGTTGTAGCTGTGTTT-3'
IL-4	5'-CCAAGTCTTCCCCCTCTG-3'	5'-TCTGTTACGGTCAACTCGGTG-3'
IL-6	5'-ACTCACCTCTTCAGAACGAATTG-3'	5'-CCATCTTTGGAAGGTTTCAGGTTG-3'
IL-12	5'-ACCTTGACCATCCAAGTCAAA-3'	5'-TTGGCCTCGCATCTTAGAAAG-3'
IL-17 α	5'-TCCCACGAAATCCAGGATGC-3'	5'-GGATGTTTCAGGTTGACCATCAC-3'
IL-18	5'-TCTTCATTGACCAAGGAAATCGG-3'	5'-TCCGGGTGCATTATCTCTAC-3'
TNF- α	5'-CCTCTCTCTAATCAGCCCTCTG-3'	5'-GAGGACCTGGGAGTAGATGAG-3'
IFN- α	5'-ATCTCAGCAAGCCCAGAGT-3'	5'-GGGTCTCAGGAGATCACAG-3'
IFN- γ	5'-TCGGTAACTGACTTGAATGTCCA-3'	5'-TCGCTTCCCTGTTTTAGCTGC-3'
36B4	5'-AGCCCAGAACACTGGTCTC-3'	5'-ACTCAGGATTTCATGGTGCC-3'
Mouse-specific primers		
IL-1 β	5'-GCAACTGTTTCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
IL-2	5'-GTGCTCCTTGTCAACAGCG-3'	5'-GGGGAGTTTTCAGGTTTCTGTA-3'
IL-4	5'-GGTCTCAACCCCCAGCTAGT-3'	5'-GCCGATGATCTCTCTCAAGTGAT-3'
IL-6	5'-GGAGCCCAACAAGAACGATA-3'	5'-ACCAGCATCAGTCCCAAGAA-3'
IL-8	5'-CAAGGCTGGTCCATGCTCC-3'	5'-TGCTATCACTTCCTTTCTGTTGC-3'
IL-10	5'-GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
IL-12	5'-TGGTTTGCCATCGTTTTGCTG-3'	5'-ACAGGTGAGGTTCACTGTTTTCT-3'
IL-22	5'-ATGAGTTTTTCCCTTATGGGGAC-3'	5'-GCTGGAAGTTGGACACCTCAA-3'
IL-17	5'-TTTAACCTCCCTTGGCGCAAAA-3'	5'-CTTTCCCTCCGCATTGACAC-3'
IL-18	5'-GACTCTTGCGTCAACTTCAAGG-3'	5'-CAGGCTGTCTTTTGTCAACGA-3'
IFN- α	5'-GGCTCTGTGCTTTCTCTGATG-3'	5'-GCGGGAATCCAAAGTCCTTC-3'
IFN- γ	5'-ATGAACGCTACACACTGCATC-3'	5'-CCATCCTTTTGCAGTTCCTC-3'
TNF- α	5'-CCCTCACACTCAGATCATCTTCT-3'	5'-GCTACGACGTGGGCTACAG-3'
36B4	5'-GACCGCTGGTTCTCCTATAA-3'	5'-AGACGATGTCACTCCAACGA-3'
Ucp1	5'-GGCCTCTACGACTCAGTCCA-3'	5'-TAAGCCGGCTGAGATCTTGT-3'
Cidea	5'-TTCAAGCCGTGTTAAGGA-3'	5'-CCTTTGGTGTAGGCTTGG-3'
Dio2	5'-CTGCGCTGTGTCTGGAAC-3'	5'-GGAGCATCTTCAACCCAGTTT-3'
Acadl	5'-GCTTATGAATGTGTGCAACTCC-3'	5'-CCGAGCATCCACGTAAGC-3'
Cpt1b	5'-GAGTGACTGGTGGGAAGAATATG-3'	5'-GCTGCTTGCACATTTGTGTT-3'
Cox4i	5'-TACTTCGGTGTGCCCTTCGA-3'	5'-TGACATGGGCCACATCAG-3'
Ldha	5'-TGCTTACGAGGTGATCAAGCT-3'	5'-GCACCCGCCAAGGTTCTTC-3'
Ldhd	5'-AGTCTCCCGTGCATCCTCAA-3'	5'-AGGGTGTCCGCACTCTTCCT-3'
Acadm	5'-AGGTTTCAAGATCGCAATGG-3'	5'-CATTGTCCAAAAGCCAAACC-3'
Ppara	5'-ACTACGGAGTTACGCATGTG-3'	5'-TTGTGCTACACCAGCTTCAGC-3'
ATP25	5'-GCTGAGGAATGTTCAAGCAGA-3'	5'-CCAAGTTCAGGGACATACCC-3'
Scd1	5'-TGGAATGCCTTTGAGATGG-3'	5'-CCAGCCAGCCTCTTGACTAT-3'
Dgat2	5'-TCATGGGTGTCTGTGGGTAA-3'	5'-CAGAGTGAAACCAAGCAACA-3'
Fasn	5'-CTCCGTGGACCTTATCACTA-3'	5'-CTGGGAGAGGTTGTAGTCAG-3'
Acacb	5'-CGCTACCAACAGTAAGGTGG-3'	5'-GCTTGGCAGGAGTTTCCTC-3'
Srebf1c	5'-TGCGGCTGTTGTCTACCATA-3'	5'-TGCTGGAGCTGACAGAGAAA-3'
Ppar α	5'-TCACCGCAAGTCCAGCCA-3'	5'-ACACCAGGCCCTTCTCTGCCT-3'
Pgc1a	5'-GGACATGTGCAGCCAAGACTCT-3'	5'-CACTTCAATCCACCCAGAAAGCT-3'
Tfam	5'-ATTCCGAAGTGTTCCTCCAGCA-3'	5'-TCTGAAAGTTTTCATCTGGGT-3'
Prdm16	5'-ACAGGCAGGCTAAGAACCAG-3'	5'-CGTGGAGAGGAGTGTCTTCAG-3'
β 3-Adrenergic receptor	5'-GGCCCTCTCTAGTTCACAG-3'	5'-TAGCCATCAACCTGTTGAGC-3'

Acacb, acetyl-coenzyme A carboxylase beta; Acadl, acyl-coenzyme A dehydrogenase, long-chain; Acadm, acyl-coenzyme A dehydrogenase, medium chain; ATP25, ATPase synthesis protein 25 mitochondrial; Cox4i, cytochrome c oxidase subunit 4I1; Cpt1b, carnitine palmitoyltransferase 1b; Dgat, diacylglycerol O-acyltransferase 1; Fasn, fatty acid synthase; IFN, interferon; qPCR, real-time quantitative PCR; Ldha, lactate dehydrogenase A; Ldhd, lactate dehydrogenase B; Pgc1a, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; Ppara, peroxisome proliferator activated receptor alpha; Ppar γ , peroxisome proliferator activated receptor gamma; Prdm16, PR domain containing; Scd1, stearoyl-CoA desaturase-1; Srebf1c, sterol regulatory element binding transcription factor 1; Tfam, transcription factor A, mitochondrial; TNF- α , tumor necrosis factor- α ; Ucp, uncoupling protein.

Figure 1 Gsdma3-mutant AE mice have decreased percentages of body fat and increased heat production. **A** and **B**: Lean mass, fat mass, and total body weight (**A**) and the fat percentages of WT and AE male mice (**B**). **C**: Weight of brown adipose tissue (BAT), s.c. fat, and visceral fat in 3-month-old male WT and AE mice. **D**: Morphology of BAT, s.c. fat, and visceral fat in 3-month-old male WT and AE mice. **E–J**: volume of O₂ consumption (VO₂; **E**), volume of CO₂ production (VCO₂; **F**), heat production (**G**), daily food intake (**H**), respiratory exchange ratio (RER; **I**), and locomotor activity (**J**), determined by metabolic cage study, of 3-month-old male WT and AE mice. $n = 7$ (**A** and **B**); $n = 6$ (**E–J**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT.

were collected for two consecutive 12-hour dark and light cycles. AE mice showed significantly higher O₂ consumption ($P = 0.0037$), CO₂ production ($P = 0.0012$), and heat production ($P = 0.0020$) than WT mice (Figure 1, E–G). AE mice also consumed more food ($P = 0.0033$) (Figure 1H). The respiratory exchange ratio (Figure 1I) and activity (Figure 1J) of AE mice were similar to those of WT mice. AE mice showed better glucose tolerance than WT mice by a GTT, although their insulin tolerance remained the same as that of WT mice (Supplemental Figure S1). The data indicated that heat production and metabolic rate are increased in AE mice.

The Heat Production Change Is Independent of Skin Barrier Disruption in AE Mice

The metabolic alteration may be caused by a progressive hair loss and skin barrier disruption phenotype in AE mice. The metabolic parameters were measured when mice were maintained in a thermoneutral condition at 30°C. The AE mice again displayed higher O₂ consumption ($P = 0.0406$), CO₂ production ($P = 0.0200$), heat production ($P < 0.0001$), and food consumption ($P = 0.0483$) than WT mice (Figure 2, A–D). Interestingly, the respiratory exchange ratio of AE mice was significantly lower than that

Figure 2 Increased heat production in AE mice is not related to skin barrier disruption. **A–F:** O₂ (A), CO₂ production (B), heat production (C), daily food intake (D), respiratory exchange ratio (RER; E), and locomotor activity (F), determined by metabolic cage study, of 3-month-old male WT and AE mice after 1 week of thermally neutralized treatment. **G:** Rectal temperature of 3-month-old male mice after cold (4°C) induction. $n = 6$ (A–F); $n = 7$ (G). * $P < 0.05$, *** $P < 0.001$ versus WT.

of WT mice, which suggests that AE mice like to use lipid as an energy resource ($P = 0.0004$) (Figure 2E). The activity of AE mice showed no differences (Figure 2F). Furthermore, the AE mice could maintain body temperature better than WT mice at 4°C (Figure 2G). AE mice also showed better glucose tolerance than WT mice by a GTT, although their insulin tolerance remained the same as that of WT mice after 1 month of feeding in a thermoneutral condition (30°C), just like in a room temperature condition (Supplemental Figure S2). All these data indicated that the increased heat production in AE mice was caused by up-regulation of thermogenesis, which is not due to disruption of skin barrier function.

Activation of BAT Thermogenesis in AE Mice

BAT is crucial for cold-induced thermogenesis for maintaining body temperature. BAT is significantly smaller in AE mice, suggesting the activation of BAT. The lipid droplets were also found to be much smaller in the BAT of AE mice than that of WT mice, as seen by hematoxylin and eosin staining, which is consistent with the active status of thermogenesis in AE mice (Figure 3A). The expression of major thermogenic markers,^{24,25} including *Ucp1* ($P = 0.0017$), peroxisome proliferator-activated receptor gamma coactivator 1- α PGC1A; (*PPARGC1A*; $P = 0.0085$), *Cidea* ($P = 0.0060$), *Dio2* ($P \leq 0.0001$), and *Tfam* ($P = 0.0159$), was significantly up-

regulated in the BAT of AE mice (Figure 3, B–F). However, the expression of the β 3-adrenergic receptor ($P = 0.1258$), which is a response to cold-induced thermogenesis through neural circuits, did not change significantly in the BAT of AE mice compared with that of WT mice. This result is consistent with our metabolic cage data. The markers of lipid oxidation and lipolysis^{26,27} were also significantly up-regulated in the BAT of AE mice (Supplemental Figure S3A). The thermogenic markers were also tested in s.c. fat, and there was no up-regulation of those markers; some markers were even down-regulated in AE mice, suggesting that there is no white adipose tissue (WAT) browning (Supplemental Figure S3B). Although skeletal muscle is another important organ for heat production, there were no significant changes in the muscles of WT and AE mice (Supplemental Figure S3C). The data above suggested that BAT activation played a key role in the up-regulation of thermogenesis in AE mice.

IL-6 Is the Key Candidate for Inducing Up-Regulation of Heat Production after *Gsdma3* Activation in the Epidermis

The activation of heat production cannot be an *in situ* effect because *Gsdma3* is exclusively expressed in skin tissues.^{15,28,29} Therefore, it is reasonable to assume that some specific factor(s) released from skin induce the activation of BAT and thermogenesis. Indeed, expression of *Dio2* was

Figure 3 Increased heat production in AE mice is due to the up-regulation of brown adipose tissue (BAT) thermogenesis. **A:** Representative histology of BAT in WT and AE mice. **B–G:** *Ucp1* (B), PGC1A (*PPARGC1A*; C), *Cidea* (D), *Dio2* (E), *Tfam* (F), and $\beta 3$ receptor (G) mRNA levels in BAT from 3-month-old male mice, measured by real-time quantitative PCR. Expression levels of target genes were normalized to *Rplp0* (alias 36B4), and data were calculated using the $2^{-\Delta\Delta C_t}$ method. $n = 6$ (A–G). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT. Scale bar = 200 μm (A). PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

significantly up-regulated when primary BAT cells from newborn WT mice were treated with serum from AE mice for 4 hours ($P = 0.0179$) (Figure 4A). Expression of *Ucp1* ($P = 0.1839$), PGC1A (*PPARGC1A*; $P = 0.2357$), and *Cidea* ($P = 0.2207$) was also increased but not significantly (Figure 4, B–D). Consistently, *Dio2* expression was also strongly up-regulated in the BAT of AE mice (Figure 3E), indicating its crucial role in heat production of AE mice.

The factors released in the serum of AE mice were identified by expressing the Gsdma3 N terminus, which was mimicked by expression of the Gsdma3 mutant form in HaCaT cells. A tet-on lentivirus system was used to generate doxycycline (DOX)-induced Gsdma3 N-terminus and C-terminus stably transfected HaCaT cell lines. These cell lines were induced for 10 hours by DOX. The expression of major cytokines (IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 α , IL-18, interferon- α , interferon- γ , and tumor necrosis factor- α) was then examined. The expression of the N terminus caused a significant up-regulation of *IL6* by quantitative RT-PCR ($P < 0.0001$) (Figure 4E). *IL6* was up-regulated after 8 hours of DOX-induced expression of the N terminus by quantitative RT-PCR ($P < 0.0001$) (Figure 4F). The IL-6 level in the supernatant of HaCaT cells was significantly up-regulated after Gsdma3 N-terminus expression ($P = 0.0027$) (Figure 4G). The expression level of major cytokines was also determined in skin of AE mice. Consistent with Gsdma3 N-terminus transfected HaCaT cells, *IL6* was extremely significantly up-regulated in the skin of AE mice; other cytokines

demonstrated slightly elevated or no significant change (Supplemental Figure S4).

IL-6 was the first identified myokine, which was secreted by muscle after exercise and promoted lipid oxidation.³⁰ Mice overexpressing IL-6 displayed improved metabolic phenotypes.^{31–33} The IL-6 level was significantly higher in the serum of AE mice than in the serum of WT mice ($P = 0.0007$) (Figure 4H). The expression level of IL-6 was also up-regulated in the skin of AE mice ($P \leq 0.0001$) (Figure 4I). Downstream of IL-6 is the Stat3 signaling pathway. The phosphorylation level of Stat3 was examined in the BAT because Stat3 is the direct downstream target after activation of the IL-6 receptor. The phosphorylation level of Stat3 was significantly up-regulated in the BAT of AE mice (Figure 4J). All these results indicated that IL-6 is the key candidate for inducing up-regulation of heat production after Gsdma3 activation in the epidermis.

Blockage of IL-6 Signaling Suppresses the Up-Regulation of Heat Production in AE Mice

Speculating that IL-6 might mediate BAT activation in AE mice, SC-144, an inhibitor, was used to block the function of GP130, which is the main subunit of the IL-6 receptor.³⁴ SC-144 has been reported to block IL-6–induced Stat3 activation.³⁵ After i.p. injection of SC-144 for 2 days, the phosphorylation level of Stat3 in BAT was significantly down-regulated in SC-144–treated mice compared with control mice (Figure 5A). Furthermore, the expression of

Figure 4 IL-6 is significantly up-regulated after Gsdma3 activation. **A–D:** *DIO2* (**A**), *UCP1* (**B**), *PGC1A* (*PPARGC1A*; **C**), and *Cidea* (**D**) mRNA levels, measured by real-time quantitative PCR (qPCR), in primary brown adipose tissue (BAT) cells after 4 hours of WT and AE serum treatment. **E:** mRNA levels of several cytokines in HaCaT cells, measured by qPCR, after 10 hours of DOX-induced Gsdma3 N-terminus and C-terminus expression. **F:** The mRNA levels of *IL6* in HaCaT cells after 8 hours of doxycycline (DOX)-induced Gsdma3 N-terminus and C-terminus expression. **G:** IL-6 levels in supernatant after 10 hours of DOX-induced Gsdma3 N-terminus (N) and C-terminus (C) expression and control measured by enzyme-linked immunosorbent assay (ELISA). **H:** IL-6 levels in WT and AE serum, measured by ELISA. **I:** The mRNA levels of *IL6* in skin from WT and AE mice, measured by qPCR. **J:** The phosphorylation and expression levels of Stat3 in BAT from WT and AE mice. The estimated amount of target cDNA was normalized to *Rplp0* (alias 36B4) in each sample before comparison. $n = 6$ (**A–F**, **H**, and **I**); $n = 3$ (**G**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT. IFN, interferon; P-Stat3, phosphorylated stat3; TNF- α , tumor necrosis factor- α ; T-Stat3, total stat3. PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

major thermogenic markers, including *UCP1*, *PGC1A* (*PPARGC1A*), *Cidea*, *Dio2*, and *Tfam*, was significantly down-regulated in BAT of SC-144-treated mice (Supplemental Figure S5). After 2 weeks of SC-144 treatment, AE mice showed a significant down-regulation of heat production ($P = 0.0115$) by metabolic cage measurement (Figure 5B). The O_2 consumption ($P = 0.0852$) and CO_2 production ($P = 0.1864$) also showed slight decreases compared with control mice but were not significantly different (Figure 5, C and D). The food intake, respiratory

exchange ratio, and activity showed no significant changes (Supplemental Figure S6). After 2 days of SC-144 i.p. injection, the GTT and insulin tolerance test showed no differences between WT and AE mice (Figure 5, E and F). To further confirm that IL-6 is an important mediator between skin and brown adipose tissue, IL-6-neutralizing monoclonal antibody was used to directly block IL-6 *in vivo*. Consistent with SC-144-treated AE mice, the heat production, volume of O_2 consumption, and volume of CO_2 production were also significantly down-regulated in IL-6

Figure 5 Blockage of IL-6 signaling may suppress the up-regulation of heat production in AE mice. **A:** The phosphorylation and expression levels of Stat3 in brown adipose tissue from WT mice injected with dimethyl sulfoxide (DMSO) and AE mice injected with DMSO and SC144, at 2 days. **B–D:** Heat (**B**), O₂ (**C**), and CO₂ (**D**) production, determined by metabolic cage study, of 3-month-old male WT and AE mice after 2 weeks of SC144 i.p. injection. **E:** Glucose tolerance test (GTT) of 3-month-old male WT and AE mice after 2 days of SC144 i.p. injection. **F:** Insulin tolerance test (ITT) of 3-month-old male WT and AE mice after 2 days of SC144 i.p. injection. $n = 6$ (**B–F**). $*P < 0.05$. P-Stat3, phosphorylated stat3; T-Stat3, total stat3.

antibody-treated AE mice (Supplemental Figure S7). These data indicated that IL-6/Stat3 signaling mediated the up-regulation of thermogenesis in AE mice.

Gsdma3 Activation Induces IL-6 Expression through the JNK Signaling Pathway

There have been several reports suggesting that mitogen-activated protein kinase signaling is involved in regulating *IL6* gene expression.^{36–38} The phosphorylation of JNK and extracellular signal-regulated kinase was significantly up-regulated after Gsdma3 N-terminus expression in HaCaT cell lines (Figure 6A), indicating that Gsdma3 activation might activate mitogen-activated protein kinase and then up-regulate IL-6 expression. Furthermore, the expression of *IL6* was significantly suppressed by JNK-specific inhibitor SP600125 in a dose-dependent manner ($P < 0.0001$) (Figure 6B), whereas the extracellular signal-regulated kinase-specific inhibitor U1026 did not affect *IL6* expression (Figure 6C). These results demonstrated that IL-6 is induced by JNK.

JNK and extracellular signal-regulated kinase activation was examined in the skin of AE mice. The phosphorylation level of JNK was significantly up-regulated in the skin of AE mice compared with that of WT mice (Figure 6D). Immunostaining also showed that phosphorylated JNK and phosphorylated c-Jun, downstream of JNK, were significantly up-regulated in the epidermis of AE mice (Figure 6E and Supplemental Figure S8). Taken together, these data

indicated that the JNK–IL-6 pathway was activated *in vivo* after Gsdma3 activation.

Discussion

Our results showed that a Gsdma3 active mutation significantly increased whole-body and BAT heat production through skin-originated IL-6 function. These data suggested a novel mechanism for skin regulation of whole-body metabolism through the humoral system. Gsdma3 activation was found to cause up-regulation of IL-6 expression through the JNK signaling pathway. This function is different from the activation of pyroptosis by Gsdmd, another GSDM family member.^{18,19}

Because members of the GSDM family localize at the barrier, such as the epidermis, intestine, and colon, and GSDMD plays an important role in innate immunity,¹⁹ other GSDM members might also be involved in immunity. The inflammasome pathway, in which GSDMD is the final executor, mainly defends against intracellular bacterial infection. The body barrier is the forefront of the microbial invasion. Thus, other members of the GSDM family might be involved in microbial resistance. In the future, we expect more discoveries using a variety of microbial infection models to fully understand the physiological function of Gsdma3/GSDMA.

Similar to AE mice, several mouse strains with skin defects have been reported to associate with metabolic phenotypes.¹ For instance, *Scd1* skin-specific knockout mice also display increased heat production.^{2,3} However, the

Figure 6 Gsdma3 activation may activate c-Jun N-terminal kinase (JNK) signaling and induce IL-6 expression. **A:** The phosphorylation and expression levels of JNK and extracellular signal-regulated kinase (ERK) in HaCaT cells after 10 hours of DOX-induced Gsdma3 N-terminus and C-terminus expression. **B:** The mRNA levels of IL-6 after JNK inhibitor SP600125 treatment. **C:** The mRNA levels of IL-6 after ERK inhibitor U1026 treatment. **D:** The phosphorylation and expression levels of JNK and ERK in the skin of 3-month-old male WT and AE mice. **E:** The immunostaining of phosphorylated JNK in skin of WT and AE mice. $n = 6$ (**B** and **C**). *** $P < 0.001$. Scale bar = 50 μm (**E**). P, phosphorylated; T, total.

Scd1-SKO mice are different from *Gsdma3*-mutant mice in the physiological mechanism for metabolic phenotypes. In *Scd1*-SKO mice, β 3-adrenergic receptor signaling in BAT, a marker of cold exposure, was elevated when the mice were housed at room temperature, suggesting increased cold perception in these mice even at room temperature.^{39,40} However, there is no difference in β 3-adrenergic receptor expression levels between WT and AE mice (Figure 3G). The AE mice could maintain body temperature better than WT mice in the 4°C condition. The heat production of AE mice was also increased at a thermoneutral temperature, which further ruled out the cold-sensing activation through thermoreceptors and the nervous system in AE mice.⁴ The mutant form of *Gsdma3* in AE mice represents the activation of *Gsdma3* because the point mutation in the C-terminal domain releases the inhibitory function of the C-terminal domain to the functional N-terminal domain.²⁰ In *Gsdmd*, this activation is achieved by the cleavage of a hinge region between the N-terminal domain and the C-terminal domain by caspase-1 and caspase-4/5/11.^{19,41,42} There still remains the unsolved question of what endogenously triggers the initial activation of *Gsdma3* in skin. Although most studies on GSDM family genes involve the process of

inflammation, this study found, for the first time, that one of the functions of active *Gsdma3* is to induce the expression of IL-6 and eventually increase whole-body heat production.

The function of IL-6 in regulating heat production has been proposed in many studies. First identified as a myokine, IL-6 is secreted from muscles after exercise.^{30,43} IL-6 can act on liver and fat to increase lipid oxidation and glucose uptake, which up-regulates the metabolic rate.^{30,43,44} Mice over-expressing IL-6 displayed an improved metabolic rate and reserved body weight gain.^{32,33} In contrast, IL-6 knockout mice showed adult obesity and leptin resistance.^{45–47} Stat3, downstream of IL-6, also played an important role in BAT-dependent thermogenesis. Disruption of Stat3 expression can prevent functional development of brown adipocytes.⁴⁸ The elevated IL-6 level in serum and related activation of BAT in AE mice are consistent with previous studies,^{30–32} which note a novel functional cross talk between skin and other major organs for metabolic regulation.

Previous studies showed highly elevated catecholamine-, corticosteroid-, and inflammatory cytokine-mediated hypermetabolic responses.^{13,49} IL-6 was one of the up-regulated cytokines after burn injury,⁵⁰ and positively correlated to body temperature increase.⁵¹ Thus, burn-

injury—induced increase of heat production in BAT may suggest that the keratinocytes secreted IL-6. The burn injury or other stimulation might activate JNK/c-Jun signaling in keratinocytes and induce IL-6 secretion as another signaling mechanism to regulate metabolism.^{52,53} These results are consistent with our finding, suggesting IL-6 is an important regulator between skin and BAT after burning injury.

In conclusion, our results uncovered Gsdma3/IL-6/Stat3-dependent cross talk between the skin and BAT. The novel mechanism for skin regulation of whole-body metabolism also provides a new way to treat other metabolic syndromes.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2019.01.012>.

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