

Leptin-deficient obesity prolongs survival in a murine model of myelodysplastic syndrome

Michael J. Kraakman,^{1,2*} Helene L. Kammoun,^{1,3*} Dragana Dragoljevic,^{1,3} Annas Al-Sharea,^{1,3} Man K.S. Lee,^{1,3} Michelle C. Flynn,^{1,3} Christian J. Stolz,^{1,3} Andrew A. Guirguis,⁴ Graeme I. Lancaster,^{1,3} Jaye Chin-Dusting,³ David J. Curtis⁴ and Andrew J. Murphy^{1,3}

¹Baker Heart and Diabetes Institute, Melbourne, VIC, Australia; ²Naomi Berrie Diabetes Center and Department of Medicine, Columbia University, NY, USA; ³Monash University, Melbourne, VIC, Australia and ⁴Australian Centre for Blood Diseases, Monash University, Melbourne, VIC, Australia

**MJK AND HLK contributed equally to this work.*

©2018 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2017.181958

Received: October 3, 2017.

Accepted: January 19, 2018.

Pre-published: January 25, 2018.

Correspondence: andrew.murphy@baker.edu.au

METHODS

Mice

Male Ob/Ob mice were purchased from the Jackson Laboratory (USA), along with littermate lean controls and were used as recipients of the bone marrow transplant at 10 weeks of age. Donor male WT C57bl/6 mice originating from Jackson Laboratory C57Bl/6 and male NHD13 mice were sourced from colonies maintained within the Alfred Medical Research Education Precinct (AMREP) Precinct Animal Centre (PAC). These donor mice (i.e. WT and NHD13) were 6-8 weeks of age when the BM was harvested for the transplantation procedure. All mice were then housed in the AMREP PAC in a pathogen-free facility under controlled conditions and exposed to a 12:12 hour light dark cycle. All animal experiments were approved by the AMREP Animal Ethics Committee and conducted in accordance with the National Health and Medical Research Council (NHMRC) of Australia Guidelines for Animal Experimentation (Ethics E/1444/2013/B). Regarding the midway MDS analysis, all mice were studied for non-endpoint procedures and 3 mice per group were randomly chosen to be euthanized for tissue analysis.

Diets

Mice were fed either a chow diet (5% of total energy from fat or a HFD) (43% of total energy from fat) (Specialty Feeds, Glen Forrest, AUS) with *ad libitum* access to food and water unless stated.

Oral glucose tolerance test

Mice were administered 2 g of glucose per kg of lean mass by oral gavage following a 5 h fast to assess glucose tolerance. Blood was obtained prior to and at the indicated intervals for the proceeding 2 h from the tip of the tail. Blood glucose concentrations were determined using a glucometer (Accu-Check, Roche, NSW, Australia).

Body composition

Mouse body composition (fat mass and lean body mass) was measured weekly with a 4-in-1 EchoMRI body composition analyzer (Columbus Instruments, USA) and standard laboratory scales.

Indirect calorimetry

Oxygen consumption (VO^2) and activity (beam breaks) were measured using a 12-chamber indirect calorimeter (CLAMs Oxymax Series) with an airflow of 0.6 L/min.

Plasma metabolite analysis

NEFAs, TAGs (Roche, Hitachi) and cholesterol (Wako, Japan) were measured according to the manufacturer's instructions using plasma obtained from a cardiac bleed. AST, ALT, alkaline phosphatase, albumin and bilirubin (total) were measured by Veterinary Pathology ASAP Laboratories from the same plasma (Melbourne, Australia).

Complete blood count

Complete blood counts were measured in whole blood obtained from tail bleeding using an automated hematology analyzer (Sysmex XS-1000i; Kobe, Japan).

Bone marrow transplantation

Recipient mice were given 100mg/L neomycin 2 weeks before and after the transplant procedure. Recipient mice were irradiated (from a cesium gamma source) with 2 doses of 5.5 Gy separated by at least 4 hours. The following day 5×10^6 BM cells from recipient mice were injected intravenous. Mice were given a 5-week reconstitution recovery period.

Tissue processing

BM cells were flushed from femurs and tibias. For spleen and liver, a small section was crushed through a 40 μ m filter with PBS. Epididymal VAT was minced with scissors in RPMI and digested in liberase solution (0.03mg/mL) for 30 mins at 37°

C with shaking. Blood was collected into EDTA containing tubes and RBC lysed. Cells were resuspended in 1X HBSS (0.1% BSA w/v, 5mM EDTA) before staining.

Flow cytometry

Blood leukocytes

Blood leukocytes were identified from whole blood as described previously.¹ Monocytes were identified as CD45^{hi}CD115^{hi} and further identified as Ly6-C^{hi} and Ly6-C^{lo}; neutrophils were identified as CD45^{hi}CD115^{lo}Ly6-C/G^{hi}. T and B cells were identified as TCRβ^{hi}B220^{lo} and TCRβ^{lo}B220^{hi} respectively, gated from the CD45^{hi}CD115^{lo}Ly6-C/G^{lo} cells. Immature immune cells were identified as CD45^{hi}CD115^{lo}Ly6-C/G^{lo}TCRβ^{lo}B220^{lo}cKit⁺.

Hematopoietic stem and progenitor cells

Following red blood cell lysis, cells were stained with a cocktail of antibodies before analysis by flow cytometry as previously described.² Briefly, lineage committed cells were identified as CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly6-C/G (either all FITC or APC conjugated and from eBioscience or Biolegend) positive, with antibodies against Sca1 (Biolegend) and cKit (eBioscience) to identify progenitor cell populations and HSPCs (Lineage⁻, Sca1⁺, cKit⁺) and with antibodies to CD16/CD32 (FcγRII/III) and CD34 (BD Biosciences) to separate CMP (Lineage⁻, Sca1⁻, cKit⁺, CD34^{int}, FcγRII/III^{int}) and MEP (Lineage⁻, Sca1⁻, cKit⁺, CD34^{lo}, FcγRII/III^{lo}) and MkPs (Lineage⁻, Sca1⁻, cKit⁺, CD34^{int}, FcγRII/III^{int}CD41⁺). Cell proliferation was assessed by DAPI incorporation using the cytofix/cytoperm kit (BD Biosciences) according to manufacturer's protocol.

Myeloid cells

Inflammatory macrophages were identified as CD45^{hi}CD11b^{hi}F4/80^{hi}CD11c^{hi}. Tissue resident macrophages in hematopoietic tissues (BM and spleen) were identified as CD45^{hi}CD11b^{hi}F4/80^{hi}CD169⁺.

Platelet reticulation

As previously described³, Undiluted EDTA-anti-coagulated blood (5 μ L) within 30 mins of collection was mixed with an anti-CD41-APC antibody (eBiosciences) and the fluorescent dye thiazole orange (final conc. 1 μ g/mL) and incubated at room temperature in the dark for 20 min. Reticulated platelets were identified as CD41⁺thiazole orange^{hi}.

All aforementioned antibodies have been summarized with the clone used and the catalog number in the supplementary information.

Histology

Adipose tissue, liver and spleen were fixed in 4% paraformaldehyde (PFA) before being stained with Mayer's Hemotoxylin and Eosin for histological determination of tissue structure.

Femurs were harvested, fixed in 2% paraformaldehyde overnight, and immersed in 0.5M EDTA in PBS with 1% paraformaldehyde for 14 days for decalcification prior to paraffin embedding. The paraffin-embedded sections (5 μ m thickness) were deparaffinized, rehydrated, and stained in mayer's hematoxylin for 8 mins followed by a 2min stain in scott's tap water. Slides were then stained in eosin for 6mins, dehydrated, cleared in xylene and mounted with DPX.

Stained sections were imaged microscopically (Olympus BX50) and captured using computer software (QCapture Pro 6.0). Adipocyte size was quantified as previously described using Image Pro J⁴.

Transmigration Assay

Preparation of ckit⁺ stem cells. Bone marrow were flushed from WT or NHD13 mice through a 40 μ m cell strainer to obtain single cell suspension RBCs were the lysed and resuspended in FACS buffer ready for staining. A cocktail of antibodies were used to stain ckit⁺ stem cells (Lin⁻ckit⁺) before using the FACSaria I to sort for transmigration assay. Purity of cells collected were above 90%.

Preparation of lean and obese conditioned media. Visceral adipose tissue (VAT) were dissected from 30 week old WT mice either on a chow (lean) or HFD (obese). VATs were diced in IMDM media and incubated at 37°C for 4h to allow for the release of cytokine/ chemokines. Media were then collected and filtered through to get rid of VAT and spun at 3,000rpm for 5min. Supernatants were collected for transmigration assay.

Transmigration assay. 24 well Costar Transwell plates (6.5mm diameter Insert, 8.0mm polycarbonate membrane) (Corning Incorporated, USA) were used for transmigration assay. Briefly, sorted BM ckit⁺ cells from WT and NHD13 mice were added into inserts with the bottom of the wells containing either lean or obese conditioned media. ckit⁺ stem cells were incubated at 37°C for 6h to allow for transmigration to occur. Cells transmigrated into the conditioned media were counted on the Sysmex XS-1000i Automated Hematology Analyzer (Sysmex Corporation, Japan).

Statistics

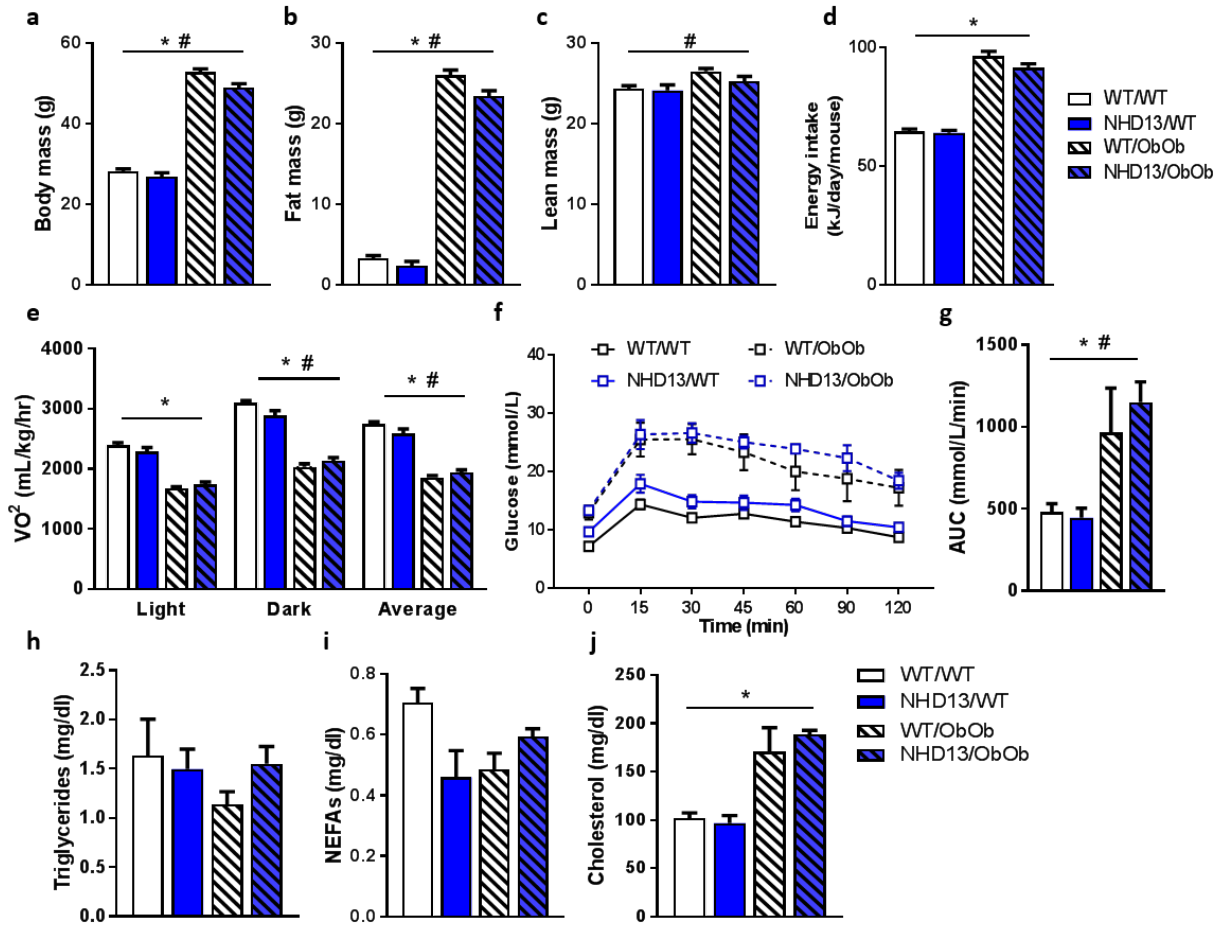
Data are presented as mean \pm SEM and analyzed by 2-way ANOVA or student t test as stated in the figure legends (Sigma STAT V5.0). When performing 2-way ANOVA, if data was not normally distributed it was log-transformed and reanalyzed using 2-way ANOVA. Survival was presented as a Kaplan-Myer curve and analyzed through a Mantel-Cox test on Graphpad Prism 7.01. Differences with a p-value less than 0.05 were deemed to be statistically significant.

References:

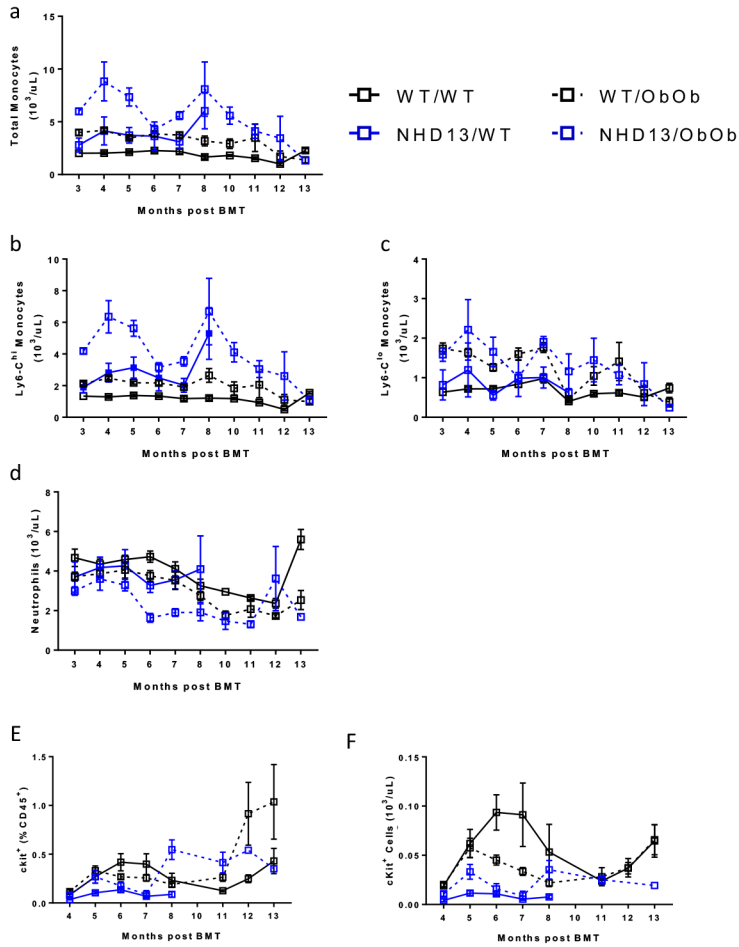
1. Murphy AJ, Akhtari M, Tolani S, et al. ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. *J Clin Invest.* 2011;121(10):4138-4149.
2. Nagareddy PR, Murphy AJ, Stirzaker RA, et al. Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis. *Cell Metab.* 2013;17(5):695-708.
3. Kraakman MJ, Lee MK, Al-Sharea A, et al. Neutrophil-derived S100 calcium-binding proteins A8/A9 promote reticulated thrombocytosis and atherogenesis in diabetes. *J Clin Invest.* 2017;127(6):2133-2147.

4. Murphy AJ, Kraakman MJ, Kammoun HL, et al. IL-18 Production from the NLRP1 Inflammasome Prevents Obesity and Metabolic Syndrome. *Cell Metab.* 2016;23(1):155-164.

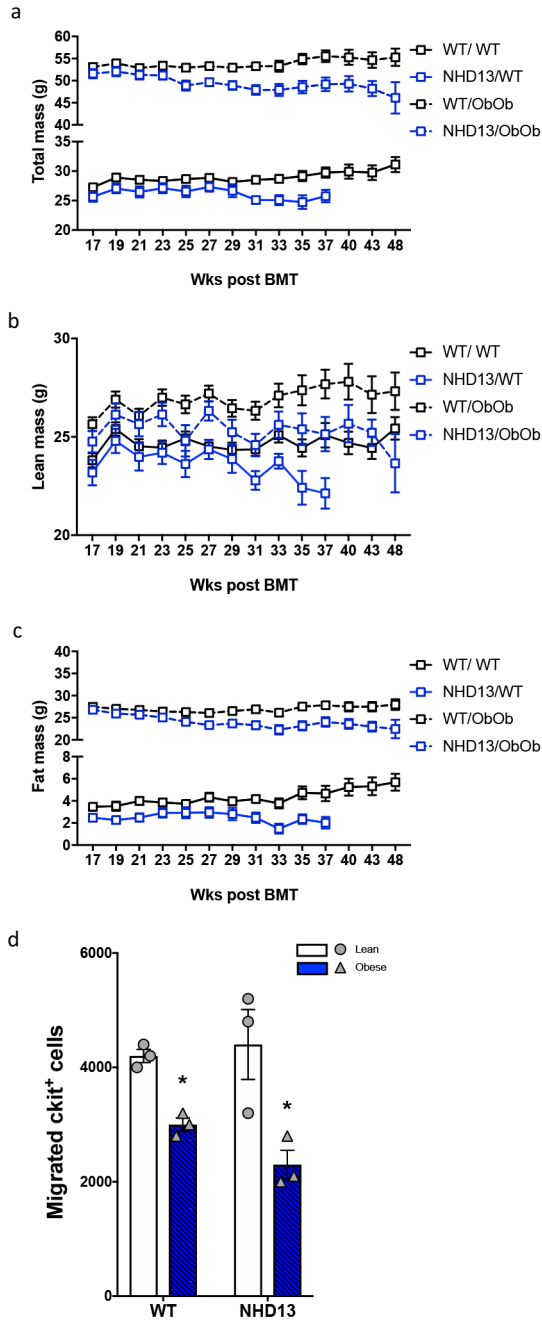
Supplemental Figures:



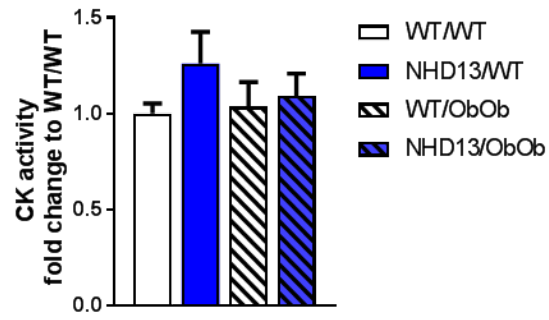
Supplementary Figure 1: Ob/Ob mice exhibit a conserved metabolic phenotype after 7 months of myelodysplastic syndrome. (a-c) Body composition at 7 months after bone marrow transplant with weight **(a)**, fat **(b)** and lean mass **(c)** obtained by EchoMRI. **(d)** Energy intake measured over 2 weeks of food weighing. **(e)** Oxygen consumption in the light and dark cycle obtained through CLAMs analysis. **(G-H)** A glucose tolerance test performed through oral gavage of 2g glucose/kg of lean body mass was performed on fasted animals and expressed as the 120 minutes curve **(f)** or the incremental area under the curve **(g)**. **(i-k)** Triglycerides **(h)**, non-esterified fatty acids **(i)** and cholesterol **(j)** measured in plasma from fasted mice. **a-h**; n=12-16; **i-k**; n=3. All data expressed as mean \pm SEM. * $p < 0.05$, for obesity effect; # $p < 0.05$, for MDS effect as analyzed by 2-way ANOVA.



Supplementary Figure 2: Total cell counts. (a) Total, **(b)** Ly6-C^{hi} and **(c)** Ly6-C^{lo} monocytes. **(d)** Neutrophils. **(e)** Percentage and **(f)** total ckit⁺ cells. n=12-16. All data expressed as mean ± SEM.



Supplementary Figure 3: Body weight. (a-c) Body composition over time with (a) total weight, (b) lean mass and (c) fat mass obtained by EchoMRI. (d) ckit⁺ cell migration assay to lean or obese fat conditioned media. a-c; n=12-16; d; n=3. All data expressed as mean \pm SEM. * p<0.05, for obesity effect.



Supplementary Figure 4: Creatine Kinase activity in plasma. Creatine kinase activity was measured in plasma samples. n=5-8. All data expressed as mean \pm SEM.

Antibody	Clone	Supplier	Catalog Number
CD45	30-F11	Biolegend®	103126
CD115	AFS98	eBioscience™	17-1152-82
Ly6-C/G	RB6-8C5	Biolegend®	108428
TCRβ	H57-597	Biolegend®	109212
B220	RA3-6B2	Biolegend®	103212
CD19	H1B19	Biolegend®	302206
CD11b	N418	Biolegend®	117306
CD3e	145-2C11	Biolegend®	100322
TER-119	TER-119	Biolegend®	116212
CD2	RM2-5	Biolegend®	100112
CD8	YTS156.7.7	Biolegend®	126612
CD4	RM4-5	Biolegend®	100516
Sca-1	D7	Biolegend®	108120
cKit	2B8	Biolegend®	105826
CD16/CD32	2.4G2	BD bioscience	560540
CD34	HM34	Biolegend®	128606
CD11c	N418	Biolegend®	117326
F4/80	BM8	eBioscience™	25-4801-82
CD169	3D6.112	Biolegend®	142418
CD41	eBioMWRReg30	eBioscience™	17-0411-82

Supplementary Information. Antibody clone and catalog number list.