

The helminth glycoprotein omega-1 improves metabolic homeostasis in obese mice through type-2 immunity-independent inhibition of food intake

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Short title: Helminth glycoprotein omega-1 and metabolic homeostasis

List of abbreviations

1	AAM	Alternatively activated macrophage
2	ALAT	Alanine aminotransferase
3	BAT	Brown adipose tissue
4	DC	Dendritic cell
5	EE	Energy expenditure
6	GTT	Glucose tolerance test
7	HEK	Human embryonic kidney
8	HFD	High-fat diet
9	HOMA-IR	Homeostatic model assessment of insulin resistance
10	IFN- γ	Interferon gamma
11	IL	Interleukin
12	ILC	Innate lymphoid cell
13	ITT	Insulin tolerance test
14	LBM	Lean body mass
15	LFD	Low-fat diet
16	NAFLD	Non-alcoholic fatty liver disease
17	NASH	Non-alcoholic steatohepatitis
18	pLe X - ω 1	Recombinant omega-1 from Le X -glyco-engineered <i>Nicotiana benthamiana</i> plants
19	PMA	Phorbol myristate acetate
20	pWT- ω 1	Recombinant omega-1 from wild-type <i>N. benthamiana</i> plants
21	SEA	<i>Schistosoma mansoni</i> soluble egg antigens
22	STAT	Signal transducer and activator of transcription
23	SVF	Stromal vascular fraction
24	Th	T helper
25	TNF	Tumor necrosis factor

26 UCP1 Uncoupling protein 1

27 WAT White adipose tissue

28 WT Wild-type

30 **Abstract**

31 Type 2 immunity plays an essential role in the maintenance of metabolic homeostasis and its disruption
32 during obesity promotes meta-inflammation and insulin resistance. Infection with the helminth parasite
33 *Schistosoma mansoni* and treatment with its soluble egg antigens (SEA) can induce a type 2 immune
34 response in metabolic organs and improve insulin sensitivity and glucose tolerance in obese mice, yet a
35 causal relationship remains unproven. Here, we investigated the effects and underlying mechanisms of the
36 T2 ribonuclease omega-1 (ω 1), one of the major *S. mansoni* immunomodulatory glycoproteins, on metabolic
37 homeostasis. Male C57Bl6/J mice were fed a high-fat diet for 12 weeks followed by bi-weekly injection of
38 SEA, ω 1 or vehicle for 4 additional weeks. Whole-body metabolic homeostasis and energy expenditure were
39 assessed by glucose/insulin tolerance tests and indirect calorimetry, respectively. Tissue-specific immune cell
40 phenotypes were determined by flow cytometry. We show that treatment of obese mice with plant-
41 produced recombinant ω 1, harboring similar glycan motifs as present on the native molecule, decreased
42 body fat mass and improved systemic insulin sensitivity and glucose tolerance in a time- and dose-
43 dependent manner. This effect was associated with an increase in white adipose tissue (WAT) type 2 T
44 helper cells, eosinophils and alternatively-activated macrophages, without affecting type 2 innate lymphoid
45 cells. In contrast to SEA, the metabolic effects of ω 1 were still observed in obese STAT6-deficient mice with
46 impaired type 2 immunity, indicating that its metabolic effects are independent of the type 2 immune
47 response. Instead, we found that ω 1 inhibited food intake, without affecting locomotor activity, WAT
48 thermogenic capacity or whole-body energy expenditure, an effect also occurring in leptin receptor-deficient
49 obese and hyperphagic *db/db* mice. Altogether, we demonstrate that while the helminth glycoprotein ω 1
50 can induce type 2 immunity, it improves whole-body metabolic homeostasis in obese mice by inhibiting food
51 intake via a STAT6-independent mechanism.

52

53 **Key words:**

54 Helminths, immunometabolism, type 2 immunity, macrophages, insulin sensitivity, food intake

56 **Author summary**

57 The obesity-induced chronic low-grade inflammation, notably in adipose tissue, contributes to insulin
58 resistance and increased risk of type 2 diabetes. We have previously shown that infection with parasitic
59 helminth worms was associated with protection against obesity-related metabolic dysfunctions both in mice
60 and humans. We have also reported that treatment of obese mice with an extract of *Schistosoma mansoni*
61 eggs (SEA) improves insulin sensitivity and glucose tolerance, a beneficial effect that was associated with a
62 helminth-specific type 2 immune response in metabolic organs. Here, we studied the effects of omega-1
63 (ω 1), a single immunomodulatory molecule from SEA, on metabolic health in obese mice, and investigated
64 the role of the host immune response elicited. We found that ω 1 induced a helminth-characteristic type 2
65 immune response in adipose tissue and improved both insulin sensitivity and glucose tolerance in obese
66 mice. Yet, in contrast to SEA, ω 1's immunomodulatory properties were dispensable for its metabolic effects.
67 Instead, we show that ω 1 inhibited food intake, a feature accounting for most of the improvements in
68 metabolic health. Together, our findings indicate that helminth molecules may improve metabolic health
69 through multiple distinct mechanisms, and further characterization of such molecules could lead to new
70 therapeutic strategies to combat obesity.

72 **Introduction**

73 Obesity is associated with chronic low-grade inflammation in metabolic organs (1). This so-called meta-
74 inflammation plays a prominent role in the etiology of insulin resistance and type 2 diabetes (1-3), and is
75 associated with increased numbers of pro-inflammatory macrophages, notably in white adipose tissue
76 (WAT) (4) and liver (5). In WAT, these macrophages mainly originate from newly-recruited blood monocytes
77 that differentiate into pro-inflammatory macrophages upon entering the inflammatory milieu (4) and/or
78 being activated by elevated local concentration of free fatty acids (6). These pro-inflammatory macrophages
79 produce cytokines, such as tumor necrosis factor (TNF) and interleukin 1-beta (IL-1 β), which directly inhibit
80 canonical insulin signaling [as reviewed in (2)] and contribute to tissue-specific insulin resistance and whole-
81 body metabolic dysfunctions. In the liver, activation of Kupffer cells, the tissue-resident macrophages,
82 promote the recruitment of pro-inflammatory monocytes and neutrophils which trigger hepatic
83 inflammation and insulin resistance through the production of pro-inflammatory cytokines and elastase,
84 respectively (5, 7, 8). In contrast, a type 2 cytokine environment predominates in lean metabolic tissues
85 under homeostatic conditions, notably in WAT where IL-4, IL-5 and IL-13 produced by type 2 innate
86 lymphoid cells (ILC2s), T helper 2 (Th2) cells and/or eosinophils promote alternatively activated macrophages
87 (AAM) (9, 10). According to the current paradigm, AAMs are the final effector cells of this type 2 immune
88 response, contributing to the maintenance of WAT insulin sensitivity by underlying molecular mechanism(s)
89 that are still largely unknown (2, 11).

90 Parasitic helminths are the strongest natural inducers of type 2 immunity (12). Interestingly, several
91 studies have reported an association between helminth-induced type 2 immunity and improved whole-body
92 metabolic homeostasis in both humans and rodents [as reviewed in (11)]. We also showed that chronic
93 treatment with *S. mansoni* soluble egg antigens (SEA) promoted eosinophilia, Th2 cells, type 2 cytokines
94 expression and AAMs in WAT, and improved both tissue-specific and systemic insulin sensitivity in obese
95 mice (13). SEA drives dendritic cell (DC)-mediated Th2 skewing at least partly through glycosylated molecules
96 [(14), and reviewed in (15)], particularly the T2 RNase glycoprotein omega-1 [ω 1; (16, 17)]. Interestingly,
97 acute treatment with human embryonic kidney 293 (HEK-293)-produced recombinant ω 1 was recently

98 shown to decrease body weight and improve whole-body glucose tolerance in obese mice, through ILC2-
99 mediated type 2 immunity and induction of WAT beigeing (18). In this study, the metabolic effect of ω 1 was
100 reported to be glycan-dependent, yet we have previously shown that the glycosylation pattern of HEK-293-
101 produced ω 1 differs significantly from the *S. mansoni* native molecule, which notably harbors immunogenic
102 Lewis-X (Le^X) glycan motifs (17, 19). By exploiting the flexible N-glycosylation machinery of *Nicotiana*
103 *benthamiana* plants, we successfully produced large amounts of recombinant ω 1 glycosylation variants,
104 either carrying Le^X motifs on one of its glycan branches or not (20).

105 In the present study, we investigate the effects and underlying immune-dependent mechanisms of
106 both SEA and two plant-produced ω 1 glycovariants on whole-body metabolic homeostasis in obese mice.
107 Remarkably, we demonstrate that while SEA improved metabolic homeostasis in obese mice through a
108 STAT6-dependent type 2 immune response, recombinant pLe^X- ω 1 did so independent of its type 2 immunity-
109 inducing capacity, by reducing food intake in a leptin receptor-independent manner.

111 **Methods**

112 **Animals, diet and treatment**

113 All mouse experiments were performed in accordance with the Guide for the Care and Use of Laboratory
114 Animals of the Institute for Laboratory Animal Research and have received approval from the university
115 Ethical Review Board (Leiden University Medical Center, Leiden, The Netherlands; DEC12199) or the
116 Institutional Animal Care and Use Committee (IACUC, New York University School of Medicine, New York,
117 USA; protocol ID IA16-00864). All mice were housed in a temperature-controlled room with a 12 hour light-
118 dark cycle with *ad libitum* access to food and tap water. Group randomization was systematically performed
119 before the start of each experiment, based on body weight, fat mass, and fasting plasma glucose levels. At
120 the end of the experiment, mice were sacrificed through an overdose of ketamine/xylazine.

121 8-10 weeks old male wild-type (WT) and 7 weeks old male *db/db* mice, both on C57BL/6J
122 background, were purchased from Envigo (Horst, The Netherlands) and housed at Leiden University Medical
123 Center. WT mice were fed a low-fat diet (LFD, 10% energy derived from fat, D12450B, Research Diets, Wijk
124 bij Duurstede, The Netherlands) or a high-fat diet (HFD, 45% energy derived from fat, D12451) for 12 weeks,
125 and *db/db* mice were fed a chow diet (RM3 (P), Special Diet Services, Witham, UK) throughout the
126 experimental period. SEA was prepared as described previously (21). Recombinant ω 1 was produced in *N.*
127 *benthamiana* plants through transient expression of ω 1 alone (pWT- ω 1) or ω 1 in combination with
128 exogenous glycosyltransferases to yield Le^X glycan motifs (pLe^X- ω 1), as described previously (20). SEA,
129 pWT/pLe^X- ω 1 (10-50 μ g) or vehicle control (sterile-filtered PBS) were injected i.p. every 3 days for 1 or 4
130 weeks, as indicated in the legends of the figures. For fast-refeeding experiments, WT HFD-fed mice received
131 an i.p. injection of 50 μ g pLe^X- ω 1 or vehicle control after an overnight fast (5pm-9am), followed by refeeding
132 and frequent measurements of food intake and body weight during 24 hours. For assessing the contribution
133 of reduced food intake on the immunometabolic effects of pLe^X- ω 1, WT HFD-fed mice were single-housed
134 and, in a pair-fed group of PBS-injected mice, daily food availability was adjusted to the calorie intake of the
135 pLe^X- ω 1-treated group.

136 8-10 weeks-old male wild-type (WT) and *Stat6*^{-/-} mice, both on C57BL/6J background, were
137 purchased The Jackson Laboratory (Bar Harbor, ME, USA), housed at New York University School of
138 Medicine, and either put on a HFD (60% energy derived from fat; D12492; Research Diets, New Brunswick,
139 NJ, USA) or LFD (10% energy derived from fat; D12450J; Research Diets) for 10 weeks. To exclude effects of
140 genotype-dependent microbiota differences on metabolic and immunological outcomes, the beddings of WT
141 and *Stat6*^{-/-} mice were frequently mixed within similar diet groups throughout the run-in period. After 10
142 weeks, HFD-fed mice were randomized as described above and treated every 3 days for 4 weeks with 50 µg
143 SEA, pLe^X-ω1 or vehicle-control.

144

145 **Body composition and indirect calorimetry**

146 Body composition was measured by MRI using an EchoMRI (Echo Medical Systems, Houston, TX, USA).
147 Groups of 4-8 mice with free access to food and water were subjected to individual indirect calorimetric
148 measurements during the initiation of the treatment with recombinant ω1 for a period of 7 consecutive days
149 using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH, USA).
150 Before the start of the measurements, single-housed animals were acclimated to the cages for a period of 48
151 hours. Feeding behavior was assessed by real-time food intake. Oxygen consumption and carbon dioxide
152 production were measured at 15 minute intervals. Energy expenditure (EE) was calculated and normalized
153 for lean body mass (LBM), as previously described (13). Spontaneous locomotor activity was determined by
154 the measurement of beam breaks.

155 At sacrifice, visceral white adipose tissue (epididymal; eWAT), subcutaneous white adipose tissue
156 (inguinal; iWAT), supraclavicular brown adipose tissue (BAT) and liver were weighed and collected for further
157 processing and analyses.

158

159 **Isolation of stromal vascular fraction from adipose tissue**

160 eWAT was collected at sacrifice after a 1 minute perfusion with PBS through the heart left ventricle and
161 digested as described previously (13). In short, collected tissues were minced and incubated for 1 hour at

162 37°C in an agitated incubator (60 rpm) in HEPES buffer (pH 7.4) containing 0.5 g/L collagenase type I from
163 *Clostridium histolyticum* (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 2% (w/v) dialyzed bovine serum
164 albumin (BSA, fraction V; Sigma-Aldrich). The disaggregated adipose tissue was passed through a 100 µm cell
165 strainer that was washed with PBS supplemented with 2.5 mM EDTA and 5% FCS. After centrifugation (350 x
166 g, 10 minutes at room temperature), the supernatant was discarded and the pellet was treated with
167 erythrocyte lysis buffer (0.15 M NH₄Cl; 1 mM KHCO₃; 0.1 mM Na₂EDTA). Cells were next washed with
168 PBS/EDTA/FCS, and counted manually.

169

170 **Isolation of leukocytes from liver tissue**

171 Livers were collected and digested as described previously (13). In short, livers were minced and incubated
172 for 45 minutes at 37°C in RPMI 1640 + Glutamax (Life Technologies, Bleiswijk, The Netherlands) containing 1
173 mg/mL collagenase type IV from *Clostridium histolyticum*, 2000 U/mL DNase (both Sigma-Aldrich) and 1 mM
174 CaCl₂. The digested liver tissues were passed through a 100 µm cell strainer that was washed with
175 PBS/EDTA/FCS. Following centrifugation (530 x g, 10 minutes at 4°C), the supernatant was discarded, after
176 which the pellet was resuspended in PBS/EDTA/FCS and centrifuged at 50 x g to pellet hepatocytes (3
177 minutes at 4°C). Next, supernatants were collected and pelleted (530 x g, 10 minutes at 4°C). The cell pellet
178 was first treated with erythrocyte lysis buffer and next washed with PBS/EDTA/FCS. CD45⁺ leukocytes were
179 isolated using LS columns and CD45 MicroBeads (35 µL beads per liver, Miltenyi Biotec) according to
180 manufacturer's protocol and counted manually.

181

182 **Processing of isolated immune cells for flow cytometry**

183 For analysis of macrophage and lymphocyte subsets, both WAT stromal vascular cells and liver leukocytes
184 were stained with the live/dead marker Aqua (Invitrogen, Bleiswijk, The Netherlands) or Zombie-UV
185 (Biolegend, San Diego, CA, USA), fixed with either 1.9% formaldehyde (Sigma-Aldrich) or the eBioscience™
186 FOXP3/Transcription Factor Staining Buffer Set (Invitrogen), and stored in FACS buffer (PBS, 0.02% sodium
187 azide, 0.5% FCS) at 4°C in the dark until subsequent analysis. For analysis of cytokine production, isolated

188 cells were cultured for 4 hours in culture medium in the presence of 100 ng/mL phorbol myristate acetate
189 (PMA), 1 µg/mL ionomycin and 10 µg/mL Brefeldin A (all from Sigma-Aldrich). After 4 hours, cells were
190 washed with PBS, stained with Aqua, and fixed as described above.

191

192 **Flow cytometry**

193 For analysis of CD4 T cells and innate lymphoid cell (ILC) subsets, SVF cells were stained with antibodies
194 against B220 (RA3-6B2), CD11b (M1/70), CD3 (17A2), CD4 (GK1.5), NK1.1 (PK136) and Thy1.2 (53-2.1;
195 eBioscience), CD11c (HL3) and GR-1 (RB6-8C5; both BD Biosciences, San Jose, CA, USA), and CD45.2 (104;
196 eBioscience, Biolegend or Tonbo Biosciences, San Diego, CA, USA). CD4 T cells were identified as CD45⁺
197 Thy1.2⁺ Lineage⁺ CD4⁺, and ILCs as CD45⁺ Thy1.2⁺ Lineage⁻ CD4⁻ cells, in which the lineage cocktail included
198 antibodies against CD11b, CD11c, B220, GR-1, NK1.1 and CD3.

199 CD4 T cell subsets and cytokine production by ILCs were analyzed following permeabilization with
200 either 0.5% saponin (Sigma-Aldrich) or eBioscienceTM FOXP3/Transcription Factor Staining Buffer Set. Subsets
201 were identified using antibodies against CD11b, CD11c, GR-1, B220, NK1.1, CD3, CD45.2, CD4, Thy1.2, IL-4
202 (11B11), IL-13 (eBio13A), Foxp3 (FJK-16s; all eBioscience), IL-5 (TRFK5) and IFN-γ (XMG1.2; both Biolegend).

203 For analysis of macrophages, eosinophils, monocytes and neutrophils, cells were permeabilized as
204 described above. Cells were then incubated with an antibody against YM1 conjugated to biotin (polyclonal;
205 R&D Systems, Minneapolis, MN, USA), washed, and stained with streptavidin-PerCP (BD Biosciences) or
206 streptavidin-PerCP-Cy5.5 (Biolegend), and antibodies directed against CD45 (30-F11, Biolegend), CD45.2,
207 CD11b, CD11c [HL3 (BD Biosciences) or N418 (Biolegend)], F4/80 (BM8; Invitrogen or Biolegend), Siglec-F
208 (E50-2440; BD Biosciences), and Ly6C (HK1.4; Biolegend).

209 All cells were stained and measured within 4 days post fixation. Flow cytometry was performed
210 using a FACSCanto or LSR-II (both BD Biosciences), and gates were set according to Fluorescence Minus One
211 (FMO) controls. Representative gating schemes are shown in Figure S1 and all antibodies used are listed in
212 Table S1.

213 **Plasma analysis**

214 Blood samples were collected from the tail tip of 4h-fasted mice (food removed at 9 am) using chilled
215 paraoxon-coated capillaries. Fasting blood glucose level was determined using a Glucometer (Accu-Check;
216 Roche Diagnostics, Almere, The Netherlands) and plasma insulin level was measured using a commercial kit
217 according to the instructions of the manufacturer (Chrystal Chem, Zaandam, The Netherlands). The
218 homeostatic model assessment of insulin resistance (HOMA-IR) adapted to mice (22) was calculated as
219 $([\text{glucose (mg/dl)} * 0.055] \times [\text{insulin (ng/ml)} \times 172.1]) / 3857$, and used as a surrogate measure of whole-body
220 insulin resistance.

221

222 **Glucose and insulin tolerance tests**

223 Whole-body glucose tolerance test (ipGTT) was performed at week 3 of treatment in 6h-fasted mice, as
224 previously reported (13). In short, after an initial blood collection by tail bleeding ($t = 0$), a glucose load (2
225 g/kg total body weight of D-Glucose [Sigma-Aldrich]) was administered i.p., and blood glucose was measured
226 at 20, 40, 60, and 90 min after glucose administration using a Glucometer. For *db/db* mice, blood samples
227 were collected at 0, 20, 40 and 90 min after glucose administration, and plasma glucose levels were
228 measured using the hexokinase method (HUMAN, Wiesbaden, Germany).

229 Whole-body insulin tolerance test (ipITT) was performed determined at week 1 or week 3 of
230 treatment in 4h-fasted mice, as described previously (13). In short, after an initial blood collection by tail
231 bleeding ($t = 0$), a bolus of insulin (1 U/kg (lean) body mass [NOVORAPID, Novo Nordisk, Alphen aan den Rijn,
232 Netherlands]) was administered i.p., and blood glucose was measured at 20, 40, 60, and 90 min after insulin
233 administration using a Glucometer.

234

235 **Statistical analysis**

236 All data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed using
237 GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA, USA) with unpaired t-test, or either

238 one-way or two-way analysis of variance (ANOVA) followed by Fisher's post-hoc test. Differences between
239 groups were considered statistically significant at $P < 0.05$.

241 **Results**

242

243 ***S. mansoni* soluble egg antigens (SEA) improve metabolic homeostasis in obese mice by a STAT6-
244 dependent mechanism**

245 In order to investigate the role of type 2 immunity in the beneficial metabolic effects of SEA, we used mice
246 deficient for STAT6 (*Stat6*^{-/-}), a key transcription factor involved in signature type 2 cytokines interleukin (IL)-
247 4/IL-13 signaling and maintenance of Th2 effector functions (23, 24). As previously reported (13), we
248 confirmed that chronic treatment with SEA for 4 weeks increased IL-5 and IL-13-expressing Th2 cells (Fig. 1A-
249 B), eosinophils (Fig. 1C) and YM1⁺ AAMs (Fig. 1D) in WAT from HFD-fed obese WT mice while, as expected,
250 this type 2 immune response was abrogated in *Stat6*^{-/-} mice. SEA slightly reduced body weight (Fig. 1E) and
251 similarly affected body composition (Fig. S1A) in both WT and *Stat6*^{-/-} obese mice, without affecting food
252 intake (Fig. 1F). In line with our previous study, we showed that SEA reduced fasting plasma insulin levels
253 (Fig. S1C) and HOMA-IR (Fig. 1G), and improved whole-body glucose tolerance in WT obese mice.
254 Remarkably, this beneficial metabolic effect was completely abolished in *Stat6*^{-/-} mice (Fig. 1H-J), indicating
255 that SEA improves whole-body metabolic homeostasis in obese mice through STAT6-mediated type 2
256 immunity.

257

258 **FIG 1**

259

260 **Plant-produced recombinant ω 1 glycovariants increase adipose tissue Th2 cells, eosinophils and
261 alternatively-activated macrophages, without affecting innate lymphoid cells**

262 One of the major type 2 immunity-inducing molecules in SEA is the T2 ribonuclease glycoprotein ω 1 (17). To
263 study the effect of ω 1 on metabolic homeostasis and the role of its immunomodulatory glycans, we
264 generated two recombinant glycosylation variants using glycol-engineered *N. benthamiana* plants: one
265 carrying wild-type plant glycans (pWT- ω 1) and one harboring terminal Le^x motifs (pLe^x- ω 1; (20)). For both
266 ω 1 glycovariants, 4 weeks treatment markedly increased WAT CD4 T cells in HFD-fed obese mice, with pWT-

267 ω 1 being more potent than pLe^X- ω 1, while total ILCs were unaffected (Fig. 2A-B). Interestingly, a specific
268 increase in WAT IL-5 and IL-13-expressing Th2 cells was seen for both ω 1 glycovariants, while the other CD4
269 T cell subsets, *i.e.* regulatory T cells (Treg) and Th1 cells, were not affected (Fig. 2C). In addition, we
270 confirmed that HFD reduced WAT IL-5⁺/IL-13⁺ ILC2s, as previously reported (9), an effect that was even
271 further pronounced with ω 1 glycovariants (Fig. 2D). The type 2 cytokines IL-5 and IL-13 produced by either
272 ILC2s and/or Th2 cells have been reported to maintain WAT eosinophils (9). Congruent with our data on Th2
273 cells, we found a potent increase in WAT eosinophils upon ω 1 treatment that was of similar extent for both
274 glycovariants (Fig. 2E). Finally, both pWT- ω 1 and pLe^X- ω 1 increased WAT YM1⁺ AAMs while obesity-
275 associated CD11c⁺ macrophages were not affected (Fig. 2F-G). This ω 1-induced WAT type 2 immunity was
276 dose-dependent (Fig. S3) and already observed after one week of treatment, when ILC2s were also not
277 affected (Fig. S4).

278

279 **FIG 2**

280

281 AAMs are considered the effector cells of WAT type 2 immunity in the maintenance of tissue insulin
282 sensitivity (2), although the mechanisms are not fully understood. Monocyte-derived macrophages can
283 irreversibly be labelled upon tamoxifen administration in *Cx3cr1*^{CreERT2-IRES-EYFP} *Rosa26*^{LoxP-stop-LoxP-tdTomato}
284 (*Cx3cr1*^{CreER} *Rosa26*^{tdTomato}) mice, as described elsewhere (25). In order to characterize newly recruited, ω 1-
285 induced adipose tissue macrophages (ATMs) during obesity, we performed RNA sequencing on FACS-sorted
286 tdTomato⁺ macrophages from eWAT SVF of obese *Cx3cr1*^{CreER} *Rosa26*^{tdTomato} mice that were treated with PBS
287 or pLe^X- ω 1, the glycovariant that resembles native ω 1 most (Fig. S5A). Genes associated with alternative
288 activation, *e.g.* *Tmem26*, *Slc7a2*, *Chil3* and *Arg1*, were upregulated ($\log_2\text{FC} > 2$) in ATMs from pLe^X- ω 1-
289 treated mice as compared to controls, while genes associated with pro-inflammatory or obesity-associated
290 macrophages, *e.g.* *Igfbp7*, *Cxcl12*, *Bgn*, *Dcn* and *Cd86*, were downregulated ($\log_2\text{FC} < -2$; Fig. S5B-C).
291 Macrophage function is increasingly recognized to be supported by their metabolism to meet energy
292 demands, and as such, AAMs display increased oxidative phosphorylation (26). In PD-L2⁺ WAT macrophages

293 (Fig. S5D), pLe^X-ω1 indeed increased mitochondrial mass, while displaying decreased mitochondrial
294 membrane potential and similar total reactive oxygen species production (Fig. S5E-G), a metabolic
295 phenotype in line with alternative macrophage activation.

296 Similar to WAT, maintenance of insulin sensitivity in the liver is also associated with type 2 immunity
297 (27), whereas obesity-driven activation of Kupffer cells increases the recruitment of pro-inflammatory
298 monocytes and triggers hepatic insulin resistance (2, 7). In our conditions, while ω1 glycovariants increased
299 Th2 cells in the liver, we surprisingly did not find alternative activation of Kupffer cells (Fig. S6A-D). Instead,
300 ω1 glycovariants increased the number of CD11c⁺ pro-inflammatory Kupffer cells, hepatic expression of pro-
301 inflammatory cytokines *Ccl2*, *Tnf* and *Il1b*, and newly recruited monocytes (Fig. S6D-F), with a more potent
302 effect in pLe^X-ω1-treated mice. Taken together, these data indicate that both ω1 glycovariants potently
303 induce type 2 immunity in obese mice, triggering an alternative activation profile in WAT, but not liver
304 macrophages.

305

306 **ω1 glycovariants reduce body weight, fat mass and food intake, and improve whole-body metabolic
307 homeostasis in obese mice**

308 We next investigated the metabolic effects of ω1 glycovariants and showed that they both induced a
309 rapid and gradual body weight loss in HFD-fed mice (Fig. 3A-B), which was exclusively due to a decrease in
310 fat mass (Fig. 3C). The ω1 glycovariants significantly reduced visceral eWAT mass, but had no or only
311 marginal effects on subcutaneous iWAT, brown adipose tissue (BAT) and liver mass (Fig. S2D). This reduction
312 in fat mass was not due to increased beiging, as ω1 glycovariants neither increased expression of
313 thermogenic genes in both eWAT and iWAT (Fig. S7A-B), nor whole-body energy expenditure (Fig. S7C). In
314 addition, ω1 glycovariants did not affect hepatic steatosis (Fig. S6G-I) but rather increased the expression of
315 fibrotic gene markers (Fig. S6J), without detectable collagen accumulation (Fig. S6K-L). An increase in
316 circulating alanine transaminase levels was also observed (Fig. S6M), indicating that ω1 may also have some
317 cytotoxic effects in the liver, as previously reported (28, 29).

318 Remarkably, we found that both ω 1 glycovariants induced a significant decrease in food intake (Fig.
319 3D-E), while locomotor activity was not affected (Fig. 3F-G). Treatment with both ω 1 glycovariants
320 significantly reduced fasting blood glucose, plasma insulin levels (Fig. S2E-F) and HOMA-IR (Fig. 3H) in obese
321 mice, with a trend towards a stronger effect with pLe^X- ω 1, indicating improved insulin sensitivity. Congruent
322 with these data, we observed a significant improvement in whole-body glucose tolerance (Fig. 3I-J) and
323 insulin sensitivity (Fig. 3K-L) in both pWT- ω 1 and pLe^X- ω 1-treated obese mice. Of note, the effects of ω 1
324 glycovariants on food intake, plasma metabolic parameters and whole-body insulin sensitivity were dose-
325 dependent (Fig. S3) and already observed after one week of treatment (Fig. S4). Altogether, these data show
326 that both recombinant ω 1 glycovariants improve whole-body metabolic homeostasis in insulin-resistant
327 obese mice.

328

329 **FIG 3**

330

331 **pLe^X- ω 1 improves metabolic homeostasis in obese mice by a STAT6-independent mechanism**

332 We next investigated the role of type 2 immunity in the metabolic effects of ω 1, using pLe^X- ω 1 as
333 the most potent and native-like glycovariant. As expected, while 4 weeks pLe^X- ω 1 treatment (Fig. 4A)
334 increased WAT Th2 cells, eosinophils and YM1⁺ AAMs in obese WT mice, this type 2 immune response was
335 abrogated in obese *Stat6*^{-/-} mice (Fig. 4B-D). However, treatment with pLe^X- ω 1 still reduced body weight (Fig.
336 4E-G) and food intake (Fig. 4H), and affected body composition (Fig. S2G) in *Stat6*^{-/-} obese mice to the same
337 extent as in WT mice. In addition, both plasma insulin levels and HOMA-IR were markedly decreased in both
338 genotypes (Fig. S2H-I and Fig. 4I). The improvements in whole-body glucose tolerance (Fig. 4J-L) and insulin
339 sensitivity (Fig. 4M-O) were also still observed in *Stat6*^{-/-} mice, indicating that pLe^X- ω 1's type 2 immunity-
340 inducing capacity does not play a major role in restoration of metabolic homeostasis in obese mice. Of note,
341 in contrast to its implication in maintenance of WAT metabolic homeostasis, IL-13 signaling has recently also
342 been shown to play a role in the development of liver fibrosis (30, 31). Interestingly, the increase in liver IL-5⁺
343 and IL-13⁺ Th2 cells in response to pLe^X- ω 1 was also abrogated in *Stat6*^{-/-} mice (Fig. S6N-O), and the

344 expression of fibrotic gene markers were markedly reduced in *Stat6*^{-/-} mice as compared to WT mice (Fig.
345 S6O). Taken together, these results show that pLe^X- ω 1 improves whole-body metabolic homeostasis
346 independent of STAT6-mediated type 2 immunity, while promoting early markers of hepatic fibrosis at least
347 partly through an IL-13-STAT6-mediated mechanism.

348

349 **FIG 4**

350

351 **pLeX- ω 1 improves metabolic homeostasis through leptin receptor-independent inhibition of food intake
352 in obese mice**

353 As ω 1 significantly reduced food intake in obese mice, we next investigated its impact on feeding behavior.
354 We found that a single intraperitoneal injection of pLe^X- ω 1 in overnight fasted obese mice markedly reduced
355 food intake during refeeding for at least 24 hours, resulting in decreased body weight gain as compared to
356 PBS-injected mice (Fig. 5A-C). To determine whether reduced food intake drives the beneficial metabolic
357 effects of ω 1, we treated HFD-fed mice with pLe^X- ω 1 or PBS, and included a pair-fed group that received
358 daily adjusted HFD meals based on the food intake of the pLe^X- ω 1-treated animals (Fig. 5D). While pLe^X- ω 1
359 expectedly induced IL-13⁺ Th2 cells, eosinophils and YM1⁺ AAMs in WAT, reducing caloric intake in pair-fed
360 mice did not affect WAT type 2 immunity (Fig. 5E-G). Yet, food restriction in the pair-fed group decreased
361 body weight, fasting blood glucose and plasma insulin levels, and HOMA-IR as well as whole-body glucose
362 tolerance to the same extent as in pLe^X- ω 1-treated animals (Fig. S2K-L and Fig. 5H-I).

363

364 **FIG 5**

365

366 The central regulation of feeding behavior and whole-body energy homeostasis involves complex neuronal
367 networks, notably in the hypothalamus and brain stem. (32, 33). To investigate whether pLe^X- ω 1
368 accumulates in the brain to directly regulate hypothalamic neurons controlling food intake, we performed in
369 vivo imaging experiments with pLe^X- ω 1 conjugated to a hybrid tracer (¹¹¹In-DTPA-Cy5-pLe^X- ω 1). Both Single

370 Photo Emission Computed Tomography (SPECT) imaging and radioactivity biodistribution revealed that ^{111}In -
371 DTPA-Cy5-pLe X - ω 1 mainly accumulated in abdominal organs, *e.g.* adipose tissues, liver and intestines, and
372 peritoneal draining lymph nodes, whereas no substantial amounts of radioactivity could be detected in the
373 hypothalamus and other brain regions 24h after tracer administration (Fig. S8A-B). Hence, ω 1 does not
374 distribute to the brain and likely regulates food intake through peripheral effects.

375 The hypothalamus and brain stem also integrate signals from both the enteric nervous system and
376 circulating hormones derived from adipose tissue and other peripheral tissues. Leptin is by far the best
377 studied peripheral hormone that regulates food intake, increasing satiety by triggering STAT3-mediated
378 pathways in the hypothalamic arcuate nucleus (32). In order to study the role of leptin signaling in the
379 metabolic effects of pLe X - ω 1, we used leptin receptor-deficient *db/db* mice that are hyperphagic and
380 naturally develop obesity and severe metabolic dysfunctions (34). In this model, pLe X - ω 1 also increased WAT
381 IL-13 $^+$ Th2 cells, eosinophils and YM1 $^+$ AAMs (Fig. 6A-D). Furthermore, pLe X - ω 1 still reduced body weight (Fig.
382 6E-F), fat mass gain (Fig. S2M-N) and food intake (Fig. 6G), indicating that leptin signaling is not involved in
383 the anorexigenic effect of ω 1. Lastly, plasma insulin levels (Fig. S2O-P), HOMA-IR (Fig. 6H) and whole-body
384 glucose tolerance and insulin sensitivity (Fig. 6I-L) were still significantly improved.

385 Collectively, our results show that ω 1 improves whole-body metabolic homeostasis independent of
386 its type 2 immunity-inducing capacity, but by inhibiting food intake through a leptin receptor-independent
387 mechanism.

388

389 **FIG 6**

391 **Discussion**

392 Obesity-associated metaflammation promotes insulin resistance, while metabolic homeostasis is maintained
393 by type 2 immunity (1). Since helminths are well known for inducing a potent type 2 immune response, their
394 putative beneficial effects on insulin sensitivity and glucose homeostasis, together with the identification of
395 specific helminth-derived molecules capable of driving such type 2 immune responses, have gained
396 increasing attention (11, 15, 35). The assumption has been that induction of type 2 immunity is the main
397 mechanism by which helminths and helminth-derived molecules can improve metabolic homeostasis. The
398 glycoprotein ω 1, a T2 ribonuclease which is secreted from *S. mansoni* eggs, is one of the major
399 immunomodulatory components in SEA and has previously been shown to condition DCs to prime Th2
400 responses, at least partly through its glycan-mediated uptake and intracellular RNase activity (16, 17). Here,
401 we report that two plant-produced recombinant ω 1 glycovariants induced a rapid and sustained reduction in
402 body weight and improved whole-body insulin sensitivity and glucose tolerance in obese mice. This
403 improvement was associated with a strong type 2 immune response in WAT, characterized by a significant
404 increase in Th2 cells, eosinophils and AAMs. Contrary to SEA, ω 1 still improved metabolic homeostasis in
405 *Stat6*-deficient obese mice, indicating that its type 2 immunity-inducing capacity does not play a major role.
406 Indeed, we find that ω 1 regulates energy consumption independent of leptin receptor signaling, which
407 drives most of its metabolic effects. Altogether, these findings indicate that helminth-derived molecules may
408 act through multiple distinct pathways for improving obesity-associated metabolic dysfunctions and further
409 characterization of these molecules may lead to new therapeutic strategies for combating obesity.

410 A recent study from Hams et al. reported that acute treatment of HFD-fed obese mice with HEK-293-
411 produced recombinant ω 1 induced long-lasting weight loss, and improved glucose tolerance by a mechanism
412 involving IL-33 and ILC2-mediated WAT type 2 immunity and adipose tissue beiging (18). In contrast to this
413 report, while we also observed increased IL-33 mRNA expression in eWAT (*data not shown*), we found no
414 increase in WAT ILC2s after either one, or four weeks of treatment with both plant-produced ω 1
415 glycovariants. Moreover, we did not find evidence of WAT beiging in both eWAT and iWAT from obese mice.
416 Lastly, we also found that STAT6-mediated type 2 immunity was dispensable for the metabolic effects of ω 1.

417 It should be noted that despite similar RNase activity when compared to native ω 1 (20), the recombinant ω 1
418 produced by HEK-293 cells and the glycol-engineered molecules from *N. benthamiana* plants harbor
419 significantly different N-glycosylation patterns (17), which may partly explain the different outcomes
420 between studies. Interestingly, as compared to pWT- ω 1, we observed a trend for a stronger effect on insulin
421 sensitivity and food intake with pLe^X- ω 1, of which the glycans resemble the ones of native helminth ω 1 the
422 most.

423 In our study, both ω 1 glycovariants were found to induce a type 2 immune response in WAT,
424 characterized by a significant increase in Th2 cells, eosinophils and AAMs. In addition, as previously
425 described for SEA (13), we showed that the ω 1-induced increase in type 2 cytokines are clearly derived from
426 CD4⁺ T cells, suggesting that DC-mediated Th2 skewing is required, rather than ILC2 activation, to induce
427 WAT eosinophilia and AAM polarization. Of note, it was previously shown that pLe^X- ω 1, compared to pWT-
428 ω 1, induced a stronger Th2 polarization *in vivo* using a footpad immunization model in mice (20). In our
429 conditions, both glycovariants induced a similar increase in the percentage of Th2 cells in metabolic tissues
430 from obese mice, whereas pLe^X- ω 1 increased total CD4⁺ T cells to a greater extent in the liver and to a lesser
431 extent in WAT when compared to pWT- ω 1. Altogether, this suggests that the different glycans on ω 1
432 glycovariants might lead to tissue-specific targeting of ω 1 and resulting differences in total Th2 cells.

433 While the type 2 immune response seems not to be significantly involved in the beneficial metabolic
434 effects of ω 1, we found that treatment with both ω 1 glycovariants reduced food intake, with a trend for
435 pLe^X- ω 1 being more potent than pWT- ω 1. This anorexigenic effect, which was not observed previously when
436 mice were chronically infected with *S. mansoni* or treated with SEA (13), was dose-dependent and also
437 observed in *Stat6*-deficient mice. Importantly, since both locomotor activity and lean body mass were not
438 affected by ω 1, this inhibition of food intake could not be due to illness induced by the treatment. Using
439 fast-refeeding and paired feeding experiments, we clearly showed that ω 1 rapidly inhibited food intake, an
440 effect that mainly contributed to the improvements in metabolic homeostasis. Of note, in the study from
441 Hams *et al.* using HEK-produced recombinant ω 1 in the same concentration range as us, the effect of ω 1 on

442 feeding behavior and its putative contribution to the observed decrease in body weight and improvement of
443 glucose tolerance in obese mice have not been specifically investigated (18).

444 Anorexia is one of the clinical manifestation of infection with different helminth species in both
445 animals and humans. As such, deworming children infected with the hookworms *Ascaris lumbricoides* and/or
446 *Trichuris trichuria* has been reported to increase appetite (36), suggesting a relationship between helminth
447 infection and food intake. In rodents, infection with *Taenia taeniaformis* and *N. brasiliensis* both induced
448 anorexia by modulating neuropeptide expression in the hypothalamus (37, 38), indicating that helminths
449 and/or helminth products may regulate feeding behavior. The mechanism by which $\omega 1$ inhibits food intake is
450 however still unknown and will require further neuroscience-driven approaches to be elucidated. Regulation
451 of food intake by the central nervous system is a complex process involving both local and peripheral neuro-
452 immuno-endocrine inputs that are mainly integrated in the hypothalamic arcuate nucleus and the brain
453 stem *nucleus tractus solitarius* (32, 33). In our study, we did not detect accumulation of radioactively-
454 labelled $\omega 1$ in the brain 24 hours after intraperitoneal injection, suggesting that the glycoprotein may exerts
455 its anorexigenic effects via peripheral rather than central action(s). Upon meal ingestion, several
456 anorexigenic peptides and hormones are produced by metabolic organs, including adipose tissues and the
457 intestine, and can either directly act on specific neurons after crossing the blood-brain barrier or signal from
458 the periphery via vagal nerve-mediated pathways that contribute to satiety regulation (39, 40). Leptin is a
459 key adipose tissue-derived anorexigenic hormone which signals through the leptin receptor in the arcuate
460 nucleus of the hypothalamus to reduce food intake (32). During obesity, hypothalamic inflammation triggers
461 leptin resistance, resulting in increased energy consumption in obese mice (41-43). However, despite some
462 evidence of improved systemic leptin sensitivity by $\omega 1$ (*data not shown*), we found that its anorexigenic and
463 metabolic effects were still present in leptin receptor-deficient mice, allowing us to exclude a significant
464 contribution of peripheral/central leptin signaling. Among the peripheral signals that regulate feeding
465 behavior, it would be interesting to explore the involvement of a gut-brain axis, notably through vagal nerve
466 ablation (40). Recently, *N. brasiliensis* infection and its products were also shown to increase production of
467 the neuropeptide Neuromedin U by mucosal neurons, allowing the host to mount an effective type 2

468 immune response (44-46). Neuromedin U also has anorexigenic effects (47), thus it is tempting to speculate
469 that some helminth molecules may indirectly trigger anorexia through neuro-immune interactions in the gut.

470 It is worth mentioning that ω 1 also increased IL-13 producing Th2 cells in the liver, but, unlike SEA
471 (13), promoted CD11c expression in Kupffer cells while not affecting the expression of YM1, suggesting that
472 macrophages are rather polarized towards a pro-inflammatory state in this tissue. An increase in hepatic
473 expression of fibrotic gene markers and circulating ALAT levels was also observed, both indicating increased
474 liver damage induced by ω 1. Interestingly, the ω 1-induced increase in IL-13⁺ Th2 cells and IL-13 gene
475 expression in the liver were markedly reduced in *Stat6*-deficient mice, which was accompanied by a
476 decreased expression of fibrotic gene markers. Collectively, these findings confirmed previous studies
477 describing that IL-13 plays a role in the development of liver fibrosis (30, 31), and that ω 1 has cytotoxic
478 effects in the liver (28, 29).

479 In conclusion, we report here that the helminth glycoprotein ω 1 improved metabolic homeostasis in
480 insulin-resistant obese mice by a mechanism not dependent of its type 2 immunity-inducing capacity, but
481 rather mostly attributable to leptin receptor-independent inhibition of food intake. Further studies are
482 required to unravel such underlying mechanisms, notably exploring the role of gut hormones on peripheral
483 and/or central regulation of feeding behavior. Of note, with regards to its putative therapeutic potential for
484 metabolic disorders, it is important to underline that despite beneficial effects on whole-body metabolic
485 homeostasis, ω 1 also induced early markers of mild hepatic fibrosis, partly through a type 2 immunity-
486 mediated mechanism. Finally, by contrast to ω 1, the complex mixture of SEA does not have detrimental
487 effects on the liver and improves metabolic homeostasis through a STAT6-mediated type 2 immune
488 response, suggesting that it may contain some other unidentified molecules, such as Dectin 2 ligands (48),
489 with potentially beneficial immunometabolic properties.

490

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617 **Figure legends**

618 **Figure 1. *S. mansoni* soluble egg antigens improve metabolic homeostasis in obese mice by a STAT6-
619 dependent mechanism.** WT and *Stat6*-/- mice were fed a LFD (white bars) or a HFD for 12 weeks and next
620 received intraperitoneal injections of PBS (black bars) or 50 µg *S. mansoni* soluble egg antigens (SEA; red
621 bars) every 3 days for 4 weeks (A). At sacrifice, epididymal WAT was collected and SVF was isolated and
622 analyzed by flow cytometry. The complete gating strategy is shown in Figure S1. Frequencies of IL-5 and IL-
623 13 expressing Th2 cells (B) in WAT were determined after PMA/ionomycin/Brefeldin A restimulation.
624 Abundances of eosinophils (C) and YM1⁺ macrophages (AAMs; D) were determined. Body weight (E) was
625 measured after 4 weeks of treatment. Food intake (F) was monitored throughout the experimental period.
626 HOMA-IR (G) was calculated using fasting blood glucose and plasma insulin levels at week 4. An i.p. glucose
627 tolerance test was performed at week 3. Blood glucose levels were measured at the indicated time points
628 (H-I) and the AUC of the glucose excursion curve was calculated (J). Data shown are a pool of two
629 independent experiments. Results are expressed as means ± SEM. * $P<0.05$ vs HFD, # $P<0.05$ vs WT (n = 9-12
630 mice per group).

631

632 **Figure 2. Plant-produced recombinant ω1 glycovariants increase adipose tissue Th2 cells, eosinophils and
633 alternatively-activated macrophages, without affecting innate lymphoid cells.** Mice were fed a LFD (white
634 bars) or a HFD for 12 weeks, and next received intraperitoneal injections of PBS (black bars) or either 50 µg
635 pWT-ω1 (blue bars) or 50 µg pLe^X-ω1 (green bars) every 3 days during 4 weeks (A). At the end of the
636 experiment, eWAT was collected, processed and analyzed as described in the legend of Figure 1. Numbers of
637 CD4 T cells, ILCs (B), eosinophils (E) and macrophages (F) per gram tissue were determined. Frequencies of
638 CD4 T helper subsets (C) and cytokine-expressing ILCs (D) were determined. Percentages of CD11c⁺YM1⁻ and
639 CD11c⁻YM1⁺ macrophages (G) were measured. Data shown are a pool of at least two independent
640 experiments. Results are expressed as means ± SEM. * $P<0.05$ vs HFD, \$ $P<0.05$ vs pWT-ω1 (n = 6-19 mice
641 per group in B, E-G, and 3-9 mice per group in C and D).

642

643 **Figure 3. ω 1 glycovariants reduce body weight, fat mass and food intake, and improve whole-body**
644 **metabolic homeostasis in obese mice.** Mice were fed a LFD (white bars) or a HFD for 12 weeks, and next
645 received biweekly intraperitoneal injections of PBS (black bars) or 50 μ g pWT- ω 1 (blue bars) or pLeX- ω 1
646 (green bars) during 4 weeks. Body weight (A-B) was monitored throughout the experimental period. Body
647 composition (C) was measured after 4 weeks of treatment. Food intake (D-E) and locomotor activity (F-G)
648 were assessed using fully automated single-housed metabolic cages during the first week of treatment.
649 HOMA-IR at week 4 (H) was calculated. Intraperitoneal glucose (I-J) and insulin (K-L) tolerance tests were
650 performed during week 3. Blood glucose levels were measured at the indicated time points (I, K) and the
651 AUC of the glucose excursion curve were calculated (J, L). Data shown are a pool of at least 2 independent
652 experiments. Results are expressed as means \pm SEM. * $P<0.05$ vs HFD (n = 11-20 mice per group in A-C, H-L,
653 and 4-8 mice per group in D-G).

654

655 **Figure 4. pLe^X- ω 1 improves metabolic homeostasis in obese mice by a STAT6-independent mechanism.** WT
656 and *Stat6*^{-/-} mice were fed a LFD (white bars) or a HFD for 12 weeks and next received biweekly
657 intraperitoneal injections of PBS (black bars) or 50 μ g pLe^X- ω 1 (green bars) during 4 weeks (A). At the end of
658 the experiment, eWAT was collected, processed and analyzed as described in the legend of Figure 1. The
659 frequencies of cytokine-expressing CD4 T cells (B) were determined. Abundances of eosinophils (C) and YM1⁺
660 macrophages (D) were determined. Body weight (E-G) and food intake (H) were monitored throughout the
661 experimental period. HOMA-IR at week 4 (I) was calculated, and intraperitoneal glucose (J-L) and insulin (M-
662 O) tolerance tests were performed as described in the legend of Figures 1 and 3. Results are expressed as
663 means \pm SEM. * $P<0.05$ vs HFD, # $P<0.05$ vs WT (n = 3-5 mice per group).

664

665 **Figure 5. pLe^X- ω 1 inhibits fasting-induced refeeding and improves metabolic homeostasis through**
666 **inhibition of food intake in obese mice.** Mice were fed a HFD for 12 weeks and fasted overnight prior to
667 intraperitoneal injections of either PBS (black bars) or 50 μ g pLe^X- ω 1 (green bars; A). Food intake (B) and
668 body weight changes (C) were next monitored during 24 hours after refeeding. Mice were fed a HFD for 12

669 weeks, single-housed, and next received biweekly intraperitoneal injections of PBS or 50 µg pLe^X-ω1 during 4
670 weeks. In one group (H+pair-fed; orange bars), the amount of food available for PBS-treated mice was
671 adjusted daily in order to match the food intake of the pLe^X-ω1-treated group (D). At the end of the
672 experiment, eWAT was collected, processed and analyzed as described in the legend of Figure 1. The
673 frequencies of IL-13-expressing CD4 T cells (E), eosinophils (F) and YM1⁺ macrophages (G) were determined.
674 Body weight change (H) was determined after 4 weeks. HOMA-IR (I) was calculated at week 4 and an i.p.
675 glucose tolerance test (J-K) was performed at week 3, as described in the legend of Figure 1. Results are
676 expressed as means ± SEM. *P<0.05 vs HFD or as indicated (n = 3-5 mice per group).

677

678 **Figure 6. The metabolic effects of pLe^X-ω1 are independent of leptin signaling in hyperphagic obese mice.**
679 7 weeks-old obese *db/db* mice received biweekly intraperitoneal injections of PBS (white bars) or 50 µg pLe^X-
680 ω1 (green bars) during 4 weeks (A). At the end of the experiment, eWAT was collected, processed and
681 analyzed as described in the legend of Figure 1. The frequencies of IL-13-expressing CD4 T cells (B),
682 eosinophils (C) and YM1⁺ macrophages (D) were determined. Body weight (E) and food intake (G) were
683 monitored throughout the experimental period, and body composition (F) was measured after 4 weeks.
684 Intraperitoneal glucose (H-I) and insulin (J-K) tolerance tests were performed as described in the legend of
685 Figures 1 and 3. Results are expressed as means ± SEM. *P<0.05 vs PBS (n = 5-6 mice per group).

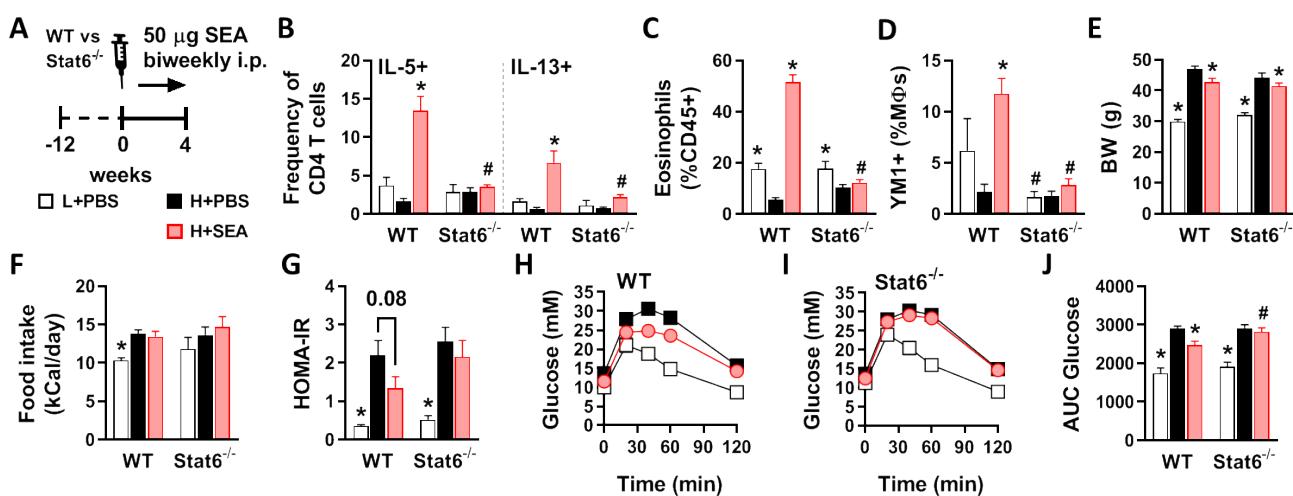


Figure 1

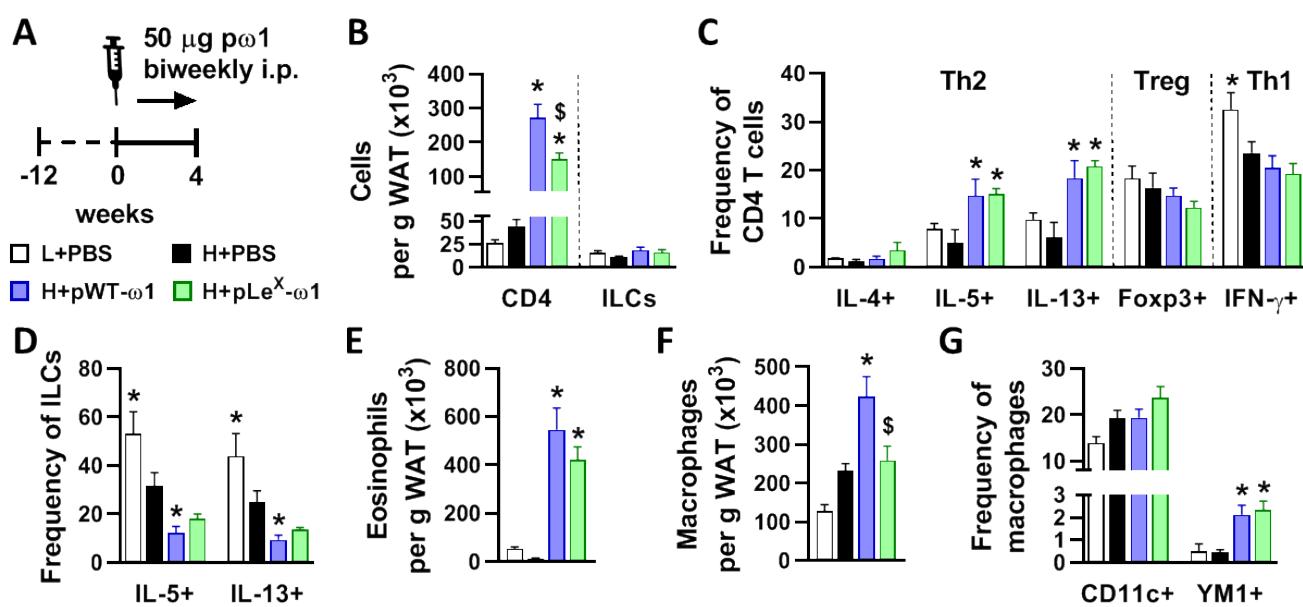


Figure 2

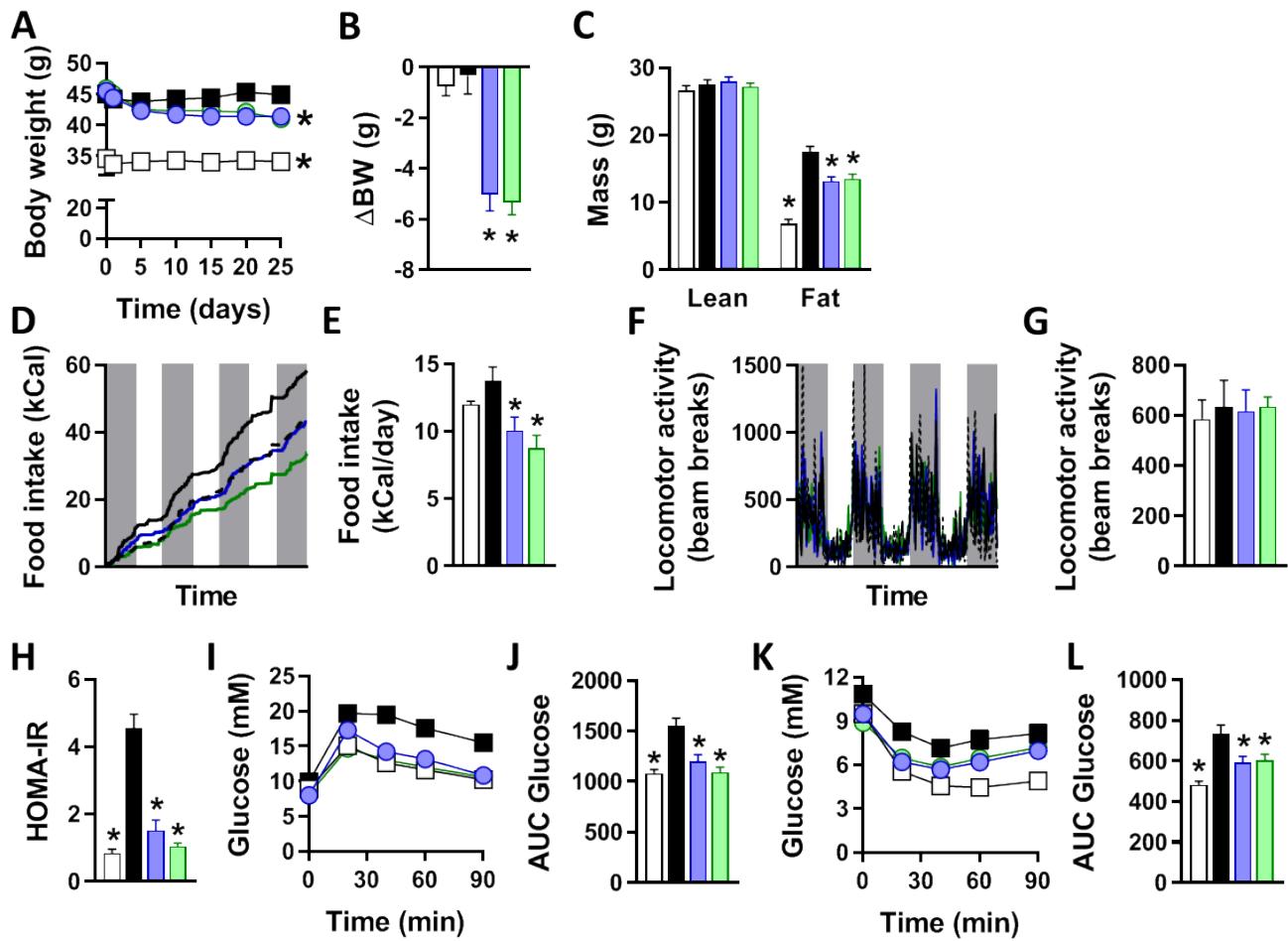


Figure 3

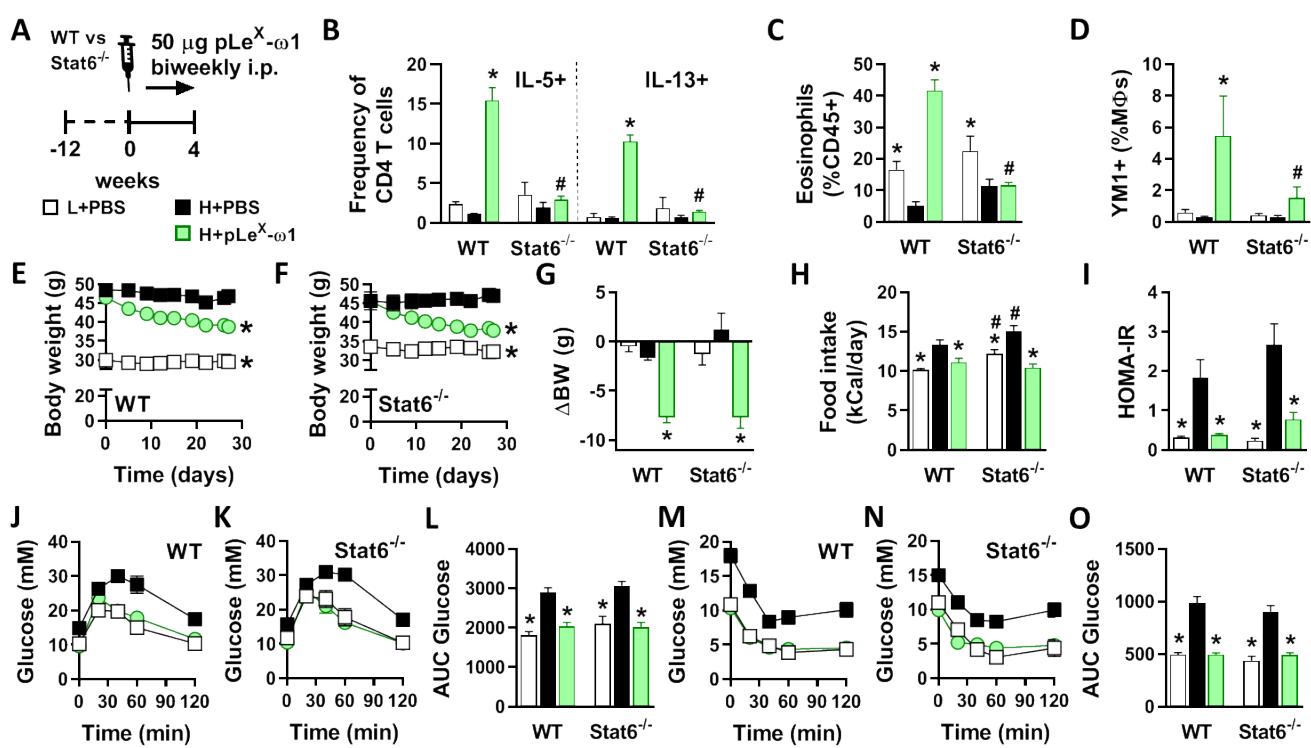


Figure 4

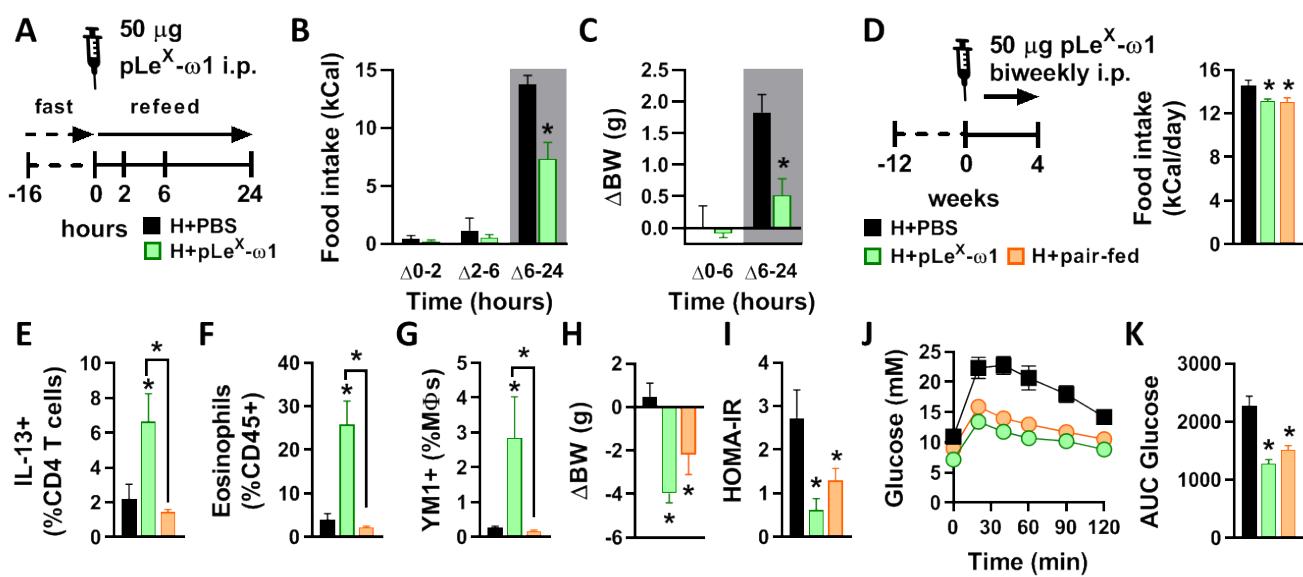


Figure 5

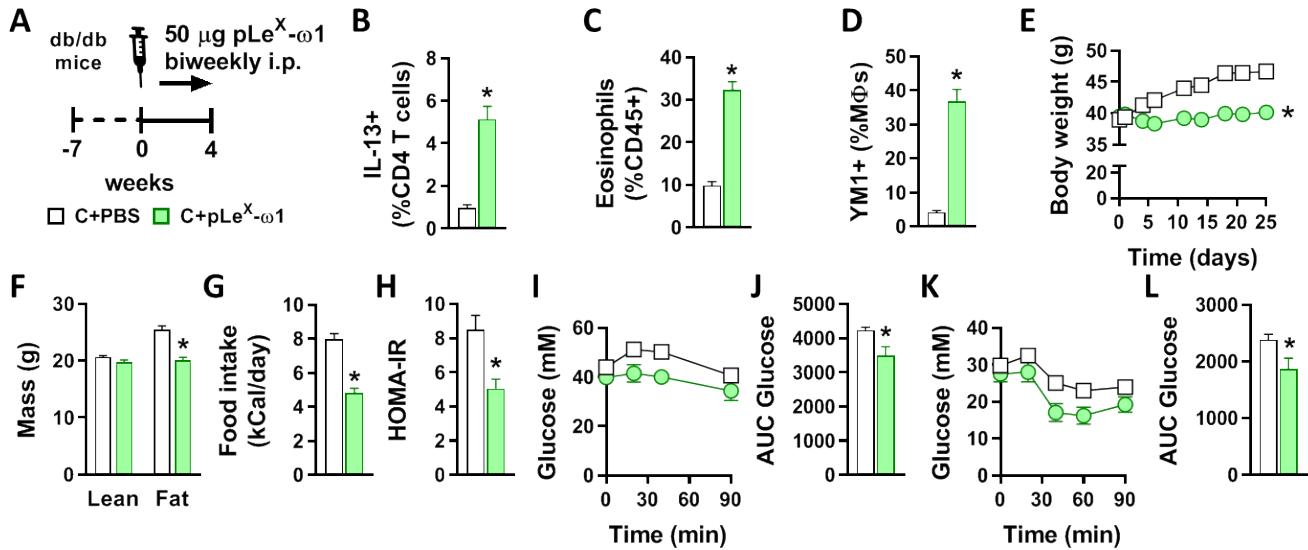


Figure 6