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1 **Autoimmunity to HSP60 during diet induced obesity in mice**

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19

1 **Abstract**

2 **Background/Objectives:** Adaptive immunity has been implicated in adipose tissue  
3 inflammation, obesity and its adverse metabolic consequences. No obesity-related autoantigen  
4 has yet been identified, although heat shock protein 60 (HSP60) has been implicated in other  
5 autoimmune diseases.

6 **Subjects/Methods:** We investigated whether feeding a high fat diet to C57BL/6J mice would  
7 cause autoimmunity to HSP60 and whether immunomodulation with peptides from HSP60  
8 would reverse the resulting obesity or metabolic dysfunction.

9 **Results:** Obese mice had higher circulating levels of HSP60 associated with increased T-  
10 lymphocyte proliferation responses and the emergence of circulating IgG1 and IgG2c antibody  
11 levels against HSP60. Treatment with escalating doses of a mixture of three proven  
12 immunomodulatory HSP60 peptides did not reduce weight but completely reversed the increase  
13 in VLDL/LDL levels and partially reversed the glucose intolerance in obese mice.

14 **Conclusions:** Obese mice mount an autoimmune response to HSP60, which partly underlies the  
15 resulting metabolic disturbances.

16

17 Key words: Autoimmunity, heat shock proteins, glucose tolerance test, lipoproteins, mice,  
18 obesity

19

20 Abbreviations: AT, adipose tissue; HSP60, heat shock protein 60; ND, normal diet; HFD, high  
21 fat diet; Th(1/2), Thelper(1/2) lymphocyte; Treg, regulatory T lymphocyte; SVF, stromal  
22 vascular fraction

23

24

## 1 **Introduction**

2 Obesity is increasing worldwide, accompanied by rising levels of type-2 diabetes and the  
3 metabolic syndrome, fatty liver disease, breast and colon cancer, musculoskeletal disorders and  
4 cardiovascular diseases, including atherosclerosis and stroke <sup>1,2</sup>. Many harmful effects of obesity  
5 have been attributed to adipose tissue (AT) inflammation <sup>3</sup>, with both innate and adaptive  
6 immunity implicated <sup>4</sup>. The evidence that T-lymphocytes contribute to AT inflammation  
7 includes: 1) T cells accumulate in AT even before macrophages <sup>5-7</sup>. 2) Restricted V<sub>α</sub> repertoires  
8 imply antigen-specific clonal expansion <sup>8</sup>. 3) Deletion of MHC Class II molecules globally or  
9 on macrophages reduces obesity, insulin resistance and AT inflammation <sup>9,10</sup>. 5) Conversely,  
10 enhancement of antigen-presenting cell function favours AT inflammation and promotes insulin  
11 resistance <sup>11</sup>. This evidence suggests an autoimmune component in obesity but no culprit  
12 autoantigens have so far been identified.

13 HSP60 is an evolutionarily-conserved mitochondrial chaperonin that can translocate to  
14 the cytosol and cell membrane and be released into the circulation under conditions of stress <sup>12</sup>.  
15 HSP60 has been associated with the autoimmune component of several inflammatory diseases,  
16 including atherosclerosis <sup>12</sup>. More recently, release of HSP60 from AT was demonstrated as well  
17 as its ability to cause insulin resistance and pro-inflammatory cytokine (TNF- $\alpha$ , IL-6 and IL-8)  
18 release by adipocytes <sup>13</sup>. Also, circulating HSP60 levels were found higher in obese individuals  
19 than lean controls <sup>13</sup>. All these observations make HSP60 a candidate autoantigen in obesity,  
20 although this has not yet been demonstrated. We therefore investigated whether high fat diet  
21 (HFD) feeding gives rise to autoimmunity against HSP60 in mice and whether  
22 immunomodulation with HSP60-specific peptides can reduce obesity or the related metabolic  
23 impairment.

24

25

## 1 **Materials and Methods**

2 More detail is given in the Supplementary Methods file available at the International Journal  
3 of Obesity's website. Briefly: C57BL/6J mice (6 weeks old) purchased from Charles River  
4 Laboratories were fed normal chow (ND) or a HFD supplemented with 21% lard and 0.15%  
5 cholesterol (Special Diets Services) for 16-20 weeks to induce obesity. For peptide treatment,  
6 6 week old mice were pre-dosed subcutaneously with HSP60 peptides (GL Biochem,  
7 Shanghai) starting at 0.1 µg/mouse. The dose was increased 10 fold every week up to 100  
8 µg/mouse, which was given weekly 3 more times, then every two weeks until the end of study.  
9 HFD was started at 11 weeks of age (after the third top dose) and lasted for 20 weeks<sup>14</sup> followed  
10 by euthanasia by cervical dislocation under Home Office Licence 70/22957. The Guide for  
11 the care and use of laboratory animals, Eighth edition (2011)  
12 (<http://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf>) was  
13 followed. Procedures were carried out under Home Office Licences 30/3064 and 70/22957. All  
14 animals survived until culled and were included in the analysis.

15

16 After euthanasia, epididymal fat pads were collected, weighed and the stromal vascular  
17 fraction (SVF) was isolated by collagenase digestion. For analysis of macrophage populations,  
18 1 million SVF cells were examined by flow cytometry analysis using antibodies against  
19 CD11b, F4/80, CD11c and CD206. T-cell populations were analysed using antibodies against  
20 CD45, CD3ε, CD4, CD25 and FoxP3.

21 Serum HSP60 levels were measured with a mouse HSP60 ELISA (NeoScientific).  
22 Serum anti-HSP60 antibody levels were measured with a custom-made ELISA using  
23 recombinant, endotoxin-depleted murine HSP60 protein (Enzo Life Sciences) bound to Nunc  
24 Immuno MaxiSorp 96 well plates. For the HSP60 reactive T cell proliferation assay, total cell

1 pellets from homogenized spleens were pulsed with <sup>3</sup>H-thymidine for 18 hours after pre-  
2 treating with buffer control, recombinant HSP60 or peptides.

3           Glucose tolerance tests were performed after 16 weeks of ND or HFD. After 6 hours  
4 fast, 2 g/kg body weight of glucose was injected intraperitoneally and glucose concentration in  
5 blood from tail snips was measured 0, 15, 30, 60, and 90 minutes later. Insulin tolerance test  
6 was conducted one week later. After 4 hours fast, rapid acting human insulin (NovoRapid;  
7 Novo Nordisk A/S) was injected intraperitoneally to give a final dose of 1 U/kg body weight.  
8 Blood glucose was measured at the same time points. Mouse Ultrasensitive Insulin ELISA kit  
9 (Alpco) was used to determine fasting insulin levels.

10           For normally-distributed variables (Kolmogorov-Smirnov test), a two-tailed, unpaired  
11 Student's t-test was used to determine significant differences between 2 means. For multiple  
12 comparisons, a one-way ANOVA or two-way ANOVA was performed, as appropriate,  
13 followed by a Bonferroni correction. Differences were considered significant if  $p < 0.05$ .

14

15

## 1 **Results**

2

3 *Increased circulating HSP60 levels, antibodies against HSP60, and T-cell responses to HSP60*  
4 *in HFD fed C57BL/6J mice*

5 C57BL/6J mice fed HFD for 16 weeks were 3g heavier than ND mice (Fig. 1A) and  
6 their epididymal fat pads were almost 3 times heavier (Fig. 1B). Plasma levels of LDL/VLDL  
7 were significantly increased (74%) by HFD, although HDL levels were unaltered (Fig. 1C).  
8 HFD significantly increased circulating HSP60 levels by 12% (Fig. 1D), suggesting that  
9 obesity is a stress factor capable of stimulating HSP60 release.

10 As evidence for an autoimmune reaction to HSP60, recombinant HSP60 increased  
11 splenic T lymphocyte proliferation relative to buffer controls by 20-40% more in HFD than  
12 ND mice. This increase was significant at 0.1 µg/ml and 10 µg/ml and it was at borderline  
13 significance (p=0.063) at 1.0 µg/ml concentration (Fig. 1E). The response to HSP60 was much  
14 weaker than the positive control ConA (13 fold, not shown), not surprisingly because the mice  
15 were not hyper-immunized. Circulating anti-HSP60 levels were undetectable in ND mice but  
16 HFD significantly elevated both anti-HSP60 IgG1, which is mainly dependent on Thelper2  
17 (Th2) lymphocytes, and IgG2c, which is driven by Th1 lymphocytes<sup>15</sup> (Fig. 1F). Differences  
18 between HFD and ND mice were highly significant above 1/16 dilution; much lower than  
19 expected for hyper-immune serum (typically >1/1000). Clearly, short periods of obesity  
20 triggered autoimmunity against murine HSP60, albeit at low-level.

21

22 *Effect of HSP60 peptide treatment on obesity and related metabolic dysfunction.*

23 To investigate the impact of HSP60 autoimmunity, we used subcutaneous HSP60  
24 peptide treatment with a combination of equal concentrations of three peptides known to cause  
25 favourable immunomodulation in models of autoimmune diabetes or atherosclerosis. These

1 were vlgggcallrcipaldslkpaned <sup>16</sup>, aelkkqskpvt <sup>17</sup> and dgealstlvlnrlkvg <sup>18</sup>. Each of these peptides  
2 had good solubility in water (results not shown). We used a dose escalation protocol that has  
3 proved safe and effective against experimental autoimmune encephalomyelitis <sup>14</sup>.

4 HSP60 peptide treatment did not reverse HFD-induced weight gain (Fig. 2A) or the  
5 increase in epididymal fat mass (Fig. 2B), which were greater in this experiment owing to the  
6 longer period of HFD feeding. However, peptide treatment reversed the significantly increased  
7 circulating LDL/VLDL levels in HFD mice (Fig. 2C).

8 Glucose tolerance was significantly impaired in HFD compared to ND mice. HSP60  
9 peptide treatment rendered HFD mice significantly more glucose tolerant, although not as  
10 tolerant as ND mice (Fig. 2D). Fasting insulin levels were elevated to the same degree relative  
11 to ND ( $0.64 \pm 0.05$ ) by HFD, irrespective of peptide treatment ( $1.01 \pm 0.22$  and  $1.18 \pm 0.22$ ) and  
12 could not account for the improved glucose tolerance. Insulin sensitivity, measured in an  
13 insulin tolerance test, was also similar in ND, HFD and peptide treatment groups (Fig. 2E).

14 To investigate whether HSP60 peptide treatment suppressed obesity-associated  
15 inflammation, we subjected SVF cells to flow cytometry. We recorded a significant increase  
16 in macrophages (Fig. 2F), no change in total CD4<sup>+</sup> or CD4<sup>-</sup> T-lymphocytes (not shown) and a  
17 decrease in Treg cells (Fig. 2G) in HFD compared to ND mice but there was no effect of HSP60  
18 peptide treatment (Fig. 2F, G). We also did not observe any difference in splenic T-cell  
19 proliferation responses to recombinant HSP60 in HFD-fed mice with and without HSP60  
20 peptide treatment. Nor did we find any difference in the levels of IL2, IL4, IL6, IL10, IL17,  
21 IFN $\gamma$  or TNF $\alpha$  released spontaneously or in response to HSP60 challenge (results not shown).  
22 However, HSP60 peptide treatment increased the titre of anti-HSP60 IgG1 (Fig. 2H) but not  
23 IgG2c antibodies (Fig. 2I), which implies an enhancement of Th2 driven antibody production.

24

25



## 1 **Discussion**

2

3 An autoimmune component of obesity has been postulated <sup>8,9</sup> (see Introduction) but  
4 our study is the first to identify a contributing autoantigen. Obese mice clearly mounted a weak  
5 autoimmune response to HSP60 at both the T and B cell levels after a short period of HFD.  
6 Consistent with this, lean humans have antibodies to HSP60, presumably arising from other  
7 stressors <sup>12</sup>, but the level of autoantibodies is increased by obesity that is clearly much more  
8 prolonged than in our mice <sup>13</sup>. Moreover, we could reverse the hypercholesterolaemic effect of  
9 HFD and partially improve glucose tolerance with immunomodulatory HSP60 peptide  
10 treatment, implying that autoimmunity to HSP60 contributed to the metabolic disturbances  
11 caused by obesity, despite weight not being reduced. A similar decoupling of obesity and  
12 metabolic disturbances has been noted in other studies when suppressing adaptive immunity,  
13 for example in RAG-1 deficient mice, which lack mature T and B lymphocytes <sup>8</sup>. Given  
14 observations that LDL production is increased by obesity <sup>19</sup> and that HSP60 autoimmunity  
15 contributes to atherosclerosis <sup>12</sup>, the reduction in LDL levels achieved by HSP60 peptide  
16 treatment is of interest. A recent study showing that regulatory T-cells can reduce the levels  
17 of blood cholesterol in low density lipoprotein deleted mice might provide a mechanisms for  
18 our results <sup>20</sup>, but this requires further investigation.

19 HFD increased AT macrophages and decreased Treg as previously reported by others  
20 (reviewed by Lolmede *et al.*<sup>4</sup>) but we did not see any changes with HSP60 peptide treatment.  
21 Neither did we find any influence of peptide-induced splenic T-cell proliferation or cytokine  
22 release. We did, however, see a switch towards Th2 driven IgG1 production, which implies  
23 that HSP60 peptide treatment caused immunomodulation rather than immune tolerization,  
24 which will require more extensive investigation. In summary, we showed for the first time that

1 low-level autoimmunity to HSP60 contributes to, and HSP60 peptide treatment partially  
2 reverses, metabolic disturbances in a murine obesity model.

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4

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7

8 Supplementary information is available at the International Journal of Obesity's website

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49

## 1 **Legends to Figures**

2

### 3 **Figure 1. Effect of HFD on weight gain, metabolism and auto-immunity to HSP60.**

4 Groups of mice (n=7) were fed HFD or ND for 16 weeks. **(A)** Body weights (p=0.007) and **(B)**  
5 epididymal fat pad weights (p=0.004) were significantly increased by HFD. **(C)** Circulating  
6 LDL/VLDL levels were also significantly increased (p=0.005). **(D)** Mouse HSP60 levels  
7 measured in serum samples by ELISA were significantly higher in HFD compared to ND fed  
8 mice (p=0.039; n=19 each). **(E)** T cell proliferation (expressed relative to the medium alone  
9 negative control) increased significantly in HFD compared to ND splenocytes when stimulated  
10 by 0.1 µg/ml (p=0.013) or 10 µg/ml (p=0.038) of recombinant mouse HSP60. **(F)** Both anti-  
11 HSP60 IgG1 and IgG2c circulating antibody levels were significantly elevated by HFD  
12 (p<sub>max</sub>=0.008 for IgG1; p<sub>max</sub>=0.00009 for IgG2c).

13

### 14 **Figure 2. Effect of HSP60 peptide treatment on obesity, metabolic dysfunction and auto-** 15 **immunity to HSP60**

16 **(A)** Mice (n=14) given HFD or HFD with HSP60 peptides increased weight to the same extent  
17 relative to ND (p=0.000002 for HFD, p=0.00045 for HSP60) **(B)** Epididymal fat pad weights  
18 were similarly doubled by HFD or HFD with HSP60 peptides (p=0.0012 for HFD, p=0.00009  
19 for HSP60) **(C)** HFD increased LDL/VLDL (p=0.0003) and HDL levels (p=0.00045) and the  
20 HSP60 treatment significantly normalised LDL/VLDL levels (p=0.0088) but not HDL levels.  
21 **(D)** HFD significantly impaired glucose tolerance (p=3.4x10<sup>-18</sup>, 2-Way ANOVA) and the  
22 impairment was partially but significantly reversed by HSP60 peptide treatment (p=0.0079).  
23 In a subgroup (n=7 each): **(E)** No differences were found in insulin tolerance between the three  
24 groups. **(F)** From flow cytometry, epididymal stromal vascular fraction CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>  
25 macrophages were increased (p=0.013) and **(G)** CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T

1 cells were decreased significantly ( $p=0.003$ ) with HFD or HFD plus HSP60 peptides. **(H)** There  
2 was an increase in the level of anti-HSP60 IgG1 antibodies observed in both HFD groups, but  
3 only the increase in the peptide treatment group was significant ( $p=0.022$ ) **(I)** Anti-HSP60  
4 IgG2c antibodies were increased in HFD mice, but this was significant only in the HFD alone  
5 group ( $p=0.048$  for 1/16 dilution).

6

7