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Role of Leptin in Immune Dysfunction in WNIN Obese Rats

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Abstract

Aim: Role of leptin in bringing about systemic immune response under obesity has so far been speculative. In the present study we addressed this question using genetically obese rats developed at our centre- WNIN/Ob and WNIN/GR-Ob.

Methodology: Both the lean (+/+) and homozygous obese animals (-/-) received either phosphate buffered saline or leptin. While another set of fed lean and obese served as controls. Effect of leptin on immune function was assessed in terms of splenic lymphocyte proliferative response to Concavalin A (Con A), splenic CD4/CD8 ratio and Nitric oxide (NO) production by macrophages. Further, we also studied if the effect of leptin on immune function was associated with changes in receptor OBR (Leptin receptor) and or JAK2 (Janus tyrosine kinase 2) total protein expression.

Results: Lean animals of both the strains (WNIN/GR-Ob & WNIN/Ob lean) responded to leptin treatment, in terms of increased CD4/CD8 ratio and lipopolysaccharide stimulated peritoneal macrophage Nitric Oxide (NO) production ($P<0.05$), while splenic lymphocyte proliferative response ($P<0.05$) to Concavalin A upon leptin treatment was observed only in WNIN/GR-Ob lean animals. Furthermore, significant increase in obese receptor (OBR) protein expression and a trend for JAK2 protein expression ($P=0.06$) upon leptin treatment were seen in WNIN/GR-Ob and WNIN/Ob lean animals respectively.

Conclusion: The data thus show that leptin resistance could be one of the factors associated with immune dysfunction in obese condition.

Keywords: Leptin resistance; WNIN/Ob; WNIN/GR-Ob; Leptin treatment

Introduction

Immune dysfunction leading to increased susceptibility to infectious diseases is often seen in both obese animal and human subjects [1]. However, no specific factor/mechanism has been attributed to this, though a correlation between reduction in total body fat and impaired immunity was established in a wide range of species including humans, suggesting the role for adipose tissue in altered immune function [2,3]. One such potential endocrine mediator is leptin. Leptin, a cytokine like molecule, binds to the long form of leptin receptor (ObRb) and mediates its function through Janus Kinase 2-Signal transducers and activators of transcription 3 (JAK2-STAT3) [4]. Leptin activates proliferation, upregulates phagocytosis and enhances the secretion of proinflammatory cytokines such as TNF-α, IL-6 and IL-12 by macrophages [5].

Starvation and malnutrition, both of which are characterized by low leptin levels are associated with alteration in immune responses and thymic atrophy and these were reversed by leptin administration [6,7]. Interventional studies involving leptin administration to obese subjects with leptin sufficiency or excess (obesity) showed no direct link between leptin and the proinflammatory state associated with obesity, suggesting that there is a threshold level for leptin to perform its function and above which it leads to leptin resistance [8]. Consistent with this, serum leptin levels in overweight or obese postmenopausal women showed no association with immune parameters suggesting leptin resistance [9].

At our institute, mutant obese rat strain (WNIN/Ob) was identified from the existing inbred Wistar strain of rats (WNIN) in 1994 and it exists in three phenotypes: homozygous lean (+/+), heterozygous carrier (+/-) and homozygous obese (-/-). These infertile homozygous obese animals are maintained through mating of fertile heterozygous carriers (+/-).These obese animals have 47 per cent of fat in the body and are hyperphagic, euglycemic, hyperinsulinaemic, hypertriglyceridaemic and leptin resistant. An accidental mix up of WNIN/Ob rats with WNIN/GR rats [a lean sub strain of WNIN rats showing impaired glucose tolerance (IGT) established at our facility in 1987 led to WNIN/GR-Ob strain which is also maintained by mating heterozygous carrier (+/-) rats. Both these strains have an average life span of about 1½ year and after one year of life they develop opportunistic infections, tumors, kidney abnormalities, and quite a few of them develop cataract and retinal degeneration. Obesity is inherited as autosomal incomplete dominant trait in this strain [10-12]. Stock of WNIN/ Ob male and female animals is infertile. However, infertility was reversed in only male animals by food restriction, testosterone administration or by adrenalectomy [13]. Female obese rats have anatomical deficiencies like low ovary weight with polycystic condition and thread like oviducts. The animals don't cycle normally and have either prolonged oestrous/dioestrous cycle. The estrogen peak which proceeds prior to ovulation is totally missing in these animals and their prolactin levels are high. Thus estrogen does not have any affect on the leptin sensitivity.

In our earlier studies on immune status, we showed altered basal immunity and reduced immune response to Hepatitis B vaccine in these mutant models (WNIN/Ob; WNIN/GR-Ob) [14,15]. In the current study the effects of leptin on immune function was analyzed

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after starvation and then by injecting leptin. Earlier studies showed that starvation induces a significant reduction in serum leptin concentrations and impaired immune functions [16]. Effect of leptin on immune function was assessed in terms of splenic lymphocyte proliferative response to Concavalin A (Con A), splenic CD4/CD8 ratio and Nitric oxide (NO) production by macrophages was studied. Further, we also studied whether the effect of leptin on immune function is associated with changes in receptor OBR (Leptin receptor) and or JAK2 (Janus tyrosine kinase 2) total protein expression in these obese models.

Materials and Methods

Animals and experimental design

Twenty four (n=24) three months old obese and lean female animals of WNIN/Ob and WNIN/GR-Ob strains were obtained from National Centre for Laboratory Animal Sciences (NCLAS). Female animals were taken for the study as our earlier studies in these obese animals showed no difference in immune dysfunction between male and female animals. Further, female obese rats are not used for breeding and as we require large number of animals for the experiment, we took female animals only. Animals were housed individually with proper temperature (22 ± 2°C), humidity (50-55%) and light control (12 hr light and 12 hr darkness) in the animal facility and were provided with *ad libitum* rat chow prepared in house and water. The study design was approved by the Institutional Animal Ethical Committee (IAEC).

WNIN/Ob and WNIN/GR-Ob obese and lean animals were divided into three groups [16]. Group I-Obese/Lean Starvation with leptin: Obese and lean animals in this group were deprived of food for 48 h as it affects the immune function. Further, as per our earlier observations 48h of fasting is required to get standard basal values. Starved animals received i.p. injections of leptin (1 µg/g initial body weight of recombinant rat leptin procured from National Hormone Peptide Program (NHPP, USA)) in Phosphate Buffered Saline (PBS) at 0900 and 1800 h for a period of two days. Before the animals were sacrificed, leptin was injected to study the phosphorylated forms of leptin receptor and its signaling molecules.

Group II-Obese/Lean Starvation with PBS: Animals in this group were deprived of food for 48 h and received i.p. injections of Phosphate Buffered Saline (PBS) at 0900 and 1800h for a period of two days.

Group III-Obese/Lean Free fed: Animals were allowed *ad libitum* access to standard diet.

Blood was collected from the retro-orbital sinus vein and the animals were sacrificed 15h after the last injection so as to complete the 48h of fasting. The following parameters were carried out as described below.

Immune parameters

Body weights: Body weights of the animals were taken before and after starvation and leptin treatment period.

Splenic lymphocyte proliferation assay: Spleen was aseptically removed and lymphocyte proliferation assay was performed as described, previously [17]. Briefly, splenocytes were dissociated using a stainless steel screen and adjusted to 1×10^9 cells/L RPMI 1640 medium supplemented with 40,000 µg/L gentamycin and 5% FBS (Sigma, Missouri, USA). 200 µL of the cell suspension was added to each well in a 96 well polystyrene plate and incubated for 48 h at 37°C, 5% CO_2 in the absence or presence of 2.5 µg/mL of Con A (Himedia, Mumbai, India). After two days the cultures were pulsed with 0.5 µci of tritiated thymidine (specific activity 240 Bq/mmol; BRIT; Mumbai, India). Twenty-four hours later, the cells were harvested onto a glass fiber filter. Radioactivity was then measured using a liquid scintillation counter (Packard Tri-Carb Liquid Scintillation Counter, Minnesota, USA) after the filters had been kept overnight at room temperature. Each test was performed in triplicates. Splenic lymphocyte proliferative response was expressed in terms of CPM of Con A stimulated (T)/CPM of unstimulated cells(C).

Lymphocyte subpopulation measurement: The splenic lymphocyte subpopulation was measured by immunofluorescent antibody staining procedure using flow cytometry [18] (Partec PAS; Germany). Briefly, an aliquot (1–2 millions) of freshly isolated cells was washed with FACS buffer (PBS with 5% FBS and 0.1% sodium azide) and was stained with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-rat CD4 (clone OX-35) and phycoerythrin (PE) anti-rat CD8a (clone OX-8) from BD Biosciences (California, USA). Cells were incubated with antibody for 30 min at 4°C and then washed three times with FACS buffer. Cells were analyzed with a flow cytometer. The samples were gated using forward vs. 90° light scatter to exclude granulocytes and monocytes from the splenocytes population. For each test sample, 20,000 cells were analyzed.

Isolation and culture of peritoneal macrophages: Peritoneal macrophages were obtained by washing the peritoneal cavity with 15 ml of RPMI 1640 medium [19]. Washed out medium was centrifuged (1200 g, 10 min, 4°C) and the pellet containing macrophages was resuspended in 1 mL of RPMI 1640 medium enriched with fetal bovine serum (5%) and gentamycin. Cell viability was determined by the trypan blue exclusion test and was >95%. Macrophage-rich cultures were obtained after 2 h incubation (37°C, 5% CO_2) of 1×10^6 cells/mL in 24-well polystyrene culture plates. Non-adherent cells were removed by washing the plate twice with RPMI 1640. The resulting adherent population consisted of >95% peritoneal macrophages. The peritoneal macrophages were cultured with and without lipopolysaccharide (LPS-1 µg/mL) and incubated for 48 h. The culture supernatants were then taken for the estimation of NO and TNF-α release.

Nitric oxide and TNF-α production by peritoneal macrophages: Nitrite (NO_2^-) which is the stable end product of NO was measured by a colorimetric assay using Griess reagent. Nitrite concentration was calculated from NaNO2 standard curve [20]. The culture supernatant was collected and stored at -80°C until further analysis of TNF-α by ELISA (R&D systems, Minneapolis, USA).

Preparation of total protein lysate: The frozen splenic tissues were homogenized in 500 µL of ice-cold lysis buffer (100 mM HEPES (pH 7.5), 0.1 mM EDTA, 5 mM sodium fluoride, 0.05 M β-glycerophosphate disodium salt hydrate, 0.05 M sodium orthovanadate, 10 µM sodium molybdate and 2 µl of protease inhibitor cocktail (Sigma P 8430, USA)) using the motor-driven Polytron which was operated at maximum speed for 30 seconds. Centrifuge the homogenates for 5 minutes at 200 x g (1000-1200 rpm). Incubate the samples for 15 minutes on ice. After adding 10% of Nonidet P-40, the homogenates were vortexed and centrifuged to collect the supernatant. The protein samples were aliquoted and stored at -80°C [21]. The protein concentrations of the samples were determined by the bicinchoninic acid assay.

Western blotting: Earlier studies have reported that pOBR & pJAK2 proteins are equally indicative of leptin sensitivity [11,22,10]. Therefore, to study whether the effect of leptin on immune function is associated with changes in protein expression associated with leptin signaling, leptin receptor OBR (Leptin receptor) and or JAK2 (Janus tyrosine kinase 2) total protein expression by western blot were analyzed in these obese models.

200 µg of Protein samples with sodium dodecy l sulfate-polyacrylamide gel electrophoresis sample buffer were boiled for 5 minutes. Proteins were separated by Tris Hcl sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Nitrocellulose membranes (Millipore, St Louis, USA) with a Trans-Blot transfer cell (Bio-Rad Laboratories, Richmond, CA). Membranes were blocked for 1 hour with 1% bovine serum albumin in TBS-T (50 mM Tris, pH 8.0, and 80 mM NaCl, 5 mM KCl with 0.05% Tween 20), and washed four times with TBS-T. All the antibodies except for JAK2 were procured from Santa Cruz Biotechnology, Inc., USA. Further, the membranes were incubated with ObR (Cat No. sc-8325), p-ObR (Cat No. sc-16421), p-JAK2 (Cat No. sc-16566-R) and JAK2 (Cat No. 3230, Cell signaling technologies, USA.) antibodies respectively in 1% bovine serum albumin in TBS-T for 2 Hours and washed three times for 15 minutes with TBS-T. Blots were then incubated with secondary antibody for 1 hour in 1% BSA in TBS-T and again washed three times for 20 minutes each time in TBS-T. Immunoreactivity was visualized by chemiluminescence detection (BioRad) and quantified by densitometry (BioRad).

Results

Data are shown as the Mean ± S.E. Statistical comparisons on the three groups in each phenotype (obese and lean) were made by one way ANOVA including all possible two-way interactions, and were compared with the others by Fisher's protected least significant difference test at P=.05. For data which either didn't approximate to a binomial distribution or the sample size was too small to determine distribution, a Kruskal-Wallis test with Dunn's Multiple Comparison Test was performed. The data for starvation plus PBS, leptin or free feeding in the lean group were compared with those in the obese group using unpaired Student's t-test [16]. Statistical analysis was conducted using SPSS 11.0 software statistical package.

Body weight

Obese mutant rats differ in their litter size (6-12) compared to their lean counterparts. So, it is difficult to get obese rats of standard weights as the normal rats. Therefore, the change in body weight is expressed as %, considering the initial body weight as 100%. Body weights were reduced upon starvation in both obese and lean groups (P=.05), but body weight did not fall further after leptin treatment (Tables 1 and 2).

Serum leptin levels

Leptin concentrations in sera were higher in both WNIN/Ob and WNIN/GR-Ob obese groups than in lean groups. In WNIN/GR-Ob lean, serum leptin concentrations were significantly reduced upon starvation (*P*=.05) and were not increased further upon leptin treatment (free feeding 1.14 ± 0.25, starvation-PBS 0.15 ± 0.006, starvation-leptin 0.17 ± 0.008). On the other hand in obese animals though serum leptin levels were unaltered by starvation, it was significantly reduced upon leptin treatment free feeding 37.2 ± 0.96, starvation-PBS 35.2 ± 1.1, starvation-leptin 25.0 ± 2.04) (*P*=.05) (Figure 1).

In WNIN/Ob, serum leptin levels were significantly reduced in both obese (free feeding 27 ± 1.73, starvation-PBS 21 ± 1.37, starvation-leptin 24.6 ± 2.90) and lean (free feeding 0.29±0.08, starvation-PBS 0.12 ± 0.00, starvation-leptin 0.17 ± 0.003) animals upon starvation (*P*=.05) and were not significantly increased upon leptin treatment (Figure 2).

CD4/CD8 Ratio

In WNIN/GR-Ob, starvation significantly reduced the CD4/CD8 ratio in both obese and lean animals (obese group: free feeding 1.66 ± 0.09, starvation-PBS 1.45 ± 0.03; Lean group: free feeding 2.01±0.01, starvation-PBS 1.74 ± 0.01) (*P*=.05). However, leptin treatment increased CD4/CD8 ratio in lean animals only (obese group: starvation-leptin 1.6±0.06; Lean group: starvation-leptin 2.07 ± 0.07) (*P*=.05) (Figure 3).

In WNIN/Ob lean animals, starvation caused a significant reduction in CD4/CD8 ratio which was reversed to free fed group upon leptin treatment (free feeding 2.08 ± 0.06, starvation-PBS 1.83 ± 0.04, starvation-leptin 2.13 ± 0.06) (*P*=.05). On the other hand neither starvation nor leptin treatment had any effect on the CD4/CD8 ratio

	Body weight (g)		
Groups	**Before starvation**	**After starvation**	**change in body weight (g)**
Obese			
Starvation-leptin	453.5 ± 7.6[a]	427.0 ± 6.5[b]	-26.5 ± 3.5*
Starvation-PBS	459 ± 8.9[a]	437.7 ± 6.2[b]	-21.3 ± 6.1*
Free feeding	463.7 ± 4.0[a]	466 ± 2.7a	2.3 ± 0.0
Lean			
Starvation-leptin	249.5 ± 4.4[a]	232.7 ± 8.6[b]	-16.8 ± 2.5*
Starvation-PBS	250.0 ± 8.3[a]	233.6 ± 4.6[b]	-16.4 ± 3.1*
Free feeding	247.5 ± 2.2[a]	249.7 ± 3.9[a]	2.2 ± 0.5

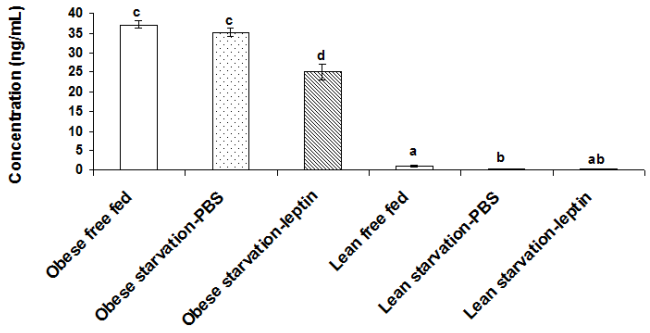
Values are mean ± S.E; n=8. a, b indicate changes in the body weight before and after starvation (by students T-test). Means that do not share common letter is significantly different at 0.05 level respectively. * indicates comparison between starved PBS or leptin treated vs. free fed in obese or lean groups. **P*=.05 by one-way ANOVA

Table 1: Changes in body weight with starvation in WNIN/GR-Ob.

	Body weight (g)		
Groups	**Before starvation**	**After starvation**	**change in body weight (g)**
Obese			
Starvation-leptin	436.4 ± 8.7[a]	414.0 ± 15.4[b]	-22.4 ± 4.63*
Starvation-PBS	433.3 ± 8.0[a]	410.4 ± 12.1[b]	-22.9 ± 3.54*
Free feeding	441.8 ± 16.8[a]	442.5 ± 19.5[a]	0.7 ± 0.0
Lean			
Starvation-leptin	233.8 ± 7.4[a]	216.8 ± 4.8[b]	-17.0 ± 3.45*
Starvation-PBS	233.8 ± 5.2[a]	217.3 ± 4.8[b]	-16.5 ± 1.58*
Free feeding	224.0 ± 6.6[a]	224.5 ± 6.9[a]	0.5 ± 0.0

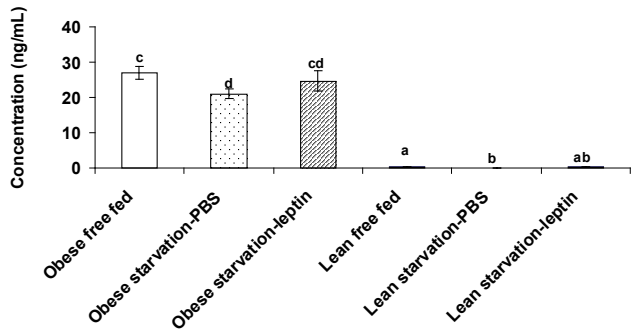
Values are mean ± S.E; n=8. a, b indicate changes in the body weight before and after starvation (by students T-test). Means that do not share common letter is significantly different at 0.05 level respectively. * indicates comparison between starved PBS or leptin treated vs. free fed in obese or lean groups. **P*=.05 by one-way ANOVA

Table 2: Changes in body weight with starvation in WNIN/Ob.



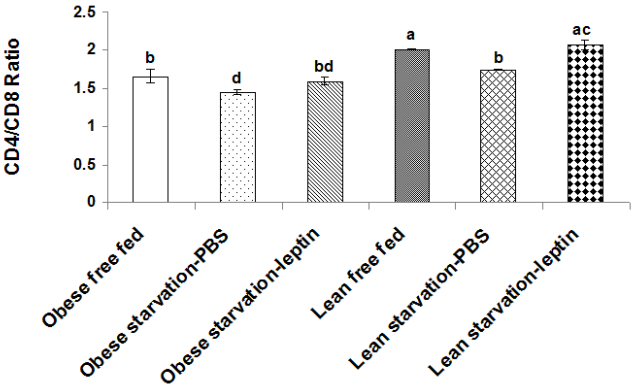
Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P*=.05 level respectively (by one-way ANOVA)

Figure 1: Serum Leptin Levels in WNIN/GR-Ob.



Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P*=.05 level respectively (by one-way ANOVA)

Figure 2: Serum Leptin Levels in WNIN/Ob.



Values are Mean ± S.E.M; n= 8. Means that do not share common letter is significantly different at *P*=.05 level respectively (by one-way ANOVA)

Figure 3: CD4/CD8 ratio in WNIN/GR-Ob.

in obese animals (free feeding 1.43 ± 0.2, starvation-PBS 1.36 ± 0.1, starvation-leptin 1.46 ± 0.06) (Figure 4).

Splenic lymphocyte proliferative response to concavalin A

In WNIN/GR-Ob, the lymphocyte proliferative response was unaltered in both obese and lean control animals upon starvation (obese group: free feeding 9.96 ± 2.43, starvation-PBS 6.09 ± 1.59; Lean group: free feeding 6.3 ± 0.071, starvation-PBS 3.37 ± 0.38). However, it was significantly increased and reduced upon leptin treatment in lean and obese animals respectively (Lean group: starvation-leptin 11.4 ± 0.44; obese group: starvation-leptin 4.7 ± 1.09) (Figure 5).

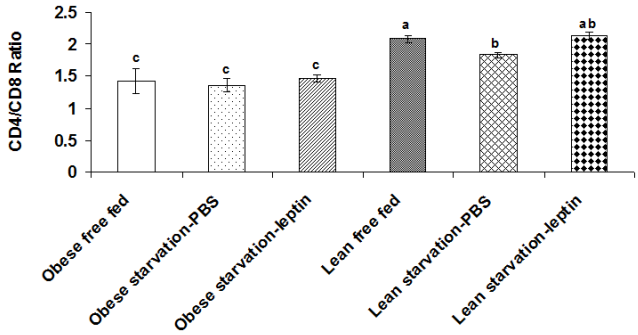
In both WNIN/Ob lean (free feeding 9.62 ± 0.981, starvation-PBS 7.9 ± 1.73, starvation-leptin 9.9 ± 2.31) and obese groups (free feeding 15.7 ± 4.8, starvation-PBS 10.73 ± 1.83, starvation-leptin 10.6 ± 1.44), neither starvation nor leptin treatment altered the lymphocyte proliferative response to Con A (Figure 6).

LPS stimulated peritoneal macrophage NO production

In WNIN/GR-Ob, though starvation did not alter NO production, leptin did increase NO production in both lean (free feeding 27 ± 1.73, starvation-PBS 21 ± 1.37, starvation-leptin 24.6 ± 2.90) and obese animals (free feeding 27 ± 1.73, starvation-PBS 21 ± 1.37, starvation-leptin 24.6 ± 2.90) (Figure 7). Similar observations were found in WNIN/Ob strain (Lean group: free feeding 9.1 ± 1.7, starvation-PBS 6.6 ± 1.1, starvation-leptin 11 ± 0.88; obese group: free feeding 9.0 ± 1.9, starvation-PBS 9.9 ± 1.48, starvation-leptin 9.5 ± 0.45) (Figure 8).

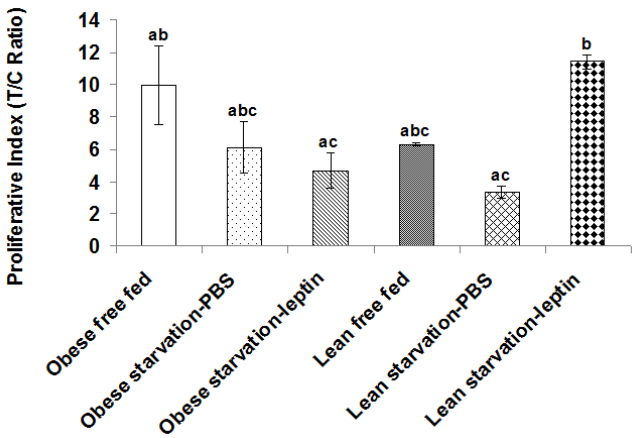
Immunoblotting

Leptin-induced tyrosine phosphorylation of OBR and JAK2 in



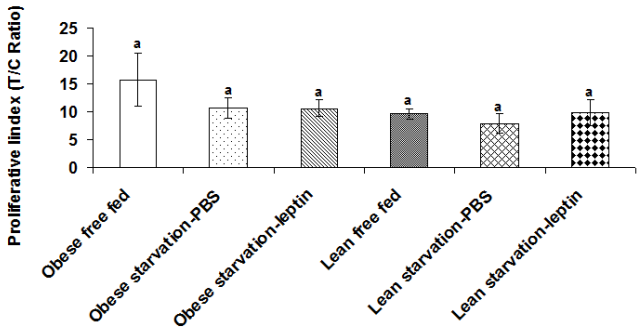
Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P*=.05 level respectively (by one-way ANOVA)

Figure 4: CD4/CD8 ratio in WNIN/Ob.



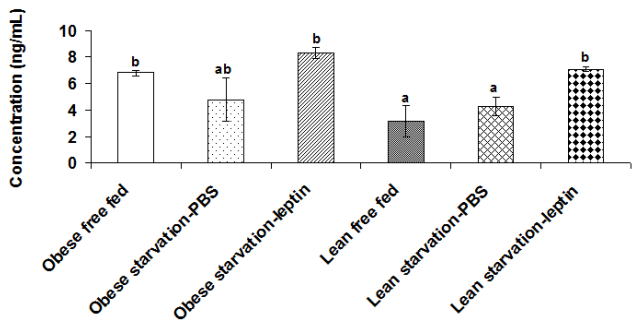
Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P*=.05 level respectively (by one-way ANOVA)

Figure 5: Splenic lymphocyte proliferative response to Con A in WNIN/GR-Ob.



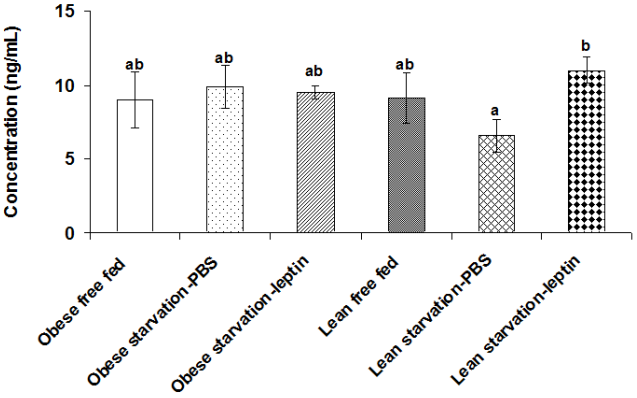
Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P=.05* level respectively (by one-way ANOVA)

Figure 6: Splenic lymphocyte proliferative response to Con A in WNIN/Ob.



Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P=.05* level respectively (by one-way ANOVA)

Figure 7: LPS stimulated peritoneal macrophage NO production in WNIN/GR-Ob.



Values are Mean ± S.E.M; n=8. Means that do not share common letter is significantly different at *P=.05* level respectively (by one-way ANOVA)

Figure 8: LPS stimulated peritoneal macrophage NO production in WNIN/Ob.

splenic cells was not detected in both WNIN/Ob and WNIN/GR-Ob animals which may be due to insufficient sensitivity in our assays. In WNIN/GR-Ob obese animals, starvation caused a significant increase (*P*=.05) in total OBR expression which tended to be reduced by leptin treatment (*P*=.06). However, in lean animals though OBR expression was unaltered upon starvation, it was upregulated by leptin treatment (*P*=.05) (Figure 9). Further, there was no significant difference in the JAK2 protein expression in obese and lean animals in free fed state. Neither starvation nor leptin treatment had any effect on JAK2 protein expression in obese animals. However, though not significant, leptin treatment led to increased JAK2 protein expression in lean animals (*P*=.03) (Figure 10).

In WNIN/Ob, OBR and JAK2 protein expression were comparable between obese and lean free fed animals. However, upon starvation in lean animals, though OBR expression was significantly reduced, JAK2 protein was unaltered. Further, in lean animals, though the OBR expression was unaltered, JAK2 protein expression tended to be higher (*P*=.06) upon leptin treatment (Figures 11 and 12). In obese animals, neither starvation nor leptin treatment had any effect on OBR & JAK2 protein expression.

Discussion

Our earlier reports in WNIN/Ob and WNIN/GR-Ob obese animals

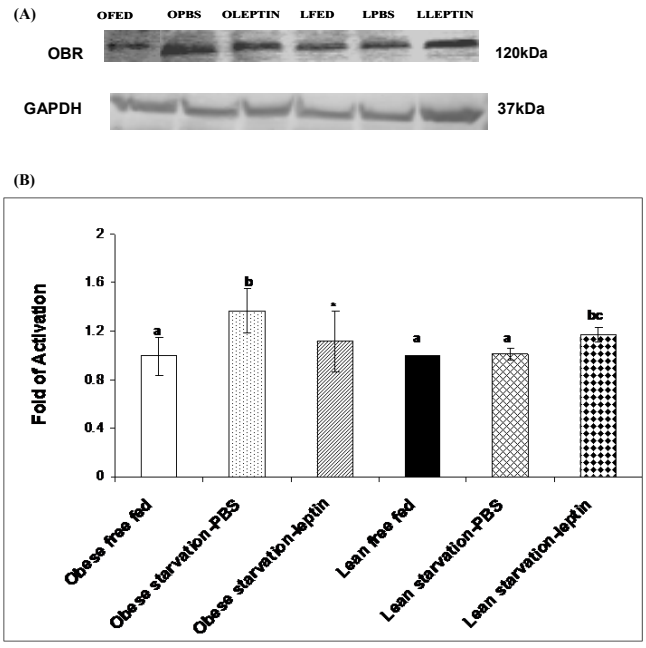


Figure 9: OBR protein expression in WNIN/GR-Ob. A. Western blot of OBR protein in Obese free fed (Ofed), Obese starvation-PBS (OPBS), Obese starvation-leptin (OLeptin), Lean free fed (Lfed), Lean starvation-PBS (LPBS) and Lean starvation-Leptin (LLeptin). B. Quantification of OBR protein expression by densitometry. Means that don't share common letter is significantly different at 0.05 levels respectively. *P=.06 between Obese starvation-leptin and obese starvation-PBS.

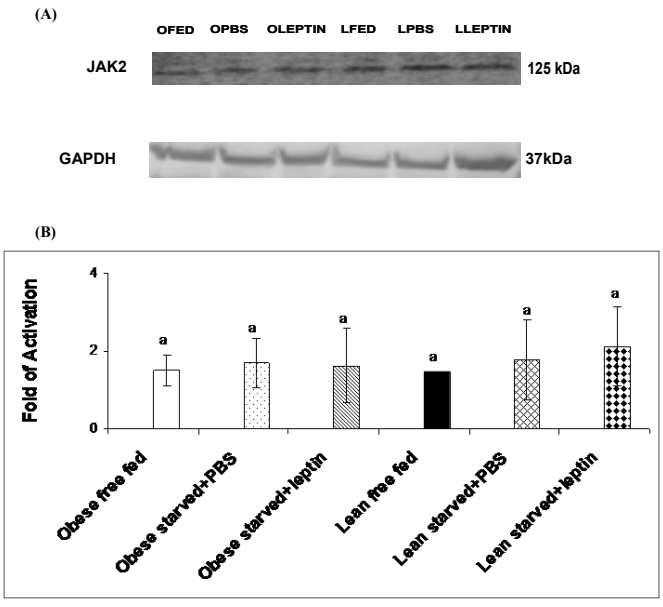


Figure 10: JAK2 protein expression in WNIN/GR-Ob. A. Western blot of OBR protein in Obese free fed (Ofed), Obese starvation-PBS (OPBS), Obese starvation-leptin (OLeptin), Lean free fed (Lfed), Lean starvation-PBS (LPBS) and Lean starvation-Leptin (LLeptin). B. Quantification of OBR protein expression by densitometry. Means that don't share common letter is significantly different at 0.05 level respectively (by one-way ANOVA).

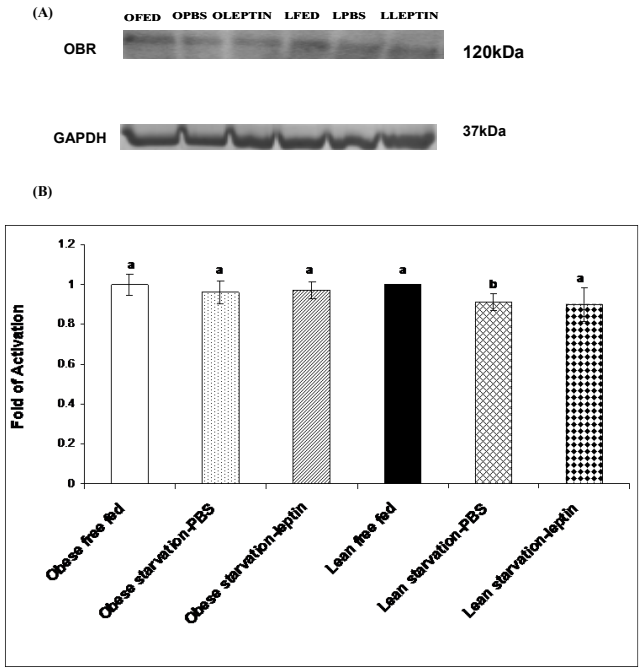


Figure 11: OBR protein expression in WNIN/Ob. A. Western blot of OBR protein in Obese free fed (Ofed), Obese starvation-PBS (OPBS), Obese starvation-leptin (OLeptin), Lean free fed (Lfed), Lean starvation-PBS (LPBS) and Lean starvation-Leptin (LLeptin). B. Quantification of OBR protein expression by densitometry. Means that don't share common letter is significantly different at 0.05 level respectively (by one-way ANOVA).

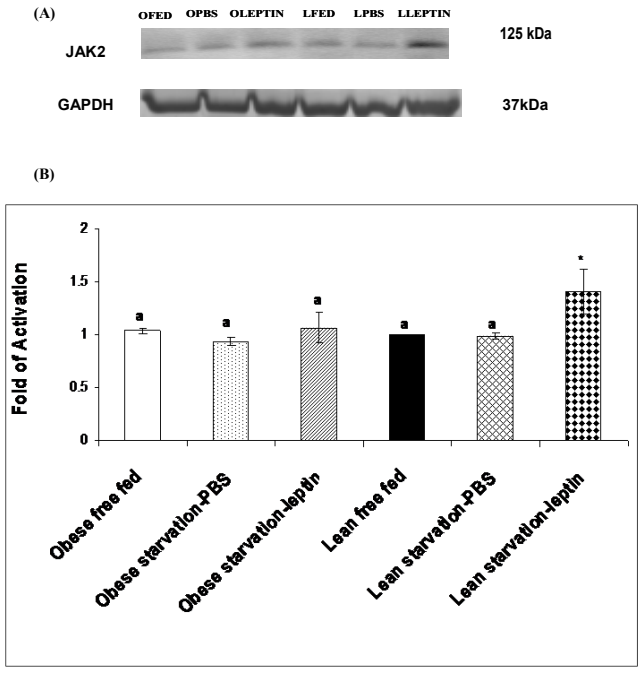


Figure 12: JAK2 protein expression in WNIN/Ob. A. Western blot of JAK2 protein in Obese free fed (Ofed), Obese starvation-PBS (OPBS), Obese starvation-leptin (OLeptin), Lean free fed (Lfed), Lean starvation-PBS (LPBS) and Lean starvation-Leptin (LLeptin). B. Quantification of OBR protein expression by densitometry. Means that don't share common letter is significantly different at 0.05 level respectively (by one-way ANOVA). *P=.06 between Lean starvation-leptin and Lean starvation-PBS.

demonstrated altered basal immunity and impaired immune response to Hepatitis B vaccine in these obese animals [14,15]. However, we could not speculate at that time the factors responsible for this altered immunity. The present study was carried out to precisely point out the reason(s) for such an altered immunity and the evidence presented here points out leptin resistance (despite high serum leptin levels) as the major cause for such a condition.

In euglyucemic WNIN/Ob strain, the body weight and serum leptin concentrations of free fed obese animals were higher compared to lean counterparts. Starvation caused a reduction in body weight and serum leptin levels in both the obese and lean animals which are in line with the observations found in diet induced obese mice (DIO) [16]. Further, similar to the studies in DIO mice [16], leptin treatment did not alter serum leptin levels significantly in both obese and lean animal which could be attributed to the very rapid metabolic rate of leptin [23,24].

In addition, to the effect of leptin on body weight and serum leptin levels, we have observed changes in immune function. However, till date several studies have discussed on how leptin may be required for or may enhance the immune function, very few have considered the fact that obese individuals are hyperleptinaemic and this excess leptin would have potential impact on the immune cells. Consistent with this hypothesis, we observed that the splenic CD4/CD8 ratio was upregulated in lean animals upon leptin treatment. On the other hand, obese animals did not respond to leptin treatment which is in contrast to the findings observed in leptin deficient Ob/Ob obese mice and genetically obese human subjects wherein leptin administration improved lymphocyte numbers, which has been suggested to be due to inhibition of stress induced apoptosis [7,25,26].

Furthermore, we observed that leptin administration did increase LPS-induced NO production in lean animals, whereas the obese animals did not respond to it. Earlier, it has been reported that leptin increases IFN-γ-induced NO and prostaglandin E_2 (PGE_2) production by increasing the expression of related inducible enzymes in murine macrophage J774A.1 cell line, where functional leptin receptor (Ob-Rb) were expressed [27]. However, further studies are needed to elucidate whether the mechanisms behind LPS-induced NO production by leptin is same as the IFN-γ-induced NO and prostaglandin E_2 (PGE_2) production, as the presence of leptin receptor has also been evidenced in peritoneal macrophages [28].

Thus, unlike genetically obese Ob/Ob mice which were responsive to leptin treatment, WNIN/Ob obese animals did not respond to leptin treatment suggesting a defect in leptin receptor or leptin signaling. In the current study leptin receptor (OBR) expression was unaltered; but there was a trend towards increased total JAK2 protein expression upon leptin administration, which was observed only in lean animals. In contrast to our observation, in Diet Induced Obese (DIO) rats the p-JAK2 protein expression was found to be reduced [29].

In impaired glucose tolerant WNIN/GR-Ob animals, starvation induced a significant reduction in body weight and serum leptin levels which is similar to that seen in WNIN/Ob lean animals. On the other hand, in WNIN/GR-Ob obese animals though starvation caused a significant reduction in the body weight, serum leptin levels were unchanged. Further, though the splenic CD4/CD8 ratio was reduced upon starvation in both WNIN/GR-Ob obese and lean animals, it was increased in lean animals upon leptin treatment. Similar observations were found in WNIN/Ob animals too. Additionally, in line with WNIN/Ob, starvation did not alter the NO production in both obese and lean animals, however leptin treatment did increase the NO production in lean animals.

Though starvation did not alter the lymphocyte proliferative response in both WNIN/GR-Ob obese and lean animals, leptin treatment had differential effect on proliferative response with preferential upregulation in lean animals and down regulation in obese animals respectively. In line with our observations, long-term leptin exposure to NK cells *in vitro*, has been shown to inhibit NK cell immune functions such as cytotoxic lysis of tumor cells, IFN-γ-secretion, and cell proliferation, which could in part be explained by peripheral leptin resistance [30]. Upon starvation, obese animals showed a significant increase in leptin receptor expression with no change in serum leptin levels. However, leptin treatment led to a reduction in both leptin receptor expression and serum leptin levels suggesting ligand induced leptin receptor down regulation which may be due to preferential trafficking of leptin receptor to lysosomes. In line with our observations, chronic administration of either low or high dose leptin for a period of 28 days in rats was shown to lead to leptin resistance by down regulation of OBR mRNA and protein in the hypothalamus [31]. Further, it was reported that following meal ingestion in subjects with already high fasting leptin levels, there was no change in leptin levels suggesting down regulation of leptin receptors [32]. Though, there was no significant change in total JAK2 protein expression in both obese and lean animals in both free fed state and upon leptin administration, coexpression studies are required, to elucidate the role of JAK2 in ligand induced ObR internalization. Further, the factors which aid in rapid internalization needs to be elucidated.

The downregulation of OBR expression observed in WNIN/GR-Ob obese animals upon leptin treatment was further evidenced by reduced lymphocyte proliferative response to Con A. In contrast to obese animals, leptin treatment increased OBR expression as well as CD4/CD8 ratio, lymphocyte proliferative response to Con A and NO production in lean animals.

The differences in response observed in both WNIN/Ob and WNIN/GR-Ob obese and lean animals to starvation and leptin treatment could be attributed to the genetic background of the respective strains. Further, the affect of starvation was less in these obese animals compared to the normal rats which could be attributed to the low reduction in initial body weights compared to normal rats [33]. It was also observed that WNIN/GR-Ob were more prone to changes in immune function upon starvation than WNIN/Ob. However at phenotypic level, both these strains differ only in their glycemic status, with WNIN/Ob being euglycemic and WNIN/GR-Ob exhibiting impaired glucose tolerance [10]. In our earlier study, we emphasized, impaired glucose tolerance (IGT) as an additional risk factor to optimal immune function in obesity. Further, a recent study stated IGT as an independent risk factor for postload leptin changes, which lead us to speculate IGT to be either a cause or consequence of leptin resistance [34]. However, the mechanism needs to be fully elucidated.

Conclusion

The present study corroborates and establishes the immunomodulatory role of leptin. However, the non responsiveness of obese animals to leptin suggests leptin resistance as one of the causal factors to be involved in obesity associated immune dysfunction. Further, we attribute the presence of leptin above a threshold level to leptin resistance arising out of JAK2 and OBR total expression in WNIN/Ob and WNIN/GR-Ob obese animals respectively.

It looks as if one needs to sensitize the leptin receptor or its signaling molecule to increase the uptake of leptin, and that way to improve the immune function under obesity condition. Amylin, a 37 peptide hormone that is co-secreted with insulin with pancreatic B-cells, in conjugation with leptin was shown to increase leptin receptor expression and also to activate STAT3 expression, probably through JAK2 activation [35]. It was also seen that caloric restriction for a period of 30 days could also induce leptin responsiveness in leptin resistant DIO animals by way increased leptin receptor expression and receptor [36]. The WNIN/Ob animals on food restriction were shown to have reduced physical (increased body weight, hypoactivity, hyperphagia) and biochemical indices (insulin and leptin resistance, hypertriglyceridemia, hypercholesterolemia) of obesity and even reversal of male infertility (unpublished data). Studies will be initiated soon; to see whether immune function improves in these animals, either by chemical sensitization or by food restriction. Positional cloning studies which were going in our lab to map the locus of mutation in these animals, point out to a defective function of OBR, where in an upstream SNP alteration at the exon region was seen in homozygous phenoypes(-/-). The observation is under validation by probe analysis in all the three phenotypes (lean (+/+), carrier (+/-) and obese (-/-) of both the strains.

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Authors' Contributions

'Prathibha Bandaru' designed the study, managed the analyses of the study, managed the literature searches, performed the statistical analysis, wrote the first draft of the manuscript and was involved in finalizing the manuscript. 'Hemalatha Rajkumar' designed the study and was involved in finalizing the manuscript. 'Venkata Prasad Upadrasta' managed the analyses of the study and statistical analysis. 'Giridharan Nappanveettil' designed the study and was involved in the finalizing manuscript. All authors read and approved the final manuscript.

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