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Characterization of iNKT cells in obese and non-obese individuals in Riyadh, Saudi Arabia

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ABSTRACT

Obesity is related with variations in the functionality of immune cells such as macrophages and natural killer cells (NK), leading to high risk for infections and different forms of cancer. However, invariant natural killer T (iNKT) cells are a subset of innate-like T cells that are the first cells to respond during an infection and can contribute to tissue homeostasis, inflammation and also damage by unbalanced production of pro- and anti-inflammatory adipocytokines which could affect adipose tissue remodeling processes. This study aimed to examine the relationship between the level of circulating iNKT cells and the expression of killer-cell immunoglobulin-like receptors (KIRs) from both obese and non-obese individuals. The study was conducted in King Saud University, Riyadh, Saudi Arabia. iNKT cells and CD56+CD3+ T cells were studied in samples of peripheral blood. The study comprised of 2 groups: non-obese Saudi men and women with BMI < 30 and obese Saudi men and women with BMI > 30. Peripheral blood mononuclear cells were isolated and examined. Results show that the percentage of iNKT cells in peripheral blood in obese was lower compared with non-obese. We also determined the level and number of CD3+CD56+ and found that their percentage was higher in obese compared with non-obese. These results are of great interest to research, considering obesity as one of the main causes of chronic low-grade inflammation, insulin resistance and type 2 diabetes mellitus; obesity and its consequental disorders are common in our population and other populations worldwide.

KEYWORDS: obesity, KIRs, iNKT cells, Saudi Arabia.

1. INTRODUCTION

Obesity is defined by the World Health Organization (WHO) as an "abnormal or exaggerated fat accumulation that presents a risk to health". As a metabolic syndrome, obesity has become a major public health and psychosocial problem because it is assosciated with an increased risk of developing chronic diseases. Some studies have reported a strong relationship between obesity-related chronic diseases and cells of the immune system [1]. Several groups have studied the effect of obesity on different immune cells and their functions including those of both innate and adaptive immune systems.

Based on some systematic reviews, which have examined the prevalence of overweight and obesity, Saudi Arabia has become one of the fastest growing economies. In 2003, it was the 27th largest economy and in 2014, the 19th largest economy in the world. Increased income has also increased the consumption of fast food. Fast food sales in Saudi Arabia reached $4.5 billion in 2015 [2].

In Saudi Arabia 30% of children are either overweight or obese, and in the children between the ages of 5-18 years in Saudi Arabia, 23.1% are overweight, 9.3% are obese, and 2% are severely obese. In adults (between 30-60 years) the obesity rates are higher in women (75% to 88%) than in men (70% to 85%) [2]. In recent years, the percentage of obesity in Saudi Arabia increased about 16.4% in 1992, 34.9% in 1997, 37.8% in 2002, 41.6% in 2007 and 46.9% in 2012 [3]. The obesity rate is exponentially growing with an overall overweight/ obesity prevalence of 20% in 1996, to 35% in 2005, to 59.4% in 2013, up to 75% if populations of Arabian Gulf countries are collectively included, with an estimated 20,000 deaths per year due to obesity and related co-morbidities [4].

In Saudi Arabia, the Eastern region has been found to have the highest percentages (19% to 35.6%) of overweight and obesity in children [5, 2]. Increased rates of obesity in the whole region over the other parts of Saudi Arabia have been previously reported, with the prevalence of obesity ranging from 33.9% in Hail to 11.7% in Jizan [6]. Moreover the prevalence rates of obesity in Hail, Baqaa, Ash Shinan and Ghazala are 64%, 70%, 69%, 35% and 55%, respectively. These values are relatively higher than most reported prevalence rates worldwide [7].

In this study we focus on the characterization of invariant natural killer (iNKT) cells. The iNKT cells constitute a small population of immune cells that share functional and phenotypic characteristics of both T cells and natural killer cells [8, 9]. iNKT cells are typically thought to regulate human immune responses and to play a major role in both development and progression of autoimmune disease, cancer and infection. Acting as a bridge between the innate and adaptive immune systems, their activity can be classified as primarily innate rather than adaptive [10, 11]. They can have both protective and harmful roles in many pathological conditions. Actions include the production of different cytokines including those associated with Th1, Th2 or Th17 T-helper cell responses promoting and initiating these responses. iNKT cells can also demonstrate direct cytotoxic activity and may also enhance the induction and activation of other innate and adaptive immune cells that promote viral clearance [12, 13]. In bacterial infection, iNKT cell activation contributes to the innate control of *S. pneumoniae* as well as protective antibody responses [12]. In addition, iNKT cells are beneficial in the resolution of infection, by killing infected airway cells, and protect the lungs from inflammatory damage through IL-22 production [14, 12]. iNKT cells have also been reported to inhibit the action of immunosuppressive components such as myeloid-derived suppressor cells and tumor-associated macrophages [8]. Notably a reduction in iNKT cell number in peripheral blood has been correlated with several autoimmune or inflammatory conditions, obesity and cancers [12].

Although the main activity of iNKT cells is in protection and regulation of immune function, including inhibiting development of autoimmune disease, tumor surveillance and protection against infections, iNKT cells can play a role in disease pathology [15]. Both naturally occurring and experimentally induced variations in iNKT cell number and function have been associated with increased risk of bacterial infection, cancer and autoimmunity [16, 17]. In autoimmune diseases such as Multiple sclerosis (MS), several studies indicate the number of circulating iNKT cells in MS patients are decreased [9, 17]. In type 1 diabetes (T1D) iNKT cells play an important role in disease development through the production of subnormal levels of IL-4 [18]. The first data obtained in patients with T1D showed a decreased number of iNKT cells indicating that the frequency of iNKT cells might be extremely low in the peripheral blood of patients with T1D when compared to those of healthy control [19].

iNKT cells express a range of receptors from several different families, which regulate their activity; one key group is that of the killer cell immunoglobulin-like receptor (KIR) family. KIRs are members of the immunoglobulin superfamily of receptors and are encoded on chromosome 19q13.4, where the KIR gene cluster consists of up to 17 highly homologous and closely linked genes and pseudogenes. KIRs can be catagorized into two different groups according to their activity, an inhibitory group marked by a long (L) intracytoplasmic tail and two or three extracellular immunoglobulin domains. Secondly, an activating group which has two or three extracellular immunoglobulin domains and a short (S) intracytoplasmic tail. The KIR activating receptors function *via* the adapter molecule DAP12 whereas KIR inhibitory receptors inhibit NK cell

function *via* immunoreceptor tyrosine-based inhibitory motifs.3,4. HLA ligand specificities for only four of the inhibitory KIRs have been clearly defined: KIR2DL2 and KIR2DL3 for the HLA-CAsn80 (C1) group of alleles, KIR2DL1 for the HLA-CLys80 (C2) group and KIR3DL1 for the Bw4 group of HLA-B (and some A) alleles. Specificities of the remaining inhibitory and activatory receptors are less well defined. Two main groups of KIR haplotypes that differ in number and types of KIR genes have been defined in the human population. Group A haplotypes include several inhibitory KIRs that lack all activating KIR genes except KIR2DL4, whereas group B haplotypes encode several inhibitory and activating KIRs [20]. Haplotype AA homozygotes therefore lack most of the activating receptors while AB or BB (BX) subjects have both activating and inhibitory genes. Cells that express class I HLA molecules are protected from iNKT cell killing, whereas cells that down-regulate or lose class I HLA molecule expression are susceptible to iNKT cell killing. Thus, KIR inhibitory receptors block NK cell killing activity by binding class I HLA molecules and delivering inhibitory signals. However, lack of expression of class I HLA molecules leads to activation of iNKT cell killing activity through delivery of activating signals [21].

The role of iNKT cells in obesity has not been fully defined. The aim of this study is to investigate the relationship between the level of circulating iNKT cells and obesity, and to examine KIR expression on the surface of iNKT cells from both obese and non-obese adult individuals in Saudi Arabia.

2. MATERIALS AND METHODS

2.1. Sample collection

2.1.1. Obese and non-obese subjects

A total of 62 Saudi men and women subjects were enrolled in this study, and were categorized into obese and non-obese subjects; the eligibility criteria are based on: age, gender, height and weight. And all of the study samples were Saudi citizen who were recruited from the blood bank in King Khalid University Hospital, Riyadh, Saudi Arabia. The study was approved by the medical ethics committee of blood banks in King Khalid University and all participants were consulted and they expressed their consent in accordance to the study protocols approved by the ethical committee of King Saud University. The donors were classified into 2 groups: **Group 1**: non-obese, included 27 Saudi men and women with BMI < 30 (age 18-45 years). **Group 2**: obese, included 29 Saudi men and women with BMI > 30 (age 18-45 years). Blood samples, both patient and control, were collected by venipuncture into tubes containing EDTA.

2.2. Reagents

mAbs used in the study were: anti-human anti-iNKT-APC; anti-human CD3-PerCP; anti-human CD56-VioBright; anti-human CD158a-PE; anti-human CD158b-PE; anti-human CD158e-PE, mouse anti human-IgG1and IgG2a isotype controls (Miltenyi Biotec, Friedrich-Ebert-Straße 68, Germany) and HLA-C1/C2 primers.

2.3. PBMCs isolation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-paque density gradient centrifugation (GE Healthcare, product number, 17-1440-03). Briefly blood was gently layered onto an equal volume of Ficoll-Paque TM Plus and centrifuged at 400 g (1800 rpm) for 20 min at room temperature. PBMCs were then collected from the interface and washed twice with phosphate-buffered isotonic saline (PBS; Sigma, product number, p4417) and spun down at 400 g (1800 rpm) for 10 min.

2.4. Flow cytometry

The proportions of iNKT cells and KIR expression in the samples were measured using flow cytometry by 4-colour staining with commercial monoclonal antibodies: anti-iNKT-APC, CD3-PerCP and CD56-VioBright according to the manufacturer's recommendations. To examine the expression of KIR receptors on iNKT cells, cells were stained with 5 µl of mouse anti-human anti-iNKT-APC, 5 µl of mouse anti-human CD3-PerCP, 5 µl of mouse anti-human CD56-VioBright, 5 µl of mouse anti-human CD158a-PE, mouse anti-human CD158b-PE, and mouse anti-human CD158e-PE. The antibodies were incubated with the cells on ice and in the dark for 20 minutes. Then, the cells were washed twice with cold PBS to remove any unbound antibody and centrifuged for 10 min at 400 g at 4 °C. The cells were resuspended in 0.2 ml of cold PBS before analyzing them on the flow cytometer.

2.5. DNA extraction

Genomic DNA was isolated from whole blood using the commercial kits, DNeasy Blood & Tissue Kit (QIAGEN). The steps were followed according to the instructions supplied by the manufacturer (DNeasy Blood & Tissue Kit instructions).

2.6. KIR Ligand genotyping

The diversity of the genotypes of KIR ligands was examined by polymerase chain reaction with sequence-specific primer (PCR-SSP) technique that detects the presence or the absence of KIR ligands in each obese and non-obese samples.

2.7. Sequence-specific primer polymerase chain reaction (SSP-PCR)

For each reaction, 50-100 ng of DNA was used in a 15 µL final volume. For 16 *KIR* genes and *HLA-C1* and *HLA-C2* group typing, the same primers were used as reported by Tajik *et al*. (2009) [22]. For each reaction, positive, negative, and internal controls were used. All PCR reactions were performed with the thermocycler apparatus T100™ (Thermal Cycler from Bio-Rad Laboratories, Inc. Life Science Research, California 94547, United States, and Veriti® Thermal Cycler from Thermo Fisher Scientific, United States).

The primer sequence of HLA-C1/C2.

	Forward	Reverse	Size
HLA-C1	GAGGTGCCCGCCCGGCGA	CGCGCAGTTTCCGCAGGT	332 bp
HLA-C2	GAGGTGCCCGCCCGGCGA	CGCGCAGTTTCCGCAGGT	332 bp

For each sample reaction, we used 5 µl Go Taq Green Master Mix from Promega (3 mM MgCl2), 1µl Primers (forward and reverse) with 0.5 µl kir2DL4 as positive control, 2.5 µl Mili-Q water and 1µl DNA.

2.8. Statistical analysis

For the phenotype and functional studies different types of tests were used; unpaired t-test and paired t-test were used when the data assume a Gaussian distribution. In contrast, for the data that did not assume a Gaussian distribution a Mann-Whitney U test was used. Groups with a p value of < 0.05 were considered to be statistically significantly different. Statistical analysis was done using software Graph pad prism version 7 for the phenotyping and functional studies.

3. RESULTS

3.1. The percentage of CD56+CD3+ and iNKT cells in peripheral blood from obese and non-obese subjects

The mean percentage of CD3+CD56+ cells in blood from obese patients (8.65%) compared with the non-obese group (5.60%) was not statistically different, $P = 0.0691$ (Table 1). Similarly, comparison of the mean percentage of iNKT cells observed in the obese group (0.24%) with the non-obese group (0.26%) did not show a statistical difference ($P = 0.588$) (Table 1 and Figure 1).

3.2. Comparison of iNKT cells' CD158a, b and e expression

As KIR receptors have a major role in the function of iNKT cells, KIR expression on iNKT cells between obese and non-obese groups was compared. The mean percentage of iNKT cells expressing CD158a in the obese group was 13.36% in comparison with 10.75% in the non-obese control group (Figure 2, Table 1). However this was not significantly different. Similarly, the mean percentage of iNKT cells expressing CD158b in the obese group (5.56%) was not significantly different from that in the non-obese control group (3.08%). For CD158e 4.47% of iNKT cells in blood from the obese group showed expression in comparison to 5.88% of iNKT cells from blood of the non-obese group (Table 1 and Figure 3). Again this was not shown to be significantly different. .

3.3. KIR ligand HLA-C1/C2 on iNKT

The HLA-C1/C2 ligand genotyping was performed by using duplex sequence-specific primer-directed

Table 1. The percentage of CD56+CD3+ and iNKT cells in obese and non-obese groups (Mean). All differences were not significant. (Mann-Whitney test).

Number of samples	Non-obese 27	Obese 29	P value
CD56+CD3+ %	5.60	8.65	0.0691
iNKT Cells %	0.26	0.24	0.5880
iNKT + CD158a+ %	10.75	13.36	0.6870
iNKT + CD158b+ %	3.08	5.56	0.3978
iNKT + CD158e+ %	5.88	4.47	0.7737
iNKT + CD158a+ HLA-C2+ %	11.2	13.57	0.7871
iNKT + CD158a+ HLA-C2- %	12.46	8.8	0.3280
iNKT + CD158b+ HLA-C1+ %	1.92	4.085	0.2773
iNKT + CD158b+ HLA-C1- %	3.44	7.22	0.6984

polymerase chain reaction (SSP-PCR) method. All ligands were examined (positive or negative) and the band was detected (Figure 4).

As KIR activity is also dependent on the respective HLA alleles expressed we compared expression of KIR with their respective ligands. The percentage of iNKT cells expressing CD158a+ in the absence of *HLA-C2* was 8.8% in samples from the obese group compared with 12.46% in the non-obese group. In the presence of *HLA-C2* the highest percentage positive expression, 13.57%, was found in obese group but no significant differences were observed between these groups. The percentage of iNKT cells expressing CD158b+ in the absence of *HLA-C1* in the obese group (3.10%) compared to non-obese group (2.06%) was not significantly different. In the presence of *HLA-C1* A the percentage found in the obese group (7.22%) compared to the non-obese (3.44%) was not statistically significant (Figure 5).

4. DISCUSSION

Obesity has become one of the biggest problems in the world in recent years, as its rate is increasing with the incidence of some diseases and infections that affect health and the immune system. Obesity classified as a metabolic syndrome, has been associated with some immune disorders as well as with some inflammatory and autoimmune diseases like multiple sclerosis, lupus, Crohn's, and type 1 diabetes, and has been specifically linked to rheumatoid arthritis and psoriatic arthritis [23, 24]. Different studies have also associated the infiltration of inflammatory cells in the adipose tissues leading to the secretion of proinflammatory molecules, especially with some cells in innate and adaptive immune system [25]. Despite evidence linking obesity to alterations in immune function, little is known about the specific effects of different immune cell subpopulations such as iNKT cells.

iNKT cells are a small subset of T lymphocyte. iNKT cells have a role in immune response even when its number and percentage in the blood is small (0.01-1%), producing a variety of cytokines [15, 9]. Many studies reported increase in the number of iNKT cells in acute dengue [26] and malarial infections [27]. On the other hand, the iNKT cells number was decreased in laryngal cancer [8], influenza and hepatitis viral infections [12, 28]. The situation is controversial for MS [9], SLE [16], symptomatic atherosclerosis [29] and for T2D patients [18]. In obesity a decrease in the number of adipose tissue and peripheral blood has been reported [30, 31].

In the present study we investigated the relationship between the level of circulating iNKT cells and the level of expression of the membranous KIRs receptors in obese and non-obese individuals. A change in number and activity of iNKT cells could have a profound effect on immune function and the development of autoimmune or inflammatory diseases, and changes in the number of iNKT cells

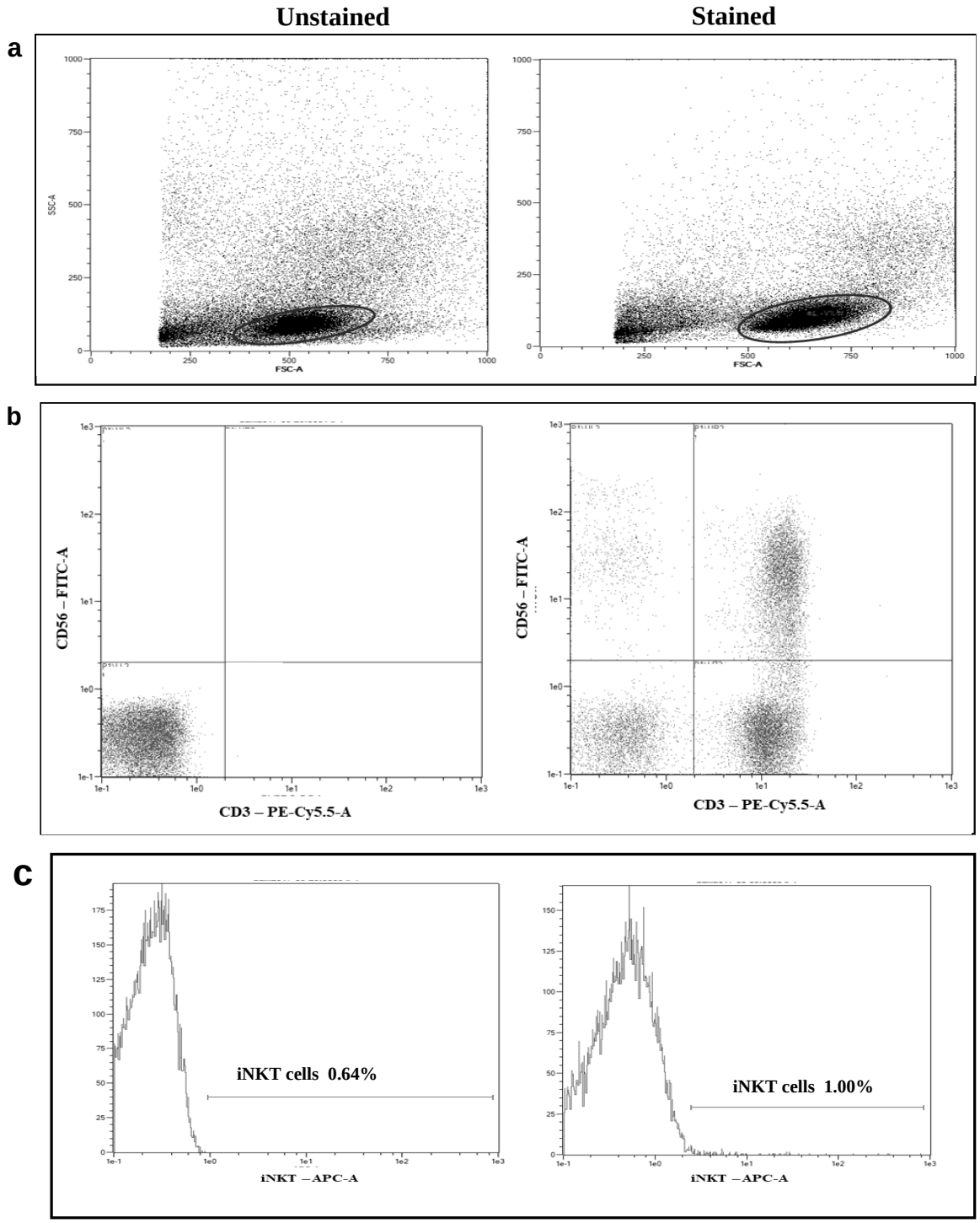

Figure 1. Flow cytometric analysis of CD56+, CD3+ and iNKT cells. (**a**) The gating of the lymphocyte population (circled) in unstained and stained samples. Lymphocytes were gated based on FSC and SSC. (**b**) The percentage of CD56+ CD3+ cells in unstained and stained samples in the lymphocyte gate. Peripheral blood mononuclear cells were stained with anti-CD56-FITC and anti-CD3-PE-Cy5.5. (**c**) The histogram shows the percentage of invariant natural killer (iNK) T cells from lymphocyte in unstained and stained samples. Peripheral blood mononuclear cells were stained with anti iNKT APC.

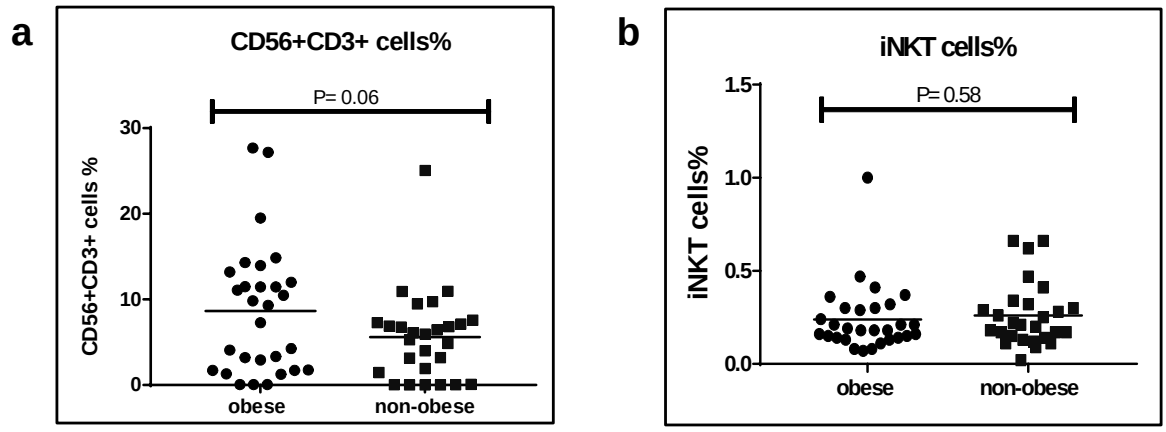

Figure 2. (**a**) Shows the percentage of gated CD56+CD3+ cells and (**b**) iNKT cells in obese and non-obese groups. The horizontal lines represent the mean value. Lower level of CD56+CD3+ were observed in non-obese compared with obese, and lower level of iNKT cells were observed in obese compared with non-obese. No statistically significant differences were found.

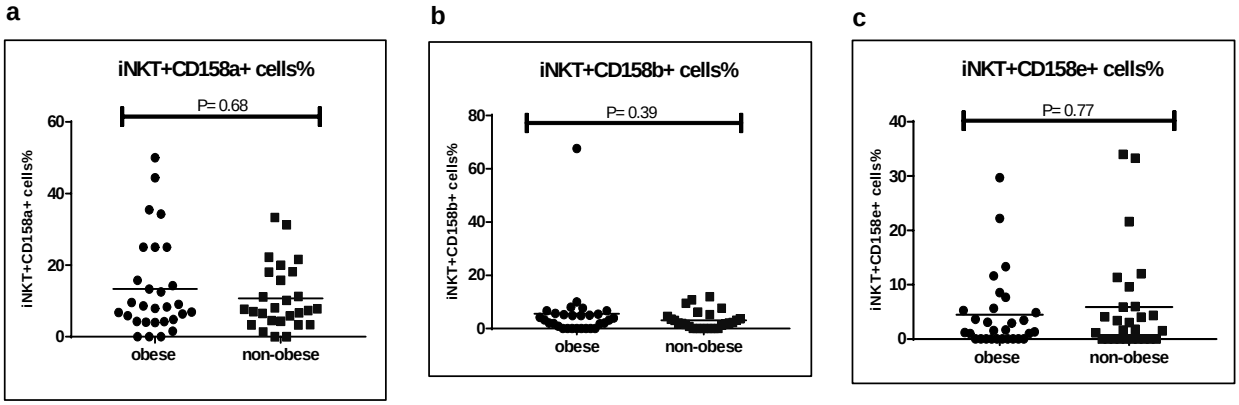

Figure 3. The percentage of gated iNKT CD158 cells in PBL from obese and non-obese. (**a**) The percentage of gated iNKT in the presence of CD158a cells in obese and non-obese. Similar level of iNKT CD158a+ cells was found in both groups. No statistically significant differences were found. (**b**) The percentage of gated iNKT in the presence of CD158b cells in obese and non-obese. Similar level of iNKT CD158b+ cells was found in both groups. No statistically significant differences were found. (**c**) The percentage of gated iNKT in the presence of CD158e cells in obese and non-obese. Similar level of iNKT CD158e+ cells was found in both groups. No statistically significant differences were found. The horizontal lines represent the mean value.

have been reported in different types of diseases [32]. In our study, although we found that the percentage of iNKT cells in peripheral blood in obese subjects appeared lower compared with those in the non-obese group, which is in agreement with other studies reported [33, 31, 30, 34], the difference in the number of iNK cells between the obese and none-obese in our study was not statistical significant. Additionally, in agreement with the results of others [35, 36] the percentage of CD3+CD56+ was not found to be significantly different between the two groups.

The frequency of functional KIRs (CD158a, CD158b and CD158e) on CD3+CD56+ cells and iNKT cells in obese and non-obese groups was investigated; there was no significant difference found between the groups.

Combinations of KIR genes and HLA have been associated with diseases such as autoimmune

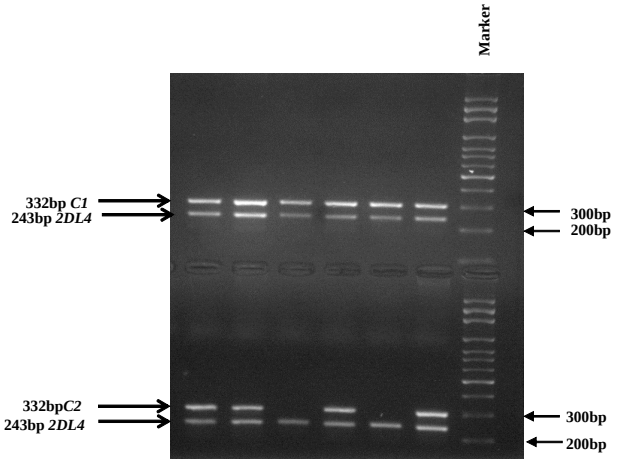

Figure 4. Genotyping of HLA-C1C2 (332bp) ligands by duplex sequence-specific primer-directed polymerase chain reaction (SSP-PCR) method. Agarose gel 3% pictures showing the duplex SSP-PCR typing of 6 DNA samples. The arrows indicate two ligand *C1- C2*-specific amplicons in each sample, and *KIR2DL4* bands were used as positive control.

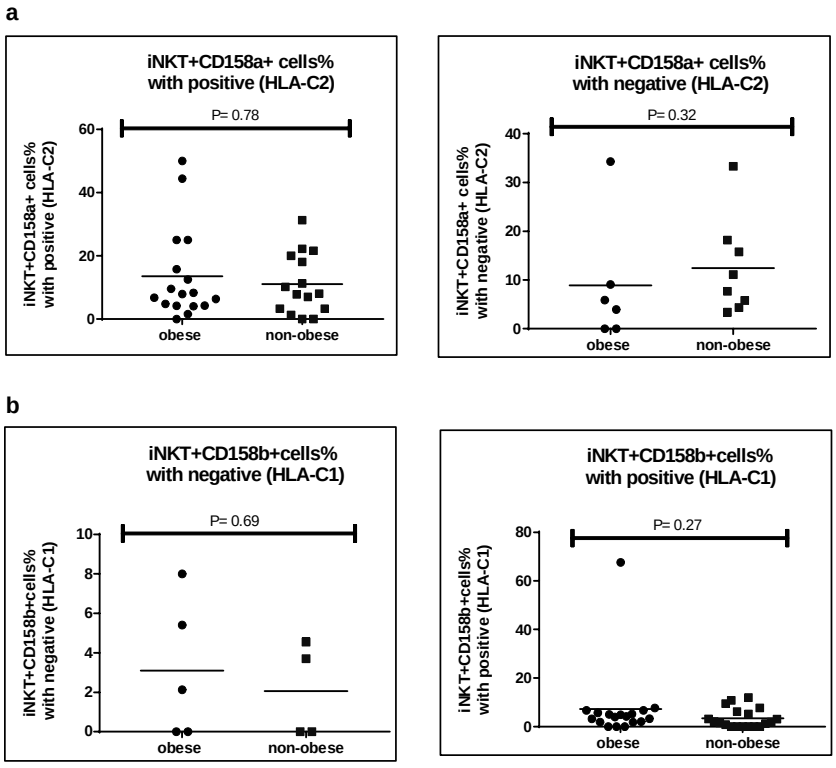

Figure 5. The percentage of gated iNKT cells expressing CD158a and CD158b in the absence and presence of *HLA-C2 and HLA-C1*, respectively in obese and non-obese. **a**) The percentage of gated iNKT cells expressing CD158a in the absence and presence of *HLA-C2*. The horizontal lines represent the mean value. Lower level of iNKT cells expressing CD158a in the presence of C2 were observed in non-obese compared with obese, and lower level of iNKT cells expressing CD158a cells in the absence of C2 were observed in obese. No statistically significant differences were found. **b**) The percentage of gated iNKT cells expressing CD158b in the absence and presence of HLA-C1. The horizontal lines represent the mean value. Lower level of iNKT cells expressing CD158b cells in the presence and in the absence of C1 were observed in non-obese compared with obese. No statistically significant differences were found.

diseases, infections, inflamation, and cancer; the interaction of CD158b with specific HLA-C antigens on a target cells inhibits cytotoxicity and prevents target cell lysis and death.

CONCLUSION

Collectively these results do not indicate that there is a significant change in iNKT cell frequency or KIR receptor expression between obese and non-obese people in Saudi Arabia. However it still remains possible that other iNKT cell activities are different between these groups or that iNKT cell activity is different in obese patients with differing underlying causes. In our present study we have not been able to stratify the obese patient group into those with underlying type two diabetes and those without. We believe that these results would be of great interest for future studies considering the associated diseases to obesity, mainly T2D which is frequent in Saudi Arabia and has become a most common disorder of this population and other similar obese populations worldwide.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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