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Effector Memory CD8+ and CD4+ T Cell Immunity Associated with Metabolic Syndrome in Obese Children

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ABSTRACT

Purpose: We investigated the association of effector memory (EM) CD8+ T cell and CD4+ T cell immunity with metabolic syndrome (MS).
Methods: Surface and intracellular staining of peripheral blood mononuclear cells was performed. Anti-interleukin-7 receptor-alpha (IL-7Rα) and CX3CR1 antibodies were used to stain the subsets of EM CD8+ T cells, while anti-interferon-gamma (IFN-γ), interleukin-17 (IL-17), and forkhead box P3 (FOXP3) antibodies were used for CD4+ T cell subsets.
Results: Of the 47 obese children, 11 were female. Children with MS had significantly higher levels of serum insulin (34.8±13.8 vs. 16.4±6.3 μU/mL, $p<0.001$) and homeostasis model assessment of insulin resistance (8.9±4.1 vs. 3.9±1.5, $p<0.001$) than children without MS. Children with MS revealed significantly higher frequencies of IL-7Rα^{low} CD8+ T cells (60.1 ±19.1% vs. 48.4±11.5%, $p=0.047$) and IL-7Rα^{low}CX3CR1+ CD8+ T cells (53.8±20.1% vs. 41.5 ±11.9%, $p=0.036$) than children without MS. As the serum triglyceride levels increased, the frequency of IL-7Rα^{low}CX3CR1+ and IL-7Rα^{high}CX3CR1− CD8+ T cells increased and decreased, respectively (r=0.335, $p=0.014$ and r=−0.350, $p=0.010$, respectively), in 47 children. However, no CD4+ T cell subset parameters were significantly different between children with and without MS.
Conclusion: In obese children with MS, the changes in immunity due to changes in EM CD8+ T cells might be related to the morbidity of obesity.

Keywords: Child; Obesity; Metabolic syndrome; Hypertriglyceridemia; CD4; CD8; T-Lymphocytes

INTRODUCTION

According to global health observatory data from the World Health Organization, 18% of children and adolescents aged 5–19 years were overweight and obese in 2016 [1]. Among school-age children aged 7–18 years in South Korea, the prevalence of obesity has increased gradually from 8.4% in 2008 to 14.4% in 2018 [2]. According to a systematic review of the

worldwide literature, the median prevalence of metabolic syndrome (MS) in children was 3.3% in the whole population, 11.9% in overweight children, and 29.2% in obese children [3].

There are many criteria for defining MS in children [4]. To define childhood MS, the criteria, which include obesity, central obesity, hypertension, elevated glucose levels, hypertriglyceridemia, and decreased high-density lipoprotein (HDL)-cholesterol levels in children, established by the International Diabetes Federation (IDF), are commonly used [5].

T cell immunity, a type of adaptive immunity, has been recently postulated to be related to changes in immunity in obesity and the pathogenesis of obesity-related comorbidities, especially diabetes mellitus (DM) type 2 [6-8]. However, there are no reports on the changes in adaptive immunity in obese children. Thus, in this study we aimed to investigate the differences in T cell immunity between children with and without MS.

MATERIALS AND METHODS

Human subjects

From January 2016 to December 2018, a total of 89 obese children who were managed at the obesity clinic for children and adolescents at Jeju National University Hospital were enrolled in the study. Children with underlying diseases and/or under 10 years of age were excluded from the study. Eventually, clinical and laboratory data were collected from 47 obese children.

The IDF criteria were applied to identify MS in the included children [5]. According to the IDF criteria, childhood MS was defined as central obesity (waist circumflex greater than the 90th percentile) with at least two of the following four criteria: 1) fasting glucose level >100 mg/dL, 2) triglyceride level ≥150 mg/dL, 3) HDL-cholesterol level ≤40 mg/dL, and 4) systolic blood pressure >130 mmHg or diastolic blood pressure >85 mmHg. Hypertension was defined as a systolic or diastolic blood pressure greater than the 95th percentile for sex and age.

Flow cytometric analysis

To evaluate CD4+ and CD8+ T cell immunity in obese children, whole blood samples were collected in heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were extracted from the whole blood samples using Ficol-Paque Premium (GE Healthcare, Chicago, IL, USA) gradients and stained with fluorescent antibodies against surface and/or intracellular markers. For the staining of surface and intracellular CD4+ T cell subset markers, the PBMCs were stimulated by a combined cocktail of phorbol myristate acetate (50 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (1 μg/mL; Sigma-Aldrich), and GolgiPlug (BD Biosciences, San Jose, CA, USA) for 4 hours. Control PBMCs were incubated with phosphate-buffered saline and GolgiPlug (BD Biosciences) for 4 hours. Anti-APC-Cy7-CD3 and anti-Alexa Fluor 700-CD4 (BD Biosciences) antibodies were used for surface staining of the stimulated cells. The fixation and permeabilization of cells were performed using forkhead box P3 (FOXP3) Fix/Perm Buffer (BioLegend, San Diego, CA, USA). For the intracellular staining of CD4+ cell subsets, cells were incubated for 30 minutes in the dark with a cocktail of anti-PE-FOXP3 (BioLegend), anti-Alexa Fluor 488-IL-17A (eBioscience, San Diego, CA, USA), and anti-PE-Cy7-IFN-r (BD Biosciences) antibodies. For surface staining of CD8+ T cell subsets, the PBMCs were stained with antibodies against APC-Cy7-CD3, Pacific Blue-CD8, PE-Cy-7-CCR7, PE-Cy5-CD45RA (BD Biosciences), FITC-interleukin-7 receptor-alpha (IL-7Rα) (R&D Systems, Minneapolis, MN, USA), and PE-CX3CR1 (BioLegend). Finally, the cells were

assayed using an LSRFortessa® flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analyses

SPSS version 24.0 (IBM Co., Armonk, NY, USA) was used for the statistical analyses. The results are presented as the mean±standard deviation (SD) for each group. The two groups were statistically compared using the Mann–Whitney U-test, Fisher's exact test, and Spearman's correlation analysis. Statistical significance was set at $p<0.05$.

Ethics statement

This study was approved by the Institutional Review Board of the Jeju National University Hospital (JNUH 2014-07-005). Informed consent was obtained from all children and their parents.

RESULTS

Clinical and laboratory characteristics

Of the 47 children, 11 were female. The mean age (mean±SD) was 12.9±2.1 years for 15 children without MS and 14.0±2.4 years for 32 children with MS (p=0.119). The group with MS had a significantly higher body mass index (BMI) (31.4±4.4 vs. 27.9±5.2, p=0.032), BMI Z-score (3.2±1.0 vs. 2.4±1.2, p=0.026), hypertension percentage (63.3% vs. 0%, $p<0.001$), insulin levels (34.8±13.8 μU/mL vs. 16.4±6.3 μU/mL, $p<0.001$), homeostasis model assessment of insulin resistance (8.9±4.1 vs. 3.9±1.5, $p<0.001$), and triglyceride levels (142.4±71.2 mg/dL vs. 88.8±40.2 mg/dL, p=0.002) than the group without MS (**Table 1**). Between the two groups, there were no significant differences in the aspartate aminotransferase, alanine aminotransferase, glucose, hemoglobin A1$_c$, total cholesterol, HDL-cholesterol, and low-density lipoprotein-cholesterol values.

Table 1. Demographic and clinical characteristics of 47 obese children with or without metabolic syndrome based on the criteria established by the International Diabetes Federation

Variable	Metabolic syndrome		
	Negative (n=15)	Positive (n=32)	p-value*
Sex (n=47), male/female	13/2	23/9	
Age (yr)	12.9±2.1	14.0±2.4	0.119
BMI (kg/m²)	27.9±5.2	31.4±4.4	0.032
BMI Z-score	2.4±1.2	3.2±1.0	0.026
Hypertension (%)†	0	63.3	<0.001
AST (IU/L)	42.4±17.4	65.4±64.8	0.185
ALT (IU/L)	76.9±45.7	129.1±137.5	0.161
Glucose (mg/dL)	94.5±6.2	103.4±32.0	0.136
Insulin (μU/mL)	16.4±6.3	34.8±13.8	<0.001
HOMA-IR	3.9±1.5	8.9±4.1	<0.001
HbA1$_c$ (%)	5.3±0.2	5.8±0.9	0.019
Total cholesterol (mg/dL)	173.1±12.4	178.5±35.2	0.565
Triglyceride (mg/dL)	88.8±40.2	142.4±71.2	0.002
HDL-cholesterol (mg/dL)	47.1±5.9	43.5±9.0	0.116
LDL-cholesterol (mg/dL)	107.8±12.6	113.5±31.6	0.509

Values are presented as number only or mean±standard deviation.
Metabolic syndrome was defined according to criteria established by the International Diabetes Federation [5].
BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, HOMA-IR: homeostasis model assessment of insulin resistance, HbA1$_c$: hemoglobin A1$_c$, HDL: high-density lipoprotein, LDL: low-density lipoprotein.
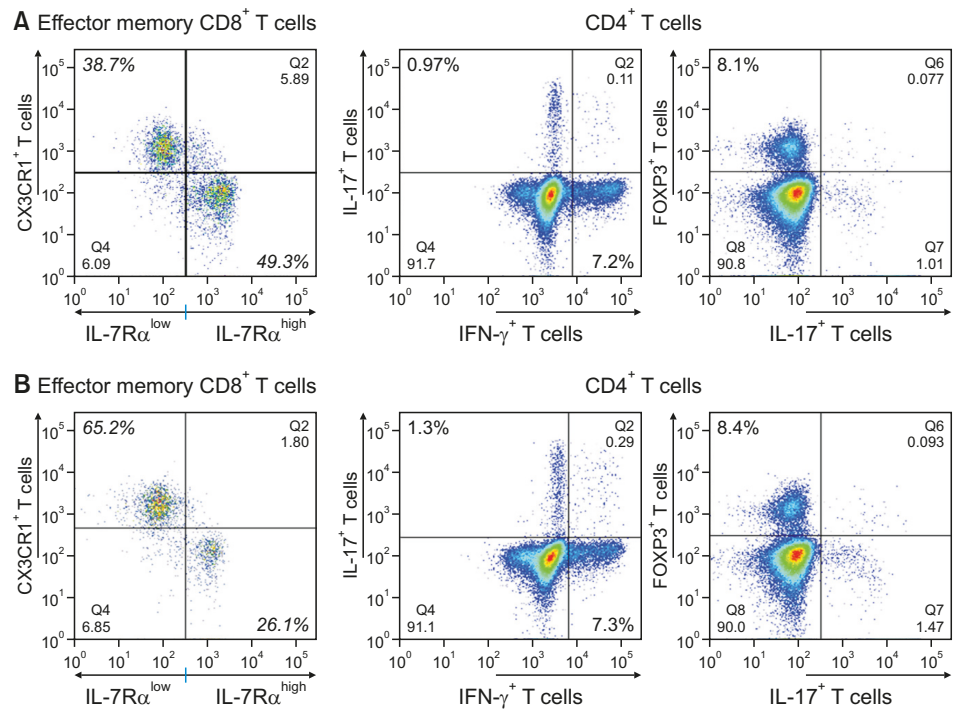*Mann–Whitney U-test was used for all parameters except hypertension. †Fisher's exact test.

Fig. 1. Frequency of CD4⁺ T cells (helper T cells) and effector memory CD8⁺ T cells (cytotoxic T cells) in two obese children without (A) or with (B) metabolic syndrome. As shown in **Tables 2** and **3**, the frequency of IL-7Rα^{low} CX3CR1⁺ CD8⁺ T cells and IL-7Rα^{high} CX3CR1⁻ CD8⁺ T cells in obese children with metabolic syndrome was significantly higher and lower, respectively (p=0.036 and p=0.028), than those in obese children without metabolic syndrome. The parameters related to CD4⁺ T cells did not show a significant difference between the two groups.

IL-7Rα: interleukin-7 receptor-alpha, IFN-γ: interferon-gamma, IL-17: interleukin-17, FOXP3: forkhead box P3.

Table 2. Frequency of CD8⁺ T lymphocytes in obese children with or without metabolic syndrome

EM CD8⁺ T cell (n=47)	Metabolic syndrome		
	Negative (n=15)	Positive (n=32)	p-value*
IL-7Rα^{low}	48.4±11.5%	60.1±19.1%	0.047
IL-7Rα^{low} CX3CR1⁺	41.5±11.9%	53.8±20.1%	0.036
IL-7Rα^{high} CX3CR1⁻	46.5±12.3%	35.3±17.8%	0.028

Values are presented as mean±standard deviation.
CD8⁺ T cells are cytotoxic T cells that include IL-7Rα^{low}/CX3CR1⁺ and IL-7Rα^{high}/CX3CR1⁻ effector memory (EM) T cells.
IL-7Rα: interleukin-7 receptor-alpha.
*Mann–Whitney U-test.

Surface and/or intracellular staining for CD8⁺ and CD4⁺ T cell subsets

Children with MS revealed significantly higher frequencies of IL-7Rα^{low} CD8⁺ T cells (60.1±19.1% vs. 48.4±11.5%, p=0.047) and IL-7Rα^{low}CX3CR1⁺ CD8⁺ T cells (53.8±20.1% vs. 41.5±11.9%, p=0.036) than children without MS (**Table 2**, **Fig. 1**). Children with MS demonstrated a significantly lower frequency of IL-7Rα^{high}CX3CR1⁻ effector memory (EM) CD8⁺ T cells (35.3±17.8% vs. 46.5±12.3%, p=0.028) than children without MS (**Table 2**, **Fig. 1**). As the serum triglyceride levels increased, the frequency of IL-7Rα^{low}CX3CR1⁺ CD8⁺ T cells and IL-7Rα^{high}CX3CR1⁻ CD8⁺ T cells increased and decreased, respectively (r=0.335, p=0.014 and r=−0.350, p=0.010, respectively), in 47 children (**Fig. 2**). However, the CD4⁺ T cell subset parameter was not significantly different between children with and without MS (**Table 3**, **Fig. 1**).
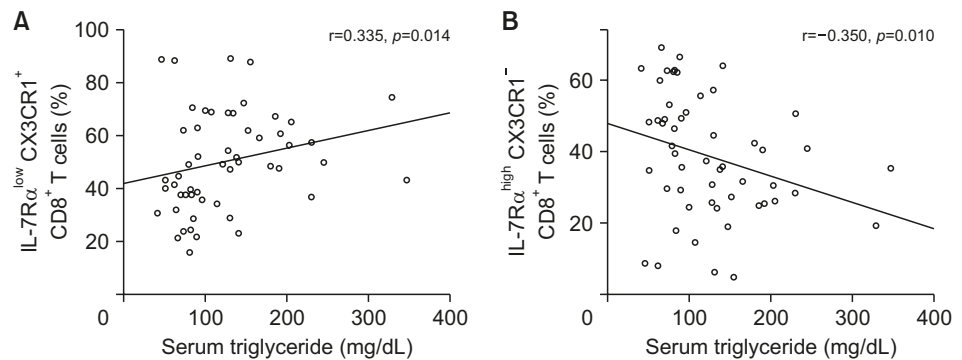
Fig. 2. Correlation between the levels of serum triglyceride and the frequency of (A) IL-7Rα^{low} CX3CR1$^+$ CD8$^+$ T cells or (B) IL-7Rα^{high}CX3CR1$^-$ CD8$^+$ T cells. As the serum triglyceride level increased, the frequency of IL-7Rα^{low}CX3CR1$^+$ CD8$^+$ T cells and IL-7Rα^{high}CX3CR1$^-$ CD8$^+$ T cells increased and decreased, respectively (r=0.335, *p*=0.014 and r=−0.350, *p*=0.010, Spearman's correlation analysis). IL-7Rα: interleukin-7 receptor-alpha.

Table 3. Frequency of CD4$^+$ T lymphocytes in obese children with or without metabolic syndrome

CD4$^+$ T cell (n=32)	Metabolic syndrome		
	Negative (n=7)	Positive (n=25)	*p*-value*
IFN-γ$^+$	8.9±7.1%	10.1±4.7%	0.635
IL-17$^+$	1.4±1.1%	1.8±1.1%	0.338
FOXP3$^+$	8.7±2.7%	7.7±2.5%	0.410

Values are presented as mean±standard deviation.
The subsets of CD4$^+$ T cells include IFN-γ$^+$ CD4$^+$ T cells (Th1 cells), IL-17$^+$CD4$^+$ T cells (Th17 cells), and FOXP3$^+$ regulatory T cells.
IFN-γ: interferon-gamma, IL-17: interleukin-17, FOXP3: forkhead box P3.
*Mann–Whitney U-test.

DISCUSSION

Some studies have reported a connection between adaptive immunity and insulin resistance such as in type 2 DM in animal models and humans [9]. Several studies employing animal models of type 2 DM induced by a high-fat diet have shown that CD4$^+$ and CD8$^+$ T cells might affect phenotypic changes in innate immunity such as M2 macrophage dominance and M1 macrophage infiltration into adipose tissues or visceral fat depots [10-12]. M1 and M2 macrophages have proinflammatory and anti-inflammatory effects, respectively. However, Sultan et al. [13] have reported that inflammation of adipose tissue mediated by T cells did not cause insulin resistance.

MS is greatly associated with insulin resistance. Based on studies reporting the relationship between adaptive immunity and insulin resistance, we hypothesized that there might be significant differences in CD8$^+$ and CD4$^+$ T cell immunity in the blood between children with and without MS. The CD8$^+$ T cell subsets included IL-7Rα^{low} EM CD8$^+$ T cells. CD8$^+$ T cells in human peripheral blood are expressed as two subsets, IL-7Rα^{high} and IL-7Rα^{low}, which have different responses to IL-7 for cell survival [14]. DNA methylation plays a major role in controlling IL-7Rα expression in T cells, which is mediated by the promoter activity of the IL-7Rα gene [15]. IL-7Rα^{low} EM CD8$^+$ T cells increase CX3CR1 expression on CD8$^+$ T cell membranes for binding to CX3C-chemokine ligand 1 (CX3CL1), a CXC3CR1 ligand fractalkine on endothelial cells near inflamed tissues [16]. CX3CR1 is a receptor for CX3CL1, which is known to function as an adhesive and chemoattractant molecule [17]. The present study demonstrated a significantly higher frequency of IL-7Rα^{low} and IL-7Rα^{low}CX3CR1$^+$ EM CD8$^+$ T cells in children with MS than in those without MS (**Table 2, Fig. 1**).

As the serum triglyceride level increased, the frequency of IL-7Rα^{low}CX3CR1$^+$ and IL-7Rα^{high}CX3CR1$^-$ CD8$^+$ T cells increased and decreased, respectively. Serum triglyceride levels were positively correlated with the frequency of IL-7Rα^{low}CX3CR1$^+$ CD8$^+$ T cells (**Fig. 2**). Hypertriglyceridemia is highly associated with metabolic abnormalities such as insulin resistance, nonalcoholic fatty liver disease, and advanced bone age in obese children [18,19]. MS can be associated with inflammatory status. Unfortunately, we did not evaluate serum inflammatory markers such as C-reactive protein or erythrocyte sedimentation rate. Thus, the differences in serum inflammatory markers between the two groups could not be assessed.

CD4$^+$ T cell subsets include proinflammatory IFN-γ$^+$CD4$^+$ (Th1 cells) and IL-17$^+$CD4$^+$ T cells (Th17 cells) and anti-inflammatory FOXP3$^+$ regulatory T cells. No CD4$^+$ T cell subset parameters were significantly different between children with and without MS (**Table 3**). Surendar et al. [20] reported that blood concentrations of both TH1-produced cytokines (IL-2, IL-12, and IFN-γ) and TH2-secreted cytokines (IL-4, IL-5, and IL-13) were higher in the sera of patients with MS than in those without MS.

This study has some limitations. First, cytokine analysis could not be performed for all obese children because all sera samples were inadvertently thawed following freezing due to an accidental shutdown of electric current to the deep freezer. Second, we did not analyze the tissues of obese children. Collecting tissues from children is difficult because of ethical issues.

In conclusion, obese children with MS showed a change in immunity due to changes in EM CD8$^+$ T cells, which may be related to the morbidity of obesity. Serum triglyceride levels were positively correlated with the frequency of IL-7Rα^{low}CX3CR1$^+$ CD8$^+$ T cells. Such correlation may also play a role in the pathogenesis of obesity morbidities, which are associated with hypertriglyceridemia.

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