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ORIGINAL ARTICLE

# Diminished immune response to vaccinations in obesity: Role of myeloid-derived suppressor and other myeloid cells

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## KEYWORDS

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**Summary** Obesity is a chronic inflammatory condition associated with an increased production of cytokines and exacerbated immune response. However, obese subjects are susceptible to infections and respond poorly to vaccines. This study evaluated the immune responses of obese mice and the underlying mechanisms by exploring the roles of myeloid cells. Diet-induced obese (DIO) mice were prepared from C57BL/6J mice fed a high-calorie and high-fat diet for 12 weeks. Humoral and cellular immune responses of DIO mice to a hepatitis B vaccine containing the hepatitis B surface antigen (HBsAg) were assessed in sera and via a lymphoproliferative assay, respectively. The effects of CD11b<sup>+</sup>GR1<sup>+</sup> myeloid-derived suppressor cells (MDSC) and CD11b<sup>+</sup>GR1<sup>-</sup> non-MDSC on T cell proliferation and cytokine production were compared via a cell culture system. The production of cytokines, expression of activation and exhaustion markers, and proportions of apoptotic T cells were estimated with flow cytometry. Increased T and B lymphocyte proliferation and higher interferon- $\gamma$  and tumor necrosis factor- $\alpha$  levels were detected in spleen cells and liver

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non-parenchymal cell cultures of DIO mice compared to controls ( $p < 0.05$ ). However, antibody to HBsAg (anti-HBs) levels and HBsAg-specific T cell proliferation were significantly lower in DIO mice compared to controls ( $p < 0.05$ ). The addition of MDSC, but not non-MDSC, induced a decrease in HBsAg-specific T cell proliferation, lower cytokine production, decrease in T cell activation, and increase in T cell exhaustion and apoptosis ( $p < 0.05$ ). MDSC play an important role in mediating impaired antigen-specific immunity.

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## Introduction

The World Health Organization has reported that in 2008, 1.5 billion adults, 20 and older, were overweight. Of these over 200 million men were obese. Overweight and obesity are the fifth leading risk for global deaths [1]. At least 2.8 million adults die each year as a result of being overweight or obese. In addition, 44% of the diabetes burden, 23% of ischemic heart disease burden, and between 7% and 41% of certain cancer burden are attributable to overweight [1]. Obesity and its comorbidities, including insulin resistance, type 2 diabetes mellitus, atherosclerosis, coronary heart disease, and non-alcoholic fatty liver diseases (NAFLD), have already reached worldwide epidemic proportions [2,3]. Obesity is also associated with an increased risk of numerous types of cancer, as described by a meta-analysis conducted on a total of 282,000 patients with over 133 million person-years of follow-up [4].

The pathological processes involved in obesity are associated with chronic inflammation that is systemic in nature. Obesity-induced inflammation appears to be initiated within adipose tissue, as it expands due to excess fat and caloric intake, and involves the activation of inflammatory pathways in cells that sense fatty acids and cytokine signaling [5,6]. These receptors were originally thought to be involved in only sensing pathogens, but recently were found to also sense fatty acids [7–9].

Despite harboring an inflammatory mucosal milieu, obese subjects exhibit impaired immune responses to specific antigens, such as vaccines. Reduced tetanus antibody titers have been documented in overweight children [10], and impaired immunogenicity of hepatitis B vaccine has been reported in obese persons [11]. Moreover, lower magnitudes of vaccine-induced immunity have been also documented in murine models of obesity [12–15].

However, there is a lack of information regarding the mechanisms underlying the upregulation of

certain inflammatory immune responses and down-regulation of antigen-specific immune responses in obesity. A series of studies have suggested that altered functional capacities of lymphocytes, natural killer cells, natural killer T cells, and dendritic cells (DC) may account for the differences in immune function in obesity [13–18]. However, little is known about the role of macrophages and myeloid cells in obesity, which constitutes an evolutionarily conserved defense system that is located at the ports of entry for pathogens and form the first line of defense against environmental threats to the body. In order to dissect the mechanisms responsible for the impaired responses of obese subjects to vaccines, attention needs to be focused on macrophages and myeloid cells, as they traditionally produce abundant amounts of inflammatory mediators, such as tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$ , and are well known for maintaining the inflammatory mucosal milieu [19–21]. Recent studies in cancer patients and animal models of cancer and chronic infections have identified a heterogeneous immature myeloid cell population that is induced by inflammation, and causes immunosuppression [22–24]. These immature myeloid cells are regarded to as myeloid-derived suppressor cells (MDSC). MDSC co-express Gr-1 and CD11b in rodents, whereas traditional macrophages or typical myeloid cells that produce inflammatory cytokines express CD11b, but not Gr-1 (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells) [19–24].

We postulated that MDSC may have a role in the diminished antigen-specific adaptive immune response in obesity. To address this issue, we prepared an animal model of obesity by providing high-fat and high-calorie diet to C57BL/6J mice. Antigen-specific humoral and cellular immune responses to the hepatitis B (HB) vaccine were evaluated and compared between obese mice and control mice, which received normal laboratory chow. Since the antigen-specific humoral antibody and cellular immune responses levels were significantly lower in obese mice compared to control

mice, we verified the functional capacities of MDSC and CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells (non-MDSC) to determine the functional diversity of different myeloid cells in obesity.

## Methods and procedures

### Production of diet-induced obese (DIO) mice and their characterization

Seven-week-old male C57BL/6J mice were purchased from Nihon Clea (Tokyo, Japan), housed individually in polycarbonate cages, and maintained in a temperature- and humidity-controlled room (23 ± 1 °C) with a 12-h light/dark cycle. After 1 week of acclimatization, mice were fed a high-fat and high-calorie diet consisting of 20% protein, 20% carbohydrate, and 60% fat with an energy density of 520 kcal/100 g (D12492, Research Diets, Inc.; New Brunswick, NJ, USA) to produce the diet-induced obese (DIO) model. Control mice were fed a standard laboratory chow consisting of 26% protein, 60% carbohydrate, and 13% fat with an energy density of 360 kcal/100 g. All mice received humane care, and the study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Ehime University, Japan.

Blood glucose (Glucose PILOT; Aventir Biotech, LLC, Carlsbad, CA, USA) and serum cholesterol levels (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were measured with commercially available kits. Fatty acid concentrations were measured in mice sera via liquid chromatography (Shikoku Chuken, Matsuyama, Japan). Mice were anesthetized with diethyl ether and sacrificed via cervical dislocation for tissue specimen collection. Liver, spleen, and fatty tissue weights were determined. Histological assessments of liver were conducted via histochemistry.

### Immunization schedule and assessment of humoral immunity

DIO mice and control mice were immunized once with an intraperitoneal hepatitis B (HB) vaccine containing hepatitis B surface antigen (HBsAg; 1 and 2 µg) (Heptavax-II, subtype adw, Banyu Pharmaceutical, Tokyo, Japan). Sera antibody levels against HBsAg (anti-HBs) were measured 4 weeks after immunization via the chemiluminescence enzyme immunoassay method (Shikoku Chuken, Matsuyama, Japan) as described previously [15,25]. Values were expressed as mIU/ml.

### Isolation of spleen cells and liver non-parenchymal cells (NPC), T lymphocytes, B lymphocytes, and DC

The methodologies for isolating spleen cells, liver NPC, T lymphocytes, B lymphocytes, and DC were previously described in detail elsewhere [15,25,26]. To prepare a single cell suspension, spleens were removed aseptically, cut into pieces, passed through a 40-µm-pore nylon filter (BD Falcon, Durham, NC, USA). To retrieve liver NPCs, liver tissues were cut into pieces, homogenized, passed through a 70-µm-pore steel mesh (Morimoto Yakuin Co., Matsuyama, Japan), and suspended in 35% percoll (Sigma Chemical, St. Louis, MO, USA). After centrifugation for 15 min at 450 × g at room temperature, a high-density cell pellet was collected and suspended in culture medium [15,26]. T and B lymphocytes were purified from single-cell suspensions using the negative-selection column method (Mouse Pan T isolation kit, Pan B cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). DC were isolated from these single cell suspensions via density centrifugation and depletion of T lymphocytes, B lymphocytes, and macrophages, as previously described [26].

### Isolation and characterization of CD11b<sup>+</sup>Gr1<sup>+</sup> MDSC and CD11b<sup>+</sup>Gr1<sup>-</sup> non-MDSC from mouse spleen and liver samples

MDSC (CD11b<sup>+</sup>Gr1<sup>+</sup>) and non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells) were isolated from single cell suspension of spleen and liver NPC using a cell sorting system [25,26]. Briefly, spleen cells and liver NPC were stained with allophycocyanin (APC) anti-mouse Gr-1 (clone RB6-8C5) and phycoerythrin (PE) anti-mouse CD11b (clone M1/70) (BD Biosciences, San Jose, CA, USA). MDSC and CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells were sorted into CD11b<sup>+</sup>Gr1<sup>+</sup> and CD11b<sup>+</sup>Gr1<sup>-</sup> cells, respectively, via the BD FACSAria™ Cell Sorting System (Becton Dickinson). All sorted cells had purity above 98%.

PE anti-mouse cytotoxic T lymphocyte antigen 4 (CTLA-4) (clone UC10-4F10-11), PD-1 (clone J43), FasL.1 (clone KAY-10), CD62L (clone MEL-14), CD40L (clone MR1) (BD, Biosciences), and CD28 (clone 37.51) (eBioscience, San Diego, CA, USA) were used to evaluate the expressions of activation and exhaustion markers on T cells. The expressions of different surface antigens on MDSC and T lymphocytes were presented as relative proportions in the total cell population.

For assessments of intracellular IFN- $\gamma$ , cells were lysed with a fixation and permeabilization kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and stained with PE anti-mouse IFN- $\gamma$  (clone XMG 1.2) (BD Biosciences). Flow cytometry was performed on a Becton Dickinson fluorescence activated cell sorter (FACScalibur) and data acquisition was performed using CellQuest Software (Becton Dickinson, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo software (TreeStar Corporation, Ashland, OR, USA).

### T cell proliferation assay

Different immunocytes from spleen cells and liver NPC were cultured with various immune modulators (concanavalin A, Con A [Sigma Chemical]), lipopolysaccharides (LPS [Sigma Chemical]), MDSC, or non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells), with or without antigens, as previously described [15,25,26]. All cultures were performed in 96-well U-bottomed plates (Corning Inc., New York, NY, USA). <sup>3</sup>[H]-Thymidine (1.0  $\mu$ Ci/ml; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was diluted in sterile RPMI-1640, added to the cultures for the last 16 h, and harvested automatically with a multiple cell harvester (Labo Mash; Futaba Medical, Osaka, Japan) onto a filter paper (Labo Mash 101-10; Futaba Medical). The incorporation levels of <sup>3</sup>[H]-thymidine were determined via a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA, USA). T cell proliferation levels were presented as counts per minute (cpm). The cpm in culture containing only T cells was considered as background proliferation, and expressed as a stimulation index (SI) of 1.0. T cell proliferation levels were estimated by dividing the cpm in cultures containing T cells with Con A, LPS, MDSC, CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells, or antigen with the cpm of control cultures. A stimulation index > 3.0 was considered as a significant proliferation.

### Cytokine production and measurements

Different immunocytes were cultured with Con A and LPS for 48 h to assess cytokine production. Cytokine levels in culture supernatants were calibrated by the mean fluorescence intensities of the standard negative control, standard positive control, and samples using Cytometric Bead Array software (BD Biosciences Pharmingen, San Jose, CA, USA) on a Macintosh computer (SAS Institute, Cary, NC, USA). Cytokine levels were shown as pg/ml.

### Apoptosis assays

T cells were plated in six-well plates at a density of  $1 \times 10^6$  cells/ml. The cells were stimulated with Con A (1  $\mu$ g/ml) for 24 h with or without MDSC and non-MDSC. After culture, cells were washed, resuspended in  $1 \times$  binding buffer ( $1 \times 10^5$  cells/100  $\mu$ l), and stained with FITC-Annexin V (BD Biosciences, San Jose, CA, USA) and 7-amino-actinomycin D (7-AAD, BD Biosciences). Using CellQuest Pro software, three subsets of cells, based on intensity of staining with Annexin V and 7-AAD, were identified: Annexin V<sup>-</sup>/7-AAD<sup>-</sup> (live cells), Annexin V<sup>+</sup>/7-AAD<sup>-</sup> (early apoptotic cells), and Annexin V<sup>+</sup>/7-AAD<sup>+</sup> (late apoptotic and necrotic cells) [27]. Subsequently, the percentage of each population was calculated.

### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD). Differences were determined via the Student *t*-test, if the data were normally distributed, and Mann-Whitney rank-sum test, if data were skewed. Statistical significances was considered at  $p < 0.05$ .

## Results

### Murine model of DIO mice

A murine model of DIO mice was developed by feeding a high-fat and high-calorie diet to C57BL/6J mice for 12 weeks, as previously described [15]. Body and fat tissue (both subcutaneous and visceral) weights were significantly higher in DIO mice than that of age- and sex-matched control mice ( $p < 0.05$ ) (Table 1). Additionally, liver and spleen weights were significantly higher in DIO mice compared to control mice ( $p < 0.05$ ). In addition to being a murine model of obesity, DIO mice demonstrated significantly higher levels of fasting blood glucose and cholesterol compared to those of control mice that were fed a standard laboratory chow ( $p < 0.05$ ). The accumulation of fat was present in the liver of all DIO mice (data not shown). Taken together, we developed a mouse model of DIO, which was characterized by obesity, NAFLD, and diabetes mellitus.

### Increased proportions of MDSC and non-MDSC in the spleen and liver of DIO mice compared to control mice

The proportion of MDSC to whole spleen cells increased significantly in DIO mice ( $2.02 \pm 0.48$ ) compared to those of control mice ( $1.21 \pm 0.30$ ) ( $N=5$ ). The ratio of CD11b<sup>+</sup>Gr1<sup>-</sup> non-MDSC

**Table 1** Characteristics of diet-induced obese and control mice.

Parameters	Control mice	Diet-induced obese mice
Body weight (g)	28.7 ± 1.2	40.1 ± 2.1 <sup>a</sup>
Total adipose tissue weight (g)	1.26 ± 0.8	9.5 ± 0.9 <sup>a</sup>
Liver weight (g)	1.28 ± 0.05	2.19 ± 0.18 <sup>a</sup>
Spleen weight (mg)	60 ± 3.9	180.7 ± 10.5 <sup>a</sup>
Fasting blood sugar (mg/dl)	73.8 ± 5.3	141.7 ± 8.7 <sup>a</sup>
Serum cholesterol (mg/dl)	106.2 ± 9.05	220.4 ± 13.76 <sup>a</sup>

Data are presented as means and standard deviations (SD). Normal C57BL/6J mice were fed either normal laboratory chow ( $n = 10$ ) or a high-calorie fat-diet ( $n = 10$ ) for 12 weeks. Mice were characterized 12 weeks after beginning the experiment.

<sup>a</sup>  $p < 0.05$  vs. control mice receiving normal laboratory chow.

was also increased in spleen of DIO mice ( $5.07 \pm 1.11\%$ ) compared to control mice ( $3.14 \pm 0.81\%$ ) ( $N = 5$ ). The numbers of MDSC populations in control and DIO mice were  $1.28 \pm 0.2 \times 10^6$  and  $3.35 \pm 0.31 \times 10^6$  cells/spleen ( $N = 5$ ), respectively. The numbers of non-MDSC population were  $2.98 \pm 0.41 \times 10^4$  cells/spleen and  $8.42 \pm 0.77 \times 10^4$  cells/spleen from control and DIO mice, respectively ( $p < 0.05$ ).

MDSC proportions were significantly higher in the livers of DIO mice compared to those of control mice ( $12.3 \pm 1.2$  vs.  $6.01 \pm 0.9\%$ , respectively;  $p < 0.05$ ). The ratio of CD11b+Gr1- non-MDSC was also increased in liver of DIO mice compared to control mice ( $9.16 \pm 1.69\%$  versus  $6.47 \pm 1.29\%$ ,  $N = 5$ ). The total numbers of MDSC were also increased in the liver of DIO mice. In control mice fed with a normal diet, the numbers of MDSC/liver was  $17.75 \pm 5.72 \times 10^4$  ( $N = 5$ ). The numbers of MDSC in DIO mice liver were  $107.75 \pm 19.75 \times 10^4$  cells ( $N = 5$ ).

### Increased proliferative responses, and proinflammatory cytokine production by immunocytes from DIO mice

The proliferative capacities of liver-derived T cells to Con A and B cells to LPS were significantly higher in DIO mice compared to control mice ( $p < 0.05$ ) (Fig. 1A and B). In addition to the increased proliferative responses of lymphocytes, DIO mice-derived liver NPC produced significantly higher levels of IFN- $\gamma$  and TNF- $\alpha$  following stimulation with Con A and LPS compared to those of control mice (Fig. 1C and D).

### Decreased responses of DIO mice to the HB vaccine

#### Decreased humoral responses of DIO mice to the HB vaccine in vivo

Despite harboring an inflammatory mucosal milieu, as well as demonstrating increased liver and spleen

T and B lymphocyte proliferative responses, and proinflammatory cytokine production, DIO mice exhibited significantly lower levels of immune responses to HBsAg-based vaccine. As shown in Fig. 2, DIO mice immunized with 1  $\mu$ g of HBsAg produced significantly lower levels of anti-HBs compared to control mice immunized with same dose of a HBsAg-based vaccine ( $26 \pm 7$  vs.  $198 \pm 21$  mIU/ml, respectively;  $p < 0.05$ ). When DIO mice were immunized with a double dose of the HB vaccine (2  $\mu$ g of HBsAg), anti-HBs levels increased to  $47 \pm 12$  mIU/ml, but were still significantly lower than those produced by control mice immunized with 1  $\mu$ g of HBsAg ( $198 \pm 21$  mIU/ml) (Fig. 2A).

#### Decreased HBsAg-specific cellular immune responses of vaccinated DIO mice

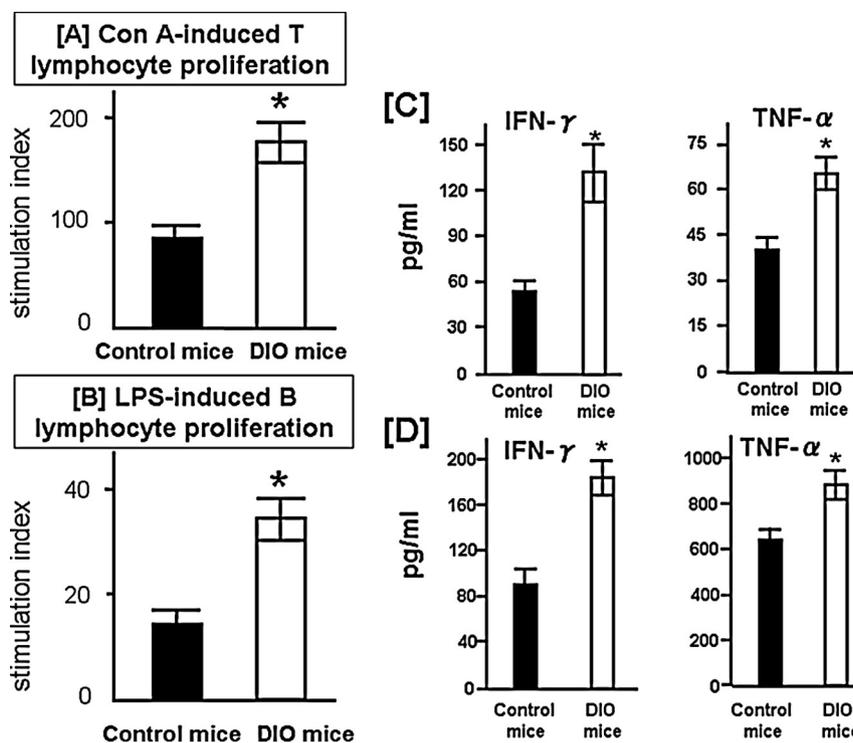
As shown in Fig. 2B, proliferation levels were significantly lower in T lymphocytes from DIO mice than those of control mice ( $p < 0.05$ ). Decreased HBsAg-specific T cell proliferation was also evident irrespective of the HBsAg dose used to stimulate HBsAg-specific lymphocytes in vitro (10  $\mu$ g/ml and 100  $\mu$ g/ml) (Fig. 2B).

#### Suppression of the antigen-specific immune response of DIO mice via MDSC, and an upregulation via CD11b<sup>+</sup>Gr1<sup>-</sup> non-MDSC myeloid cells

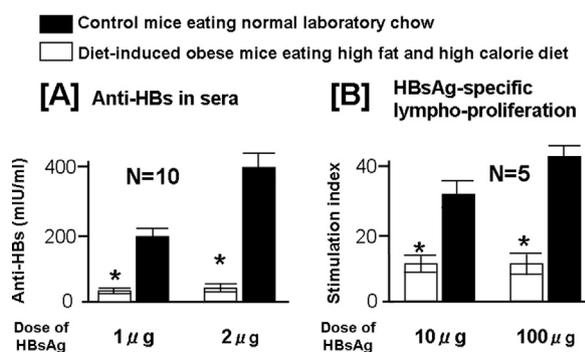
As shown in Fig. 3, liver MDSC from DIO mice significantly suppressed HBsAg-specific lymphocyte proliferation. Conversely, CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells from the livers of DIO mice resulted in an increase of HBsAg-specific lymphocyte proliferation (Fig. 3).

#### Increased immune suppressive capacities of liver MDSC of DIO mice

When the suppressive capacities of liver MDSC of DIO mice and control mice were compared, it was



**Figure 1** Diet-induced obese (DIO) mice fed a high-fat and high-calorie diet for 12 weeks had increased lymphocyte proliferation and higher cytokine production. Liver T and B lymphocytes were stimulated by concanavalin A (Con A, 1  $\mu$ g/ml) and lipopolysaccharides (LPS, 1  $\mu$ g/ml) for 48 h. The proliferation levels of T lymphocytes (A) and B lymphocytes (B) are represented by a stimulation index (SI), as described in the Method. Liver non-parenchymal cells (NPC) were cultured with Con A (C) and LPS (D). Interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  levels were measured in culture supernatants of Con A and LPS-induced T and B lymphocyte cultures. Data are presented as mean and standard deviation (SD) of five separate experiments. \* $p < 0.05$  vs. control mice.



**Figure 2** Diet-induced obese (DIO) mice had decreased humoral and cellular immune responses due to immunization with hepatitis B vaccine containing the hepatitis B surface antigen (HBsAg). (A) Antibody levels against HBsAg (anti-HBs) were measured in the sera of DIO and control mice following immunization with 1  $\mu$ g and 2  $\mu$ g of HBsAg. (B) T lymphocytes proliferation in response to stimulation with 10  $\mu$ g/ml and 100  $\mu$ g/ml of HBsAg were evaluated via T cell proliferation assays. Data are represented by a stimulation index, as described in Methods. Data are expressed as the mean and standard deviation of five separate experiments. \* $p < 0.05$  vs. control mice.

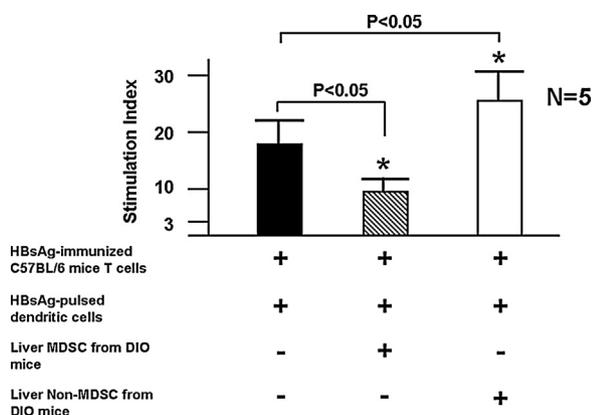
found that the immunosuppressive capacities of liver MDSC from DIO mice (SI of  $9.12 \pm 1.52$  SI) were significantly higher than MDSC of control mice (SI of  $14.3 \pm 1.2$ ) ( $p < 0.05$ ).

### Immune suppression by spleen MDSC from DIO mice

MDSC of spleen also had immune suppressive capacities. The proliferative response of T lymphocytes was  $57.90 \pm 1.97$  SI when these were cultured with Con A. However, addition of 5%, 15%, and 30% MDSC in this culture reduced the proliferative response of T lymphocytes to  $44.80 \pm 1.21$  SI,  $38.60 \pm 4.81$  SI and  $30.13 \pm 2.80$  SI, respectively. However, addition of non-MDSC to Con A-stimulated T lymphocyte culture increased T cell proliferative responses to  $61.80 \pm 2.61$  SI,  $80.75 \pm 5.23$  SI and  $90.83 \pm 3.33$  SI, with 5%, 15% and 30% non-MDSC, respectively.

### Immunosuppressive mechanisms of action of DIO-derived liver MDSC

Representative flow cytometry patterns of intracellular IFN- $\gamma$  on T cells following stimulation with Con

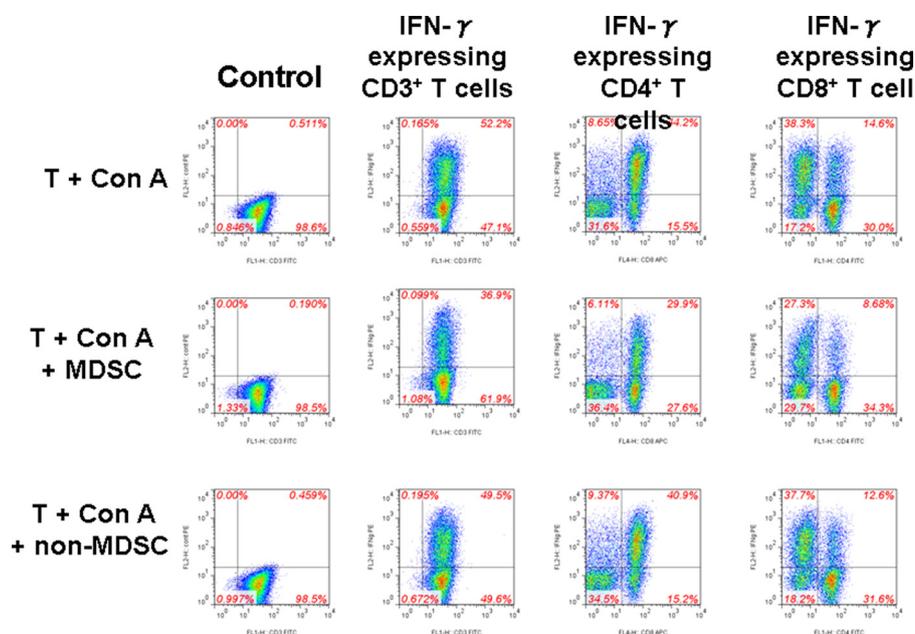


**Figure 3** Suppressed T cell proliferation via liver myeloid-derived suppressor cells (MDSC) and enhanced T cell proliferation via liver CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells (non-MDSC). T cells ( $2 \times 10^5$ ) from the spleens of hepatitis B surface antigen (HBsAg)-immunized C57BL/6 mice and HBsAg-pulsed dendritic cells ( $1 \times 10^4$ ) from the spleens of normal mice were cultured in an antigen-specific lymphoproliferative assay. Liver MDSC or non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells) were added into the cultures. T cell proliferation levels are represented by a stimulation index, as described in the Methods. Data of five separate experiments are shown. \* $p < 0.05$  vs. T cell proliferation levels in cultures without MDSC or non-MDSC.

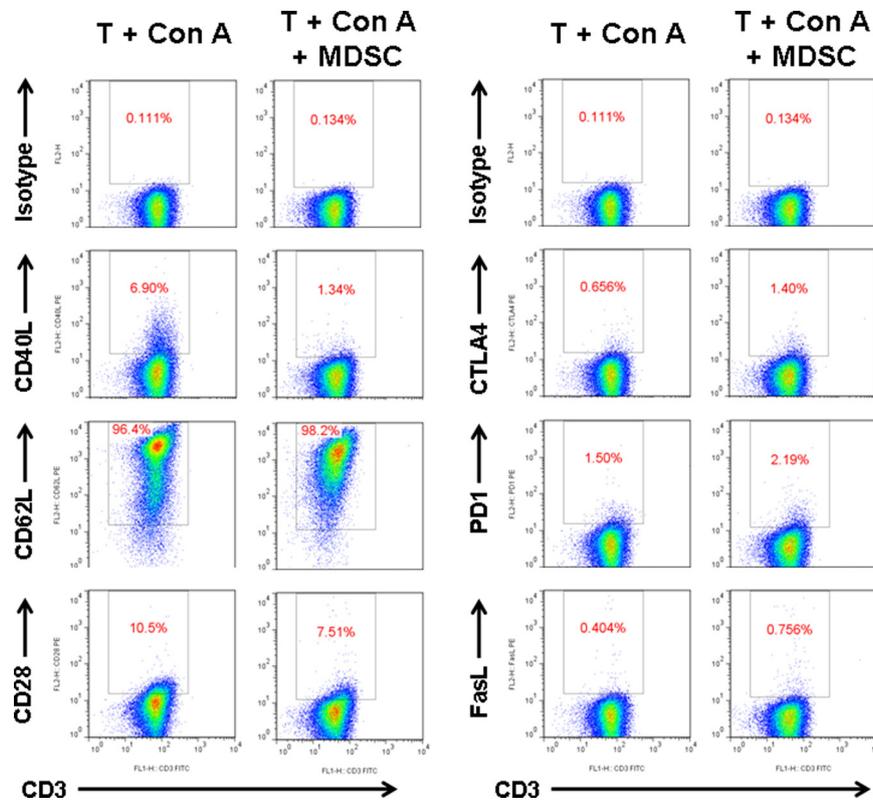
A, and the effects of MDSC and CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells, are presented in Fig. 4. Approximately 52% of spleen T cells expressed IFN- $\gamma$  following stimulation with Con A (upper panel, Fig. 4). However, that proportion decreased to 37% when liver MDSC from DIO mice were added into the culture (middle panel, Fig. 4). Furthermore, the addition of non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells) to T cell cultures did not induce any significant changes in intracellular IFN- $\gamma$  expression on T cells (lower panel, Fig. 4). This phenomenon of reduced intracellular IFN- $\gamma$  expression on T cells was detected in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the presence of MDSC (Fig. 4).

Moreover, MDSC from DIO mice reduced the expression levels of activating markers (CD40L, CD62L and CD28) and increased the expression of exhaustion markers (CTLA-4, PD-1 and FasL) on T lymphocytes (Fig. 5).

Additional mechanisms responsible for the immunosuppressive effects of MDSC were determined by examining the effects of MDSC on T cells apoptosis. When T cells were cultured with MDSC, there was an increase in the proportions of T cells expressing marker of early apoptosis and late apoptosis (Fig. 6). However, T cell cultures with non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells) did not exhibit such increases in the levels of early apoptotic marker.



**Figure 4** Decreased expressions of intracellular IFN- $\gamma$  in CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T lymphocytes in a culture containing myeloid-derived suppressor cells (MDSC). T lymphocytes were cultured with concanavalin A (Con A) and MDSC or non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells), as described in the Methods. Representative flow cytometry patterns of intracellular IFN- $\gamma$  in T cells cultured in Con A and MDSC or CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells are shown. The dot blots in the upper right quadrants reveal cells expressing intracellular IFN- $\gamma$ .



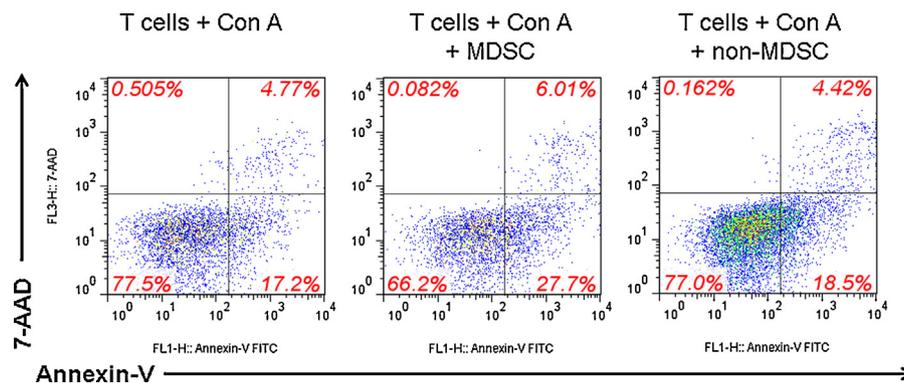
**Figure 5** The effects of myeloid-derived suppressor cells (MDSC) on T cell activation and exhaustion markers. T cells were stimulated with Concanavalin A (Con A) (1  $\mu$ g/ml) for 48 h with or without MDSC. Representative dot plots of CD40L, CD62L, and CD28, CTLA-4, PD1, and FasL antigens on T lymphocytes cultured with or without MDSC are shown.

## Discussion

Mice with DIO are a useful animal model of obesity when different cellular and molecular mechanisms related to obesity cannot be properly assessed in humans. Obesity and its complications have reached epidemic proportions worldwide. Although there is considerable information regarding diet,

the influence of genetic factors, and roles of lifestyle factors on obesity, there is a lack of information regarding the influence of immunological mechanisms in obesity [28–30].

The present study demonstrated that there is an obvious dichotomy regarding the nature of immune responses in obesity. Antigen non-specific and polyclonal immunity, which are proposed to cause



**Figure 6** The proportions of apoptotic T cells increased in cultures containing liver myeloid-derived suppressor cells (MDSC), but not liver non-MDSC (CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells). T cells were cultured with either MDSC or liver CD11b<sup>+</sup>Gr1<sup>-</sup> myeloid cells, and the levels of annexin-V and 7-amino-actinomycin D (7-AAD) expressing T cells were assessed via flow cytometry.

cellular damage and help sustain an inflammatory mucosal milieu, appears to prevail in DIO mice [3,5–7]. This may explain the underlying etiology of NAFLD, diabetes mellitus, and atherosclerosis in obesity. However, herein, we demonstrated that an exacerbated immune response in DIO mice does not influence antigen-specific adaptive immune responses to a vaccine. In this study, a commonly used prophylactic vaccine, HB vaccine, was used. Both the humoral and cellular immune responses of DIO mice were significantly lower in DIO mice compared to control mice (Fig. 2). These findings corroborate that the immune responses to a vaccine is lower in obesity [12–15].

One of the main purposes of the present study was to elucidate the mechanisms underlying the dichotomy in the immune responses in obesity. Critical analyses of T and B lymphocytes clearly found that these immunocytes do not have a direct role in the impaired immunity of DIO mice to the HB vaccine. In one hand, increased production of IFN- $\gamma$  and TNF- $\alpha$  was documented by liver NPC of DIO mice. On the other hand, DIO-derived MDSC induced decreased intracellular IFN- $\gamma$  levels in T cells, decreased T cell activating markers and increased T cell exhaustion markers. Finally, MDSC induced T cells to undergo apoptosis. Liver NPC is composed of various immunocytes. We could not explore the relative contributions of different types of immunocytes in DIO mice. However, non-MDSC population of DIO mice was not immune suppressive. Rather, non-MDSCs of DIO mice seems to be related to immune stimulation status of these mice as they increased T lymphocyte proliferation in Con-A induced T lymphocyte proliferation assays. Also, non-MDSC population of DIO mice did not suppress antigen-specific immunity. It appears exploration of functions of MDSC and non-MDSC populations as well as NK cells, and NKT cells in DIO mice would provide important information as to why increased cytokine production was maintained in spite of immune suppressive activities of MDSC in DIO mice.

This study has shown that MDSC of DIO mice is capable of suppressing HBsAg-specific immunity. An antigen-specific immunity is the final outcome of a complex interaction between T lymphocytes, B lymphocytes and antigen-presenting cells (APC) with antigen in immunological synapse. Although addition of MDSC from DIO mice showed low response to HBsAg-specific immunity, it is not clear if MDSC caused anomaly in antigen uptake, antigen cleaving, and migration of APC or antigen presentation or formation of memory lymphocytes. This study has shown that obesity is related to impaired vaccine-induced humoral and cellular immunity.

Also, it pointed that MDSC is one of the candidate underlying low antigen-specific immunity of DIO mice. Further analysis of all sorts of immunocytes and interaction between immunocytes would need to be explored to get real insights about this complex issue. We have already shown that impaired function of DC, the professional APC, in obesity [15]. However, it remains to clarify if MDSC has any role on DC function in DIO mice. Impaired antigen processing and presentation by DC due to influence of MDSC of DIO mice may provide a clue to this issue.

The role of non-MDSC is also an important variable in the context of immunity in DIO mice. We found that increased T cell stimulation by non-MDSC in antigen-specific cell culture system (Fig. 2) and decreased T lymphocyte apoptosis in cultures with non-MDSC. These observations may be related to inflammatory mucosal milieu of DIO mice. However, in addition to non-MDSC, the role of NK cells and NKT cells in DIO mice should be explored to develop insights about inflammatory mucosal milieu of liver of these mice.

Taken together, this study demonstrated that MDSC have an immunosuppressive role in antigen-specific immunity within the inflammatory mucosal milieu in obesity. Since this study was conducted in mice, it is important to determine the clinical relevance of these findings. Furthermore, it would be interesting to determine how dietary factors induce high proportions of MDSC in DIO mice. Lastly, insight into the cellular mechanisms that inhibit the maturation of myeloid cells and increase the accumulation of these cells in the livers of obese individuals is warranted.

In conclusion, we demonstrated that the immune system and host defense mechanism is complexly regulated by cells from the myeloid lineage in obesity. Further studies on human MDSC and non-MDSC, as well as interactions between the different myeloid cells with other critical regulators of the immune system, would provide more insight regarding the complex immune regulatory mechanisms in obesity.

## Disclosure statement

There are no conflicts of interest to declare.

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