Veterinary Immunology and Immunopathology 202 (2018) 122–129

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Circulating regulatory T cells (Treg), leptin and induction of proinflammatory activity in obese Labrador Retriever dogs

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ARTICLE INFO

Keywords:
Canine obesity
Labrador retrievers
Leptin
Treg
Cytotoxic T cells
Interferon-γ

ABSTRACT

Over-nutrition and obesity have been associated with impaired immunity and low-grade inflammation in humans and mouse models. In this context, a causal role for unbalanced T regulatory cell (Treg)-dependent mechanisms has been largely suggested.

Obesity is the most common nutritional disorder in dogs. However, it is not defined whether canine obesity may influence circulating Treg as well as if their number variation might be associated with the occurrence of systemic inflammation.

The present study investigated the immune profile of healthy adult obese dogs belonging to the Labrador Retriever breed, in comparison with the normal weight counterpart. Indeed, obesity has been described as particularly evident in this dogs. With this purpose, 26 healthy dogs were enrolled and divided into two groups based on body condition score (BCS): controls (CTR: BCS 4–5) and obeses (OB: BCS ≥ 7).

Our data indicate that adult obese Labrador Retrievers are characterised by the inverse correlation between leptin serum concentration and circulating Treg ($CD4^+CD25^{high}Foxp3^+$) levels. In addition, an increased number of cytotoxic T cell effectors ($CD3^+CD8^+$) and a higher IFN-γ production by cytotoxic T lymphocytes were observed in OB group. These results may provide new insights into the immunological dysregulation frequently associated to obesity in humans and still undefined in dogs.

1. Introduction

Canine obesity is the most frequent nutritional disorder in the canine population (German, 2006). Overweight dogs are considered clinically obese when body weight exceeds by at least 15% the optimum weight for body size (Laflamme, 2001). Although the aetiology of obesity is not yet identified, some canine breeds are frequently predisposed. Several factors related to the standard of living and life habits of the industrialized countries can contribute to the development of this nutritional alteration (Gossellin et al., 2007). Recently, obesity has been described as more evident in Labrador Retrievers in reason of a documented genetic predisposition (Raffan et al., 2016; Mankowska et al., 2017).

Similarly to humans, dog obesity can predispose or exacerbate several clinical conditions such as osteoarthritis, respiratory airway distress, renal diseases, diabetes mellitus and metabolic derangements in dogs (Impellizeri et al., 2000; Bach et al., 2007; German et al., 2009; Tvarijonaviciute et al., 2012, 2013).

Some evidence addressed the possible impact of obesity on cardiovascular apparatus in dogs. Mehlman et al. (2013) reported an increased systolic blood pressure and left ventricular free wall thickness in a small number of obese dogs. Pérez-Sánchez et al. (2015) focused on the correlation between obesity and hypertension in a retrospective study, including 139 obese dogs. Their data indicated that obesity does not represent a significant risk factor for hypertension development; rather, this latter condition has to be considered principally related to

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https://doi.org/10.1016/j.vetimm.2018.07.004
Received 26 April 2018; Received in revised form 25 June 2018; Accepted 5 July 2018

co-morbidities, as chronic kidney disease and/or endocrinopathies. In contrast, Piantedosi et al. (2016) recently demonstrated the significant association of canine obesity with cardiac and vascular dysfunctions. In this regard, Tropf et al. (2017) referred that obese dogs showed alterations in cardiac structure and function, associated to insulin resistance, dyslipidaemia, hypoadiponectinemia and increased concentrations of inflammatory markers, as compared with the normal weight counterpart.

It is of note that both the over-nutrition and obesity have been associated with impaired immunity and chronic low-grade inflammation in humans and mouse models (Nieman et al., 1999; Samartin and Chandra, 2001; Berg and Scherer, 2005; De Rosa et al., 2015). Moreover, increased concentration of acute-phase proteins, leptin and other pro-inflammatory cytokines, with reduction of the adipokine adiponectin have been described in obese subjects (Antuna-Puente et al., 2008).

As in other species, several studies demonstrated a significant increase of serum leptin during weight gain in dogs (Sagawa et al., 2002; Ishioka et al., 2002; Jeusette et al., 2005; Ishioka et al., 2007). Moreover, canine obesity has been shown to associate with increase of tumour necrosis factor (TNF)-α concentration (Gayet et al., 2004), while TNF-α reduction has been recently related to weight loss in obese dogs (German et al., 2009). Van de Velde et al. (2012) observed that weight gain and increased body condition score (BCS) were accompanied by significant higher leptin level, IgA and IgM increased concentration, augmented number of lymphocytes and higher response to mitogen stimulation of the peripheral blood mononuclear cells (PBMC) *in vitro*. However, when immune response was evaluated in stable obese condition, no changes in immune functions, neither systemic, low grade inflammation were observed by the same authors (Van de Velde et al. (2013)). Recently, rising level of the pro-inflammatory cytokine IL-6 and of monocyte chemo-attractant protein-1 (MCP-1) have been associated with increasing BCS in Labrador Retrievers (Frank et al., 2015), while decreasing concentration of IL-8 has been related with a weight loss program in dogs (Bastien et al., 2015).

However, the immune profile of dogs that spontaneously develop obesity remains largely unexplored. It is noteworthy that pro-inflammatory response modulation has been observed to depend on Regulatory T cell population (Treg), a $CD3^+CD4^+CD25^+$ T lymphocyte subset characterized by the expression of Foxp3 transcription factor (Sakaguchi, 2005). The inverse correlation between leptin serum concentration and Treg number has been consistently found in humans and mice (Matarese et al., 2002, 2010).

Literature has been suggesting the association between obesity and Treg reduction in visceral adipose tissue (Feuerer et al., 2009; Deiuliis et al., 2011). Conversely, Treg increase in the visceral fat of lean mice has been described; in this model a strong correlation between Treg and anti-inflammatory cytokine production has been observed (Feuerer et al., 2009); similar results have been referred in humans (Wagner et al., 2013). Therefore, a reduction of Treg-dependent anti-inflammatory mechanisms may be involved in the pathogenesis of the pro-inflammatory condition, largely associated with obesity.

In veterinary medicine, Treg significantly increase in canine tumour models (Biller et al., 2007; Horiuchi et al., 2009; O'Neill et al., 2009; Rissetto et al., 2010), while Treg decreasing has been observed in dog chronic infections, as Leishmaniasis (Cortese et al., 2013, 2015). However, the relationship between obesity, leptin and circulating Treg level as well as the occurrence of systemic inflammation in dogs is still unclear.

The aim of the present study is to address the correlation between obesity and immune regulation asset in adult Labrador Retriever dogs, in the light of predisposition of this breed to overweight condition.

Table 1
General characteristics of the Labrador Retriever dog population enrolled in the study. The values of blood arterial pressure and heart rate are also reported.

	Control Dogs (N = 16) BCS 4-5	Obese Dogs (N = 10) BCS > 7
M/F	9/7	2/8
SM/SF[a]	5/4	0/5
AGE (years)	2-8	3-9
Systolic Arterial Blood Pressure (mmHg ± SEM[b])	143.3 ± 9.9	145.8 ± 11.4
Heart rate (bpm ± SEM[b])	116 ± 11	119 ± 19

[a] SM and SF indicate sterilized male and female animals.
[b] SEM indicates standard mean error.

2. Materials and methods

2.1. Animal selection

Twenty-six healthy Labrador Retrievers, 15 females (9 spayed) and 11 males (5 neutered) were recruited into the study from the client-owned referral population of the Veterinary Teaching Hospital, Department of Veterinary Medicine and Animal Productions (University of Naples Federico II). Each enrolled dog was classified according to a body condition score (BCS) assessed by the same investigator, utilizing a nine-point scale BCS system (Laflamme et al., 1997). Ten dogs with a BCS ≥ 7 were considered obese (Tvarijonaviciute et al., 2012), forming the OB group; while 16 dogs with BCS 4–5 were included in the CTR group. Groups were homogeneous by age (dogs younger than 2 years or older than 10 years were excluded). Sex hormones have been suggested to be relevant for immune modulation (Roved et al., 2017). Moreover, similar hormonal background has been observed to underlie both the spayed females and neutered males in dog model (Frank et al., 2003). Thus, in order to ensure the homogeneity of sex distribution in our dog cohorts, we enrolled similar percentage of gonadectomized animals in controls (9/16; 51%) and in the obese cohort (5/10; 50%) in the presence of comparable percentage of intact females (19% in CTR versus 30% in Obese dogs) and males (25% in CTR versus 20% in Obese dogs) in the groups. Both OB and CTR dogs were considered clinically healthy, based on the clinical examination, including a measurement of systemic blood pressure (SBP) and an electrocardiographic exam. Five consecutive measurements of SBP were obtained by the same operator using an automated oscillometric system (HDO, S + B MedVet, Babenhausen, Germany) at the level of the right forelimb of conscious dogs, in sitting position within a quiet room. The highest and lowest values of systolic, mean and diastolic arterial blood pressure were excluded, and the average of the remaining three measurements was recorded. Only dogs with systolic arterial blood pressure (SABP) > 160 mmHg were considered to be hypertensive (Brown et al., 2007). A standard six-lead electrocardiogram (ECG model 08SD, BTL Italy) was conducted with dogs in right lateral recumbency. For each dog a 2 min strip (paper speed: 50 mm/s; calibration at 1 mV0 1 cm) was recorded. All dogs were evaluated for complete blood count (CBC), serum biochemistry and urinalysis.

Exclusion criteria were represented by endocrine diseases (such as diabetes, hypothyroidism, and Cushing's syndrome), hepatic failure, renal failure, heart diseases, inflammatory or infectious diseases, and systemic hypertension. Animals with evidence of para-physiological conditions, such as pregnancy or nursing, were not included. Labrador Retrievers, as unique enrolled breed, were selected for this study in an attempt to limit genetic and breed variability in body condition assessment differences across breeds and because their predisposition to obesity.

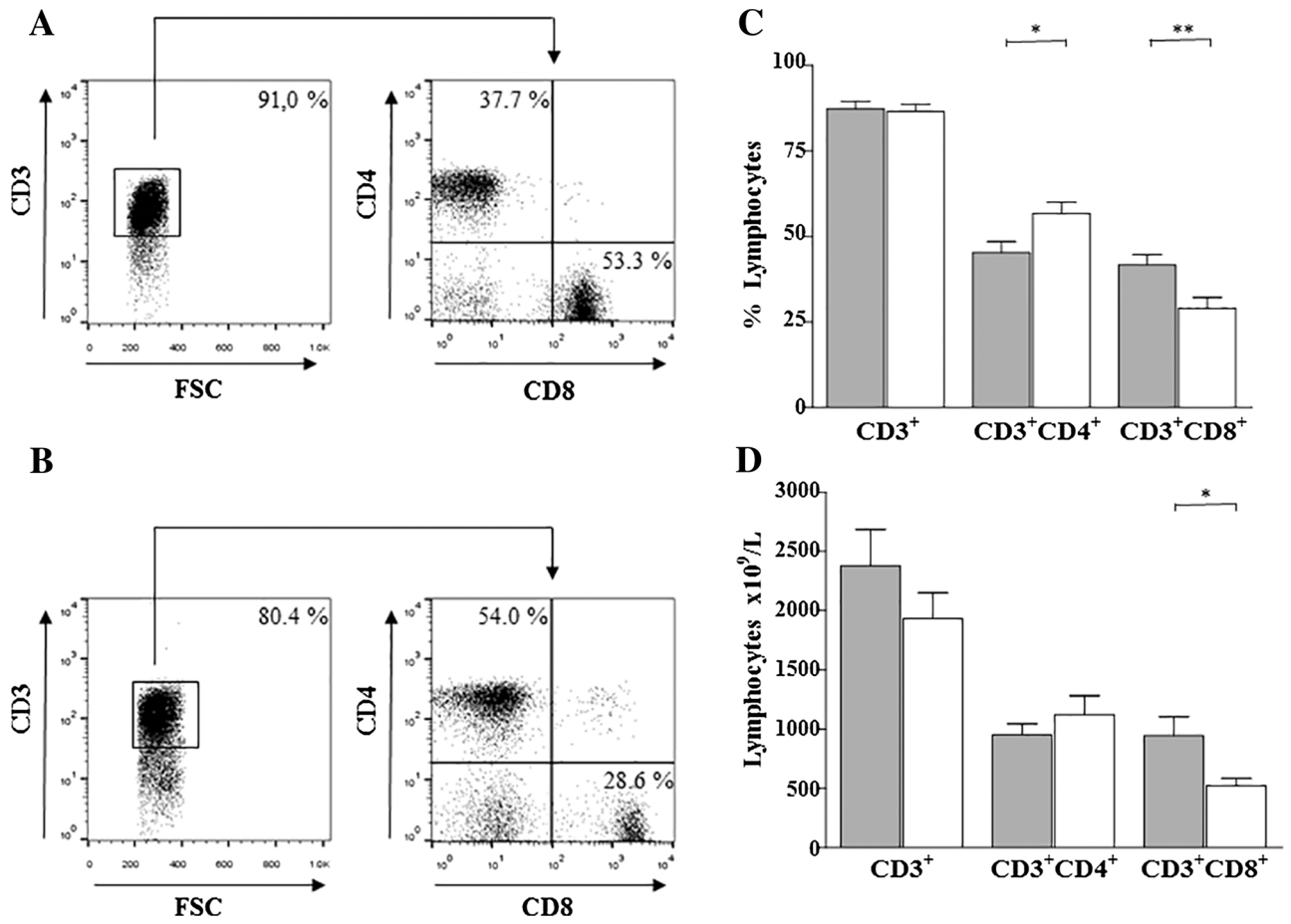

Fig. 1. Increased level of circulating $CD3^+CD8^+$ lymphocytes characterizes obese Labrador Retriever dogs. Panel A and B show flow cytometry analysis of one representative OB and CTR animal. As shown, expression of CD4 and CD8 co-receptors was analysed in the region of CD3 positive cells (identified in the lymphocyte region). Numbers indicate percent of positive cells; Panel C and D show comparative analysis of percentage and absolute number of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes in obese (OB) versus control (CTR) Labrador Retriever dogs; as indicated, significant increase of both percentage ($p < 0.01$) and number ($p < 0.05$) of cytotoxic T cells have been observed in OB dogs, while only a significant decrease in percentage of $CD3^+CD4^+$ has been revealed ($p < 0.05$); grey and white columns indicate OB and CTR dogs, respectively. Error bars indicate the mean ± SEM; * indicates $p < 0.05$; **$p < 0.01$ by two-tailed Mann Whitney test.

2.2. Sample collection

The blood collection procedure was approved by and performed according to the Ethical Animal Care and Use Committee of the University of Naples Federico II, (OPBA, CSV, University of Naples Federico II, prot. n. 2017/0069148). Blood sample collection was cruelty free, without any bloody operation and did not provide for any segregation, even partial, of the animal. A written consent was signed by the owner.

Ten millilitres of blood were collected by jugular venepuncture after 12 h of fasting. The total blood amount was divided into three fractions. The first fraction was placed in tubes containing potassium ethylene diamine tetra-acetic acid (EDTA) for CBC, performed within 30 min from the collection; the second was placed analogously in anti-coagulated tubes containing EDTA, and stored at room temperature up to 5–6 h before immunological assays; the third fraction was placed in tubes without anticoagulant, allowed to clot and centrifuged at 908 *g* for 15 min at 4 °C. Serum samples were stored at −80 °C and defrosted immediately before proceeding with biochemical profile and leptin assessment. Urine samples were collected by cystocentesis.

2.3. Complete blood count and serum biochemistry (CBC)

CBC was performed using a semi-automatic cell counter (Genius S, SEAC Radom Group). A semi-automatic chemical chemistry analyser (OLOT, Spinreact) was used to assess concentrations or activities of glucose, blood urea nitrogen (BUN), creatinine, triglycerides, total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate (ALP), total bilirubin (T-Bil), albumin and total serum proteins (TP). Serum protein electrophoresis was also performed. Urinary protein:creatinine ratio (UP:C) was calculated after their spectrophotometric determination (OLOT, Spinreact).

2.4. Leptin evaluation

Serum leptin concentration in all samples was measured by using a commercial canine-specific leptin ELISA kit (Canine Leptin ELISA Cat. EZCL-31 K, Millipore, Billerica, MA, USA) according to the manufacturer's protocol. The minimum detection limit of the assay was 0.2 ng/mL; intra- and inter- assay coefficients of variation were < 5%. Absorbance was determined using an automated microplate spectrophotometer (Epoch, BioTek Instruments Inc., Winooski, VT, USA) at 450 nm.

2.5. Monoclonal antibodies, immunofluorescence, flow cytometry and cell culture

The level of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, CD4/CD8 ratio, $CD21^+$ B cells and $CD4^+CD25^{high}Foxp3^+$Treg cells was evaluated on peripheral blood samples by immunofluorescence technique and flow cytometry analysis. All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using Forward Scatter (FSC) and

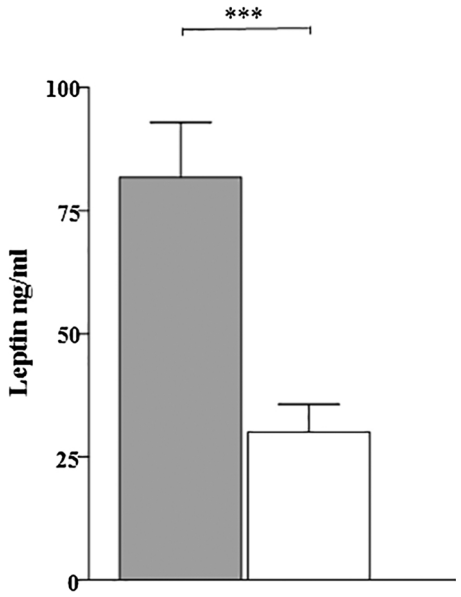

Fig. 2. Significant increase of leptin hormone in serum characterizes obese Labrador Retriever dogs as compared with the normal weight counterpart. As shown, significant difference ($p < 0.001$) in leptin serum concentration has been observed in obese (OB), as compared with control (CTR) dogs; grey and white columns indicate OB and CTR dogs, respectively. Error bars indicate the mean ± SEM; *** indicates $p < 0.001$ by two-tailed Mann Whitney test.

Side Scatter (SSC) parameters. $CD3^+CD8^+$ and $CD3^+CD4^+$ T cell subsets were always identified by a combination of canine specific anti-CD3 together with anti-CD4 or anti-CD8 mAbs on the lymphocyte region. Dead cells were excluded by evaluating FCS and SSC measurements. Indeed, due to their smaller size, dead cells and cellular debris typically have a lower level of forward scatter and are found at the bottom left corner of the dot plot. Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), PE-Cy7 and Allophycocyanin (APC) labelled monoclonal antibodies (mAbs) against dog CD3 (Clone CA17.2A12 and CD3-12), CD4 (Clone YKIX302.9), CD8 (Clone YCATE55.9), IFN-γ (Clone CC302), IL-4 (Clone CC303) and isotype-matched controls were purchased from Serotec Ltd (London, UK). Intracellular detection of Foxp3 was performed by using a cross-reactive murine Foxp3 antibody (Clone FJK-16 s, eBioscience, San Diego, CA) and the permeabilization buffer was provided by the detection Kit (Foxp3 Staining Set, eBioscience). Treg detection was based on the $CD3^+CD4^+CD25^+$ and Foxp3 staining FACS strategy, as described (Biller et al., 2007; Cortese et al., 2013; Alfinito et al., 2010). Specifically, Treg cells were identified as the high CD25 expressing $CD4^+CD3^+$ population expressing Foxp3 at a percentage > 98%, as described (Baccher-Allan et al., 2001; Alfinito et al., 2010). To analyse the production of IFN-γ and of IL-4, the purified peripheral blood mononuclear cells (PBMC) were cultured overnight in the presence of Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin (Sigma-Aldrich, St. Louis, MO). This approach has been widely indicated for the study of cytokine profile in human and animal models (Cortese et al., 2013). Cells were cultured in RPMI 1640 (Biochrom K.G., Berlin, Germany) supplemented with 5% heat inactivated foetal bovine serum and 2 mM glutamine (Biochrom) at 37 °C in 5% CO_2/95% air. To avoid extra-cellular cytokine export, the cell cultures were incubated in the presence of 5 μg/ml of Brefeldin-A (Sigma-Aldrich, St. Louis, MO), as previously described (Terrazzano et al., 2005; Papadogiannakis et al., 2009). Intracellular staining was performed by using a fixing/permeabilization kit (Caltag, Burlingame, CA) and following the manufacturer's recommendations. In order to optimize the identification of CD4 T cells in the presence of PMA induced down-modulation of CD4 co-receptor, staining with anti-CD4 antibody has been performed on permeabilized cells, thus allowing binding of intracellular CD4 molecules. This strategy has been by us observed to allow optimal detection of CD4 molecules in PMA treated cultures (our unpublished results). Flow cytometry and data analysis were performed by using a two-laser equipped FACSCalibur apparatus and the CellQuest analysis software (Becton Dickinson, Mountain View, CA).

3. Statistical analysis

Statistical analysis was performed by Mann-Whitney test (GraphPad Prism, San Diego, CA, USA). Results were considered significant at $p < 0.05$.

4. Results

4.1. Comparative analysis of clinical and biochemical parameters in normal weight and obese adult Labrador Retriever dogs

Table 1 shows the characteristics of the animal population enrolled in the study. The dogs were considered healthy based on clinical exam and they were not hypertensive (Table 1). The results of metabolic panel have been summarised in Supplementary Fig. 1. As shown, a mild no significant increase in cholesterol and triglycerides serum levels were observed in the OB as compared with the CTR group. There were no significant differences in the other biochemical parameters as well as in haematological and UP:C values between the two groups.

Regarding the ECG results, in the OB group 9 animals showed respiratory sinus arrhythmia (ASR) and only one dog had sinus tachycardia. In two obese dogs, there was evidence of ST segment depression, suggestive of myocardial hypoxia, and only one animal showed features of left ventricular enlargement. In the CTR group all dogs showed the presence of ASR. Average electrical axis was within the normal range in all cases and there were no significant differences in heart rates between the two groups (OB group 119 ± 19; CTR group 116 ± 11). No arrhythmias were found in both groups.

4.2. Cytotoxic T lymphocyte increase characterises obese adult Labrador Retriever dogs, as compared with the normal weight counterpart

In order to investigate the immune profile of the obese adult Labrador Retrievers in comparison with the CTR normal weight counterpart, we first analysed the number of leukocytes, neutrophils and lymphocytes in the cohorts of dogs enrolled in the study. As shown in Supplementary Fig. S2, all the animals, regardless the group belonging to, revealed normal number of the white cell subsets by us analysed. Thus, none significant difference between the OB and the CTR groups was observed.

When the T cell population was evaluated (Fig. 1) a significant increase in both the number (943.4 ± 1161.7 versus 521.7 ± 60.21; $p < 0.05$) and percentage (41.64 ± 2.99 versus 28.85 ± 3.23; $p < 0.01$) of cytotoxic ($CD3^+CD8^+$) T lymphocytes was observed in OB dogs as compared with the CTR counterpart. Accordingly, a significant decrease in the percentage of helper ($CD3^+CD4^+$) T lymphocytes in OB dogs was revealed (45.28 ± 3.22 versus 56.69 ± 3.24; $p < 0.05$). Therefore, significant increase of $CD3^+CD8^+$ lymphocytes seems to characterise our cohort of OB adult Labrador Retrievers as compared with the normal weight dogs.

4.3. Increased leptin serum concentration accompanied by reduced Treg and increased Interferon-γ production by cytotoxic T lymphocytes characterises obese Labrador Retriever dogs as compared with the normal weight counterpart

To investigate on the relationship between leptin hormone levels, obesity condition and immune profile in our dog cohorts, we evaluated the level of leptin in the serum of OB and CTR animals. As shown in Fig. 2, ELISA assay revealed a significant increase of serum leptin in OB

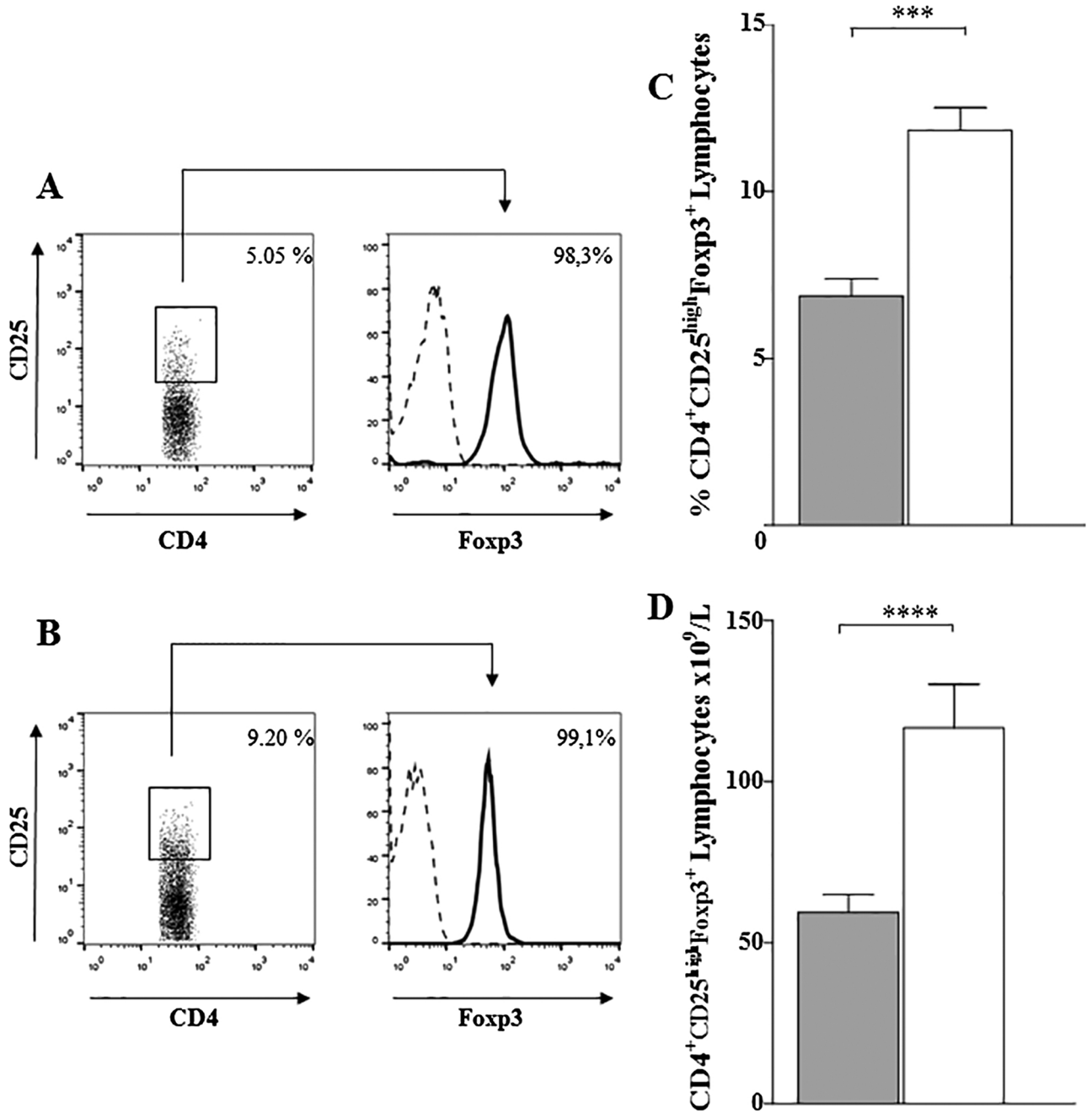

Fig. 3. Obese Labrador Retrievers show significant reduction of circulating regulatory T cells (Treg). Panel A and B show flow cytometry analysis of one representative OB and CTR animal. Expression of CD25 was analysed in the region of CD4 positive cells (identified in the CD3 region). Numbers indicate percent of positive cells; as shown, more than 98% of the CD4 T cells expressing high levels of CD25 are positive for Foxp3 transcription factor; this strategy has been largely described by other and our group in order to identify functional Treg cells (Baccher-Allan et al., 2001; Alfinito et al., 2010). see material and method section for details. Panel C and D show comparative analysis of percentage and number of Treg in obese (OB) versus control (CTR) Labrador Retriever dogs; as indicated, significant decrease of both percentage ($p < 0.0001$) and number ($p < 0.005$) were observed in OB versus CTR dogs; grey and white columns indicate OB and CTR dogs, respectively. Error bars indicate the mean ± SEM; *** indicates $p < 0.005$; **** indicates $p < 0.0001$ by two tailed Mann-Whitney test.

dogs in comparison with the normal weight group (81.72 ± 11.18; ng/ml versus 30.06 ± 5.64; ng/ml; $p < 0.001$).

In addition, we analysed both the number and percentage of Treg subset in OB and CTR adult Labrador Retrievers. Fig. 3 shows that both number (59.43 ± 5.44; versus 116.6 ± 13.5; $p < 0.005$) and percentage (6.8 ± 0.5; versus 11.84 ± 0.67; $p < 0.0001$) of circulating Treg were significantly reduced in OB dogs as compared with the CTR counterpart.

Moreover, to assess whether the observed immunological features of OB animals (high number of $CD3^+CD8^+$ T lymphocytes, increased concentration of leptin hormone and reduced level of Treg cells) might correlate with occurrence of increased pro-inflammatory activity, we analysed *in vitro* IFN-γ and IL-4 production by $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes of obese dogs as compared with the normal weight group. As shown in Fig. 4, percentage of cells producing IFN-γ seems to be increased in both helper and cytotoxic T cell subsets in OB Labrador Retriever dogs, when compared to the normal-weight counterpart. However, such difference reaches statistical significance only considering IFN-γ production by $CD3^+CD8^+$ effectors (55.36 ± 3.55 versus 42.93 ± 4.76; $p < 0.05$). As shown, IL-4 production was likely

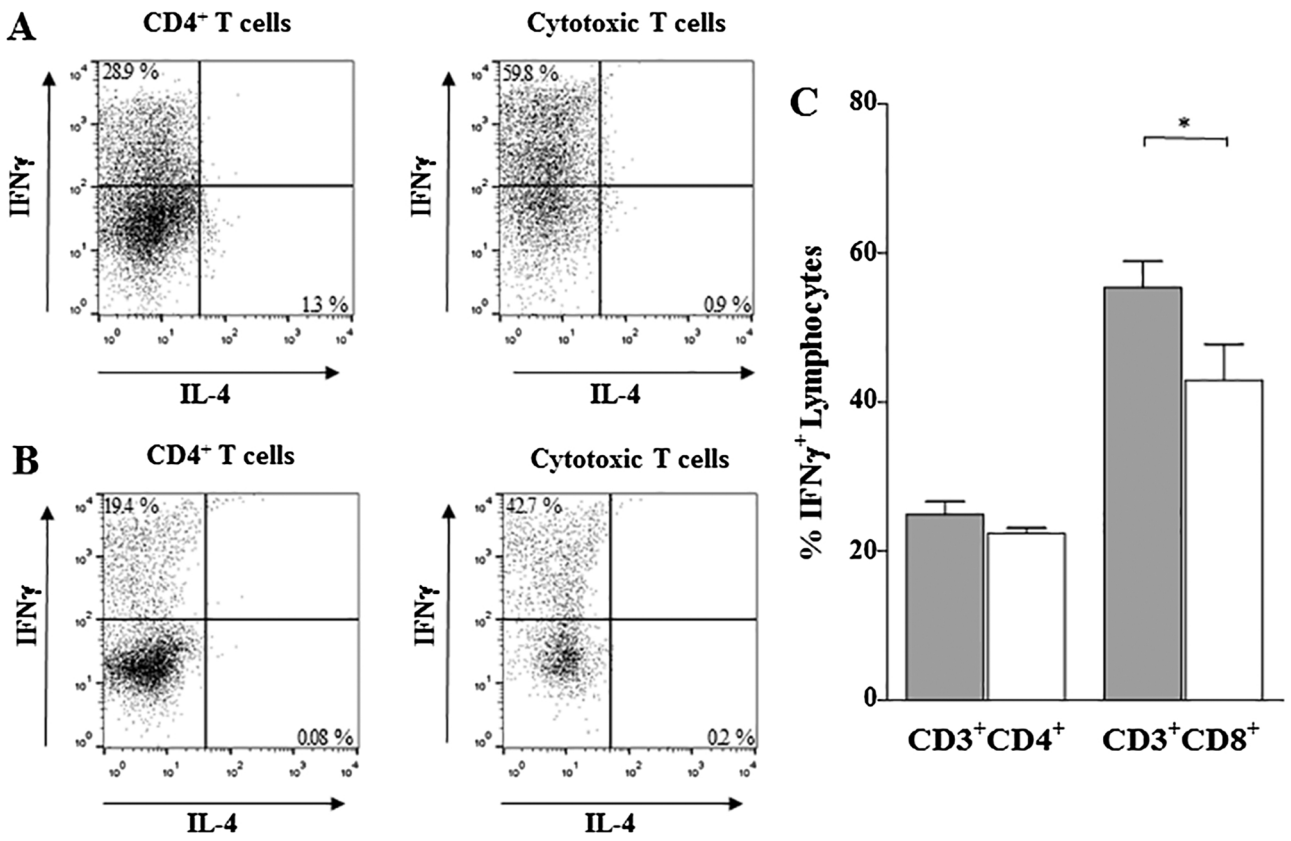

Fig. 4. Cytotoxic T cells of obese Labrador Retriever dogs were observed to produce increased level of Interferon-γ, as compared with the normal weight counterpart. Panel A and B show flow cytometry analysis of one representative OB and CTR animal. Intracellular expression of IFN-γ and IL-4 was analysed in the region of $CD3^+CD4^+$ (left panels) or cytotoxic T cells (right panels) after an ON culture of PBMC in the presence of PMA plus Ionomycin (see material and methods section for details); numbers indicate percent of positive cells; no significant production of IL-4 has been observed.
As shown, significant difference ($p < 0.05$) in IFN-γ production by cytotoxic T lymphocytes *in vitro* has been observed in obese (OB), as compared with control (CTR) dogs; grey and white columns indicate OB and CTR dogs, respectively. Error bars indicate the mean ± SEM; * indicates $p < 0.05$ by two tailed Mann-Whitney test.

undetectable in our experimental condition in both helper and cytotoxic T cell subsets for all enrolled animals, as previously described (Terrazzano et al., 2005; Cortese et al., 2013).

5. Discussion

This study reveals that in adult obese Labrador Retrievers, compared with the normal weight counterpart, high serum leptin levels associate with decreased circulating Treg and increased cytotoxic T cell effectors showing higher *in vitro* IFN-γ production.

Association of obesity with insulin resistance and alterations of cardiovascular system has been largely described in human, mouse and dog models, but the role of a deranged regulation of pro-inflammatory activity in the pathogenesis of obesity-related diseases needs further investigation.

Our obese Labrador Retriever dogs showed total cholesterol and triglycerides values within normal ranges for canine species, although hyperlipidaemia has been frequently described in obese dogs (Peña et al., 2008; Park et al., 2015). In this context, several studies (Tvarijonaviciute et al., 2012; Piantedosi et al., 2016) refer that only a sub group of obese animals can be considered to be affected by *obesity-related metabolic dysfunction* (ORMD), characterised by simultaneously presence of at least two of the following parameters: triglycerides > 200 mg/dL, total cholesterol > 300 mg/dL, glucose > 100 mg/dL and SABP > 160 mmHg. Moreover, no data are available on the pro-inflammatory activity regulation in obese dogs not affected by ORMD. Here, we specifically focused such issue by analysing the phenotypical and functional immune profile of adult obese Labrador Retrievers unaffected by ORMD. These animals were characterized by high serum leptin levels, decrease of circulating Treg and increase of cytotoxic T cell effectors highly producing *in vitro* IFN-γ when compared with the normal weight counterpart. Thus, a derangement in the regulation of pro-inflammatory activity *in vitro* seems to characterize obese subjects in the absence of clinical alterations.

Notably, the observed immunological alterations in obese animals were associated with significant increase of serum leptin, the adipokine largely demonstrated to have the unique ability to modulate both, energy metabolism and immune response (De Rosa et al., 2017). Several data consistently indicate that leptin, a hormone produced by the adipose tissue, fosters experimental autoimmune encephalomyelitis (EAE) in mice by modulating Treg dependent tolerance control (Lord et al., 2002; De Rosa et al., 2006). The hypothesis that leptin levels, in the presence of a susceptible genetic background, might sustain the occurrence of immune-mediated disorders has been also proposed (De Rosa et al., 2006; Iikuni et al., 2008).

Our data highlighted the inverse correlation of leptin concentration with circulating Treg number in adult obese Labrador Retrievers. In these obese dogs, we revealed a significant increase of cytotoxic T cell effectors, highly producing IFN-γ *in vitro*. Therefore our data confirm and extend the results obtained in human and mouse model, suggesting the key role of leptin (largely produced by adipocytes) in regulating Treg level and pro-inflammatory response also in dogs. Of note, no significant effect of age, sex and breed have been described for leptin concentration in dogs (Ishioka et al., 2002, 2007).

Literature indicates that cytotoxic $CD8^+$ T cells may contribute to the mechanisms by which the established risk factors (arterial hypertension and metabolic derangements) promote cardiovascular alterations in humans and mice. Hypertension has been observed to

increase activated $CD8^+$ T cell numbers in human subjects (Youn et al., 2013; Itani et al., 2016), thus likely favouring perivascular inflammation and the following endothelial dysfunction (Itani et al., 2016; Mikolajczyk et al., 2016). Moreover, current evidence suggests that both athero-protective and pro-atherogenic $CD8^+$ T cell subsets exist. Indeed, $CD8^+$ T cells may contribute to the genesis of apoptotic cells and necrotic cores in atherosclerotic lesions and macrophages can be target cells for cytolytic $CD8^+$ T cells in atherosclerosis (Itani et al., 2016; Mikolajczyk et al., 2016). In this context, it is still unknown if numbers of $CD8^+$ T cells might correlate with their functional contribution to atherosclerosis, or whether a certain cytokine profile might contribute to shape $CD8^+$ T cell behaviour in lesion formation/progression.

Overall, our data suggested that a deranged immune-regulation, combined with enhanced pro-inflammatory responses, might characterize obese adult Labrador Retrievers in the absence of clinical and metabolic alterations. It is of note that immune-dysregulation occurrence could highlight an increased risk to develop cardiovascular disease and metabolic complications related to increased body weight. In this regard, a limit of our study is the absence of a prospective clinical evaluation to support the prognostic relevance of immune derangement in obese Labrador Retrievers. Future clinical studies and/or diet regimen approaches could be useful to ascertain the relation between obesity, the occurrence of inflammatory conditions and cardiovascular and metabolic complications in dogs.

In conclusion, these results may represent new insights into the immunological dysregulation frequently associated to obesity in humans and still undefined in dogs.

Conflicts of interest

None of Authors of this study has financial or personal relationships with other people or organisations that could inappropriately influence or bias the content of the paper.

Authorship

ATP, VR, AG and VP performed the research, analysed the data and contributed to write the paper; DP, LC and JG participated in the clinical management of the dogs, analysed the data and wrote the paper; GR, LC and GT designed the research study, analysed the data and wrote the paper.

Acknowledgments

We have to thank Giuliana Agresti, Francesca Ciotola, Giovanna Fiorillo, Beatrice Franzese, Roberta Lucà, Francesca Menna, Barbara Priore, Antonio Rubino and Antonio Zotti for their cooperation; we are also in debt with Gennaro Chierchia, Ciro Laperuta, Paolo Muzj and Antonio Sica for their technical assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2018.07.004.

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