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A study of immune tolerance control in physiological models and in immune-mediated diseases.

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Abstract

Immune response is based on a complex molecular and cellular network able to ensure protection against pathogens and simultaneously maintain tissue homeostasis. Multiple immunoregulatory processes are physiologically involved in preventing potentially deleterious immune reactions against *self* tissues. The key role of regulatory immune cell populations, as represented by $CD4^+CD25^+Foxp3^+$ Treg cells, in induction and maintenance of immunological tolerance has been largely demonstrated.

Aim of this study is to investigate on cell-dependent control of immune response in physiological conditions as well as in the context of immune-mediated diseases, also addressing the possibility to modulate deranged immune effectors.

With this purpose we focused: i. a human model of autoimmunity, as represented by a subgroup of patients affected by Myelodysplastic Syndrome (MDS), a hematological disorder characterized by immune-mediated selection and expansion of pathological stem precursors; ii. human and animal models of pharmacological and metabolic immune-modulation; iii. the functional analysis of a T cell population, characterized by the co-expression of CD3 and CD56 molecules, whose quantitative defect has been observed in autoimmune diabetes.

Immune-response has been largely recognised as a finely tuned micro-site process. Thus, the possibility to focus cell-mediated immune tolerance control in Bone Marrow (BM), the microenvironment in which immune-mediated selection of pathological stem precursor takes place, represent a powerful analysis tool to investigate on MDS pathogenesis. Our study of BM T cell repertoire revealed an inverse correlation between BM Treg levels, activation status and BM clonal expansion of $CD8^+$ T lymphocytes in MDS patients. Thus, BM Treg were proposed to represent a key element for the control of the deranged immune effectors in an inflammatory microenvironment.

Cross talk between immune response and metabolism is still largely undefined. Particularly, Treg availability has been observed *in vitro* to specifically depend on the oscillatory activity of the mammalian Target Of Rapamycin (mTOR), a Serine/Threonine kinase playing a key role in regulating cell growth and metabolism in response to nutritional cues. The employment of mTOR pharmacological inhibition for the control of tumour cell growth has been largely described. We found that dosage and administration schedule of the mTOR inhibitor Everolimus, able to ensure mTOR oscillatory activity, is relevant to induce immune-tolerance rather than inhibition of cell growth in a model of tolerance induction, as represented by allogeneic kidney transplant.

Our study also addressed the possibility to use nutraceutical compounds, by us selected for their immune-modulating effects in a veterinary model of chronic infection, to control immune effector activity *in vitro*. Our data are conceivable with the possibility to employ these substances as pharmacological co-adjuvants to modulate pro-inflammatory activity in contexts of altered immune homeostasis.

Co-expression of CD3 and CD56 molecules identifies a lymphocyte population whose functional activity is largely undefined. A severe reduction of this cells has been associated with the extent of β-cell loss in patients affected by type 1 diabetes. We found that $CD3^+CD56^+$ lymphocytes, by us named $T_{R3\text{-}56}$, represent a distinct subgroup of T lymphocytes, able to preferentially modulate effector function of cytotoxic T cells. Indeed, the co-culture of $T_{R3\text{-}56}$ with $CD8^+$ effector cells mediates significant inhibition of their cytotoxic activity and IFN- γ production. No effects were observed when cytotoxic T cells were cultured with NK, CD4 or CD8 T lymphocytes. A contact-dependent mechanism has been observed to underlie immune-modulating activity of $T_{R3\text{-}56}$ cells.

A better knowledge of cell mediated processes involved in immune-tolerance control is expected to significantly improve the availability of innovative immune-modulating strategies, thus ameliorating clinical management of immune-mediated disorders.

1. Introduction

1.1 The immune system

The immune system represents a complex network of molecular and cellular mediators ensuring effective defense against pathogens together with tissue homeostasis maintenance. It is organized in two compartments, *innate immunity* and *adaptive immunity,* that coordinate their activity in order to maximize immune functions.

The innate immunity represents the first defence-line of both vertebrate and non-vertebrate organisms. It is based on the availability of a molecular machinery able to directly recognize and consequently destroy potentially harmful agents as well as damaged *self* tissues. Indeed, a series of molecular structures, (TLR, NOD, RIG) enable innate immune effectors to rapidly recognize the Pathogen Associated Molecular Patterns (PAMP), representing molecular targets specifically expressed by foreign, potentially invading organisms. In addition, Natural Killer cells evaluate cell integrity trough a complex repertoire of surface receptors. Thus, the possibility to directly recognize a dangerous diversity inside tissues is the basis for molecular and cellular innate defense mechanisms. Direct induction of microrganism-lysis (Complement), phagocytosis, as well as apoptosis induction of infected or damaged autologous cells are the main mechanisms employed by innate immune compartment [(1)].

The adaptive immune response (Figure 1), also called acquired, is highly specific and is able to "remember" and to respond more effectively to repeated exposure to a specific pathogen. Its recognition strategy involves a high variable repertoire of receptors generated by clonal gene rearrangement strategies; these receptors are able to recognize a wide range (billions) of molecular specificities, but are unable *per se* to distinguish *self* and *non-self* structures [(2)].

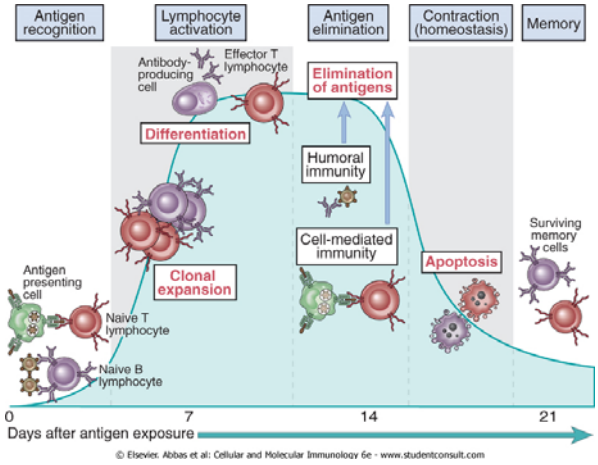

Figure 1: General organization of adaptive immune response. From Abul K. Abbas, Cellular and Molecular Immunology, Elsevier Inc.

The adaptive immunity can be distinguished in humoral immunity, mediated by antibody, and cell-mediated immunity, dependent on helper ($CD4^+$) and cytotoxic ($CD8^+$) T lymphocytes. Helper T cells (T_H) are a key element for the orchestration of immune response; indeed, they are able to improve phagocytic activity of innate cells and to optimize humoral and cytotoxic functions. Moreover, their activity is plastically dependent on the differentiation of peculiar cytokine production profile (Figure 2), usually defined as *proinflammatory* (T_H1, T_H17) or *non-inflammatory* (T_H2). The T_H1 response is characterized by the production of Interferon-gamma (IFN-γ) which optimizes the bactericidal macrophages capability, induces pathogen-opsonization and optimizes cytotoxic T lymphocyte (CTL) response. The T_H2 response is characterized by the release of Interleukin-4, IL-5, IL-10, IL-13 which results in the activation of B cells to make neutralizing non-cytolytic antibodies, leading to "*humoral non-inflammatory immunity*" [(3)].

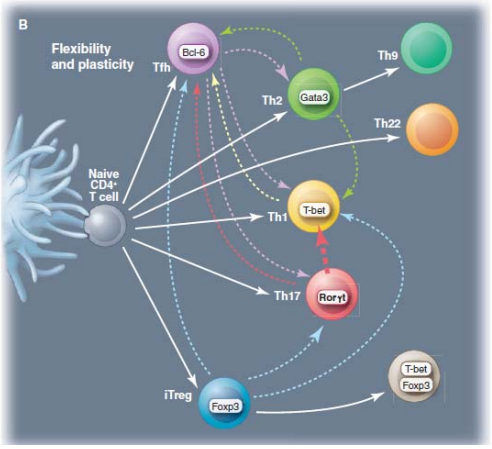

Figure 2: Plasticity of cytokine profile acquirement by helper CD4 T cells. O'Shea &W. Paul, Mechanisms Underlying Lineage Commitment and Plasticity of Helper $CD4^+$ T Cells. *Science* 2010; 327:1098-110.

T lymphocytes are unable to recognize native antigens. Indeed, only peptides, expressed on the surface of Antigen Presenting Cells (APC) in association with Major Histocompatibility Complex (MHC) molecules, are specifically recognized by the T Cells Receptor (TCR).

1.2 The T cell receptor (TCR)

The TCR is a membrane glycoprotein constituted by two polypeptide chain, called α and β chain, each showing a constant (C) and a variable (V) region. The variable region has three handles, called Complementary Determining Regions (CDR), representing the hypervariable regions that form the binding site for the antigen-MHC complexes.

The possibility of T lymphocytes to recognize a wide number of specificities is due to TCR diversity. It is generated, on clonal basis, during T lymphocytes development thanks to the presence

of RAG recombinase enzymes. Gene rearrangement of V and J genes for α chain variable region and of V, D, and J genes for β chain variable region are key events for TCR generation [(1)].

Each T clone expresses only a TCR type. Therefore, the TCR, that marks a single T clone, is characterized by the presence of the particular V gene segment used to build the receptor during the gene rearrangement processes [(1)]. When a T cell clone recognizes a foreign substance it undergoes a clonal expansion.

The TCR ligands are represented by the complex of antigenic peptide-MHC molecule expressed on APC cells. The MHC molecules are divided into two classes, the MHC class I and MHC class II that are recognized by different T cells. The cytotoxic lymphocytes bind endogenous antigens associated to MHC class I molecules; instead, the helper T cells recognize, in association with MHC class II, exogenous antigens. This recognition strategy, called restriction, allows T cells to obtain specific information about pathogen intracellular localization. Indeed, MHC Class I molecules preferential binds intracellular molecules, while MHC Class II antigens are mainly associated with extracellular antigens [(2)].

The TCR-binding to MHC-ligand complex is usually insufficient to achieve antigen-dependent naïve T cell activation. In order to allow adaptive response, a second signal (*co-stimulation*), generally delivered by innate compartment after recognition of microbial molecules, is able to place the TCR-recognized antigen, in a *danger* frame. Binding of CD28 molecule, expressed by T lymphocytes, with CD80/CD86 costimulatory molecules, expressed by APC, represents the main second signal delivering system, able to avoid harmful T cell activation against autologous, non-dangerous targets [(2)].

1.3 The Immune Tolerance

Due to inability of adaptive recognition repertoire to distinguish self from non-self/dangerous structures, a complex network of cellular and molecular mechanisms usually controls physiological adaptive immune response (Figure 3). Particularly, we define *central* immune tolerance the selection processes of adaptive repertoire taking place in primary lymphoid organs, thymus for T lymphocytes and bone marrow for B lymphocytes [(1)]. Moreover, a number of redundant mechanisms, belonging to peripheral tolerance control, usually provide to inactivate the auto-reactive T and B cell clones that, despite central selection, are physiologically present in the adaptive repertoire.

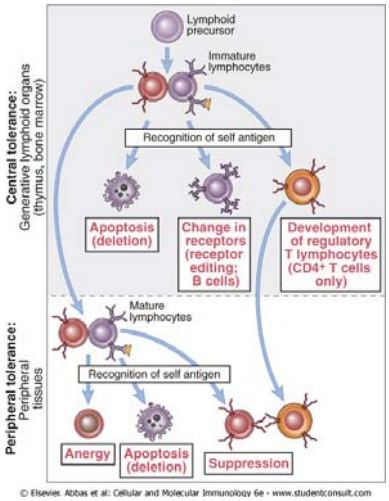

Figure 3: A scheme of immune tolerance mechanisms. From Abul K. Abbas, Cellular and Molecular Immunology, Elsevier Inc.

A key role in peripheral tolerance control is played by regulatory cell populations mainly represented by the Treg subset, characterized by the expression of the fork head box protein 3 (Foxp3) Transcription Factor [(1)].

1.4 Regulatory T cells (Treg)

Treg are a subpopulation of $CD4^+$ T lymphocytes (generally, 5-10% of CD4 population) expressing high levels of the α chain of the interleukin-2 receptor (IL-2R or CD25) together with Foxp3 [(4)]. Treg originate in thymus (natural Treg o nTreg) or in tissue by differentiation of $CD4^+$ T cells in a microenvironment characterized by high levels of Transforming Growth Factor (TGF)-β, in the absence of IL-6 production. Survival and function of Treg is dependent on the presence of IL-2 [(4)]. Indeed, expression of high affinity IL-2R on their surface and signalling through IL-2R are required for optimal Treg function. Treg act with different mechanisms: direct inhibition of APC cells, secretion of anti-inflammatory cytokines (IL-10 and TGF-β), inhibition of effector cells by direct contact, induction of T cells death by deprivation of IL-2, their growth factor. Moreover, Treg cells have been observed to regulate tissue homeostasis, also affecting neo-angiogenesis processes [(5)]. Depletion of Treg results in development of autoimmune disorders [(6)]. Thus, the control of Treg availability and function has been largely suggested as a critical element for fine tuning of immune response.

1.5 Metabolism and immunity

Recent observations indicate that nutrient availability influences immune system functions. Indeed, nutrient deprivation has been associated with defects in adaptive immunity activation and

epidemiological studies refer the failure of immunological responsiveness in hypo-nutrition conditions. Instead, high caloric intake in industrialized countries has been associated with reduced infections and increased risk of autoimmune disorders [7,8]. Moreover, adipose tissue has been observed to produce pro-inflammatory cytokines and adipocytokines that favor activation of pro-inflammatory immune cells including those with an autoreactive potential [9-11]. In this contest, a key role is performed by the mammalian Target of Rapamycin (mTOR), the main intracellular nutrient sensor [12] (Figure 4). It is a serine-threonine kinase that regulates cell growth, glycolysis and nucleotide synthesis.

A number of data indicate that effector T lymphocytes and Treg are characterized by distinct metabolic profiles [13]. Indeed, effector T cells contain inactive mTOR, unable to sustain their growth, while active mTOR molecules, present in Treg, underlie their proliferation *in vivo*. These observations are conceivable with the opposite effects exerted *in vitro* by pharmacological mTOR inhibition of T cell effectors or Treg [14]. Indeed, the mTOR inhibitor rapamycin is able to inhibit effector T cells growth, while restoring TCR-dependent Treg proliferation [15,16].

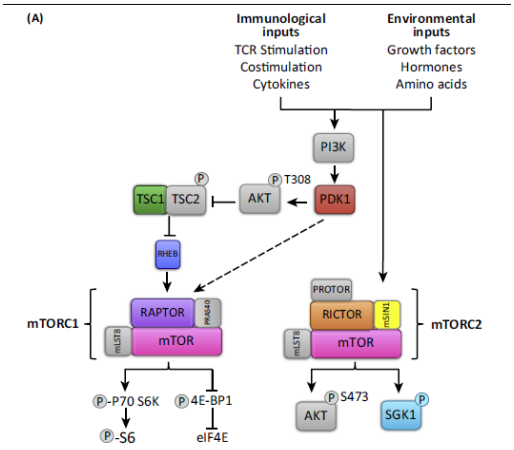

Figure 4: A simplified model of mammalian target of rapamycin (mTOR) signaling. From Pollizzi and Powell. Regulation of T cells by mTOR: the known knowns and the known unknowns. *Trends in Immunology* 2015; 36:13.

Thus, the activation of mTOR represents a key environmental signal for the plasticity of adaptive cells that may use metabolic pathways to finely tune their fate and function. In particular, the intracellular metabolic balance of Treg, strictly dependent on extracellular environment, regulates proliferation or quiescence of these cells highlighting the importance of metabolism for immune system control [13].

The standard treatment for kidney transplantation is currently represented by calcineurin inhibitors (CNI) whose chronic use can cause cardiovascular disease, as well as graft dysfunction and malignant tumors [17-19]. Pharmacological mTOR inhibition might represent a valuable

therapeutic alternative for effective immune modulation in this condition. A number of data refer the use of mTOR inhibitors (Rapamicin and/or Everolimus) to treat solid and hematological malignancies. The possibility to establish specific schedule and administration strategies in order to obtain immune modulation instead of cell death, represents a key investigation issue.

1.6 Nutraceutical compounds and pro-inflammatory activity control *in vitro*.

A deranged regulation of the immune system represents a key element for the pathogenesis of immune-mediated diseases [20,21]. In particular, the exacerbation and endurance of T_H1 response, based on a pathological production of IFN-γ, has been largely associated with inflammatory and autoimmune diseases [22,23]. Several studies have been suggesting the anti-inflammatory and antioxidant properties of some botanicals [24-28]. Moreover, previous studies evidenced the immune-modulating effects of a nutraceutical diet in dogs affected by *Canine Leishmaniosis,* a model of natural chronic infection [29]. In this context, the evaluation of *in vitro* effects of the botanical substances, contained in the nutraceutical canine food on cytokine production by human and animal lymphocytes, represents an interesting field of investigation to provide useful information about the possibility modulate human inflammatory immune response by using metabolic tools.

1.7 Myelodysplastic Syndromes: a model to study deranged immune effectors inside an inflammatory microenvironment.

Myelodysplastic Syndromes (MDS) are clonal haematological disorders characterised by emergence, dominance and expansion of dysplastic progenitor/s in the context of ineffective haematopoiesis, peripheral cytopenia/s and increased risk to develop Acute Myeloid Leukaemia (AML).

The pathogenesis of the disease is not still well defined; cytogenetic and molecular abnormality as well as an altered medullar microenvironment are involved in the selection and clonal expansion of the dysplastic precursor/s [30,31]. Several data have been suggesting the involvement of an altered immune tolerance control in MDS pathogenesis [32]. Indeed, an autoimmune attack to normal Bone Marrow (BM) precursors by deranged adaptive effectors as well as the activity of bystander T cells, recruited during an immune-response against dysplastic antigens, can be hypothesised to be relevant for the selection of dysplastic clones that are able to escape to immune-mediated damage.

The study of the Treg cells, involved in negative control of immune response, in MDS patients suggests that these cells can play two opposite pathogenic roles [33,34]. Indeed, functional defects and altered BM migration of Treg in the first phases of the disease and a Treg increase in the late

stages of MDS have been consistently found [33-35]. These data support the hypothesis that in the advanced stage of the disease, the increase of the regulatory cells might promote the suppression of the immune response against the dysplastic clones fostering AML progression while, in the early stage of MDS, Treg defects can enhance activity of cytotoxic immune effectors against normal BM precursors, favoring selection of the dysplastic clone/s. Therapeutic efficacy of immune-suppressive drugs (mainly anti-T lymphocyte sera with or without Cyclosporin A) in a group of MDS patients [36,37] strongly supports such hypothesis. The possibility to correctly identify the subgroup of MDS patients susceptible to immune-modulating therapy represents a key element to optimize clinical management of the disease. In this context, the analysis of the Treg role in the inflammatory microsite, the BM, is a key element to propose immune-modulating strategies able to control the pathological selection of dysplastic stem precursors in MDS.

1.8 Co-expression of CD3 and CD56 molecule as a marker of a new regulatory T cell subset.

The key role of deranged cytotoxic effectors in the pathogenesis of immune-mediated disorders has been largely recognized [38]. Together with the classical regulatory cells, represented by the $CD4^+CD25^+Foxp3^+$ (Treg) cells, a number of experimental data point to the involvement of other regulatory, less characterized, regulatory cell subsets in tolerance maintenance. In this context, the involvement of $CD8^+$ T regulatory cells, whose role and phenotype features are still undefined [39,40], has been consistently postulated. Moreover, it is not clear whether different $CD8^+$ regulatory T cells represent an independent T subset or if they reflect the dynamic plasticity of a single population during immune response.

We observed that co-expression of CD3 and CD56 molecules identifies a T cell subset significantly reduced in type I autoimmune diabetes. Moreover, we observed significant positive correlation between the number of $CD3^+CD56^+$ cells and the β-cell residual function [41]. Considering that auto-reactive $CD8^+$ cells represent the main T cell subset mediating disruption of insulin-producing β-cells in T1D, we investigate whether $CD3^+CD56^+$ cells are able to control $CD8^+$ T cells functions. The referred correlation of this cell subset with viral persistence in hepatitis [42,43] and with the positive outcome of *in vitro* fertilization approaches [44] strongly supports such hypothesis.

2. Aim

This study is aimed to investigate on cell-dependent mechanisms of immune response control in physiological conditions as well as in the context of immune-mediated diseases.

In order to address such issues, we investigated a human model of autoimmunity, as represented by a subgroup of patients affected by MDS, a hematological disorder characterized by immune-mediated selection and expansion of pathological stem precursors. In this model, the possibility to directly analyse cell-mediated tolerance control in the microsite of the deranged inflammation, is expected to represent a powerful tool to identify the key regulatory mechanisms to be hopefully targeted by effective immune-modulating strategies.

Recent observations indicate that the availability of nutrients and the possibility to modulate metabolic pathways might influence immune system regulation and functions. In particular, *in vitro* studies consistently indicate that activation status of mTOR, the main cell nutrient sensor, represents a key environmental signal for the plasticity of adaptive cells and that changes in metabolic pathways might be used to finely tune their fate and function.

The employment of mTOR pharmacological inhibition for the control of tumour cell growth has been largely described. However, *in vitro* data indicate that oscillatory mTOR inhibition is critical to ensure preferential Treg expansion. Thus, we asked whether dosage and administration schedule of mTOR inhibitors, able to ensure mTOR oscillatory activity, might be relevant to induce immune-tolerance, rather than cell growth inhibition in a model of tolerance induction as represented by kidney allogeneic transplant.

Moreover, our study addressed the possibility to use nutraceutical compounds, by us selected for their immune-modulating effects in a veterinary model of chronic infection, to control immune effector activity *in vitro*.

Control of deranged cytotoxic effectors is a key therapeutic target in immune-mediated disorders. In this context, the involvement of multiple cell-dependent mechanisms of immune modulation has been largely demonstrated. Here, we addressed the functional analysis of a poorly defined T cell subset, characterized by the co-expression of CD3 and CD56 molecules, recently associated with the residual pancreatic function in type I autoimmune diabetes.

A better knowledge of cell mediated processes involved in immune-tolerance control is expected to significantly improve the availability of innovative immune-modulating strategies, thus ameliorating clinical management of immune-mediated disorders.

3. Materials and methods

3.1 Immune dysregulation and Myelodysplastic Syndromes

Patients and controls

In collaboration with the Divisione di Ematologia dell'Università "Federico II" di Napoli, we examined BM and peripheral blood (PB) samples of 37 consecutive, newly diagnosed MDS patients categorised according to WHO 2016 and IPSS score. Twenty-six have been classified as Low Risk, six as Intermediate-1 (Int-1) Risk, five as Intermediate-2 (Int-2) Risk/High Risk. BM and PB samples from MDS patients were obtained during routine diagnostic procedures previous informed consent from each patient. None of the recruited patients were receiving medical treatments that could have an impact on their immune condition. To avoid any interference on immune-regulatory mechanisms, patients were devoid of immune-mediated diseases and acute or chronic viral infections. All the patients enrolled in the study received a minimal 36-month clinical follow up.

For comparative analysis of T cell repertoire, 10 PB and 3 BM samples of healthy donors have been collected. BM specimens, obtained from consenting healthy donors, were part of their marrow donation.

mAb, immunofluorescence and flow cytometry

Lymphocyte population has been gated by using FSC and SSC parameters, as well as CD45 labelling. FITC, PE, Cychrome and APC labelled mAb against CD3, CD4, CD8, CD56, CD25, CD45 and CD54 have been used to the identification of immune cell subsets and to evaluate their activation status. To study the T cell repertoire have been used mAbs anti -Vβ14, -Vβ12, -Vβ7.2, -Vβ20, -Vβ18, -Vβ7.1, -Vβ22, -Vβ13.2, -Vβ1, -Vβ17, -Vβ5.3, -Vβ5.1 -Vβ23, -Vβ4, -Vβ2, -Vβ13.1, -Vβ5.2, -Vβ8, -Vβ9, -Vβ11, -Vβ3, -Vβ13.6, -Vβ21.3F, -Vβ16. To define a $CD4^+$ and/or $CD8^+$ skewed repertoire, we considered the occurrence of a percentage of expression exceeding of three Standard Deviation (SD) that observed, for each Vβ family analysed, in ten healthy controls sex/age matched with the MDS patient cohort. Occurrence of a skewed BM CD4/CD8 repertoire with an expression frequency higher that 20% respect to peripheral blood (PB) has been considered as a BM preferential skewing. Treg subset was identified as the higher CD25 expressing $CD4^+CD3^+$ population and Foxp3 at a percentage >98%. The Foxp3 expression has been evaluated trough an intracellular staining using the anti-human Foxp3 kit (eBioscience San Diego, USA) and following the manufacturer's instructions. For the comparative analysis of CD54 expression level on BM

$CD8^+CD3^+$ and $CD4^+CD3^+$ lymphocytes, immune-fluorescence data were expressed as ratio of mean intensity fluorescence (MIF) value for the $CD4^+$ and $CD8^+$ population and the control MIF value obtained after staining of the same cell population with the isotype control mAb, as previously described [34].

Flow cytometry and data analysis were performed by a two-laser equipped FACScalibur apparatus and the CellQuest analysis software (Becton Dickinson).

Statistical analysis

Statistical evaluation of data, by *InStat 3.0* software (GraphPad Software Inc., San Diego, California, USA), was performed by Mann-Whitney test or Fisher's exact test. Two-sided *p* values less than 0.05 have been considered significant.

3.2 mTOR modulation and tolerance in kidney transplanted patients

Study population

The study was carried out on 19 renal transplant recipients, all first transplant from cadaver donors. Inclusion criteria were: age 18-65 years; transplant vintage >3 years; plasma creatinine <2 mg/dl, with stable estimated glomerular filtration rate (eGFR) in the previous three months; haemoglobin value >10 g/dl; white cell count >3000/μL (neutrophils >1500/μL); platelets >75.000/μL; and absence of rejection signs or infectious episodes in the previous three months. Individuals with previous or combined transplantation, hyperlipidaemia (baseline cholesterol and/or triglycerides values exceeding 220 and 200 mg/dl, respectively) andor evidence of autoimmune diseases or viral infections have been excluded from the study.

Study protocol

At baseline (T0), dosage of CNI was reduced empirically by 50% and the mTORC1 specific inhibitory drug Everolimus was introduced at a starting dosage of 0-50 mg/twice a day (b.i.d). Plasma levels of both drugs were checked after 1 week, and Everolimus dosage was modified opportunely to reach trough levels (TL) of 5–8 ng/ml. After a 4-week stabilization period, CNI dose was further reduced by 25% and finally withdrawn (within the fourth month), whereas Everolimus TL was increased up to 6–10 ng/ml. After 6 months all the patients were on Everolimus alone; they were evaluated again at one year from baseline (T12). Dosage of steroids was never altered throughout the study. Six of the enrolled patients continued mycophenolic acid (MFA) co-treatment that was associated to Everolimus. These patients, whose immune modulating regimen included MFA co-administration, were independently analysed throughout the study. Clinical management

of the patients was performed at Divisione di Nefrologia dell'Università "Federico II" di Napoli. All the patients signed their informed consent to the study. Twelve healthy blood donors, age- and sex-matched with the patients, were enrolled into the study as controls.

Immunofluorescence and T cell activation

To evaluate the immune profile, blood samples of kidney transplanted patients were analysed by immunofluorescence and flow cytometry by using a two-laser equipped fluorescence activated cell sorter (FACS) Calibur apparatus and CellQuest analysis software (Becton Dickinson, San Jose, CA, USA). To identify the different cell populations and the cytokine production were employed monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD56, CD25, FoxP3, Ki67, interferon (IFN)-γ, interleukin (IL)-4, IL-17 labelled to fluorochromes [Fluorescein isothiocyanate (FITC), phycoerythrin (PE), cychrome (CY) and allophycocyanin (APC)] purchased from Becton Dickinson. APC labelled anti-phospho S6 kinase mAb was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). To analyse cytokine production, Peripheral Blood Mononuclear Cells (PBMC) were isolated from whole blood through centrifugation on Lymphoprep gradients (Nycomed Pharma) and cultured overnight in the presence of phorbol myristate acetate (PMA) and ionomycin to induce the *ex vivo* cytokine production and with 5 μg/mL of Brefeldin-A (Sigma-Aldrich) to avoid extracellular cytokine export. Intracellular cytokine profile, FoxP3, Ki67 and phospho S6 kinase staining were performed with a fixation-permeabilization buffer (Becton Dickinson), following the manufacturer's instructions. $CD4^+$ and $CD8^+$ T cells were sorted by FACSJazz (Becton-Dickinson). To mimic antigen-dependent T cell activation, sorted $CD4^+$ and $CD8^+$ cells were incubated for 1 hour with anti-CD3/anti-CD28 mAb-coupled microbeads (Life Technologies AS, Oslo, Norway) at the cell/bead ratio of 1:0.2.

Statistical analysis

Statistical evaluation of the data using InStat version 3.0 software (GraphPad Software Inc., San Diego, CA, USA), was performed by Mann–Whitney test or Wilcoxon's matched pairs signed-rank test, as indicated. Two-sided P-values less than 0.05 have been considered significant. The corrected P-value (Pc) were calculated by applying Bonferroni adjustment for multiple comparisons.

3.3 Study of *in vitro* effects of Nutraceutical compounds on pro-inflammatory T cell profile

Culture Medium and Botanicals

PBMCs isolated by peripheral blood samples of 10 healthy donors were tested for their capability to produce cytokines in the presence of conditioned cell cultured medium containing botanicals compounds. Conditioned cell cultured medium was prepared by using 1gr of powder of each plant-derived substance, solubilized in an appropriate chemical vehicle. The botanical substances and the amount to be used for the *in vitro* study were selected according to the immune-modulating results previously obtained in a canine model [29]. In particular, *Ascophyllum nodosum* (pure powder of *Ascophyllum nodosum* seaweed, laminarin content min. 2.3%, and fucoidans content min. 11.4%); *Aloe vera* (*Aloe vera* gel 200:1 powder, aloe content min. 1%); *Cucumis melo* (lyophilized extract of melon, superoxide dismutase min. 1 UI/mg), *Polygonum cuspidatum* (powder obtained from dried *Polygonum cuspidatum* roots, resveratrol content min. 8%), *Camellia sinensis* (standardized decaffeinated green tea leaves powder, catechins content min. 75%), *Carica papaya* (Papaya fermented granular, rich in papain), *Glycine max* (Soy powder, 40% isoflavones), and *Grifola frondosa* (maitake carpophore dry extract, polysaccharides content min. 20%) have been solubilized in 10mL of PBS, while the *Glycine max* has been added to 30 mL of PBS to gain the full solubilization. *Haematococcus pluvialis* (standardized beadlets of *Haematococcus pluvialis* extract, astaxanthin content min. 2.5%) has been solubilized in 5 mL of dimethyl sulfoxide and 5 mL of PBS. *Echinacea purpurea* (*Echinacea purpurea* dried extract, polyphenols content min 4%), *Piper nigrum* (black pepper powder, piperine content min. 95%), *Curcuma longa* (turmeric dried extract, curcuminoids content min. 95%), and *Punica granatum* (standardized powdered extract from pomegranate, ellagic acid content min. 20%) have been solubilized in 4 mL of ethanol and 6 mL of water. To obtain a conditioned culture medium, the solubilized botanicals have been added to RPMI 1640 culture medium (Sigma-Aldrich, Milan, Italy) in the proportion of 1:10 in order to preserve the good conditions of the cell cultures.

Intracellular cytokine production was evaluated after ON incubation with Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin (Sigma-Aldrich, St. Louis, MO) alone or in the presence of culture medium containing the single substance or a mixture of all the botanicals tested. This approach has been widely described for the study of cytokine profile in human and animal models. Cytokine production was evaluated by using immune fluorescence and flow cytometry detection (FACSCalibur platform) and CellQuest Software (Becton Dickinson Pharmingen, San Jose, California).

Statistical Analysis

Statistical analysis was performed by Kruskal-Wallis followed by Dunn's multiple comparisons analysis (GraphPad Prism, San Diego, CA, USA). Results were considered significant at $p< 0.05$.

3.4 Study of $CD3^+CD56^+$ cell subset

Monoclonal antibodies, immunofluorescence, flow cytometry and cell sorting

$CD3^+CD56^+$ ($T_{R3\text{-}56}$), $CD3^-CD56^+$ (NK) and $CD4^+$ and $CD8^+$ T cells were isolated, after Ficoll hypaque–gradient centrifugation (GE-Healthcare), from PBMCs of human healthy donors by high-performance cell sorting (BD FACS-Jazz, BD Bioscience) in the IEOS-CNR Sorting Facility in Napoli, after staining with the following antibodies: anti-CD3, anti-CD56, anti-CD4, anti-CD8 or by magnetic cell separation with microbeads $CD3^+CD56^+$ isolation Kit (Miltenyi Biotec). Sorted cells were 95%–99% pure by FACS analysis. Samples were analysed by immunofluorescence and Flow Cytometry by using a two-laser equipped FACSCanto II (BD PharMingen).

FITC, PE, PE-Cy7, PE-Cy5, APC-H7 and APC-labelled mAbs against CD3, CD4, CD8, CD16, CD45, CD25, CD39, CD49d, CD45RA, CD45RO, CD54, CD56, CD57, CD62L, CD69, CD107a/LAMP-1, CD94, CCR7, CTLA-4, CXCR4, Foxp3, GITR, DNAM-1, PD1, IFN-γ, NKG2A, NKp30, NKp46, CD1d Ig fusion, Vα24 and isotype-matched controls, all from BD Pharmingen were used for cell characterisation. FITC and PE labelled mAbs against TCR Vβ epitopes, namely anti-Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2 Vβ5.3, Vβ7.1, Vβ8 Vβ9, Vβ11, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ20, Vβ21.3 Vβ22, Vβ23, all from Beckman Coulter were used for T cell repertoire analysis. To analyse the production of Interferon (IFN)-γ intracellular staining with the specific mAb was performed by using the fixing/permeabilization (BD Bioscience), following the manufacturer's instructions. To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/ml of Brefeldin-A (Sigma-Aldrich). Analysis were performed by using FlowJo Software (FlowJo, LLC). The control 345.134 IgG2a mAb, recognizing a glycoprotein widely expressed on human leucocytes was a kind gift of Dr. S. Ferrone; recombinant human soluble NCAM-1/CD56 molecule was purchased from R&D Systems, Inc.

Cell culture, CD107/LAMP-1 expression and cytokine production

To obtain activated IL-2PBMC or IL-2CD8$^+$ cells, PBMC or flow sorted CD8$^+$ T cells were cultured for 36 hours in RPMI-1640 (Thermo Scientific Scientific) supplemented with 5% AB human serum (Euroclone) in the presence of recombinant human IL-2 (Sigma) at 200UI/ml. IL-2PBMC or IL-2CD8$^+$ cells were incubated for 4 hours with anti-CD3 plus anti-CD28 mAb-coupled microbeads (Gibco by Thermo Scientific) at the cell/bead ratio of 1:1 or with the K562 cell line (ATCC) at 1:1 ratio. CD107a/LAMP-1 expression and IFN-γ production was evaluated in flow cytometry gated CD3$^+$CD56$^-$ (T cells), $T_{R3\text{-}56}$, NK, CD4$^+$ and CD8$^+$ T cells, as indicated. When indicated IL-2PBMC or IL-2CD8$^+$ T cells were co-cultured with fresh isolated $T_{R3\text{-}56}$, NK CD4$^+$ and CD8$^+$ T lymphocytes at different ratio. Brefeldin-A at 5 μg/ml (Sigma Aldrich) was added in the last 3 hours of culture for CD107a/LAMP-1 expression or for the whole culture period for IFN-γ production. To avoid cell-to-cell contact, co-culture of $T_{R3\text{-}56}$ cells with IL-2CD8$^+$ T lymphocytes was performed by using transwell inserts (Corning Life Sciences).

Proliferation assay

For the assessment of cell proliferation, cells were cultured in the presence of microbeads coated with anti-CD3 plus anti-CD28 (Gibco by Thermo Scientific). Cultures were incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2 and pulsed with 0.5 μCi/well [^{3}H] thymidine for the final 16 hours. The incorporation of the labelled nucleotide was determined by scintillation counting after automatic cell harvesting. All tests were performed in the presence of RPMI 1640 Medium supplemented with 5% heat inactivated AB human serum (Euroclone). To analyse cell division flow cytometry sorted CD4$^+$ and CD8$^+$ T cells were labelled with 5, 6-carboxyfluorescein-diacetate-succinimidyl ester (CFSE) (ThermoFischer Scientific) before the culture.

Statistical analysis

Statistical evaluation of data has been performed by Mann-Whitney test and Student's *t-test* using the *InStat 3.O* software (GraphPad Software Inc, San Diego, California, USA). Two- sided p values less than 0.05 has been considered significant.

4. Results and Discussion

4.1 Immune dysregulation and Myelodysplastic Syndromes

Treg increase and decreased expression of CD54 on $CD8^+$ T cells in BM associate with MDS progression.

Immune response is a finely tuned micro-site process. Thus, the possibility to specifically access the study of BM immune profile in MDS represents a powerful tool to investigate on Treg-mediated immune tolerance control in the pathogenesis and progression of the disease. With this purpose, we evaluated the level of Treg and activated cytotoxic T cells in the BM of 37 MDS patients classified according to IPSS score system, mirroring leukemia progression risk. Left panel of Figure 5 shows that Treg levels in the BM increase with disease progression. A significant increment ($p<0.05$) of BM Treg percentage has been observed in Int-2/High Risk patients in comparison with the Low and Int-1 Risk groups. Moreover, in order to investigate on BM cytotoxic $CD8^+$ T cells (CTL), largely associated with the occurrence of immune-mediated damage of stem precursors [45,46], we focused on activated cytotoxic effectors by evaluating surface expression of CD54 molecule, consistently associated with the occurrence of antigen dependent activation of CTL [47,48]. As shown, a clear trend of reduction of CD54 expression on BM $CD8^+$ cytotoxic T cells was observed from the Low Risk to the Int-2/High Risk stage of MDS (Figure 5, right panel). These data suggest that BM Treg levels increase with disease progression and that, instead, an higher number of activated $CD8^+$ T cells characterises the first stage of the disease.

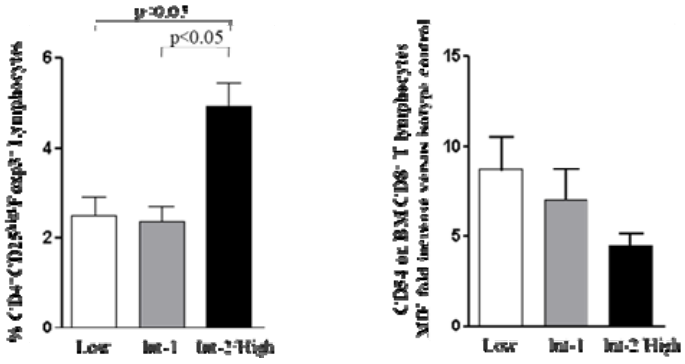

Figure 5: Treg percentage and CD54 expression on bone marrow $CD8^+$ T lymphocytes in MDS patients. Left panel indicates the percentage of Treg cells in BM of MDS patients classified according the International Prognostic Score System (IPSS). Treg are significantly increased in Int-2/High when compared to the Low and Int-1 patients ($p < 0.05$). Right panel indicates the expression level of CD54 on $CD8^+$ T lymphocytes in BM of MDS patients classified according to the IPSS; a trend of decreased expression from Low to Int-2/High patients is observed. White, grey and black columns indicate Low, Int-1 and Int-2/High Risk MDS patients, respectively.

Thus, reduced Treg level in BM might be hypothesised to associate with the occurrence of deranged activity of CTL, likely able to damage stem precursors in BM and to select pathological dysplastic precursors able to escape CTL attack. [46,49].

Reduced BM Treg levels in Low Risk MDS patients are significantly associated with increased BM recruitment of $CD8^+$ T cells

To evaluate whether activity of cytotoxic adaptive effectors in BM of MDS patients associate with immune-mediated selection of pathological precursors, we first evaluated the recruitment in BM of cytotoxic adaptive effectors in our MDS cohort calculating the ratio between the BM and peripheral blood (PB) $CD8^+$ T cells. A value >1 indicates an increased frequency of cytotoxic effectors in BM, as compared with the PB.

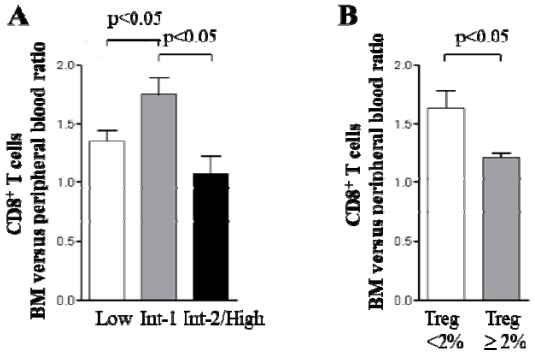

Figure 6: BM recruitment of CTL in Low Risk MDS patients inversely associates with Treg level in BM. Panel A indicates the CTL recruitment in BM of MDS patients classified according the IPSS. There is a significant increase of CTL in BM of Int-1 patients compared to Low and Int-2/High Risk individuals. White, grey and black columns indicate Low, Int-1 and Int2/high Risk MDS patients, respectively. Panel B shows the CTL recruitment in BM of Low Risk MDS patients categorized according to Treg levels in BM; Percentage < 2% or ≥ 2% were indicated with white and grey columns, respectively The BM recruitment of CTL has been evaluated by calculating the ratio between CTL percentage in BM and in PB.

As shown in panel A of Figure 6, there is a significant BM recruitment of $CD8^+$ T lymphocytes in Int-1 when compared with Low Risk group, while reduction of CTL recruitment in BM characterises the Int2/high Risk stage of MDS.

Previous data obtained by our research group indicated [34] that Treg show a clustered distribution in BM of Low Risk patients and that a cut-off of 2% allows the identification of two subgroups of Low Risk individuals. A subgroup with physiological level of BM Treg (>2%) and another one with lower BM Treg level (<2%) compared to healthy donors (data not shown). As shown in panel B of Figure 6 the subgroup of Low Risk MDS patients with lower (<2%) BM Treg level show an increased BM recruitment of $CD8^+$ T lymphocyte. No significant difference in the BM recruitment of $CD4^+$ T cell effectors was observed (not shown).

Then, BM-Treg level, seems to preferentially control the BM recruitment of CTL in MDS. These observations support the idea that lower Treg level could promote immune-mediated damage of stem precursors in BM of Low/Int-1 Risk patients. In addition, the increasing of Treg number

associated to a decreased activation status of $CD8^+$ T cells, could allow the suppression of the cytotoxic response against the dysplastic clones in the Int-2/High Risk groups [33-35].

Then, BM Treg levels preferentially control both the activation status and the BM recruitment of cytotoxic T cell effectors in MDS.

Clonal expansion and activation status of $CD8^+$ T cells in BM of Low risk MDS patients inversely correlates with BM Treg percentage.

Antigen-dependent activation of effector T lymphocytes is associated with their expansion. Diversity of T cell repertoire is dependent on rearrangement of V-D-J gene segments occurring during TCR assembly. Each single T cell clone expresses one type of TCR. So, the expression level of single Vβ gene segments of the variable region of TCR β-chain indicates the expansion, also called "skewing", of single T cell clones.

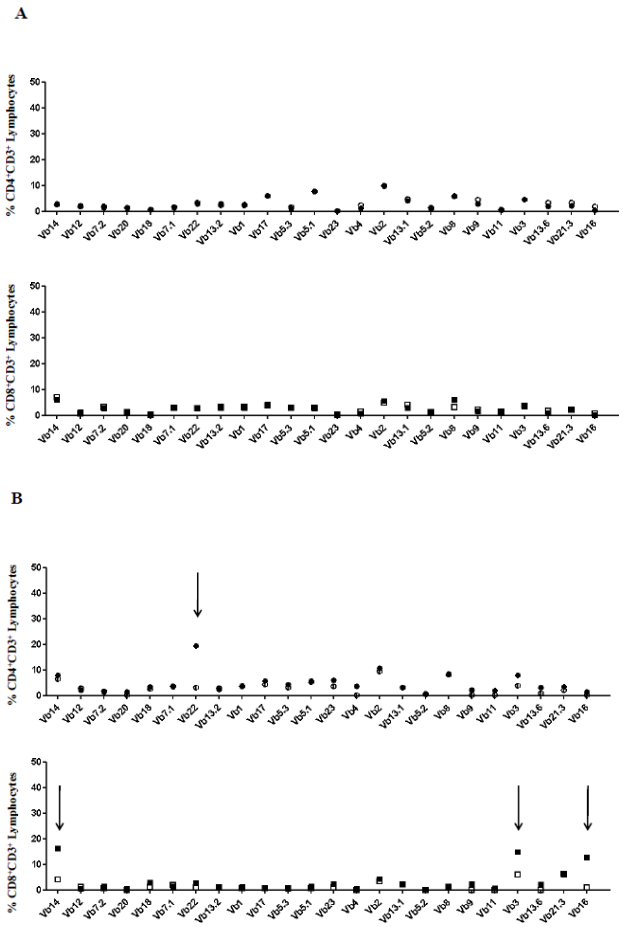

Figure 7: Comparative analysis of TCR repertoire on BM and PB of $CD4^+$ and $CD8^+$ T cells in one representative healthy donor (CTR) and one Low Risk MDS patient. Panel A shows the expression of Vβ gene segments in BM and PB of CD4 and CD8 T lymphocytes in the CTR. White and black symbols indicate BM and PB, respectively; Panel B shows the expression of Vβ gene segments in BM and PB of CD4 and CD8 T lymphocytes in a representative Low Risk MDS patient. White and black symbols indicate BM and PB, respectively; The arrows indicate the occurrence of BM T cell expansions in CD4 and CD8 lymphocyte repertoire. T lymphocyte expansion has been considered significant when the TCR Vβ expression percentage is higher than 3 standard deviations (SD) compared to TCR Vβ expression in a cohort of 10 healthy controls. Furthermore, preferential BM TCR Vβ expansions have been considered significant when the expression percentage of a TCR Vβ family in BM was greater than 20% compared to peripheral blood.

In this context, the possibility to directly analyse the BM microenvironment, represents a valuable tool to investigate on T-cell mediated mechanisms involved in the selection/expansion of pathological clones in the first phases of MDS. Indeed, the presence of a T cell expansion in BM is likely related with an ongoing T cell response against BM antigens.

Figure 7 shows the results obtained when T cell repertoire in BM and PB was compared. As shown, (Figure 7A) in one healthy donor no significant difference was observed in BM and PB CD4 and CD8 T cell repertoire (indicated by the black and white symbols); at variance, in Low Risk MDS patients the presence of preferential BM clonal expansions were observed in both $CD4^+$ and $CD8^+$ T lymphocytes (panel B). Of note, no particular Vβ segment expansion has been observed to characterise BM of MDS patients. Thus, Low Risk patients were divided in two groups, according to the number of T cell expansions detected in BM: those with <2 Vβ skewing and those showing ≥2 Vβ skewing in BM T cell repertoire.

As shown in Figure 8, Low Risk MDS patients with ≥2 Vβ BM clonal expansion have lower BM Treg cells level (panel A) and higher CD54 expression (panel C) on BM T lymphocyte as compared to patients with <2 Vβ skewing. No significant difference was observed when $CD4^+$ T lymphocyte BM clonal expansions were evaluated (panel B and D).

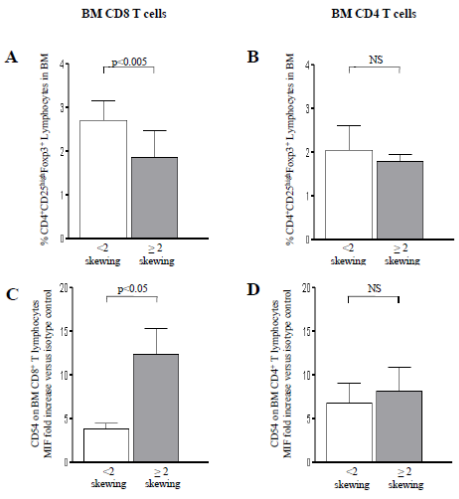

Figure 8: Treg percentage and CD54 expression on bone marrow (BM) $CD8^+$ and $CD4^+$ T lymphocytes in Low Risk MDS patients classified according the number of T cell expansions (skewing) in BM. Panel A and B indicate the Treg percentage in Low Risk MDS patients with <2 (white columns) or ≥2 (gray columns) skewing in BM $CD8^+$ and $CD4^+$ T cells, respectively. Panel C and D indicate CD54 expression on BM $CD8^+$ and $CD4^+$ T lymphocytes in Low Risk MDS patients with <2 (white columns) or ≥2 (gray columns) skewing in BM $CD8^+$ and $CD4^+$ T cells, respectively. Significant difference has been observed only for CD8 T cells.

These data confirm the relevance of BM Treg levels in the preferential regulation of both the expansion and activation status of cytotoxic T cell effectors in BM of Low Risk MDS patients.

Grouping of Low Risk MDS individuals according to their BM Treg level identifies a subgroup of patients showing a skewed CD8 T cell repertoire in BM, lesser leukaemia evolution and better survival in a minimal 36 month follow up.

In order to investigate whether the evaluation of Treg level in BM (< 2% versus ≥2%) might represent a useful criterion to identify the MDS patients in which immune-mediated mechanism are involved in pathogenesis or progression of the disease, we analysed the occurrence of $CD8^+$ and $CD4^+$ T cell expansions in BM of 26 Low Risk individuals categorised according to their BM Treg level. As shown in Table 1, a significant increase of $CD8^+$ T cell expansions in BM has been observed in patients showing lower Treg level (< 2%) at disease onset, as compared to the counterpart with BM Treg percentage >2% ($p<0.05$). No significant association of Treg level with $CD4^+$ T cell expansions in BM has been observed.

To investigate on the prognostic relevance of Treg BM level at diagnosis, we also analysed leukaemia evolution and survival, in a minimal 36 month follow up, in Low Risk MDS patients grouped according to their BM Treg level. Table 1 shows that there is a significant increment of leukaemia evolution ($p<0.05$) and death ($p<0.05$) in the sub-group of Low Risk patients with BM Treg percentage >2% at disease onset.

These observations are conceivable with the hypothesis that Treg preferentially suppress cytotoxic immune effectors in BM of MDS Low Risk patients. Moreover, their number at diagnosis, seems to inversely associate with an immune profile able to control disease progression.

Table 1. Follow up evaluation of Low risk MDS patients categorised according to Treg level in BM at disease onset[1]

	N	Age	CD8 skewed in BM ≥2	CD4 skewed in BM ≥2	Transfusion dependance	Leukemia evolution	Death
Low Risk	26	72.6±9	13	10	8	5	6
BM Treg ≤2%	14	71±5	10[2]	6[3]	5	0[4]	0[5]
BM Treg >2%	12	74.4±11	3	4	3	5	6

[1]data refer a minimum 36 month follow-up; [2] significantly different from BM Treg >2% group ($p<0.05$ by Fisher exact test; Odd Ratio 7.5 (95% CI: 1.307 to 43.047); [3] not significantly different from BM Treg >2% group; [4] significantly different from BM Treg >2% group ($p<0.05$ by Fisher exact test); Odd Ratio 0.047 (95% CI: 0.002279 to 0.9704); [5]significantly different from BM Treg >2% group ($p<0.005$ by Fisher exact test); Odd Ratio 0.034 (95% CI: 0.001679 to 0.7082);

Taken in all our observations indicate that BM Treg level at diagnosis inversely associates with an immune profile able to control disease progression. Indeed, at disease onset, Treg percentage in BM associated with decreased occurrence of Leukaemia progression and better survival. Further investigation, addressing the molecular target/s of the BM skewed CTL, will likely clarify the role of immune mediated processes in MDS pathogenesis and/or progression.

4.2 mTOR modulation and tolerance of kidney transplants

Effect of conversion from CNI to Everolimus on immune profile

Previous data indicate that Treg amount in the inflammatory microsite is a key element to modulate adaptive cytotoxic effectors and mediate immune-tolerance control. A number of experimental data [12,13] suggest mTOR modulation as a valuable strategy to increase Treg subset in the context of a deranged tolerance control. Pharmacological mTOR inhibition is currently employed in clinical trials to suppress tumor growth in multiple oncological settings. Moreover, selective Treg growth *in vitro* has been observed to depend on oscillatory mTOR activity [14]. Kidney transplant condition represent a valuable clinical model of tolerance induction. In this context, the employment of immune-modulating strategies based on CNI drugs has been largely associated with therapy-related damaging of the transplanted organ. Thus, the needing of alternative pharmacological approach to immune suppression in kidney transplanted patients is largely recognised.

mTOR inhibition has been proposed as an alternative strategy to obtain immune-modulation, also avoiding possible nephrotoxic effect. In this context we took advantage from a clinical trial at Divisione di Nefrologia dell'Università di Napoli "Federico II" based on the conversion from CNI to mTOR inhibitory immune-modulating protocol in a cohort of kidney transplanted patients. In this model we investigated on the hypothesis that dosage and clinical administration schedule of mTOR inhibitors, as represented by Everolimus, might selectively mediate the establishment of a Treg-dependent tolerance control of the transplanted organ.

With this aim we analysed the number of leucocytes, neutrophils, $CD4^+$ and $CD8^+$ T lymphocytes at T0 (at the beginning of the study) and at T12 (after 12 months of pharmacological conversion). We found that conversion from CNI to Everolimus is able to reduce the number of total leucocytes, neutrophils cells and $CD8^+$ T lymphocytes at T12 if compared to T0 data. No differences were observed in $CD4^+$ T cell numbers (not shown).

Then we evaluated the effect of Everolimus conversion on cytokine production, by analysing IFN-γ, IL-4 and IL-17 in iNKT lymphocytes, a major player in cytokine profile polarization [50], IFN-γ in $CD8^+$ T cells and IFN-γ and IL-17 in $CD4^+$ T lymphocytes. Comparison with healthy donors (CTR) was also performed.

As shown in Figure 9 (panel A-C) IFN-γ and IL-17 produced by iNKT cells remained significantly lower respect to healthy donors in patients both at T0 as well as at T12. In contrast, the high IL-4 production in CNI treated patients, evident at T0, was significantly reduced at T12 becoming similar to healthy donors (Figure 5B). The production of IFN-γ by $CD8^+$ T cells (Figure 9D) significantly decreased after the Everolimus conversion, resulting lower than controls at T12.

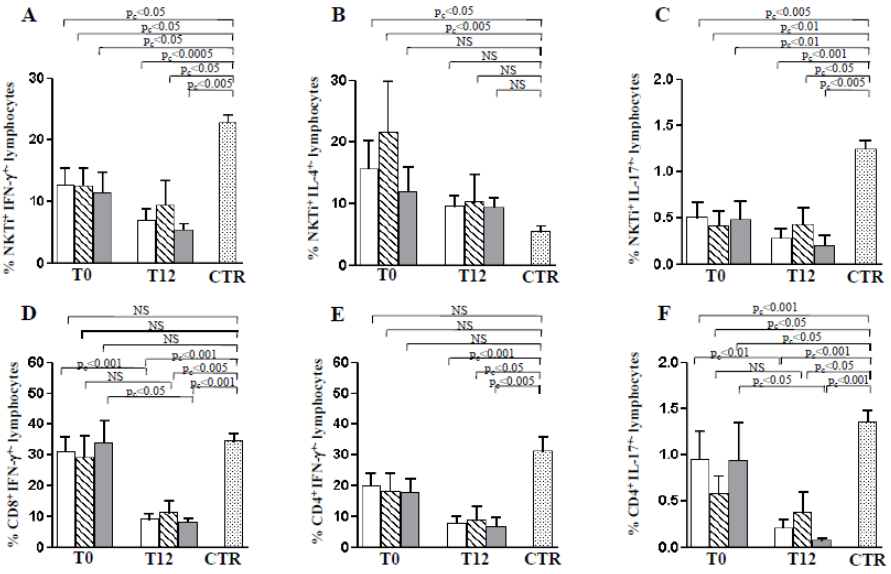

Figure 9: Cytokine secretion profile of NKTi, $CD8^+$ T and $CD4^+$ T lymphocytes in kidney-transplanted patients undergoing conversion from CNI to Everolimus. Panels A-C refer to the IFN-γ, IL-4 and IL-4 production by NKTi cells at T0 and T12 after CNI to Everolimus conversion. Panel D indicates IFN-γ production in $CD8^+$ T cells and Panels E and F indicate IFN-γ and IL-17 production by $CD4^+$ lymphocytes at T0, and T12. Data have been referred as mean±SEM. White columns indicate data obtained in all the patients enrolled in the study (N=18); striped columns indicate patients whose treatment included MFA co-administration (N=6); Grey columns indicate patients treated with immune modulating regimens not including MFA (N=12). Mann-Withney test is reported. For T0-T12 comparison of paired samples Wilcoxon matched-pairs signed-rank test has been performed. The corrected p value (p_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

Notably, the IFN-γ reduction was preferentially observed in the group of patients undergoing an immune-modulating treatment without MFA. Such evidence suggests a preferential role for mTOR-dependent mechanisms in regulating $CD8^+$ T lymphocyte pro-inflammatory cytokine production. IFN-γ by $CD4^+$ T cells was significantly lower at T12 than controls (Figure 9E). In addition, the percentage of IL-17 produced by $CD4^+$ T lymphocyte was reduced in patients than in controls at T0 and at T12 (Figure 9F). Moreover, in paired samples, the comparison of T0 and T12 values suggested that Everolimus, in absence of MFA, was able to mediate complete inhibition of IL-17 producing $CD4^+$ T cells. Thus, MFA co-administration affects Everolimus-dependent modulation of pro-inflammatory cytokines by T cells.

Effect of Everolimus on Treg number and proliferation

Treg cell homeostasis depends on mTOR activation pathways [14,51]. Therefore, we analysed the effects of the pharmacological mTOR inhibition on number and proliferation of Treg cells. As indicated in Figure 10, T12 patients treated without MFA association show a significant increase of Treg number as compared to T0 values. Notably, Treg count always remains similar to controls.

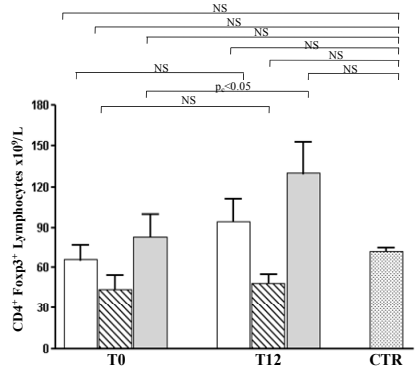

Figure 10: Treg population in kidney-transplanted patients following CNI to Everolimus conversion. Number of Treg, gated as $CD4^+Foxp3^+$T cells, at T0 and T12 were reported. Data have been referred as mean±SEM. White columns indicate data obtained in all the patients enrolled in the study (N=18); striped columns indicate patients whose treatment included MFA co-administration (N=6); Grey columns indicate patients treated with immune modulating regimens not including MFA (N=12). For comparison with controls Mann-Withney test is reported. For T0-T12 comparison of paired samples Wilcoxon matched-pairs signed-rank test has been performed. The corrected p value (p_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

Moreover, we analysed Treg growth rate by evaluating *ex-vivo* the expression of the proliferation marker Ki67. As shown in Figure 11, the conversion from CNI to Everolimus was associated with a significant increase of Ki67 expression on the Treg population. Conversely, Everolimus-MFA co-treatment mediated a significant reduction of Treg proliferation. At variance, the *ex-vivo* evaluation of Ki67 expression in $CD4^+FoxP3^-$T effector cells indicated a significant proliferation decrease at T12 (data not shown). This decreased trend was observed to be very strong in T12 patients treated with Everolimus in association to MFA. Therefore, MFA administration was observed to mediate significant growth reduction of both Treg and T cell effectors.

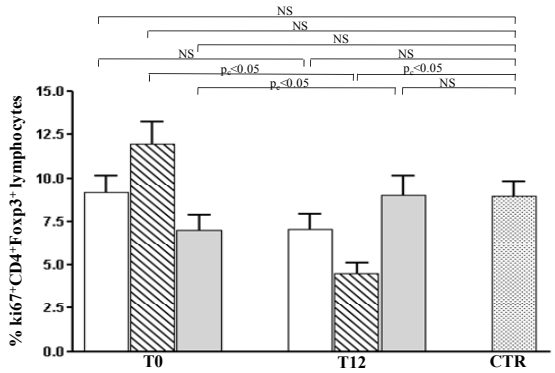

Figure 11: Ki67 expression on Treg cells gated as $CD4^+Foxp3^+$T cells in kidney-transplanted patients. Data have been referred as mean±SEM. White columns indicate data obtained in all the patients enrolled in the study (N=18); striped columns indicate patients whose treatment included MFA co-administration (N=6); Grey columns indicate patients treated with immune modulating regimens not including MFA (N=12). For comparison with controls Mann-Withney test is reported. For T0-T12 comparison of paired samples Wilcoxon matched-pairs signed-rank test has been performed. The corrected p value (p_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

Everolimus serum concentration associates with different levels of mTOR-dependent S6 kinase phosphorylation

To evaluate the activation status of mTOR complex after one year of Everolimus administration, we studied the phosphorylation of S6 kinase (p-S6), the major downstream target of mTOR activity [52]. Moreover, to assess whether phosphorylation of mTOR-dependent targets might be conditioned by drug serum concentration, we evaluated S6 kinase phosphorylation (p-S6) level in $CD4^+$ and $CD8^+$ T cells of T12 patients after 3h (T12-3h) and 12h (T12-12h) from Everolimus administration, reflecting maximal and minimal TL of the drug (data not shown). As shown (Panel A and B of Figure 12), comparative analysis by immune fluorescence of basal p-S6 levels in $CD4^+$ and $CD8^+$ T cells from samples obtained from T12 patients 3h and 12h after Everolimus administration (highest and lowest drug TL, respectively) indicated the occurrence of an oscillatory inhibition of mTORC1 kinase activity. Indeed, significant decrease of p-S6 kinase was observed in $CD4^+$ and $CD8^+$ T cells obtained after 3h, as compared with those obtained after 12h from Everolimus administration ($p<0.001$). Moreover, p-S6 kinase levels were significantly lower than controls in samples after 3h ($p<0.05$), but not after 12h from Everolimus (Panel A and B of Figure 12).

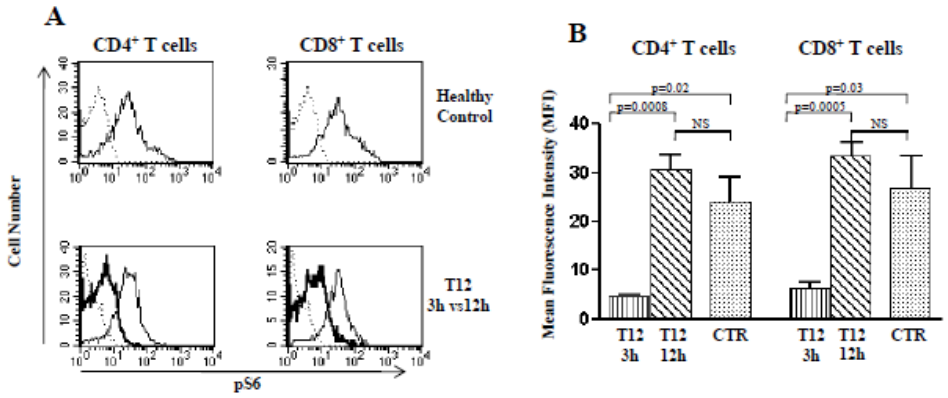

Figure 12. p-S6 kinase analysis 3 and 12 hours after Everolimus administration in T12 patients. Panel A refers to flow cytometry comparative evaluation of p-S6 kinase levels in $CD4^+$ and $CD8^+$ T cells obtained from one representative T12 patient 3h and 12h after Everolimus administration (T12-3h and T12-12h) and in one healthy control. As indicated, upper histogram plots in Panel A show the p-S6 kinase level in $CD4^+$ and $CD8^+$ T cells of one healthy donor (plane line); isotype matched control binding is indicated as dashed line. Lower histogram plots in Panel A refer to the p-S6 kinase evaluation in cells of one representative patient 3h (bold line) and 12h (plane line) after Everolimus administration; dashed line indicates isotype matched control binding. Panel B shows comparative analysis of p-S6 phosphorylation levels of $CD4^+$ and $CD8^+$ T cells after 3h (vertical depicted column) and 12h (oblique depicted column) in all four T12 patients analysed. Dotted columns indicate healthy controls (CTR). Statistical analysis was performed by Mann Whitney test.

Similar data were obtained by western blot analysis. Indeed (Figure 13), no difference in p-S6 levels in medium cultured samples obtained from T0 and T12 patients were observed in $CD4^+$ and $CD8^+$ T cells after a one-hour culture in the presence of medium alone or with anti-CD3/anti-CD28 beads. Conversely, a strong increase of TCR-dependent p-S6 up-regulation was observed in the samples obtained from T12 patients after 12h from Everolimus administration (minimal drug serum

concentration), in both the $CD4^+$ and $CD8^+$ T cells. In addition, strongly reduced pS6 up-regulation was observed upon TCR triggering in the samples obtained from the same patients 3 hours after Everolimus administration (maximal drug serum concentration).

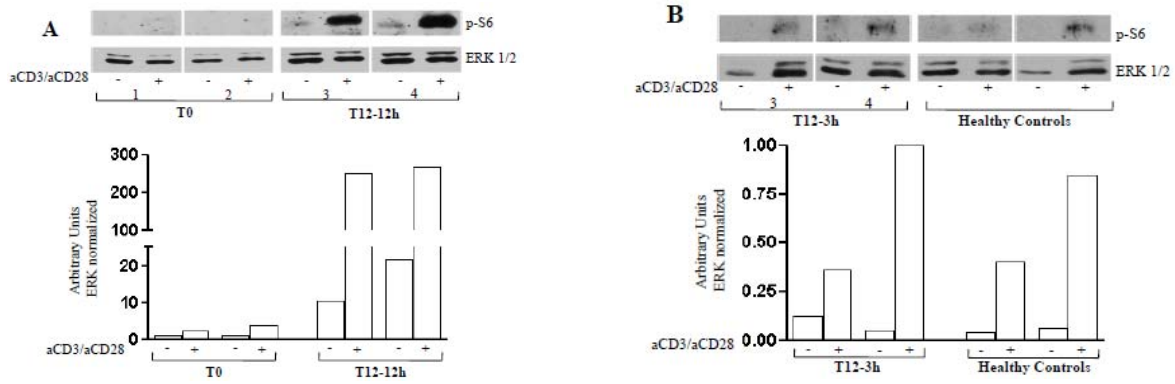

Figure 13: Western blot analysis of p-S6 kinase from protein lysates of sorted $CD4^+$ T cells. Data are from two representative T0 and T12 patients and refer to 1h culture with medium or anti-CD3/anti-CD28 mAb coupled microbeads. As shown, a tremendous increase of pS6 levels was observed in T12-12h patients after TCR triggering. As indicated, (Panel B) comparable up-regulation of pS6 was observed in T12-3h patients and controls. Arabic numbers (1-4) identify samples obtained from single patients. Data are representative of two concordant experiments.

Thus, basal phosphorylation level of S6 kinase, a major mTOR-dependent molecular target, was substantially maintained in patients treated with Everolimus. Moreover, oscillations in serum concentration of Everolimus were associated with changes in basal and activation-dependent S6 kinase phosphorylation of $CD4^+$ and $CD8^+$ T cells. Indeed, T Cell Receptor (TCR) triggering was observed to induce significantly higher S6 kinase phosphorylation in the presence of lower Everolimus serum concentrations. Taken in all, our results shed light on the complex mTOR-dependent immune-metabolic network, and propose that oscillatory inhibition of TCR-dependent mTOR activity might represent a therapeutic strategy to optimise targeted manipulation of specific adaptive effectors in kidney-transplant recipients.

4.3 Study of *in vitro* effects of nutraceutical compounds on pro-inflammatory cytokine production by T cell effectors

Nutraceutical compounds co-culture is able to modulate *in vitro* IFN-γ production by human T cells

Our previous work [29] showed, in a model of natural chronic canine infection, as represented by Canine Leishmaniosis, that combination of pharmacological treatment with an immune-modulating diet containing *Ascophyllum nodosum, Cucumis melo, Carica papaya, Aloe vera, Haematococcus pluvialis*, *Curcuma longa, Camellia sinensis, Punica granatum, Piper nigrum, Polygonum cuspidatum, Echinacea purpurea*, *Grifola frondosa* and *Glycine max* was associated with

significant restoration of Treg level and with the decrease of T_H1 inflammatory response. These effects were proposed to have a role in reducing the immune-pathological injury resulting from canine leishmaniasis. Thus, we investigated whether co-culture of human T cells with the above botanicals might exert immune-modulating effect on activated human T cells.

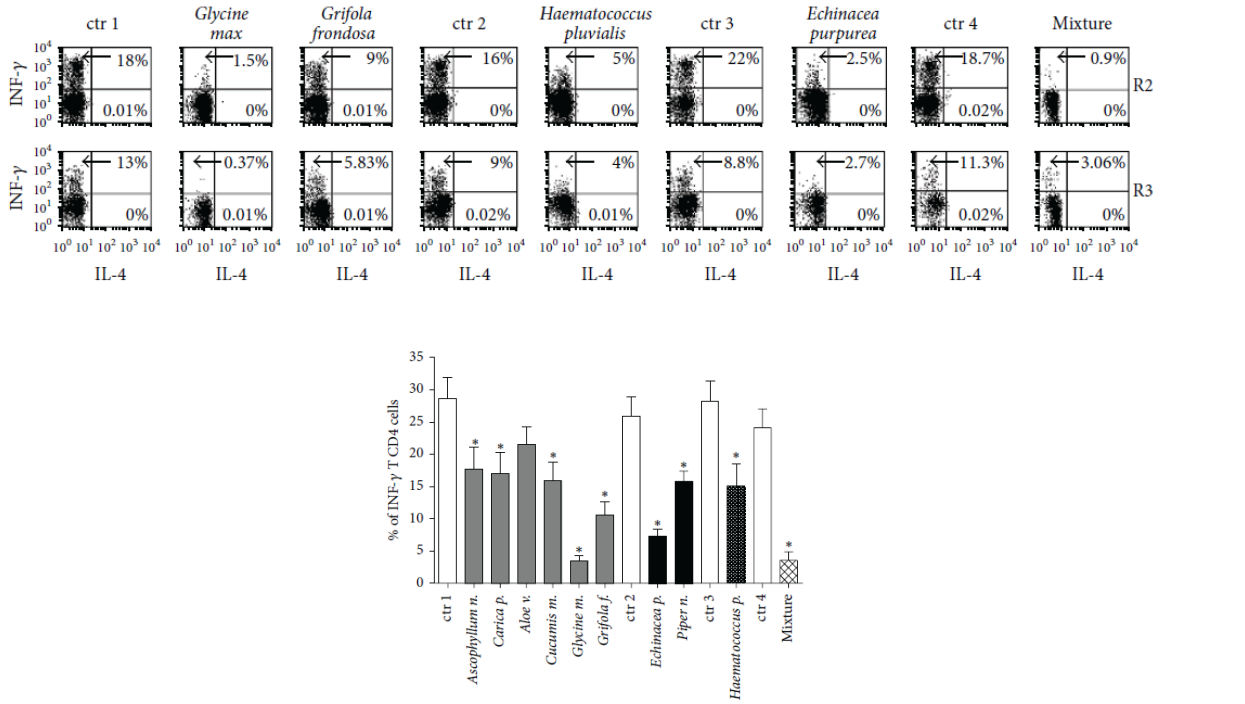

Figure 14: Effects of botanical treatment on INF-γ production by human PBMCs. Upper dot plots represent results from one representative experiment showing the percentage of INF-γ and IL–4 produced by CD4+ T lymphocytes (R2) and non-T cells (R3). The different cell treatment with *ad hoc* medium or mixture are indicated on the top. Lower histograms show the statistic representation of the INF-γ production by human CD4+ T Lymphocytes evaluated as percentage of INF- γ producing cells in 10 representative experiments, $p < 0.05$. The abbreviation "ctr" indicates the basal INF- γ production by PMBC stimulated by PMA and Ionomycin in the presence of the *ad hoc* medium, containing the same solubilizing-vehicle but free from the botanicals.

As shown in Figure 14, co-culture of the botanicals, except *Aloe vera*, were able to induce a significant decrease in IFN-γ production of human T and non-T lymphocytes (manly represented by NK cells). Instead, no changes were observed in IL-4 production, undetectable or only slightly detectable in T and non-T lymphocytes. The chemical vehicles, used to solubilize the substances, were always used as control and the resulting values were subtracted from each experimental point.

4.4 A study of cell-mediated regulation of T cell-dependent cytotoxic effector functions: characterization of the $CD3^+CD56^+$ T cell subset as a novel regulatory cell population.

Co-expression of CD3 and CD56 molecules characterises a subset of lymphocytes whose physiological function is still undefined. Recently, the absolute number of circulating $CD3^+CD56^+$ has been observed to mirror residual function of pancreatic β-cell up to one year after diagnosis of autoimmune type 1 diabetes (T1D) [41]. In order to investigate on the possible involvement of $CD3^+CD56^+$ cells, here defined $T_{R3\text{-}56}$ cells, in the T1D pathogenesis we first analysed the

phenotype of $T_{R3\text{-}56}$ cells in order to clarify whether co-expression of T and NK molecules (CD3 and CD56 molecule) might indicate belonging of $T_{R3\text{-}56}$ cells to the NKT cell lineage. Of note, NKT cells have been largely characterized as a CD1d T cell subset, able to ensure prompt cytokine production also shaping T cell cytokine profile during antigen-dependent immune response.

As shown in panel A of Figure 15, $T_{R3\text{-}56}$ cells are not CD1d-restricted for the Ag-recognition, their TCR does not express the Vα24 segment in the α chain, commonly associated with the invariant NKT subset and are unable to be activated by NK-dependent stimulation, as represented by the K562 cell line. Thus, $T_{R3\text{-}56}$ cells do not belong to the NKT cell subset.

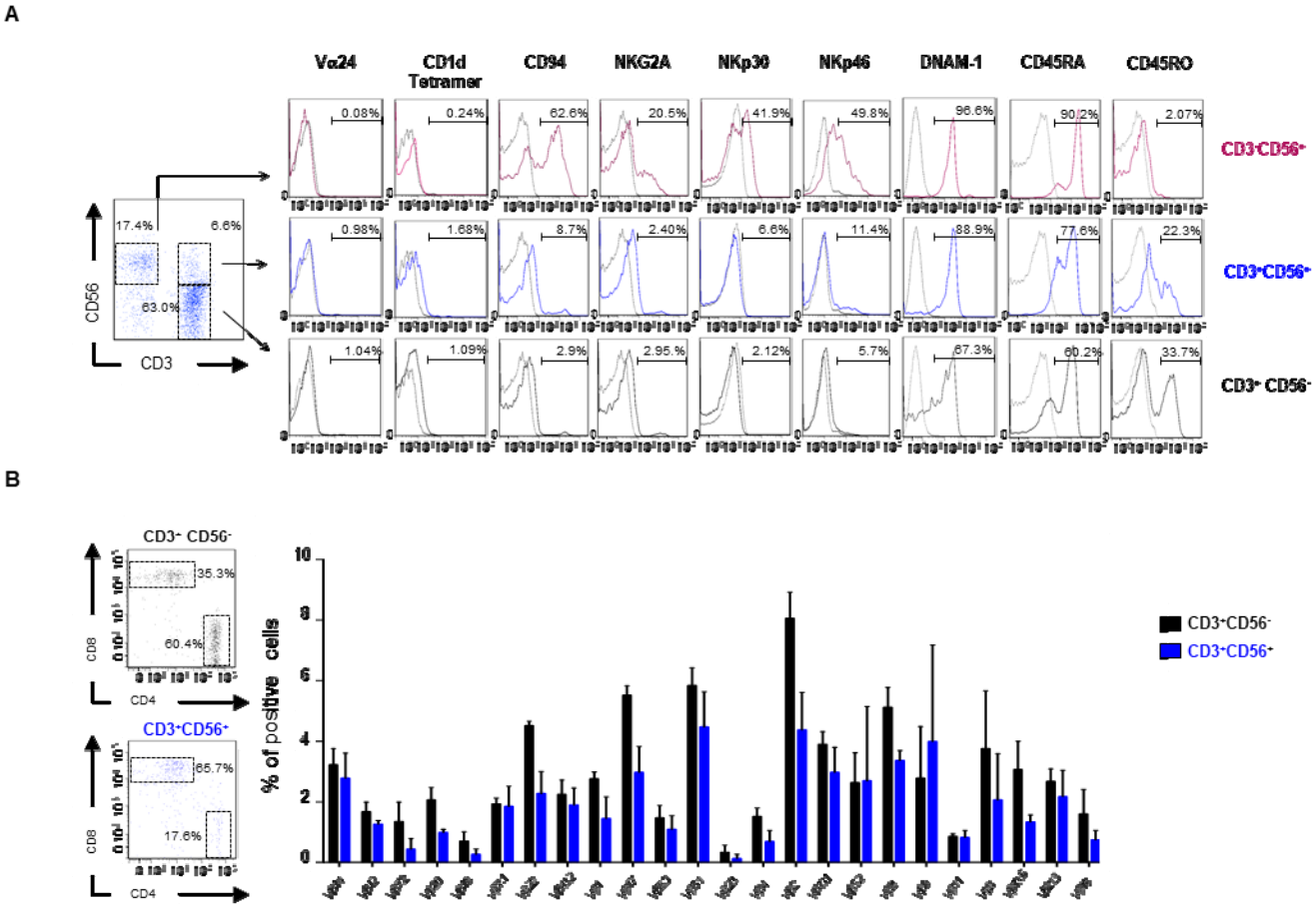

Figure 15: $T_{R3\text{-}56}$ cells are a T cell subset distinct from NK and NKT cells. A. left panel shows flow cytometry analysis and gating strategy to define NK, $T_{R3\text{-}56}$ and T cell subset on PBMC of one representative healthy subject; right panels show the expression of several molecules usually associated with NKT, NK and T cells in NK (magenta), $T_{R3\text{-}56}$ (blue) and T (black) cells. Numbers indicate the percentage of positive cells. B. Left panels show the ratio of $CD4^+$ and $CD8^+$ cells in T (black) and $T_{R3\text{-}56}$ (blue) cells. Right panel shows TCR V gene family expression in $CD3^+CD56^-$ (black) and $T_{R3\text{-}56}$ (blue) cells; data are from three independent experiments (n=3);

The $T_{R3\text{-}56}$ cells express low level of the typical molecules of NK lymphocytes (CD94, NKG2A, NKp30, Nkp46 and DNAM-1), resembling that observed on CD8 T cells. In addition, the analysis of CD45 isoforms shows that $T_{R3\text{-}56}$ cells preferentially express CD45RA molecule (Panel A of Figure 15) and do not express specific markers of Treg cells (data not shown).

Moreover, $T_{R3\text{-}56}$ cells show a heterogeneous αβ TCR repertoire, similar to that observed in classical $CD3^+$ T cells (Figure 15 B). The assessment of the CD4 and CD8 molecules reveals that a CD8 expression generally exceeding 70% characterizes the $T_{R3\text{-}56}$ cells (left panel of Figure 15 B).

To assess whether the $T_{R3\text{-}56}$ cells are responsive to TCR-dependent signals, they have been cultured for 4h with anti-CD3 plus anti-CD28 microbeads, in order to mimic TCR-dependent stimulation. After 4h of incubation, we assessed the cytotoxic activity, evaluating the expression of CD107a/LAMP-1 molecule that is associated to the release of perforin granules; IFN-γ production has been also detected.

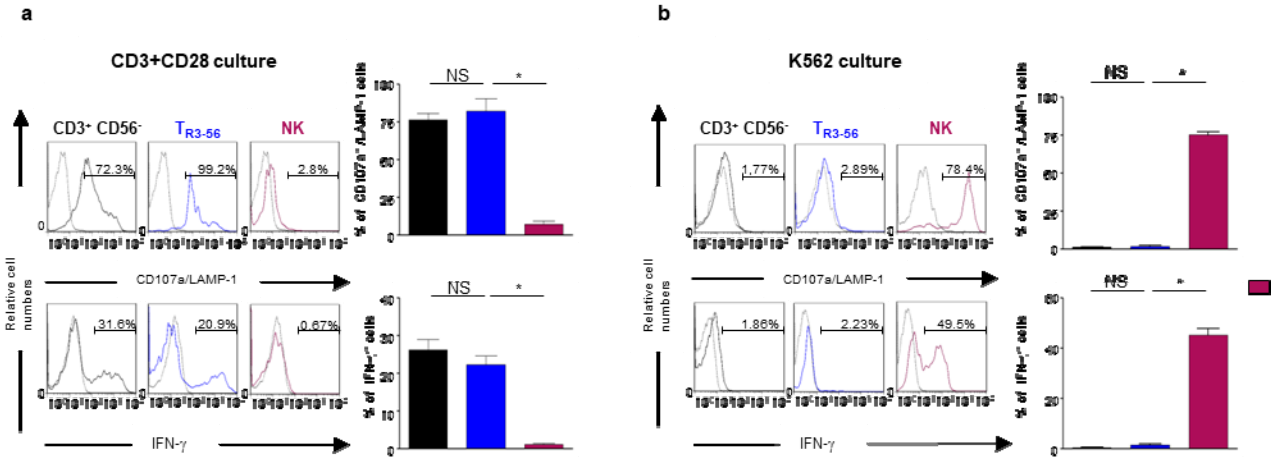

Figure 16: $T_{R3\text{-}56}$ cells are responsive to TCR-dependent signals. A. Left panels indicate CD107a/LAMP-1 and IFN-γ staining of $CD3^+CD56^-$ (black), $T_{R3\text{-}56}$ (blue), NK (magenta) cells, after 4 hours of culture in the presence of medium (dotted line) or anti-CD3 plus anti-CD28 microbeads (plain line); numbers show the percentage of positive cells; data refer one representative experiment; right panels show cumulative data from three independent experiments (n=3; average±s.e.m.); *P <0.005 (two-tailed t-test). B. Left panels show CD107a/LAMP-1 and IFN-γ staining profiles of $CD3^+CD56^-$ (black), $T_{R3\text{-}56}$ (blue), NK (magenta) cells, after 4 hours of culture in the presence of medium (dotted line) or K562 cells (plain line); numbers show the percentage of positive cells; data are from one representative experiment; right panels show cumulative data from three independent experiments (n=3; average±s.e.m.); *P <0.005 by two-tailed t-test;

Moreover, we evaluated TCR-dependent proliferation of $T_{R3\text{-}56}$ as compared with conventional CD4 and CD8 T cells by analysing their [^{3}H] Thymidine incorporation after 72h of TCR stimulation. As indicated in Figure 17, $T_{R3\text{-}56}$ cells show a reduced proliferative capability compared to $CD4^+$ and $CD8^+$ T lymphocytes.

Together, these data suggest that the $T_{R3\text{-}56}$ cell represent a T cell subset with low proliferative ability after TCR engagement.

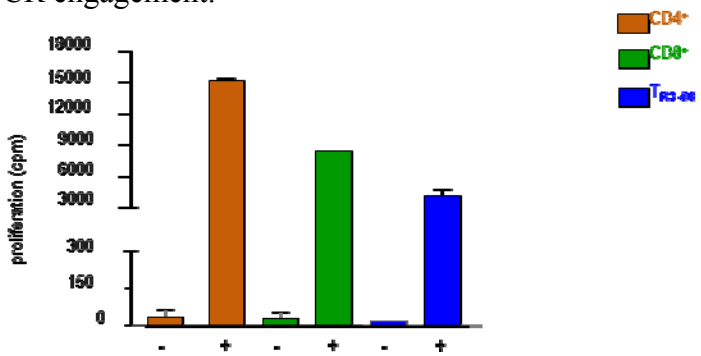

Figure 17: $T_{R3\text{-}56}$ cells show a TCR-dependent reduced proliferation, as compared to conventional T lymphocytes. ^{3}H Thymidine incorporation is referred in $CD4^+$ (orange), $CD8^+$ (green) T lymphocyte and in $T_{R3\text{-}56}$ cells (blue) after 72 h of incubation without stimulation or in the presence of anti-CD3 plus anti-CD28 microbeads (indicated by + and - symbols).

T_{R3-56} are able to modulate antigen-dependent cytotoxicity and interferon-γ production by $CD8^+$ T cells.

To evaluate the T_{R3-56} effect on T lymphocytes proliferation, we co-cultured the sorted T_{R3-56} cells with isolated $CD4^+$ and $CD8^+$ T cells stimulated with anti-CD3 plus anti-CD28 microbeads.

As shown in Figure 18, flow cytometry sorted T_{R3-56} cells reduced [^{3}H] thymidine incorporation of both the $CD4^+$ and $CD8^+$ purified T cells.

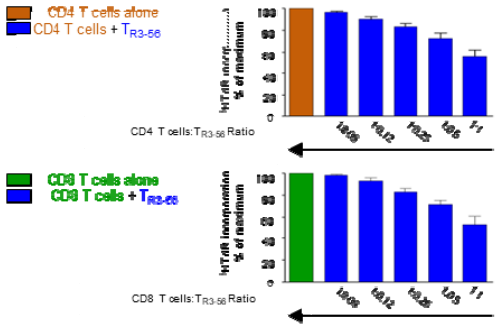

Figure 18: T_{R3-56} cells control the proliferation of both $CD4^+$ and $CD8^+$ cells. ^{3}H Thymidine incorporation is referred as % of maximum in $CD4^+$ (orange) and $CD8^+$ (green) T lymphocytes cultured for 72 hours with anti-CD3 plus anti-CD28 microbeads alone or in the presence of freshly isolated T_{R3-56} cells (blue) at the indicated ratios.

However, the analysis of cell division, assessed by CFSE staining, shows a stronger inhibition of the $CD8^+$ T cells, as compared with the $CD4^+$ T cell counterpart, (Figure 19).

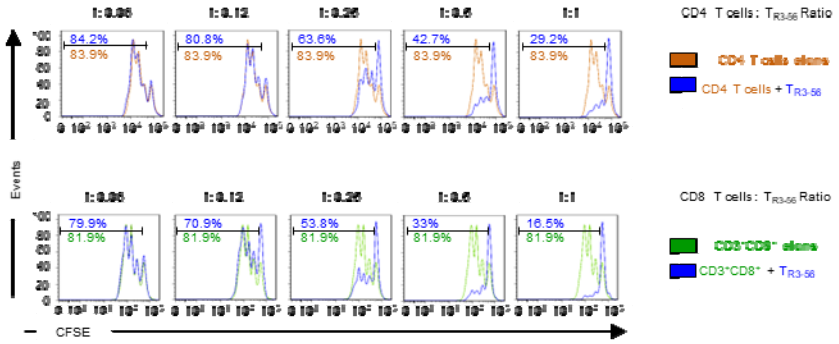

Figure 19: Freshly isolated T_{R3-56} cells preferentially affect cell division of $CD8^+$ T lymphocytes. CFSE staining of flow cytometry sorted $CD4^+$ T cells and $CD3^+CD8^+$ T lymphocytes cultured 72 hours with anti-CD3 plus and CD28 microbeads alone or in presence of T_{R3-56} cell (blue) at the indicated ratios; data are from one representative experiment.

Then, we evaluated whether T_{R3-56} are able to modulate the cytotoxic activity and IFN-γ production by cytotoxic T cells. In order to optimize T cell effector function of the cytotoxic T lymphocytes, flow sorted PBMC or $CD8^+$ sorted T cells were cultured for 36h with rhIL-2 (200IU/ml) to obtain IL-2PBMC or IL-2CD8, as described [56]; these IL-2-treated cells were then stimulated for 4h with

anti-CD3 plus anti-CD28 microbeads to induce CD107/LAMP-1 and IFN-γ production. To specifically analyse IL-2PBMC, these cells were labelled with an anti CD45 mAb before the TCR stimulation with anti-CD3 plus anti-CD28 microbeads. As shown in Figure 20, co-culture of the $T_{R3\text{-}56}$ with IL-2PBMC significantly reduces their expression of CD107/LAMP-1 and IFN-γ production. No effects have been observed in the presence of control populations as represented by NK and $CD8^+$ T cells. Of note, $T_{R3\text{-}56}$ cells are unable to significantly affect TCR-induced CD107/LAMP-1 expression and IFN-γ production of $CD4^+$ T cells (data not shown) suggesting that $T_{R3\text{-}56}$ cells exert a preferential regulatory role on the effector functions of TCR activated $CD8^+$ T cells.

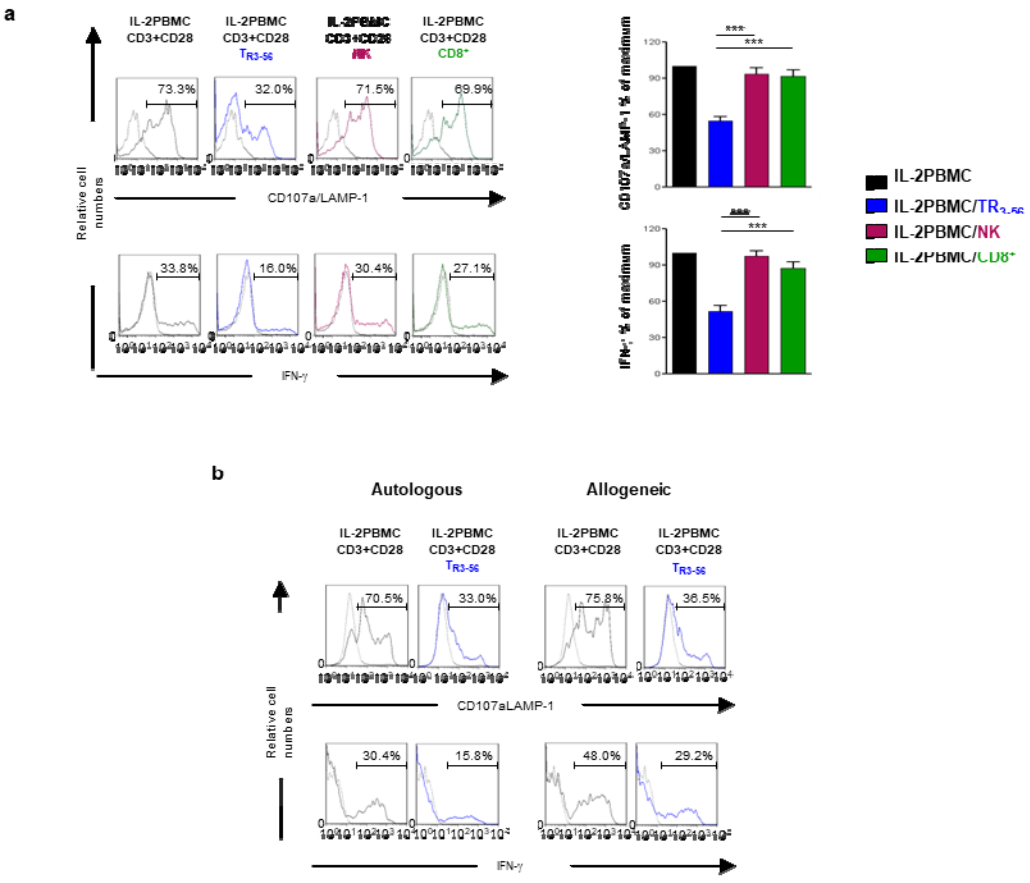

Figure 20: $T_{R3\text{-}56}$ cells significantly reduce CD107a/LAMP-1 and IFN-γ of cytotoxic T cell effectors. A. Left panels show CD107a/LAMP-1 and IFN-γ staining of $CD8^+$ T cells, gated on $CD45^+$ labelled IL-2PBMC after 4 hours of culture with anti-CD3 plus anti-CD28 microbeads alone (black), in the presence of $T_{R3\text{-}56}$ (blue), NK (magenta), $CD8^+$ T cells (green); dotted lines indicate medium culture; numbers indicate the percentage of positive cells; data are from one representative experiment. Right panels show cumulative data from nine independent experiments (n= 9; percent of maximum ± s.e.m.); ***P <0.0005 by two-tailed Wilcoxon matched pairs test. B. Upper and lower panels show CD107a/LAMP-1 and IFN-γ staining of $CD8^+$ T cells, gated on $CD45^+$ labelled IL-2PBMC after 4 hours of culture with anti-CD3 plus anti-CD28 microbeads alone (black), in the presence of autologous or allogeneic $T_{R3\text{-}56}$ cells (blue); numbers indicate the percentage of positive cells; data are from one representative experiment out of three; dotted lines indicate medium culture.

<u>$T_{R3\text{-}56}$ cells can be induced *in vitro* and require cell-to-cell contact to exert their regulatory activity</u>

To investigate on the possibility to generate *in vitro* $T_{R3\text{-}56}$ cells with regulatory properties, we cultured FACS-sorted human $CD3^+CD56^-$ lymphocytes in presence of anti-CD3 plus anti-CD28

microbeads for 10 days with regular supplementation of hrIL-2 (20IU). As shown in Panel A of Figure 21, 8 to 10% of cultured $CD3^+CD56^-$ cells acquired CD56 molecule expression; these induced T_{R3-56} cells (iT_{R3-56}) become able to suppress CDl07a/LAMP-l expression by human CTL obtained by 36h incubation of flow sorted $CD8^+$ T cells (indicated as IL-2CD8).

Moreover, ten-day culture of freshly isolated T_{R3-56} cells in the presence of TCR stimulation and regular rhIL-2 supplementation resulted in massive CD56 down-modulation associated with inability to exert regulatory activity.

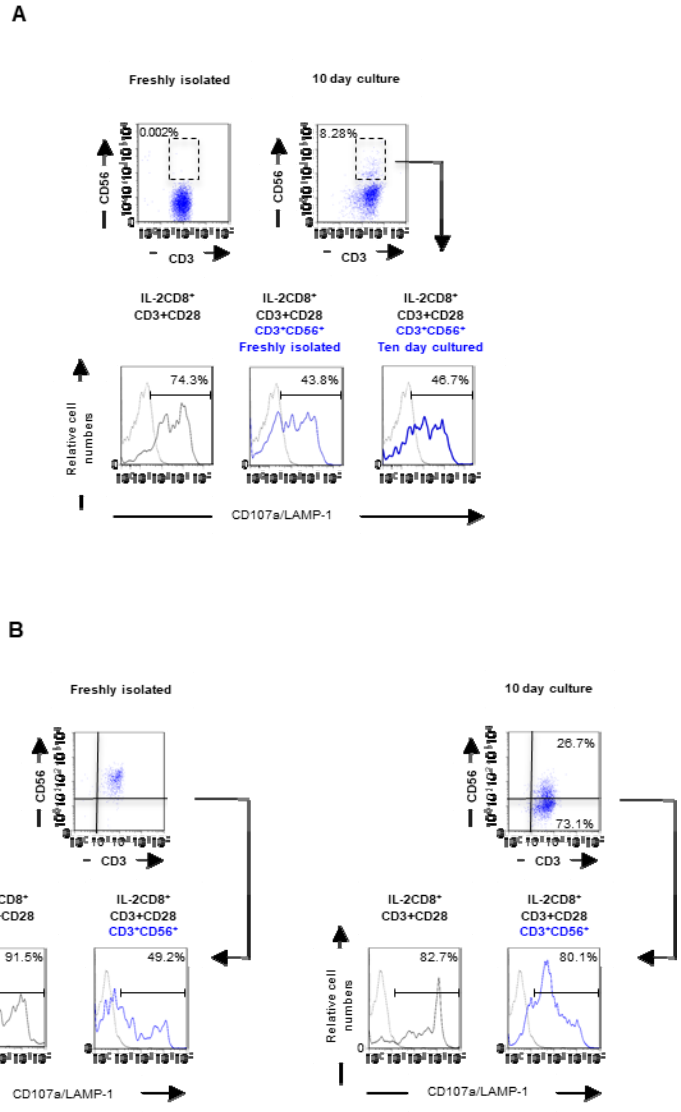

Figure 21: T_{R3-56} cells can be induced *in vitro* upon activation of flow sorted $CD3^+CD56^-$ T cells. A. Freshly isolated $CD3^+CD56^-$ cells (purity > 99%), when cultured for 10 days with anti-CD3 plus anti-CD28 microbeads and regular rhIL-2 supplementation, up-regulate CD56 expression. As indicated by lower panels, these T_{R3-56} cells "induced" *in vitro*, when co-cultured with IL-$2CD8^+$ suppress their CD107/LAMP-1 expression at the same extent than the freshly isolated T_{R3-56} cells. B. freshly isolated T_{R3-56} cells cultured for 10 days with anti-CD3 plus anti-CD28 microbeads and rhIL-2 regular supplementation are unable to maintain CD56 expression and lose their regulatory ability. Numbers indicate the percentage of positive cells; data are from one representative experiment.

To understand mechanisms underlying T_{R3-56} regulatory activity we evaluated whether direct cell-to-cell contact with TCR activated $CD8^+$ T cells was indispensable for T_{R3-56}-dependent

suppressive activity. As shown in panel A of Figure 22, the $T_{R3\text{-}56}$ cells are unable to suppress both CD107a/LAMP-1 expression and lFN-γ production when physically separated from $CD8^+$ T cells in a transwell assay.

Then, to evaluate whether the CD56 molecule is directly involved in contact-dependent suppressive signals, we cultured the $T_{R3\text{-}56}$ cells and TCR-stimulated IL-2CD8 with saturating concentration of soluble human recombinant neural cell adhesion molecule (hrNCAM/CD56) or anti-CD56 mAb. In these experimental conditions, we observed that $T_{R3\text{-}56}$ cells maintaining their capability to modulate CD107a/LAMP-1 expression and IFN-γ production by TCR-activated IL-2CD8. Suppressive capability of the $T_{R3\text{-}56}$ cells co-cultured with IL-2CD8 has been also evaluated in the presence of a blocking anti-CD49d mAb. Indeed, CD49b molecule has been observed to be expressed on the surface of $T_{R3\text{-}56}$ cells. As shown in Panel B of Figure 22, incubation of freshly isolated human $T_{R3\text{-}56}$ cells with anti-CD49d mAb was unable to affect their suppressive capacity.

These data indicate that both CD56 and CD49d-mediated contact are irrelevant for $T_{R3\text{-}56}$ cells regulatory activity.

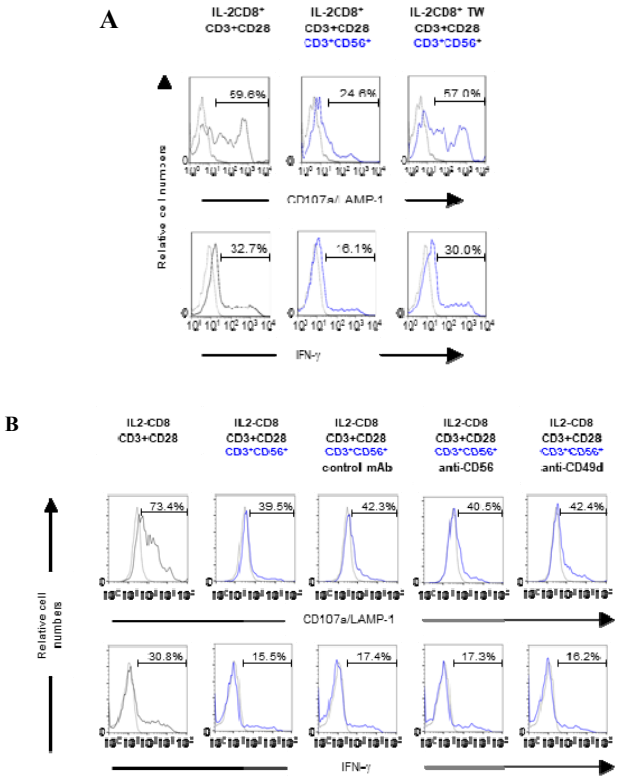

Figure 22: $T_{R3\text{-}56}$ cell regulatory functions require cell-to-cell contact and are independent on CD56 expression. A. Upper and lower panels show CD107a/LAMP-1 and IFN-γ staining of $IL2CD8^+$cultured for 4 hours with anti-CD3 plus anti CD28 microbeads alone (black), together with $T_{R3\text{-}56}$ cells (blue) or with the $T_{R3\text{-}56}$ cells in a transwell assay, as indicated. B. Upper and lower panels show CD107a/LAMP-1 and IFN-γ staining of $IL2CD8^+$cultured for 4 hours with anti-CD3 plus anti CD28 microbeads alone (black) or in the presence of $T_{R3\text{-}56}$ cells (blue); co-cultures of $T_{R3\text{-}56}$ cells with control 345.134 antibody or with the anti-CD56 or the anti-CD49d neutralizing mAb at saturating concentration were specifically indicated. Dotted lines indicate medium culture; numbers indicate the percentage of positive cells; data are from one representative experiment out of four.

5. Conclusions

This study focused the analysis of cell-mediated immune tolerance control in physiological conditions as well as in the context of immune-mediated disorders also addressing the possibility to modulate deranged immune effectors.

With this aim we took advantage from a model, as represented by MDS, allowing direct access to the microsite (BM) specifically involved in the deranged inflammatory process likely underlying the selection/expansion of pathological stem precursors. In this model, we found that, in the first stage of the disease (Low Risk), defective Treg recruitment in the inflammatory microsite (BM), associates with antigen-dependent expansion of cytotoxic immune effectors [53].

In the effort to address the possibility to specifically manipulate adaptive immune effectors, hopefully improving clinical management of immune-mediated diseases, we analysed the mechanisms underlying tolerance induction in a kidney transplantation model. We found [54] that, in this model, mTOR inhibition therapy is able to mediate significant oscillation in the mTOR kinase activity. This effect, confirming previous *in vitro* observations, [14] relates to a significant increase of Treg number and growing ability, and decreased Ki67 expression by the $CD4^{+}Foxp3^{-}$ (T cell effector) counterpart. Thus, oscillatory inhibition of TCR-dependent mTOR activity might represent a therapeutic strategy to optimise targeted manipulation of specific adaptive effectors in kidney-transplant recipients.

Previous studies suggested the immune-modulating effects of a nutraceutical diet in dogs affected by *Canine Leishmaniosis,* a model of natural chronic infection [29]. Thus, in the effort to analyze the possibility to modulate a deranged inflammatory response, we also evaluated the *in vitro* effects of the botanical substances, included in the above nutraceutical diet, on cytokine production by human lymphocytes. We found [55] that these substances are able to exert immune-modulating effects *in vitro,* thus suggesting their possible employ as pharmacological adjuvants able to control the pro-inflammatory activity in the context of an altered immune homeostasis.

Co-expression of CD3 and CD56 molecules, usually associated with T and NK lineage, identifies a cell subset, by us named $T_{R3\text{-}56}$, significantly reduced in T1D diabetes and able to mirror residual β cell activity, as measured by peptide-C availability [41]. In order to analyse the complex network of cell-mediated control of immune response we investigated on the possibility that $T_{R3\text{-}56}$ cells might be able to participate in the regulation of antigen-dependent immune activation. We found (paper submitted) that $T_{R3\text{-}56}$ cells play a key role in the control of cytotoxic T lymphocytes. Indeed, they have been observed to represent a distinct cell subset preferentially involved in the control of effector functions (cytotoxicity and cytokine production) of $CD8^{+}$ T cells. We

consistently found that regulatory ability of $T_{R3\text{-}56}$ cells is strictly relied on cell-to-cell contact and is independent on CD56-mediated interactions.

Taken in all our data confirm the key role of resident Treg in the control of antigen-dependent expansion of adaptive immune effectors in the inflammatory micro environment. Moreover, we add to the complex scenario of cell mediated immune control, a novel T cell subset, the $T_{R3\text{-}56}$ cells, by us observed to preferentially modulate $CD8^+$ T cell effector functions. The possibility that metabolic intervention strategies might be able to improve clinical management of immune-mediated disorders was also addressed.

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Reduced regulatory T cells (Treg) in bone marrow preferentially associate with the expansion of cytotoxic T lymphocytes in low risk MDS patients

The myelodysplastic syndromes (MDS) include clonal bone marrow (BM) disorders characterised by the emergence/dominance of dysplastic progenitors in the context of ineffective haematopoiesis, peripheral cytopenias and increased risk of acute myeloid leukaemia (AML) (Ades *et al*, 2014).

The link between immune dysregulation and MDS has been suggested (Glenthøj *et al*, 2016). Autoimmune attack to normal precursors as well as the activity of bystander T cells, recruited during an immune-response against dysplastic antigens, were hypothesised as relevant for the selection of dysplastic clones that are able to escape immune-mediated damage. The involvement of Natural Killer cells was also described (Terrazzano *et al*, 2012).

Basing on the evidence that innate and adaptive immune-effectors might participate in MDS development, several trials of immune-suppressive therapy have been performed. Younger age, Low Risk classification according to the International Prognostic Scoring System (IPSS) (Greenberg *et al*, 1997), the presence of the HLA-DR15 and high percentage of proliferating $CD4^+$ T cells have been considered as potential predictors of clinical response to immune-suppression (Sloand *et al*, 2008). However, valuable criteria to identify the subgroup of MDS patients susceptible to immune-modulating approaches, are currently lacking.

The T cell regulatory population (Tregs) are physiologically involved in the negative control of immune response (Sakaguchi, 2004). Increased Tregs in the late stages of MDS and the occurrence, in the first phases of the disease, of functional defects and altered migration patterns of this cell subset support the hypothesis that Tregs could play two opposite pathogenic roles in MDS (Kotsianidis *et al*, 2009; Alfinito *et al*, 2010).

Immune response has been fundamentally recognised as a finely tuned microsite process. Thus, the focus on BM immune profile represents a powerful tool for investigating Treg-mediated immune-tolerance control in the pathogenesis/progression of MDS. With this aim, we evaluated Tregs and cytotoxic T cells (CTL) in the BM of 37 MDS patients classified according to IPSS (Greenberg *et al*, 1997). The materials and methods are described in the online supporting information (Data S1). A significant increment ($P < 0{\cdot}05$) in the percentage of BM Tregs was observed in Intermediate-2 (Int-2)/High Risk patients in comparison with the Low/Int-1 groups (Fig 1A). Moreover, a reduction trend of CD54 expression, largely associated with the occurrence of antigen-dependent activation of CTL, was observed on BM-CTL from Low to the Int-2/High stages. BM CTL recruitment was then analysed by calculating the ratio between CTL percentage in the BM and peripheral blood (PB). There was a significant BM recruitment of CTL in Int-1 when compared with Low Risk group ($1{\cdot}74 \pm 0{\cdot}13$ vs. $1{\cdot}35 \pm 0{\cdot}08$; $P < 0{\cdot}05$), while reduced CTL recruitment in BM characterises the Int-2/High stage ($1{\cdot}07 \pm 0{\cdot}14$ in Int2/High vs. $1{\cdot}74 \pm 0{\cdot}13$ in Int-1; $P < 0{\cdot}05$) (Fig 1B, left).

We previously reported a clustered distribution of Tregs in BM of Low Risk patients and that a cut-off of 2% allows identification of two subgroups (Alfinito *et al*, 2010): thus, Low Risk patients were grouped accordingly (Fig 1B, right panel). As shown, the lowest (≤2%) Treg level was significantly associated with increased BM/PB CTL ratio ($1{\cdot}63 \pm 0{\cdot}14$ vs. $1{\cdot}21 \pm 0{\cdot}03$; $P < 0{\cdot}05$). No difference in BM recruitment of $CD4^+$ T cells was observed (not shown). Thus, BM-Treg level seems to preferentially control the BM recruitment of CTL in MDS.

Then, we analysed the cytotoxic T cells (TCR) repertoire in the PB and BM of healthy donors and MDS patients (Figure S1). $CD8^+$ and $CD4^+$ T lymphocytes were considered TCR-skewed when they expressed a single TCR-Vβ protein at a percentage higher than 3 standard deviations (SD) than observed in 10 healthy donors. Moreover, preferential BM-T cell expansions were defined as BM clones that showed a single TCR-Vβ protein expression exceeding the 20% of that observed in PB. Low Risk patients were then divided in two subgroups according to the number of observed BM-T cell expansions. The BM Treg percentage was significantly increased in Low Risk patients with <2 skewed Vβ families in BM CTL ($3{\cdot}04 \pm 0{\cdot}35$ vs. $1{\cdot}88 \pm 0{\cdot}58$; $P < 0{\cdot}005$; Fig 1C, left). Moreover, (Low Risk patients with ≥2 Vβ expansions in BM CTL showed significantly increased CD54 expression on CTL ($12{\cdot}99 \pm 2{\cdot}47$ vs. $3{\cdot}94 \pm 0{\cdot}42$; $P < 0{\cdot}05$; Fig 1D, left). No difference was observed when $CD4^+$ T cells were analysed (Fig 1C and D, right panels). Therefore, Tregs appear to exert a key role in the regulation of CTL activation/expansion in BM.

We previously observed that Tregs from Low Risk patients show a clustered, not homogeneous, distribution in BM and that a 2% cut-off value identifies two populations (Alfinito *et al*, 2010). Therefore, the occurrence of T cell expansions in BM of Low Risk individuals, categorised according to BM

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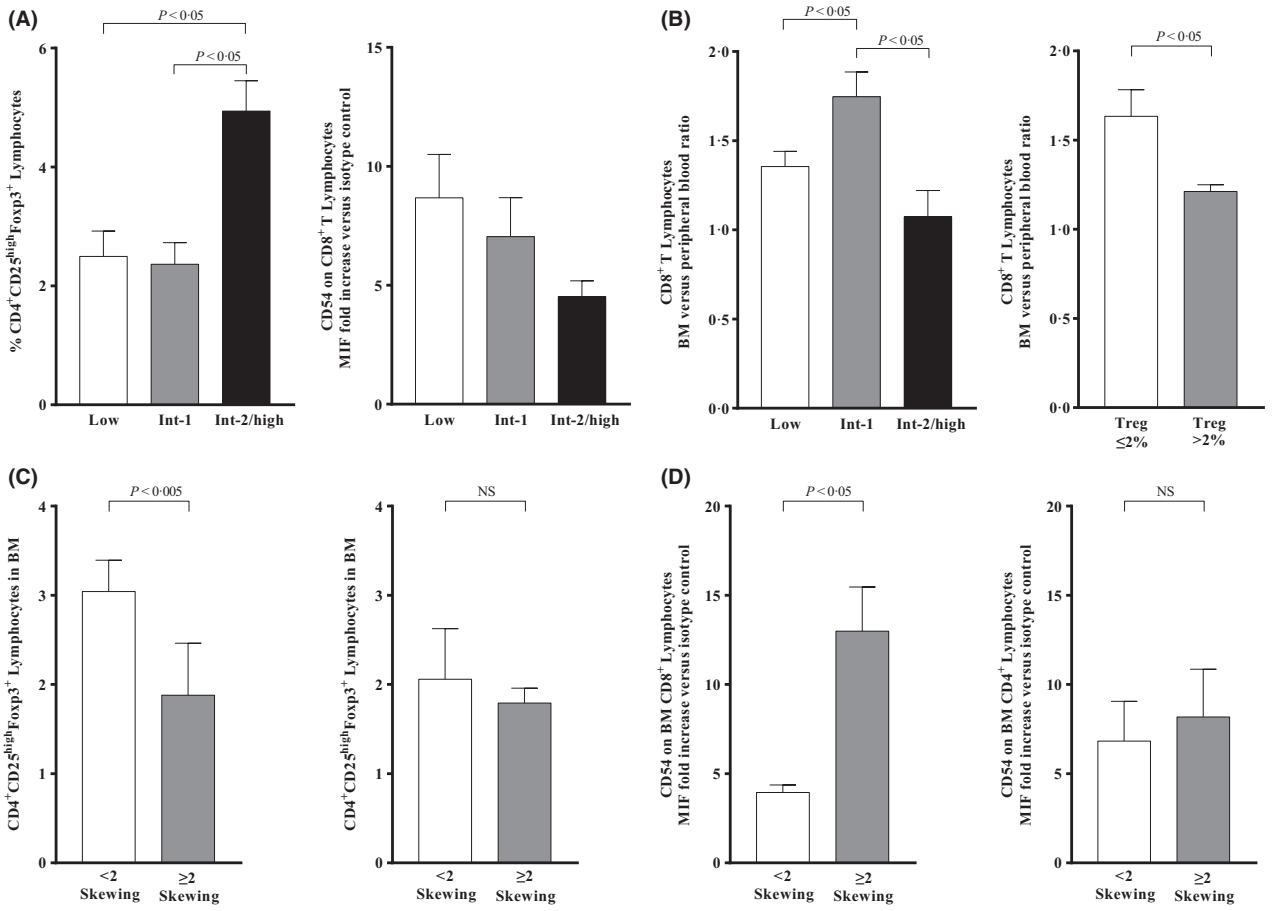

Fig 1. Treg level in BM preferentially controls BM-recruitment, activation and expansion of cytotoxic T cell effectors in Low risk MDS patients. (A) Treg percentage and CD54 expression on bone marrow cytotoxic T cells (BM-CTL) in myelodysplastic syndrome (MDS) patients classified as Low (white column), Intermediate 1 (Int-1; grey column) and Int-2/High (black column) risk, respectively; Tregs were significantly increased in Int-2/High, when compared to the Low ($P < 0{\cdot}05$) and Int-1 ($P < 0{\cdot}05$) individuals (left panel). For CD54 expression on BM-CTL, a trend of decreased expression was detected from Low and Int-1 to Int-2/High stages (right panel); (B) Left: there was a significant increase of BM-CTL in Int-1 ($P < 0{\cdot}05$), as compared with the Low Risk, while BM-CTL was decreased in Int-2/High Risk ($P < 0{\cdot}05$ as compared with the Int-1); White, grey and black columns indicate Low, Int-1 and Int-2/High risk patients, respectively; BM recruitment of CTL was evaluated by calculating the ratio between CTL percentage in BM and peripheral blood (PB); Right: BM recruitment of CTL in Low Risk patients categorised according to BM-Treg level [≤2% (white column) or >2% (grey column)], as described (Alfinito *et al*, 2010); Lower Treg percentage significantly correlated ($P < 0{\cdot}05$) with higher recruitment rate of CTL in BM; (C) Treg percentage and T-cell receptor (TCR)-Vβ skewing in BM-CTL and BM-$CD4^+$ T cells of Low Risk patients classified, according to the number of the T cell expansions, in two groups: <2 Vβ skewing (white column) vs. ≥2 Vβ skewing (grey column). An increase of Treg percentage in BM characterises Low risk patients with <2 TCR-Vβ skewed CTL, as compared those with ≥2 expansions in the BM (left); no significant difference was observed in the BM-Treg percentage when considering the $CD4^+$ T cell TCR-Vβ repertoire in the BM (right); (D) CD54 expression and TCR-Vβ skewing in BM-CTL and BM-$CD4^+$ T cells of Low Risk patients. As shown, individuals with ≥2 TCR-Vβ expansions in BM-CTL are characterised by significantly increased CD54 expression on CTL (left); no difference in CD54 expression was observed in $CD4^+$ T cells, despite the occurrence of a more skewed TCR-repertoire (right).

Treg level, was analysed. A significant increase of CTL expansions was detected in the BM of patients that showed lower (<2%) Treg level at disease onset ($P < 0{\cdot}05$; Table I). No significant association of Treg level with BM-$CD4^+$ T cell expansions was observed.

We also analysed leukaemia evolution and survival, in a minimal 36-month follow-up, in our Low Risk cohort grouped according to BM-Treg level. There was a significantly higher evolution to leukaemia ($P < 0{\cdot}05$) and death ($P < 0{\cdot}05$) in the Low Risk individuals with BM Tregs >2% at disease onset (Table I).

Taken together, our observations suggest that BM-Treg preferentially modulate CTL recruitment, activation and proliferation in BM of Low Risk MDS patients, and that their frequency at diagnosis inversely associates with an immune profile able to control disease progression.

A long-term follow-up study (Sloand *et al*, 2008) reported that responders to immune-suppression showed significantly longer survival with lower transformation to leukaemia. In addition, Tregs together with B cell progenitors were described as independent prognostic predictors in Low Risk patients, while overall survival and progression-free survival was

Table I. Follow-up evaluation of Low Risk myelodysplastic syndrome patients categorised according to regulatory T cell (Treg) level in the bone marrow (BM) at disease onset.*

	N	Age	CD8 skewed in BM ≥2	CD4 skewed in BM ≥2	Transfusion dependence	Leukaemiaevolution	Death
Low Risk	26	72·6 ± 9	13	10	8	5	6
BM Treg ≤2%	14	71 ± 5	10†	6‡	5	0§	0¶
BM Treg >2%	12	74·4 ± 11	3	4	3	5	6

*data refer to a minimum 36-month follow-up.
†significantly different from BM Treg >2% group ($P < 0·05$ by Fisher exact test; Odds Ratio (OR) 7·5 (95% confidence interval [CI]: 1·307–43·047).
‡not significantly different from BM Treg >2% group.
§significantly different from BM Treg >2% group ($P < 0·05$ by Fisher exact test); OR 0·047 (95% CI: 0·002279–0·9704).
¶significantly different from BM Treg >2% group ($P < 0·005$ by Fisher exact test); OR 0·034 (95% CI: 0·001679–0·7082).

significantly associated with lower Treg levels (Kahn *et al*, 2015). Moreover, the co-occurrence of MDS with autoimmune disorders was observed to predict longer survival and reduced leukaemia progression (Glenthøj *et al*, 2016; Komrokji *et al*, 2016). Accordingly, we found a significant association of lower Treg frequency and higher skewing in the Vβ BM-CTL TCR-repertoire, with decreased leukaemia progression and better overall survival in Low Risk MDS patients. Further investigation, addressing the molecular targets of BM skewed CTL, will hopefully clarify the role of immune-mediated processes in MDS pathogenesis and/or progression.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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Keywords: myelodysplastic syndromes, Treg, bone marrow, Vβ repertoire, cytotoxic T cells

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Analysis of $CD4^+$ and $CD8^+$ T lymphocyte V-beta repertoire in BM and PB of healthy donors as compared with MDS patients.

Data S1. Methods and Materials.

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Oscillatory mTOR inhibition and T_{reg} increase in kidney transplantation

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Summary

Intracellular metabolic pathways dependent upon the mammalian target of rapamycin (mTOR) play a key role in immune-tolerance control. In this study, we focused on long-term mTOR-dependent immune-modulating effects in kidney transplant recipients undergoing conversion from calcineurin inhibitors (CNI) to mTOR inhibitors (everolimus) in a 1-year follow-up. The conversion to everolimus is associated with a decrease of neutrophils and of $CD8^+$ T cells. In addition, we observed a reduced production of interferon (IFN)-γ by $CD8^+$ T cells and of interleukin (IL)-17 by $CD4^+$ T lymphocytes. An increase in $CD4^+CD25^+$ forkhead box protein 3 $(FoxP3)^+$ [regulatory T cell [(T_{reg})] numbers was also seen. T_{reg} increase correlated with a higher proliferation rate of this regulatory subpopulation when compared with the $CD4^+FoxP3^-$ effector counterpart. Basal phosphorylation level of S6 kinase, a major mTOR-dependent molecular target, was substantially maintained in patients treated with everolimus. Moreover, oscillations in serum concentration of everolimus were associated with changes in basal and activation-dependent S6 kinase phosphorylation of $CD4^+$ and $CD8^+$ T cells. Indeed, T cell receptor (TCR) triggering was observed to induce significantly higher S6 kinase phosphorylation in the presence of lower everolimus serum concentrations. These results unveil the complex mTOR-dependent immune-metabolic network leading to long-term immune-modulation and might have relevance for novel therapeutic settings in kidney transplants.

Keywords: everolimus, immunosuppression, kidney transplants, mTOR

Introduction

Calcineurin inhibitors (CNI) are the standard treatment in kidney transplantation [1,2]. Chronic use of CNI is associated with graft dysfunction, increased risk of cardiovascular disorders and malignancies [3,4]. The key challenge in the management of renal transplants is to reduce adverse effects while maintaining a low acute rejection rate. New therapeutic protocols have been proposed [5,6], aimed at limiting CNI use from early post-transplant phases up to their discontinuation. Inhibitors of mammalian target of rapamycin (mTOR) have been found to represent a viable alternative to CNI [7–9].

The serine/threonine kinase mTOR, which belongs to the phosphatidylinositol kinase-related kinase (PIKK) family, regulates cell growth and metabolism in response to environmental cues [10]. The mTOR structure provides binding sites for multiple proteins that regulate its activity. It associates with *raptor* and *rictor* to form mammalian target of rapamycin complex 1 (mTORC1) and mTORC2, respectively, with different regulatory activities [10].

Survival of a transplanted kidney is mainly dependent upon immune tolerance *versus* allogeneic cells [11]. Immune-regulatory systems include accessory signals intrinsic to antigen recognition and those mediated by suppressor subsets, represented primarily by $CD4^+CD25^+$ regulatory T cells (T_{reg}) expressing the forkhead box protein 3 (FoxP3) transcription factor [12–14].

Cross-talk between immune response and metabolism is still largely undefined. Defective mTOR activity severely impairs T helper type 1 (Th1), Th2 and Th17 cell differentiation [15,16] and induces the T_{reg} [17,18]. T_{reg} availability and proliferation depend specifically upon mTOR oscillatory activity [19], while FoxO1, a major transcriptional regulator of CD8 differentiation, also depends upon mTORC1 [20].

The mTORC1 inhibitor everolimus, a synthetic derivative of rapamycin, shows high oral bioavailability, stability and solubility [9,21]. Beyond its use as immunosuppressor, everolimus has been approved for treatment of solid [22,23] and haematological malignancies [24]. Its dosage is higher (by six to 10-fold) in an oncology setting than in transplantation [22,23,25]. The difference in both the dosage and administration schedule of the drug could be relevant to induce immune tolerance rather than inhibition of cancer cell growth. In order to address this issue, we investigated the immune profile of kidney-transplanted patients undergoing conversion from CNI to everolimus. We performed an *ex-vivo* analysis of leucocyte number, T cell cytokine profile, T_{reg} number and proliferation, as well as evaluation of the major mTOR-dependent molecular pathway (S6 kinase) in $CD4^+$ and $CD8^+$ T cells before (T0) and after drug conversion, throughout a 1-year follow-up (T12). A better understanding of the mTOR-dependent immune metabolic network is expected to favour manipulation of specific adaptive effectors, hopefully improving the survival of functional graft in kidney transplant recipients.

Material and methods

Study population

The study was carried out on 19 renal transplant recipients, all first transplant from cadaver donors. Inclusion criteria were aged 18–65 years; transplant vintage > 3 years; plasma creatinine < 2 mg/dl, with stable estimated glomerular filtration rate (eGFR) in the previous 3 months; haemoglobin value > 10 g/dl; white cell count $> 3000/\mu l$ (neutrophils $> 1500/\mu l$); platelets $> 75{\cdot}000/\mu l$; and absence of rejection signs or infectious episodes in the previous 3 months.

Exclusion criteria included previous or combined transplantation; panel-reactive antibodies (PRA) $> 25\%$ and/or the presence of donor-specific antibodies (DSA) at transplantation; the presence of proteinuria exceeding 300 mg/day on 24-h samples; hyperlipidaemia (baseline cholesterol and/or tryglicerides values exceeding 220 and 200 mg/dl, respectively); and evidence of autoimmune diseases or of viral infections.

Study protocol

At baseline (T0), dosage of CNI was reduced empirically by 50% and everolimus was introduced at a starting dosage of 0·50 mg/twice a day (b.i.d.). This initial dose of everolimus, lower than that (0·75 mg/b.i.d.) suggested by the ZEUS study [26], was chosen because of the difference in the clinical features of the cohort we enrolled, characterized by a long transplant vintage, stable renal function and no immunological/infectious problems in the 3 months preceding enrolment. Plasma levels of both drugs were checked after 1 week, and everolimus dosage was modified opportunely to reach trough levels (TL) of 5–8 ng/ml (with further dose modifications, if necessary). After a 4-week stabilization period, CNI dose was reduced further by 25% and finally withdrawn (within the fourth month), whereas everolimus TL were increased up to 6–10 ng/ml. After 6 months all the patients were on everolimus alone; they were evaluated again at 1 year from baseline (T12). Dosage of steroids was never altered throughout the study. Six of the enrolled patients continued mycophenolic acid (MFA) co-treatment that was associated with everolimus. These patients, whose immune-modulating regimen included MFA co-administration, were analysed independently throughout the study.

At each study step (T0 and T12), all the patients were scheduled in clinical visits; samples were withdrawn to determine the main laboratory data, including TL of immunosuppressive drugs.

The study, conducted in agreement with good clinical practice guidelines, was approved by the Ethics Committee of Federico II University of Naples (protocol number: 66/11). All the procedures were in accordance with the Declaration of Helsinki, as revised in 2008. All the patients signed their informed consent to the study. Twelve healthy blood donors, age- and sex-matched with the patients, were enrolled into the study as controls.

Immunofluorescence, cell sorting and T cell activation

Blood samples were analysed by immunofluorescence and flow cytometry by using a two-laser equipped fluorescence activated cell sorter (FACS)Calibur apparatus and CellQuest analysis software (Becton Dickinson, San Jose, CA, USA). Fluorescein isothiocyanate (FITC), phycoerythrin (PE),

cychrome and allophycocyanin (APC)-labelled monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD56, invariant natural killer T (NKTi), CD25, FoxP3, Ki67, interferon (IFN)-γ, interleukin (IL)-4, IL-17 and isotype-matched controls were purchased from Becton Dickinson. APC-labelled anti-phospho S6 kinase mAb was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

To analyse cytokine production, peripheral blood mononuclear cells (PBMC) were cultured overnight in the presence of phorbol myristate acetate (PMA), ionomycin and brefeldin-A (Sigma-Aldrich, St Louis, MO, USA), as described previously [27].

All phenotypes referred to flow cytometry analysis of the lymphocyte population gated using forward- (FSC) and side-scatter (SSC) parameters. Intracellular cytokine profile, FoxP3, Ki67 and phospho S6 kinase staining were performed with a fixation-permeabilization buffer (Becton Dickinson), following the manufacturer's instructions.

$CD4^+$ and $CD8^+$ T cells were sorted by FACSJazz (Becton-Dickinson). To mimic antigen-dependent T cell activation, sorted $CD4^+$ and $CD8^+$ cells were incubated for 1 h with anti-CD3/anti-CD28 mAb-coupled microbeads (Life Technologies AS, Oslo, Norway) at the cell/bead ratio of 1 : 0.2, as described previously [18].

To evaluate some possible oscillation in the results, two independent samples obtained for each patient at T0 and T12 were analysed at 1-week intervals and produced substantially comparable results (not shown).

Molecular signalling analysis

Independent total cell lysates, obtained from $CD4^+$ and $CD8^+$ sorted T cells, were incubated as indicated previously, and 30 μg of total proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions; proteins were transferred onto a nitrocellulose filter membrane (Protan; Schleicher & Schuell, Dassel, Germany) with a Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA, USA). Filters were then incubated with specific mAbs (anti-phospho-S6 Ser240/244 and anti-S6 5G10 clone, from Cell Signaling Technology; anti-extracellular-regulated kinase (ERK)1/2 (clone H72; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and with a peroxidase-conjugated secondary antibody (Amersham Biosciences, Picaraway, NJ, USA). Peroxidase activity was detected with the enhanced chemiluminescence (ECL) system (Amersham Biosciences) or Femto (Pierce, Rockford, IL, USA). Normalization was performed against total ERK1/2. All filters were quantified by band densitometry analysis using the ScionImage version 1·63 software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Statistical evaluation of the data using InStat version 3·0 software (GraphPad Software Inc., San Diego, CA, USA), was performed by Mann–Whitney test or Wilcoxon's matched-pairs signed-rank test, as indicated. Two-sided *P*-values less than 0·05 were considered significant. The corrected *P*-value (P_c) was calculated by applying Bonferroni adjustment for multiple comparisons, as described previously [28].

Results

The clinical evaluation of patients during the study

The demographic data of patients are reported in Table 1. One patient left the study after the sixth month because of the occurrence of an acute antibody-mediated rejection. The main laboratory data are presented in Table 2. Cholesterol was higher at T12 than at T0 (+19·8%; $P < 0{\cdot}001$) and haemoglobin concentration was lower at T12 than at T0 (−6%; $P < 0{\cdot}001$). No modification was detected in urinary protein excretion. Glomerular filtration rate was increased slightly, although not significantly, after CNI withdrawal.

The mean everolimus TL, effective in maintaining adequate immunosuppression, were at the lower step of the desired range (6–10 ng/ml). The six enrolled patients receiving CNI/MFA also continued MFA co-administration after everolimus conversion. No dose adjustment in statins or in erythropoiesis-stimulating factors was performed during the study. All the patients remained under 4 mg/day of methylprednisolone.

Side effects after the everolimus switch were mild and transient (headache, pruritus, joint pain). One patient complained of persisting aphthous stomatitis, requiring therapy and a small reduction of the everolimus dosage.

Effect of conversion from CNI to everolimus on leucocyte number and cytokine production profile

As shown in Fig. 1a, the conversion from CNI to everolimus was able to reduce the number of total leucocytes to T12 (7099 ± 476) compared to T0 (8443 ± 690). This reduction trend was observed specifically in patients undergoing everolimus treatment without MFA association (9457 ± 770 at T0 *versus* 7261 ± 628 at T12; $P_c < 0{\cdot}05$).

Table 1. Demographic and laboratory data of the patients enrolled into the study.

Patients ($n = 19$)	
Sex (M/F)	13/6
Age (years)	$49{\cdot}6 \pm 11{\cdot}7$
Weight (kg)	$74{\cdot}1 \pm 13{\cdot}2$
Transplant vintage (years)	$3{\cdot}5 \pm 1{\cdot}0$
CNI (Cya)	19/19
MPA derivatives (yes/no)	6/13
Anti-hypertensive drugs (*n*)	$2{\cdot}3 \pm 0{\cdot}8$
Statins (yes/no)	14/5

CNI = calcineurin inhibitors; Cya = cyclosporin; MPA = mycophenolic acid; M/F = male/female.

Table 2. Main laboratory data throughout the study.

	T0	T6	T12
eGFR (m/min)	65·9 ± 20·1	70·3 ± 20·8	72·3 ± 27·6
Plasma creatinine (mg/dl)	1·30 ± 0·47	1·32 ± 0·83	1·27 ± 0·47
Plasma haemoglobin (g/dl)	13·8 ± 1·4	13·3 ± 1·5	13·0 ± 1·7*
Plasma albumin (g/dl)	4·7 ± 0·3	4·50 ± 0·3	4·6 ± 0·3
Plasma cholesterol (mg/dl)	176·2 ± 29·3	211·6 ± 45·3	211·3 ± 38·4*
Plasma triglycerides (mg/dl)	126·0 ± 58·6	134·3 ± 54·0	141·2 ± 58·8
24-h urinary protein excretion (g)	0·10 ± 0·13	0·19 ± 0·24	0·18 ± 0·24
Everolimus trough levels (ng/ml)	0	6·47 ± 3·4	7·0 ± 2·1

T0 = baseline (under calcineurin inhibitors); T6 = 6 months from baseline (under everolimus); T12 = 12 months from baseline (under everolimus); eGFR = glomerular filtration rate (MDRD equation). *Indicates significant difference from T0 value.

Similar behaviour was seen for neutrophils (Fig. 1b). Indeed, the significant reduction observed at T12 (6192 ± 509 at T0 *versus* 3958 ± 406 at T12; $P_c < 0{\cdot}05$) specifically involved the subgroup of patients treated with everolimus without MFA (5776 ± 660 at T0 *versus* 3833 ± 596 at T12; $P_c < 0{\cdot}05$). Analysis of $CD8^+$ T cell number (Fig. 1d) also revealed a significant reduction of this cell subset in patients receiving the everolimus treatment without MFA (743 ± 135 at T0 *versus* 471 ± 58 at T12; $P_c < 0{\cdot}05$). No differences were observed in total lymphocyte count (Fig. 1c) or in $CD3^+CD4^+$ T cell number (data not shown) when comparing T0 *versus* T12 data. Percentage analysis confirmed this trend (data not shown).

Therefore, long-term everolimus-dependent mTORC1 inhibition reduced significantly the number of neutrophils and CD8 T cells in kidney transplant recipients. These changes were hampered by MFA co-administration.

To evaluate the effect of everolimus conversion on cytokine production, we analysed IFN-γ, IL-4 and IL-17 in NKTi lymphocytes, a major player in cytokine profile polarization [29]. IFN-γ in $CD8^+$ T cells and IFN-γ and IL-17 in $CD4^+$ T lymphocytes were also evaluated. Notably, NKTi lymphocyte numbers in controls were similar to those in patients, regardless of their immunosuppressive treatment (data not shown). Comparison with healthy controls has been included in order to underline, when present, the persistent reduced cytokine production observed in our patient cohort after conversion.

As shown in Fig. 2a, no significant changes in IFN-γ production were observed after the conversion from CNI to everolimus. Indeed, the percentage of IFN-γ-producing NKTi cells remained significantly lower in patients than in healthy donors both at T0 (12·68 ± 2·65 at T0 *versus* 22·79 ± 1·25 in controls; $P_c < 0{\cdot}05$) as well as at T12 (6·96 ± 1·74; $P_c < 0{\cdot}005$ *versus* controls), regardless of their immune-modulating regimen.

In contrast, the high IL-4 production in MFA-CNI-treated patients at T0 (21·51 ± 8·35 *versus* 5·51 ± 0·90 in controls; $P_c < 0{\cdot}005$) was reduced at T12, so that it became similar to healthy donors (Fig. 2b).

The frequency of IL-17-producing NKTi cells reduced significantly compared to controls at T0, and remained substantially unchanged at T12 (Fig. 2c).

The production of IFN-γ by $CD8^+$ T cells (Fig. 2d) decreased significantly after the everolimus conversion (30·88 ± 5·12 at T0 *versus* 9·14 ± 1·76 at T12; $P_c < 0{\cdot}001$),

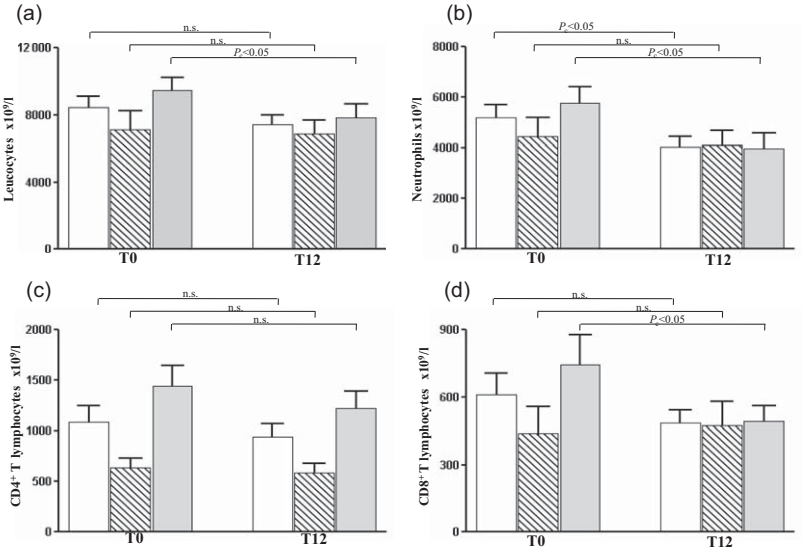

Fig. 1. Leucocytes, neutrophils and T cell subsets in kidney transplanted patients undergoing calcineurin inhibitors (CNI) to everolimus conversion. (a–d) Evaluation of leucocytes, neutrophils, CD4 and CD8 T cell subsets as indicated at T0 before therapy conversion and T12 1 year after everolimus conversion. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Wilcoxon's matched-pairs signed-rank test is reported. The corrected *P*-value (P_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

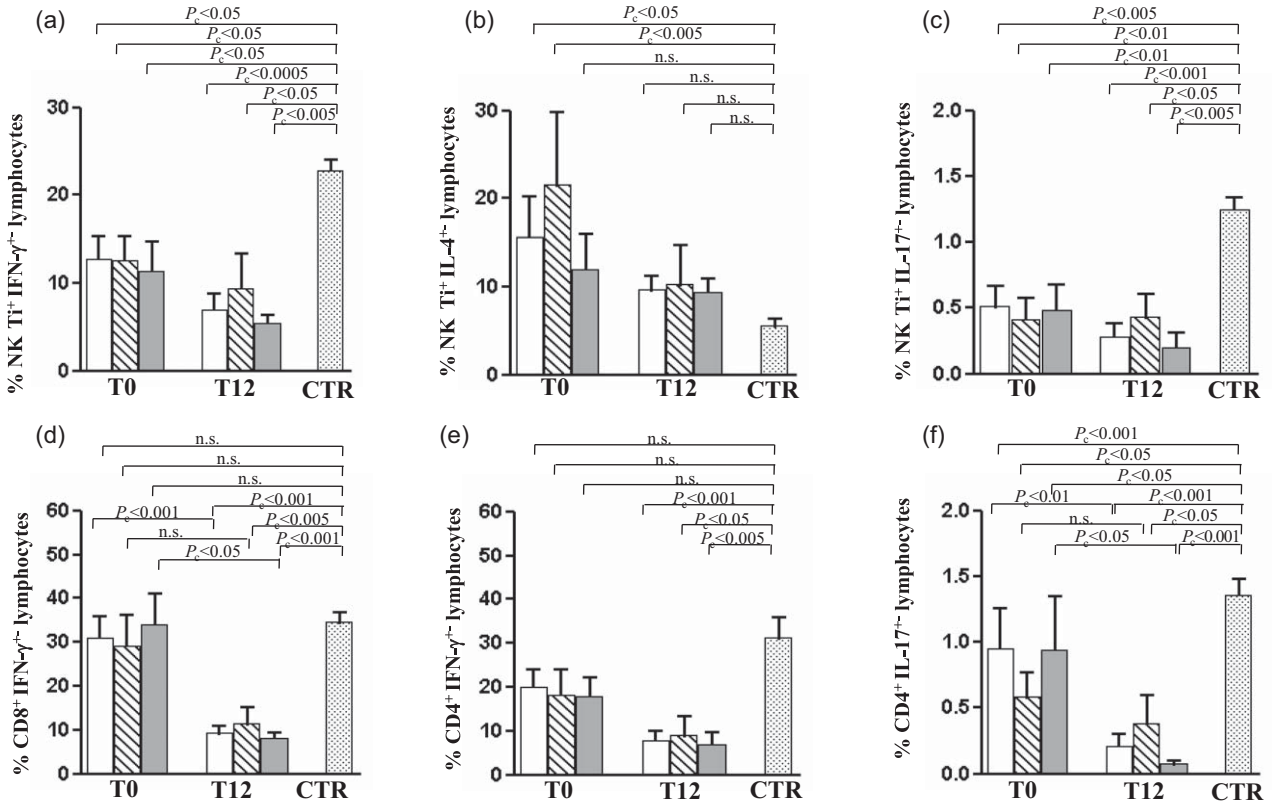

Fig. 2. Cytokine secretion profile of invariant natural killer T (NKTi), $CD8^+$ T and $CD4^+$ T lymphocytes in kidney-transplanted patients undergoing conversion from calcineurin inhibitors (CNI) to everolimus. (a–c) Interferon (IFN)-γ, interleukin (IL)-4 and IL-4 production by NKTi cells at T0 and T12 after CNI to everolimus conversion. (d) IFN-γ production in $CD8^+$ T cells and (e,f) IFN-γ and IL-17 production by $CD4^+$ lymphocytes at T0 and T12. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). Mann–Whitney test is reported. For T0–T12 comparison of paired samples, Wilcoxon's matched-pairs signed-rank test has been performed. The corrected *P*-value (P_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

significantly lower than controls at T12 ($P_c < 0{\cdot}001$). Notably, the reduction trend was observed to involve preferentially the group of patients whose treatment did not include MFA association ($34{\cdot}06 \pm 7{\cdot}01$ at T0 *versus* $8{\cdot}08 \pm 1{\cdot}25$ at T12; $P_c < 0{\cdot}05$). Therefore, MFA co-administration specifically hampered everolimus-dependent modulation of IFN-γ production in $CD8^+$ T lymphocytes. This observation suggests the relevance for mTOR-dependent mechanisms in proinflammatory cytokine production by $CD8^+$ T lymphocytes.

As shown in Fig. 2e, the production of IFN-γ by $CD4^+$ T cells was significantly lower than controls at T12 ($7{\cdot}70 \pm 2{\cdot}32$ *versus* $31{\cdot}10 \pm 4{\cdot}75$; $P_c < 0{\cdot}001$), regardless of the immune-modulating regimen.

IL-17 producing $CD4^+$ T lymphocytes (Fig. 2f) were reduced significantly in patients than in controls at T0 ($0{\cdot}95 \pm 0{\cdot}29$ *versus* $1{\cdot}36 \pm 0{\cdot}12$; $P_c < 0{\cdot}001$) and at T12 ($0{\cdot}2 \pm 0{\cdot}09$; $P_c < 0{\cdot}001$). Moreover, comparison of T0 and T12 values in paired samples revealed that everolimus treatment without MFA co-administration was able to mediate complete inhibition of IL-17 producing $CD4^+$ T cells ($0{\cdot}94 \pm 0{\cdot}4$ at T0 *versus* $0{\cdot}086 \pm 0{\cdot}02$ at T12; $P_c < 0{\cdot}05$). Thus, MFA association seemed to affect significantly everolimus-dependent modulation of IL-17 production by $CD4^+$ T lymphocytes.

No change was observed in NK-dependent IFN-γ production between controls and patients, regardless of their immunosuppressive treatment (not shown).

T_{reg} numbers and proliferation in kidney transplant recipients undergoing everolimus conversion

Because mTOR-dependent pathways affect T_{reg} homeostasis [17–19], we analysed the effect of everolimus conversion on number and suppressor activity of T_{reg} in our patients. No difference in the T_{reg} suppressor activity was observed in any of the patients, regardless of the treatment group (not shown). As shown in Fig. 3, the comparison of paired samples revealed a significant increase of T_{reg} after CNI to everolimus conversion ($89 \pm 16{\cdot}5$ at T0 *versus* $129{\cdot}9 \pm 22{\cdot}87$ at T12; $P_c < 0{\cdot}05$) only in those patients

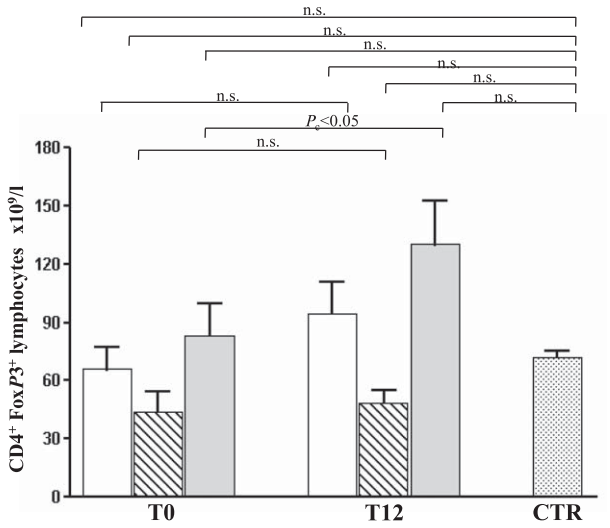

Fig. 3. Regulatory T cell (T_{reg}) population in kidney-transplanted patients following calcineurin inhibitors (CNI) to everolimus conversion. Number of T_{reg}, gated as $CD4^+$forkhead box P3 (FoxP3)$^+$ T cells at T0 and T12 were reported. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). For comparison with CTR the Mann–Whitney test is reported. For T0–T12, comparison of paired-samples Wilcoxon's matched-pairs signed-rank test was performed. The corrected *P*-value (P_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

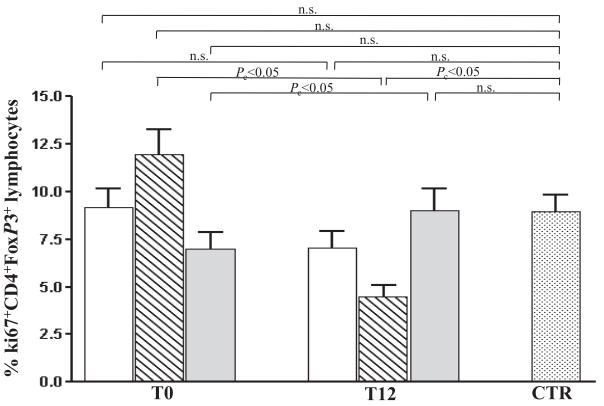

Fig. 4. Ki67 expression on regulatory T cells (T_{reg}) gated as $CD4^+$forkhead box P3 (Foxp3)$^+$ T cells in kidney-transplanted patients. Percentage is given for Ki67 expression in T_{reg}, gated as $CD4^+FoxP3^-$ T cells, at T0 and T12 after calcineurin inhibitors (CNI) to everolimus conversion. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). For comparison with CTR, the Mann–Whitney test is reported. For T0–T12, comparison of paired-samples Wilcoxon's matched-pairs signed-rank test was performed. The corrected *P*-value (P_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

whose treatment did not include MFA association. Percentage data confirmed this trend (not shown). Notably, T_{reg} numbers always remained similar to controls.

To investigate the growth ability of T_{reg}, we analysed their *ex-vivo* expression of the proliferation marker Ki67 [30]. As shown in Fig. 4, CNI to everolimus conversion associated with a significant increase of Ki67 expression on the T_{reg} population (6·99 ± 0·89 at T0 *versus* 8·99 ± 1.15 at T12; $P_c < 0{\cdot}05$). Conversely, everolimus-MFA co-treatment was observed to mediate reduction of this proliferation marker in the T_{reg} subset (11.43 ± 1·23 at T0 *versus* 4·53 ± 0·52 at T12; $P_c < 0{\cdot}05$). Therefore, MFA association hampered the positive effect of everolimus significantly on T_{reg} growth ability. As already stated, no difference in T_{reg} suppressor activity was observed between T0 and T12, regardless of the immunosuppressive treatment of the patients (not shown).

As the growth ability of T cell effectors is crucial for T cell activation, we also analysed the *ex-vivo* Ki67 expression of $CD4^+FoxP3^-$ T cells supposed to include preferentially the CD4 effector population. As shown in Fig. 5, Ki67 expression in $CD4^+FoxP3^-$ T lymphocytes was significantly higher in T0 patients than in controls (7·07 ± 0·83 *versus* 1·26 ± 0·12; $P_c < 0{\cdot}0005$), regardless of the immune-modulating regimen. A significant decrease in growing $CD4^+FoxP3^-$ T cells was observed at T12 (2·81 ± 0·47; $P_c < 0{\cdot}01$ *versus* T0; NS *versus* controls). This decreased trend was observed to be very strong in T12 patients treated with MFA–everolimus association [7·32 ± 0·94 at T0 *versus* 1·74 ± 0·33 at T12; $P_c < 0{\cdot}05$; not significant (n.s.) *versus* controls]. Indeed, the reduced level of Ki67 expression in $CD4^+FoxP3^-$ T cells of T12 patients treated with everolimus alone remained significantly higher than in controls (6·57 ± 1·30 at T0 *versus* 3·48 ± 0·66 at T12; $P_c < 0{\cdot}05$; $P_c < 0{\cdot}01$ *versus* controls). The MFA–everolimus association was observed to mediate a strong decrease of cell growth in both T_{reg} and $CD4^+FoxP3^-$ T cells. Conversely, a preferential effect on T_{reg} growth ability was observed in everolimus-treated patients. Notably, the CNI–everolimus conversion always restored the physiological difference in growing ability between T_{reg} and T cell effector subset, lost at T0 in our cohort.

Everolimus serum concentration associates with different levels of mTOR-dependent S6 kinase phosphorylation in CD4 and CD8 T cells of kidney transplant recipients

To investigate the molecular mechanisms underlying immune modulation in kidney transplant recipients shifted from CNI to everolimus, we analysed the phosphorylation

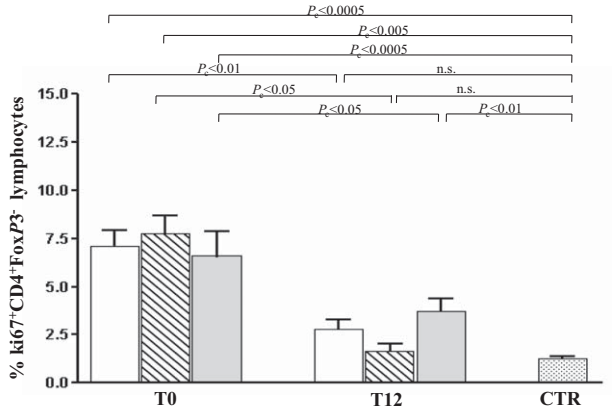

Fig. 5. Ki67 expression on $CD4^+$forkhead box P3 (Foxp3)$^-$ T cells in kidney-transplanted patients. Percentage is given for Ki67 expression in $CD4^+$ T cell effectors, gated as $CD4^+$FoxP3$^-$ T cells at T0 and T12 after calcineurin inhibitors (CNI) to everolimus conversion. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). For comparison with CTR, the Mann–Whitney test is reported. For T0–T12, comparison of paired-samples Wilcoxon's matched-pairs signed-rank test was performed. The corrected *P*-value (P_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

of S6 kinase as a major downstream target of mTORC1 activity [10]. In order to avoid MFA-dependent interference, we analysed only samples obtained from patients undergoing immune-modulating regimens not including MFA administration.

To ascertain whether phosphorylation of mTOR-dependent targets might be conditioned by drug serum concentration, we evaluated S6 kinase phosphorylation (p-S6) levels in $CD4^+$ and $CD8^+$ T cells of T12 patients after 3 (T12–3 h) and 12 h (T12–12 h) from everolimus administration, reflecting maximal and minimal TL of the drug (data not shown). T cell receptor (TCR) triggering was mimicked by incubation with anti-CD3/anti-CD28 beads, as reported [18]. Figure 6 shows Western blot analysis of sorted $CD4^+$ and $CD8^+$ T cells after a 1-h culture in the presence of medium alone or with anti-CD3/anti-CD28 beads. As shown, no difference was observed in p-S6 levels in medium-cultured samples obtained from T0 and T12 patients. Conversely, a tremendous increase of TCR-dependent p-S6 up-regulation was observed in the samples obtained from T12 patients after 12 h from everolimus administration (minimal drug serum concentration) in both the $CD4^+$ and $CD8^+$ T cells (Fig. 6a,b). As shown in Fig. 6c,d, strongly reduced pS6 up-regulation was observed upon TCR triggering in the samples obtained from the same patients 3 h after everolimus administration (maximal drug serum concentration). It should be noted that *ex-vivo* TCR triggering of the cells was always performed in the absence of the drug.

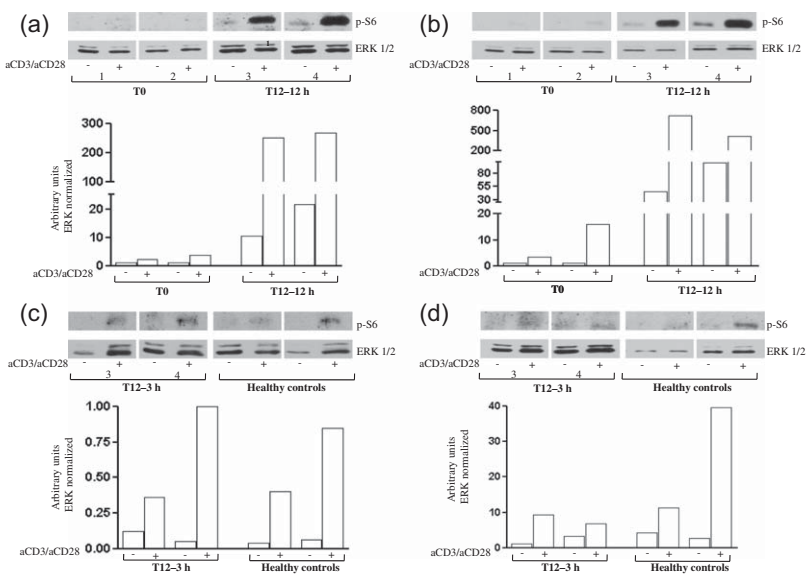

Fig. 6. Analysis of S6 kinase phosphorylation level as a measure of mammalian target of rapamycin complex 1 (mTORC1) activity in $CD4^+$ and $CD8^+$ T cells after calcineurin inhibitors (CNI) to everolimus conversion. (a–d) Comparative analysis of phospho-S6 kinase in all four patients analysed. As indicated, samples from T12 patients were obtained 12 h (T12–12 h) after everolimus administration (minimal drug TL) and 3 h (T12–3 h) after drug administration (maximal drug TL). Western blot analysis for p-S6 kinase from protein lysates of sorted $CD4^+$ and $CD8^+$ T cells from two representative T0 and T12 patients are reported; as indicated, data refer to 1 h culture with medium or anti-CD3/anti-CD28 monoclonal antibody (mAb)-coupled microbeads. As shown, a tremendous increase of pS6 levels was observed in T12–12 h patients after T cell receptor (TCR) triggering. As indicated (c,d) comparable up-regulation of pS6 was observed in T12–3 h patients and controls. Arabic numbers (1–4) identify samples obtained from single patients. Data are representative of two concordant experiments.

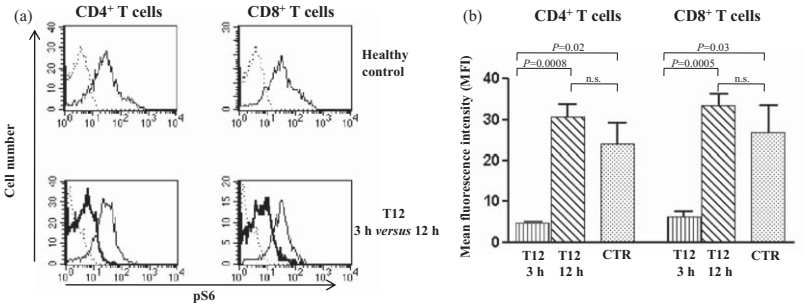

Fig. 7. p-S6 kinase analysis 3 and 12 h after everolimus administration in T12 patients. (a) Flow cytometry comparative evaluation of p-S6 kinase levels in $CD4^+$ and $CD8^+$ T cells obtained from one representative T12 patient 3 h and 12 h after everolimus administration (T12–3 h and T12–12 h) and in one healthy control. As indicated, upper histogram plots in (a) show the p-S6 kinase level in $CD4^+$ and $CD8^+$ T cells of one healthy donor (plane line); isotype matched control binding is indicated as dashed line. Lower histogram plots in (a) refer to the p-S6 kinase evaluation in cells of one representative patient 3 h (bold line) and 12 h (plane line) after everolimus administration; dashed line indicates isotype matched control binding. (b) Comparative analysis of p-S6 phosphorylation levels of $CD4^+$ and $CD8^+$ T cells after 3 h (vertical depicted column) and 12 h (oblique depicted column) in all four T12 patients analysed. Dotted columns indicate healthy controls (CTR). Statistical analysis was performed by Mann–Whitney test.

Comparative analysis by immune fluorescence of basal p-S6 levels in $CD4^+$ and $CD8^+$ T cells from samples obtained from T12 patients 3 and 12 h after everolimus administration (highest and lowest drug TL, respectively) confirmed the occurrence of an oscillatory inhibition of mTORC1 kinase activity (Fig. 7a,b). Indeed, a significant decrease of p-S6 kinase was observed in $CD4^+$ and $CD8^+$ T cells obtained after 3 h, compared with those obtained after 12 h from everolimus administration ($P < 0{\cdot}001$). Moreover, p-S6 kinase levels were significantly lower than controls in samples after 3 h ($P < 0{\cdot}05$), but not after 12 h from everolimus (Fig. 7a,b).

Thus, no significant difference in basal S6 phosphorylation levels was mediated by CNI compared with the everolimus-based immune-modulating regimen. Moreover, changes in everolimus serum concentration, due probably to the dosage and administration schedule of the drug, were observed to associate with oscillatory basal and TCR-dependent activation of mTORC1 kinase in both $CD4^+$ and $CD8^+$ T cells. This behaviour might be of particular relevance in the presence of chronic TCR stimulation, as represented by the allotransplantation setting.

Discussion

mTOR targeting was proposed as an immunosuppressor to limit CNI adverse effects in renal transplant recipients [5,6]. This condition represents a unique opportunity to study the effect of long-term mTOR inhibition therapy in a transplantation model. With this purpose in mind, we focused on mTOR-dependent immune modulation, also addressing the hypothesis that targeting the immune metabolic network could optimize clinical manipulation of specific adaptive immune effectors.

Our data indicate that an immune-modulating regimen based on everolimus administration was associated specifically with a significant decrease in leucocyte and neutrophil numbers. This effect might be accounted for by mTOR-dependent effects on cellular survival, migration and proliferation, as well as by activation of the CD11b/CD18 complex, which alters granulocyte adhesion to endothelial cells [31–34].

Neutrophils play a key role in inflammation [35]. They release proinflammatory, angiogenic and anti-inflammatory mediators and can interfere with the development of intimal hyperplasia and transplant vasculopathy [36–38]. *Ex-vivo* everolimus treatment of isolated neutrophils mediates inhibition of their vascular endothelial growth factor (VEGF) and IL-8 release, also increasing the anti-inflammatory IL-1RA [34]. Therefore, the ability of everolimus to specifically modulate such a population might be of some relevance to optimize clinical management of kidney transplants.mTOR has been described to play a key role in regulating antigen-independent proliferation of $CD8^+$ T cells [39] and in maintaining homeostasis of effector lymphocytes [40–42]; accordingly, CNI to everolimus conversion was observed to mediate a significant reduction of $CD8^+$ T cells in our cohort. The MFA–everolimus association was revealed to hamper such effects significantly.

mTORC1 is a central regulator of adaptive immunity [43,44]. It affects Th1 and Th17, while mTORC2 is required for Th2 differentiation [15–18]. Our data confirm such effects, showing that everolimus-based immune-modulating therapy is able to decrease the proinflammatory activity of adaptive effectors significantly in kidney transplant recipients. Accordingly, CNI to everolimus conversion maintained a reduced proinflammatory activity (IFN-γ and IL-17 production) in the absence of significant modification of IL-4 secretion by NKTi cells, the key regulators of cytokine polarization [29]. Moreover, everolimus treatment modulates specifically IFN-γ-producing $CD8^+$ and $CD4^+$ T cells, while maintaining the decreased IL-17

production by $CD4^+$ T lymphocytes from patients treated with CNI. These effects seemed to be favoured by immune-modulating protocols not including MFA administration (Fig.2a,f). Moreover, the inhibition of IFN-γ production by $CD8^+$ T cells without effect on NK effectors confirms the role of mTORC1-dependent pathways in T cell response [17,44] and $CD8^+$ T cell homeostasis regulation [39–42]. The major role of mTOR in regulating FoxO1-dependent $CD8^+$ T cell differentiation [20] might also account for such effects.

A number of data have suggested the critical involvement of T_{reg}-dependent immune modulation in mTOR inhibition-based immunosuppression regimens [17,45]. Moreover, both T_{reg}-hampering [46,47] as well as enhancing activity [48] has been referred for MFA-based treatment. Here, we describe that T_{reg} increase, coupled with significant effects on their growth rate, characterizes everolimus-based immunosuppression in kidney transplant recipients. Moreover, opposite effects on Ki67 expression were observed by mTORC1 inhibition alone (T12 patients treated with everolimus without MFA association) compared with immune-modulating regimens, including MFA/everolimus co-administration. The analysis of the growing ability of $CD4^+Foxp3^-$ T cell subset, probably representing the effector T lymphocyte population, allowed better characterization of MFA and everolimus-dependent effects in our cohort. As shown, a preferential effect on growing lymphocytes, regardless of the subset to which they belong, has been observed for MFA co-administration, while a specific increase of Ki67 expression in the T_{reg} subset, coupled with a significant modulation of $Ki67^+CD4^+FoxP3^-$ T cells, was observed to characterize mTORC1 inhibition. Specific amplification of T_{reg} cells able to modulate alloreactive T cell effectors might be hypothesized to account for everolimus-dependent immune-suppression in kidney transplant recipients. Conversely, preferential modulation of growing immune effectors represents a key element for immunosuppression regimens, including MFA administration. Moreover, mTOR-dependent immune-modulating effects are hampered by MFA co-administration. Thus, the inhibition of purine biosynthesis pathways, key targets of MFA, has been observed to severely impair mTOR-dependent immune modulation.

We observed that changes in everolimus serum levels correlate with oscillation in basal and activation-dependent phosphorylation of S6 kinase, a major target of mTORC1 kinase. As expected in a transplantation setting, continuous antigen stimulation is an activation trigger for T cells. Our data indicate that mTOR inhibition therapy is able to mediate significant oscillation, probably depending on drug serum concentration, in the mTOR kinase activity. This effect relates to a significant increase of T_{reg} numbers and growing ability, and decreases Ki67 expression by the $CD4^+FoxP3^-$ counterpart.

The possibility that the effects observed on T_{reg} and T cell effector homeostasis might affect selected cell clones preferentially, probably involved in alloantigen recognition, needs to be investigated. In this context, our data confirm and extend our previous *in-vitro* observations indicating that oscillatory inhibition of mTOR activity induces robust proliferation of TCR triggered T_{reg}, also inhibiting T cell effector growth [18,19]. Thus, a key role for T_{reg} modulation coupled with a decrease of neutrophils and $CD8^+$ T cells and reduced proinflammatory activity might be hypothesized to underlie mTOR inhibition in a transplantation setting.

Two opposite therapeutic indications are currently proposed for everolimus: immunosuppression [8,9,21] and cancer control [22,23,25]. In this context, we might hypothesize the possibility that dosage level (lower by six to 10-fold than in the oncology setting) and administration schedule (twice *versus* once a day in cancer therapy) could represent a therapeutic strategy to regulate mTOR-dependent intracellular pathways differentially and target immune tolerance or cancer control preferentially.

Taken together, our results shed light on the complex mTOR-dependent immune metabolic network, and propose that oscillatory inhibition of TCR-dependent mTORC1 activity might represent a therapeutic strategy to optimize targeted manipulation of specific adaptive effectors in kidney transplant recipients.

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Disclosure

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Author contribution

A. T. P., V. R., M. G. and G. M. participated in the design, interpretation and analysis of data as well as in writing the paper; S. T. and L. A. participated in the clinical management of the patients and in acquisition and evaluation of data; M. S. and A. G. participated in acquisition and evaluation of data and contributed to paper writing; M. S., G. R. and G. T. planned, directed and co-ordinated the research and revised the paper. M. S. also co-ordinated the clinical management of patients.

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Research Article

In Vitro Effects of Some Botanicals with Anti-Inflammatory and Antitoxic Activity

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Several extrinsic factors, like drugs and chemicals, can foster autoimmunity. Tetracyclines, in particular oxytetracycline (OTC), appear to correlate with the emergence of immune-mediated diseases. Accumulation of OTC, the elective drug for gastrointestinal and respiratory infectious disease treatment in broiler chickens, was reported in chicken edible tissues and could represent a potential risk for pets and humans that could assume this antibiotic as residue in meat or in meat-derived byproducts. We investigated the *in vitro* anti-inflammatory properties of a pool of thirteen botanicals as a part of a nutraceutical diet, with proven immunomodulatory activity. In addition, we evaluated the effect of such botanicals in contrasting the *in vitro* proinflammatory toxicity of OTC. Our results showed a significant reduction in interferon- (INF-) γ production by human and canine lymphocytes in presence of botanicals ($^{*}p < 0.05$). Increased INF-γ production, dependent on 24-hour OTC-incubation of T lymphocytes, was significantly reduced by the coincubation with *Haematococcus pluvialis*, with *Glycine max*, and with the mix of all botanicals ($^{*}p < 0.05$). In conclusion, the use of these botanicals was shown to be able to contrast OTC-toxicity and could represent a new approach for the development of functional foods useful to enhance the standard pharmacological treatment in infections as well as in preventing or reducing the emergence of inflammatory diseases.

1. Introduction

The immune system has the fundamental role of not only protecting and defending the organism against infections but also controlling homeostasis and health maintenance against infections, autoimmune diseases, and tumor onset [1]. Depending on the pathogen or on antigen, two different immune responses can occur: the humoral and the cellular responses [2]. Moreover, the immune system can be classified into two fundamental phases: the innate and acquired (or adaptive) responses [3]. Innate immunity is present in vertebrates and in nonvertebrates, represents the first-line defence in the species and is based on cells (i.e., macrophages, polymorphonuclear cells, and natural killer lymphocytes) and on some mechanisms, mediated by soluble substances (i.e., complement proteins, antibodies, natural compounds, etc.) that defend the plants and animals from infections [4]. Conversely, adaptive immunity is present only in vertebrates and is a host defence related to several specific cellular mechanisms that specifically recognize the

antigens and are fundamentally expressed by B and T lymphocytes, plasma cells, and antibodies [5]. The $CD4^+$ T helper (T_H) lymphocytes represent key cells in the polarization of inflammatory/noninflammatory immune response: T_H1 and T_H2 are the most common [6]. The T_H1 response is characterized by the a secretion of INF-γ, which optimizes the bactericidal macrophages capability, induces the production of opsonizing and complement-fixing antibodies, and fosters the establishment of an optimal CTL response. The T_H2 response is characterized by interleukin- (IL-) 4, IL-5, IL-10, and IL-13 release, which results in the activation of B cells to make neutralizing noncytolytic antibodies, leading to humoral immunity [6].

Exacerbation and endurance of T_H1 response have been associated with the emergence of inflammatory diseases [6] and autoimmune disorders [7]. In particular, INF-γ appears to play a pivotal role in inducing autoimmune responses [8–16].

Several extrinsic factors, like drugs and chemicals, can foster the development of autoimmunity [17–22]. In this regard, the use of tetracyclines appears to correlate with the emergence of autoimmune diseases [23–29]. Concerning this, OTC represents the main drug used to control gastrointestinal and respiratory diseases in broiler chickens. Its accumulation was demonstrated in chicken edible tissues [30] and could represent a potential risk also for pets and humans that could assume this antibiotic as a residue in meat or in meat-derived byproducts. Recently, we published two papers evidencing the *in vitro* toxicity of bone meal-derived OTC from intensive poultry farming, in terms of apoptosis induction [31], as well as the proinflammatory cytokines, that is, INF-γ, release from peripheral blood mononuclear cells (PBMCs) cultures [32]. Moreover, we evidenced that the presence of significant concentrations of OTC in gym trained human subjects was linked to the presence of food intolerances [33]. Therefore, we hypothesized a possible modulatory activity exerted by a pool of botanicals derived from medical plants, which are successfully used in several commercially available nutraceutical diets. Intriguingly, many botanicals could have the capability to modulate the immune system [34]. In this regard, it is well known that the immunomodulatory activity of acemannan, a mucopolysaccharide extracted from *Aloe vera*, related to modulation of nitric oxide release that modulate classes I and II MHC cell surface antigens involved in antigen presentation [35, 36]. The same immunomodulating activity was observed for fermented *Carica papaya* able to increase T_{reg} cells, reduce INF-γ^+CD4^+ T cells, and possibly alter the growth of several cancer cell lines [37–39]. As to Maitake mushroom (*Grifola frondosa*), many reports have shown its ability to downregulate cytokine secretion, such as Tumor Necrosis Factor- (TNF-) α and INF-γ, as well as to inhibit adhesion molecule expression and cell-mediated immunity enhancement [40–44]. Downregulation of overexpressed cytokines in different inflammatory and immune-related inflammatory conditions was also reported for curcumin extracted from turmeric (*Curcuma longa*) [45–47]. Antiproliferative and chemopreventive effects are known to be also exerted by other curcuminoids, for example, demethoxycurcumin, bisdemethoxycurcumin, and alpha-turmerone [48, 49]. Cytokine downregulation is also performed by *Glycine max* (soybean) isoflavones that interfere with leukocyte endothelial adhesion ability [50–54]. In more detail, isoflavones, that is, genistein, can suppress dendritic cell function and cell-mediated immunity.

It is noteworthy that some botanical principles, which have been investigated in this study such as astaxanthin (from *Haematococcus pluvialis*), resveratrol (from *Polygonum cuspidatum*), and *Cucumis melo*, are characterized by antioxidant and anti-inflammatory properties as well as modulation properties towards $CD8^+$ T-cell proliferation [55, 56]. Anti-inflammatory but also oxidative stress preventing activity has been also ascribed to *Cucumis melo* extract due to its high activity on superoxide dismutase [57, 58].

Recently, we published a paper evidencing the role for a nutraceutical diet in regulating the immune response in canine *Leishmaniosis* along with standard pharmacological treatment [59]. In particular, the presence of *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* in the diet correlated with a significant decrease in T_H1 response, in terms of INF-γ production. Such evidence highlighted the anti-inflammatory effects of these specific botanicals. In addition, we suggested the anti-inflammatory effects of several botanicals added to specific diets in relieving inflammatory conditions in chronic pathologies affecting dogs [59–63].

Based on these premises, the aim of our study was to investigate the potential anti-inflammatory properties of those 13 botanicals having immune-modulatory effect as supplemented diet regulating the immune response in *Leishmaniosis* [59]. In particular, we tested the *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* and their ability to counteract the proinflammatory toxicity of OTC *in vitro*.

2. Materials and Methods

2.1. Culture Medium and Botanicals. To evaluate the cellular production of cytokines, human and canine PBMCs were incubated overnight with an *ad hoc* culture medium. Briefly, the first step was the solubilization of 1 gr of powder of each plant-derived substance in an appropriate chemical vehicle depending on solubility degree. In particular, *Ascophyllum nodosum* (pure powder of *Ascophyllum nodosum* seaweed, laminarin content min. 2.3%, and fucoidans content min. 11.4% [64]), *Aloe vera* (*Aloe vera* gel 200 : 1 powder, aloin content min. 1% [65]), *Cucumis melo* (lyophilized extract of melon, superoxide dismutase min. 1 UI/mg [57]), *Polygonum cuspidatum* (powder obtained from dried *Polygonum cuspidatum* roots, resveratrol content min. 8% [66]), *Camellia sinensis* (standardized decaffeinated green tea leaves powder, catechins content min. 75% [67]), *Carica papaya* (Papaya fermented granular, rich in papain [68]), *Glycine max* (Soy powder, 40% isoflavones [69]), and *Grifola frondosa* (maitake carpophore dry extract, polysaccharides content min. 20.0%

[70]) were solubilized in 10 mL of PBS 1x, with the exception of *Glycine max* that was added to 30 mL of PBS 1x to gain the full solubilization. *Haematococcus pluvialis* (standardized beadlets of *Haematococcus pluvialis* extract, astaxanthin content min. 2.5% [71]) was solubilized in 5 mL of dimethyl sulfoxide and 5 mL of PBS 1x. *Echinacea purpurea* (*Echinacea purpurea* dried extract, polyphenols content min 4% [72]), *Piper nigrum* (black pepper powder, piperine content min. 95% [73]), *Curcuma longa* (turmeric dried extract, curcuminoids content min. 95% [74]), and *Punica granatum* (standardized powdered extract from pomegranate, ellagic acid content min. 20% [75]) were solubilized in 4 mL of ethanol and 6 mL of water.

The solubilized botanicals were added to RPMI 1640 culture medium (Sigma-Aldrich, Milan, Italy) to obtain the *ad hoc* medium in the proportion of 1 : 10 (vehicle-solubilized substance : RPMI 1640) to preserve the good quality of cellular condition in the culture.

The cytokine cell production was evaluated in presence of the *ad hoc* medium containing the solubilized individual substance or a mixture containing all the solubilized botanicals. The vehicles employed for the solubilization were used as specific controls in the same proportion of *ad hoc* medium (1 : 10, vehicle : RPMI 1640). The mixture was composed by all *ad hoc* medium from the botanicals in a variable percentage according to that contained in the commercial canine food, previously used as immunomodulating diet able to reduce INF-γ production [59]. Briefly, the mixture contained 66.3% of *Ascophyllum nodosum*, 3.1% of *Aloe vera*, 6.1% of *Cucumis melo*, 1.5% of *Polygonum cuspidatum*, 1.5% of *Camellia sinensis*, 3.1% of *Carica papaya*, 4.6% of *Glycine max*, 6.3% of *Grifola frondosa*, 1.1% of *Haematococcus pluvialis*, 3.1% of *Echinacea purpurea*, 0.6% of *Piper nigrum*, 2.3% of *Curcuma longa*, and 1.5% of *Punica granatum*. The obtained mixture was added to RPMI 1640 culture medium to obtain the *ad hoc* medium in the proportion of 1 : 10 (vehicle/mixture : RPMI 1640) to preserve the good quality of cellular condition in the culture.

Ascophyllum nodosum, *Aloe vera*, *Cucumis melo*, *Polygonum cuspidatum*, *Camellia sinensis*, and *Haematococcus pluvialis* were purchased from Italfeed S.r.l, Milano (Italy).

Carica papaya, *Glycine max*, *Echinacea purpurea*, *Punica granatum*, *Piper nigrum*, and *Curcuma longa* were purchased from Nutraceutica S.r.l, Monterenzio, Bologna (Italy) while *Grifola frondosa* was purchased from A.C.E.F. S.p.a., Fiorenzuola D'Arda, Piacenza (Italy).

All the botanicals are in form of powder and are free from genetically modified organisms (Reg. 1829/2003-1830/2003 EC), gluten, bovine transmissible spongiform encephalopathy, and food allergens (DIR 2003/89/EC and 2006/142/EC).

2.2. Human and Canine Donors and Cell Preparation. The human blood collection from 10 healthy donor volunteers (5 males and 5 females, 20–30 years old) was performed at the Haemotrasfusional Center of University of Naples "Federico II," according to standard procedures and used within the 3 hours from the collection.

Peripheral blood was collected from ten healthy dogs (5 males and 5 females, 5–9 years old and ranging between 15 and 35 kg in weight). All dogs were enrolled with the owner consent in the Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II." Human or canine PBMCs were isolated by centrifugation on Lymphoprep (Nycomed Pharma) gradients, as previously described [59, 76]. Obtained PBMCs were considered as mixed population of T and non-T lymphocytes.

2.3. Monoclonal Antibodies, Detection of Intracellular Cytokine Production, and Flow Cytometry. For the immune-fluorescent staining a panel of fluorescent-labelled monoclonal antibodies (mAbs) was used to evaluate the human CD3, CD8, INF-γ, and IL-4, as well as a panel of isotype-matched mAb controls (Becton Dickinson Pharmingen, San Jose, California). In addition, we used several fluorescent-labelled mAbs against canine CD3, CD4, CD8, CD45, INF-γ, and IL-4 molecules and isotype-matched controls (Serotec Ltd., London, UK).

To analyze the production of INF-γ and IL-4 cytokines, 2×10^6/mL purified PBMCs were incubated overnight (10–12 hours) in the *ad hoc* medium of each botanical or of mixture (see Section 2.1). In particular, to obtain the cytokine production, PBMCs were always cultured in presence of 500 ng/mL of phorbol-12-myristate-13-acetate (PMA) and 1 μg/mL of Ionomycin (Sigma-Aldrich), as described in [77]. To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/mL of Brefeldin-A (Sigma-Aldrich), as described in [77].

To test the ability of botanicals in contrasting the toxic role of OTC, we used the commercial preparation of the drug (Oxytetracycline 20%®, TreI, Reggio Emilia, Italy). 1 μg of OTC [31] was added to cell culture and incubated for overnight (10–12 hours) as previously described [32]. In addition, *Haematococcus pluvialis* or *Glycine max* or the mixture *ad hoc* medium was used in the coincubation of cells with OTC and all along the overnight (10–12 hours) culture.

At the end of overnight (10–12 hours) incubation, the above incubated cells were fixed and permeabilized by using a commercial cytokine staining kit following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA, USA). Briefly, the cell fixing and permeabilization procedure were of 20 min at 4°C each. At the end of procedure, PBMCs were washed twice by centrifugation (800 ×g) in RMPI 1640 culture medium.

PBMCs were stimulated overnight with PMA and Ionomycin, cultured in a medium containing the botanicals solubilization buffer (vehicle), and used as control points. The proportion of vehicle and RPMI 1640 was the same of *ad hoc* medium (1 : 10 ratio).

The intracellular cytokine production was evaluated by using the triple staining technique and analyzed by flow cytometry (FACSCalibur platform) and CellQuest Software (Becton Dickinson Pharmingen, San Jose, California). The analyzed cells were always gated (R1 in dot plot of Figures 1(a) and 2(a)) on forward scatter (FSC) and side scatter (SSC) FACS parameters (cell size and cell complexity, resp.) to reasonably select the region of viable lymphocytes in order to avoid any interference due to the possible presence of death cells.

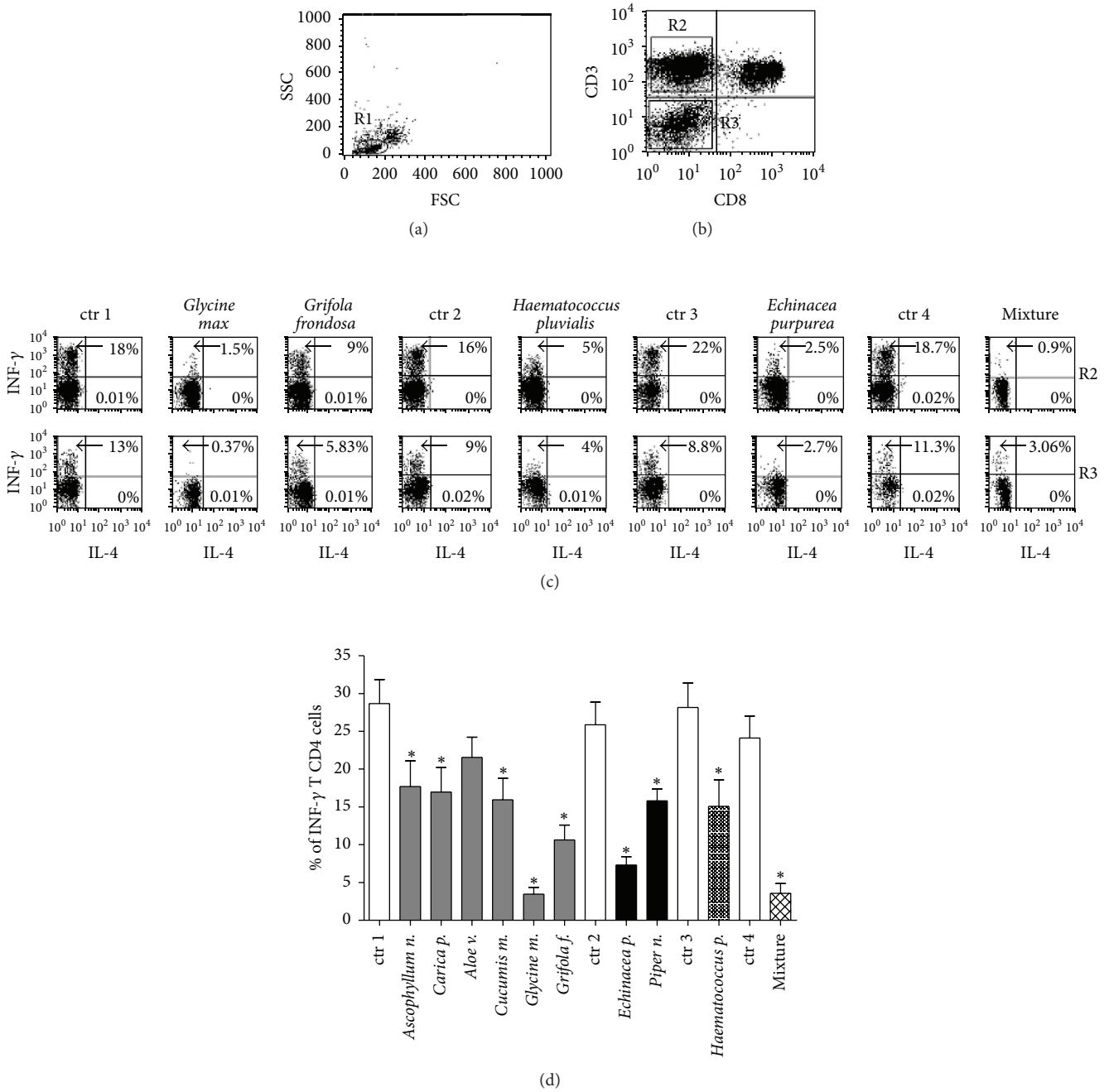

Figure 1: The effects of botanicals on cytokine production by human PBMCs. (a) shows the gating on viable lymphocytes (R1 in dot plot graph) based on FSC and SSC parameters (see Section 2); (b) represents the gating on T_H lymphocytes ($CD3^+$ $CD8^-$ as R2 in the dot plot graph) and on non-T cells ($CD3^-$ $CD8^-$ cells as R3 in the dot plot graph); and (c) shows the INF-γ and IL-4 production in human T_H lymphocytes and non-T cells incubated with *ad hoc* medium derived from botanicals or from mixture (see Section 2). Cytokine production was evaluated as percentage of INF-γ (y-axis) and IL-4 (x-axis) producing cells. The percentage of INF-γ (upper left quadrant inside the dot plots) and IL-4 (low right quadrant inside the dot plots) producing CD4 T (R2) and non-T (R3) cells are reported. The different cell incubations with *ad hoc* medium derived from botanicals or from mixture (see Section 2) are indicated on the top of each graph. (d) reports the statistic representation of 10 experiments on human $CD4^+$ T Lymphocytes evaluated as percentage of INF-γ producing cells, $^*p < 0.05$. The different cell-incubations with *ad hoc* medium derived from botanicals or from mixture (see Section 2) are indicated on the top of each column. The abbreviation "ctr" in (c) and (d) indicates the basal cytokine production by PMBCs stimulated by PMA and Ionomycin and in presence of the *ad hoc* medium based on the same solubilizing-vehicle but free from the botanicals (see Section 2); specifically, ctr 1 (*Ascophyllum n.*, *Carica p.*, *Aloe v.*, *Cucumis m.*, *Glycine m.*, and *Grifola f.*), ctr 2 (*Echinacea p.*, *Piper n.*), ctr 3 (*Haematococcus p.*), and ctr 4 (the mixture of all the botanicals).

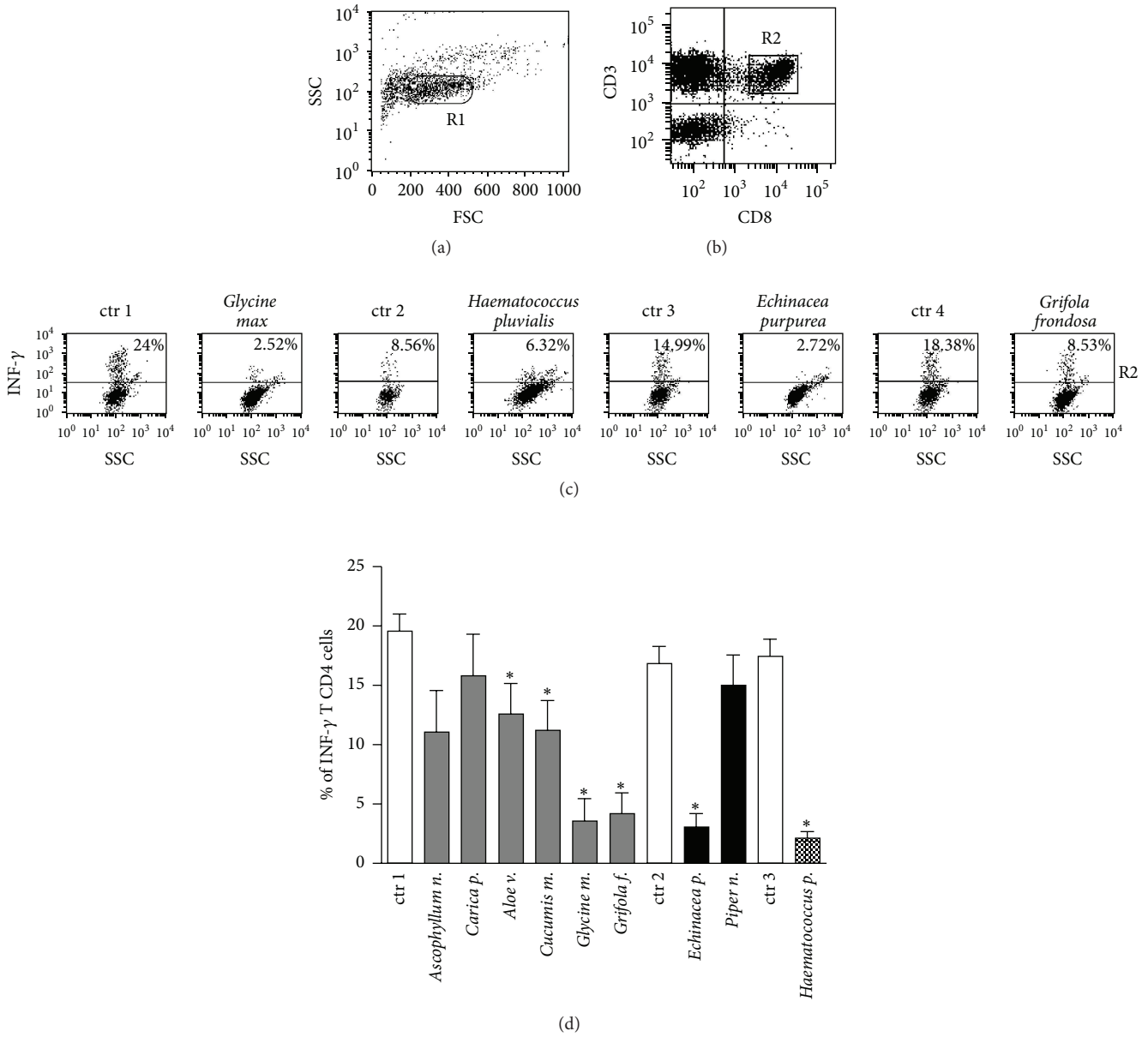

Figure 2: The effects of botanicals on INF-γ production by canine PBMCs. (a) shows the gating on viable lymphocytes (R1 in dot plot graph) based on FSC and SSC parameters (see Section 2). (b) represents the gating on $CD4^+$ T lymphocytes ($CD3^+$ $CD8^-$ as R2 in the dot plot graph). (c) reports the results from one representative experiment showing the percentage (the number in upper quadrant) of INF-γ producing canine $CD4^+$ T lymphocytes gated on R2 (y-axis); x-axis indicates the SSC parameter (see Section 2). The different coincubations of cells with *ad hoc* medium or mixture (see Section 2) are indicated on the top. (d) shows the statistic representation the INF-γ production by canine $CD4^+$ T Lymphocytes evaluated as percentage of INF-γ producing cells in 10 representative experiments, $^*p < 0.05$. The abbreviation "ctr" in (c) and (d) indicates the basal INF-γ production by PMBCs stimulated by PMA and Ionomycin and in presence of the *ad hoc* medium based on the same solubilizing-vehicle but free from the botanicals (see Section 2): specifically, ctr 1 (*Ascophyllum n.*, *Carica p.*, *Aloe v.*, *Cucumis v.*, *Glycine m.*, and *Grifola f.*), ctr 2 (*Echinacea p.*, *Piper n.*), and ctr 3 (*Haematococcus p.*).

2.4. Statistical Analysis. Data are presented as the means ± standard error of the mean (SEM) and were firstly checked for normality using the D'Agostino-Pearson normality test. The Kruskal-Wallis followed by Dunn's multiple comparisons analysis was performed. A $^*p < 0.05$ was considered significant. Statistics was performed by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and Discussion

3.1. The Anti-Inflammatory Effect of Botanicals as Significant Reduction of INF-γ Production in Human T and Non-T Lymphocytes. We focused on INF-γ production, as the main proinflammatory cytokine able to foster the T_H1 and non-T cell immune responses involved in several etiopathogenic

mechanisms at the basis of inflammatory-mediated disease [6].

As shown in Figure 1, the overnight incubation (10–12 hours) with each botanical as well as with a mix of all botanicals induced a significant decrease in INF-γ production in the T_H lymphocytes ($CD3^+$ $CD8^-$ cells gated as R1 in the dot plot graphs of Figures 1(b) and 1(c) and reported as mean ± SEM of 10 experiments in Figure 1(d)) and in non-T cells, mainly represented by NK lymphocytes ($CD3^-$ $CD8^-$ cells gated as R2 in the dot plot graphs of Figure 1(c)). In particular, the individual incubation with *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* was able to reduce INF-γ production. Intriguingly, despite the obtained slightly decrease in cytokine production, the *Aloe vera* incubation did not induce a significant reduction from the statistical point of view (Figure 1(d)). In this regard, we cannot rule out that a larger number of experiments (more than the 10 performed in this study and summarized in Figure 1(d)) or a higher concentration of substance could confirm the reduction in lymphocyte INF-γ production by the incubation with this botanical.

The basal IL-4 production was undetectable or only slightly detectable in T and non-T lymphocytes, as expected in PBMCs from healthy human donors after exposure to PMA and Ionomycin [77] and was not modulated after the overnight incubation with the botanicals (Figure 1(c)). Each specific vehicle, used to solubilize the botanicals, was used as control and the obtained value was substracted from each experimental point to obtain the correction following the formula "the value obtained from cell culture in presence of botanicals – the value obtained from cell culture in presence of the vehicle alone = corrected experimental point value." It is of note that even if the used vehicles appeared to not induce significant cell death in the culture, the flow cytometry analysis was always performed by gating on viable cells to avoid any possible interference dependent on death cells (see Figure 1(a) and Section 2.3). Moreover, the *ad hoc* medium from botanicals did not exert effect in absence of PMA and Ionomycin stimulation (data not shown).

3.2. The Anti-Inflammatory Effect of Botanicals as Significant Reduction of INF-γ Production in Canine $CD4^+$ T Lymphocytes. The individual incubation with *Ascophyllum nodosum*, *Cucumis melo*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* was able to significantly decrease the INF-γ production in the $CD4^+$ lymphocytes (dot plot graphs in Figure 2(c), summarized in Figure 2(d)). In contrast, the incubation with *Carica papaya* or with *Piper nigrum* seemed not to induce a statistically significant reduction (Figure 2(c)). Also, in this case, as referred to in human experiments, we cannot rule out that a larger number of experiments (more than the 10 performed in this study, summarized in Figure 2(d)) or a higher concentration of the substances could confirm the reduction in lymphocyte INF-γ production by the incubation with these two botanicals.

IL-4 production was undetectable in T lymphocytes, as expected in PBMCs from healthy dogs after exposure to PMA and Ionomycin [59], and was not modulated after the overnight incubation with the botanicals (data not shown).

The specific vehicles, employed to solubilize the substances, were used as controls and the resulting values were substracted from experimental points, as described (see Section 3.1). Flow cytometry analysis was always performed by gating on viable cells to avoid any possible interference dependent on death cells (see Figure 2(a) and Section 2.3).

3.3. The Anti-Inflammatory Effect of Botanicals as Significantly Contrasting Effect on INF-γ Production Dependent on OTC Exposure of Human T Lymphocytes. Notably, the individual incubation with *Haematococcus pluvialis* or with *Glycine max* was able to contrast the previously demonstrated proinflammatory effect of OTC in human T lymphocytes [32]. Indeed, the increased INF-γ production, dependent on 24-hour OTC-incubation of T lymphocytes, was strongly reduced by the coincubation with *Haematococcus pluvialis* or *Glycine max* (Figures 3(a) and 2(b), resp.). Note that the individual incubation with the botanicals, other than *Haematococcus pluvialis* and *Glycine max*, was unable to contrast OTC-toxicity (data not shown), while the mixture of all substances exerted a significant effect. Nevertheless, as referred to in previous sections, we cannot rule out that a larger number of experiments or a higher concentration of each substance could confirm the anti-OTC effect also for the other tested botanicals.

The specific vehicles, used to solubilize the substances, were considered as controls and the resulting values were substracted from experimental points, as described (see Section 3.1).

4. Conclusions

This study was inspired by two recently published *in vivo* observations in which we suggested a potential anti-inflammatory effect of some nutraceutical diets, containing the studied botanicals, in infectious and inflammatory diseases [59–61].

In particular, we observed that a diet enriched by *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* was able to reduce proinflammatory T cell responses in canine *Leishmaniosis* [59] and the clinical feature of ear inflammation in chronic otitis in dogs [60].

Here, we observed the *in vitro* effect of *Ascophyllum nodosum*, *Cucumis melo*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* in reducing *in vitro* proinflammatory cytokine production by human and canine PBMCs. These botanicals appeared to exert a potential anti-inflammatory effect that was evident in the reduction of INF-γ production in human T and non-T cells and in canine T lymphocytes. Conversely, *Aloe vera*,

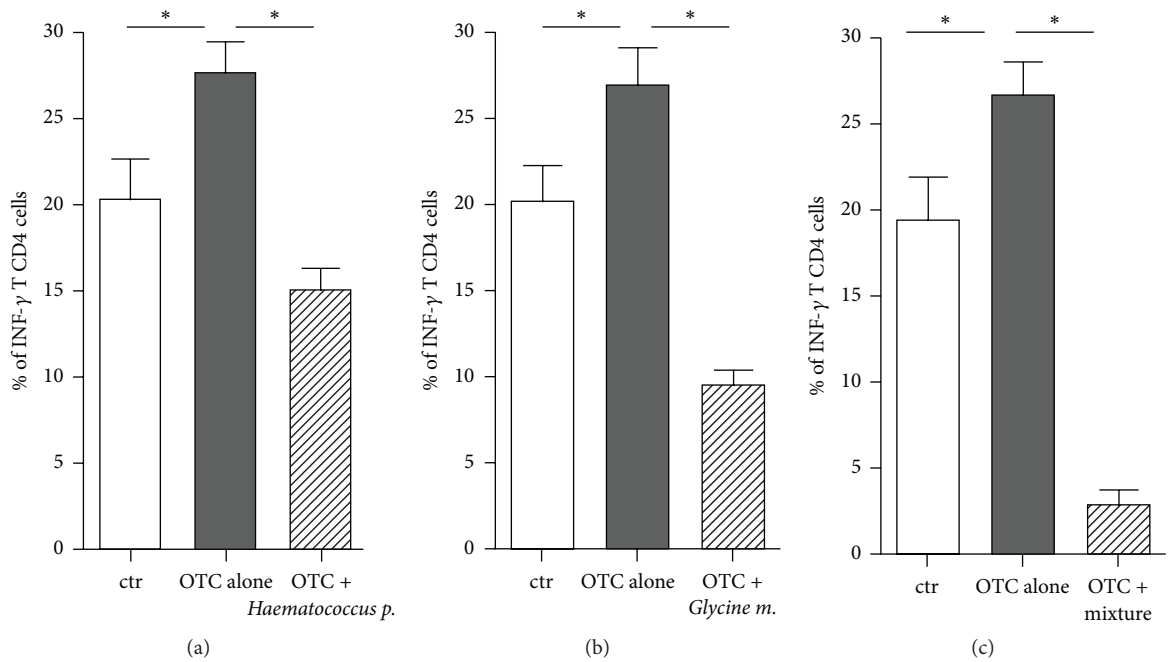

Figure 3: Statistic representation of the INF-γ production in human CD4$^+$ T Lymphocytes after the OTC exposure and the contrasting effects after botanicals challenge in 10 representative experiments. (a) *Haematococcus p.*; (b) *Glycine m.*; and (c) the mixture of all the botanicals. Cytokine production was evaluated as percentage of INF-γ producing T CD4$^+$ cells. All the incubations (basal, OTC alone, and OTC + botanical) were performed in the *ad hoc* medium based on the vehicle used to solubilize the botanical, so that the abbreviations "ctr" indicate the basal INF-γ production by PMBCs stimulated by PMA and Ionomycin and in presence of the *ad hoc* medium based on the same solubilizing-vehicle but free from the botanicals (see Section 2). $^*p < 0.05$.

Carica papaya, and *Piper nigrum* appeared to be ineffective in reducing this cytokine production. These results seem to be contradictory with the data observed in dogs [59], where the diet containing all these botanicals, including *Aloe vera*, *Carica papaya*, and *Piper nigrum*, exerted a therapeutic effect by reducing the inflammatory aspects of *Leishmaniosis*. Such apparent contradiction may be explained by the different sensitivity between *in vitro* and *in vivo*, as well as by the fact that *in vivo* botanicals are probably synergized in the combined administration as in the diet. In this regard, this latter consideration fits with *in vitro* effect obtained by the mixed incubation with all substances that induced the INF-γ decrease.

Moreover, as stated in Section 3, we cannot rule out that a larger number of experiments or a higher concentration of substances could confirm the reduction in lymphocyte INF-γ production also by *Aloe vera*, *Carica papaya*, and *Piper nigrum*.

Taken together, our observation highlighted the relevance for the use of botanicals to modulate the inflammatory responses in both dogs and humans. Indeed, exacerbation and the persistence of T_H1 response frequently result in the emergence of inflammatory diseases [6] and autoimmunity disorders [7] and the increase of INF-γ production is associated with autoimmunity in humans [8–16]. In addition, some of the botanicals used in this study were previously suggested as antioxidants and immune-modulating substances to reach the physiological status in several models of disease in human [55, 78] and animals [59, 60, 79–83].

Moreover, this study was also inspired by our recent paper, which evidenced the *in vitro* toxicity of OTC in terms of inflammatory response increase by human lymphocytes [32]. In this regard, here we evaluated the potential ability of *Ascophyllum nodosum*, *Cucumis melo*, *Carica papaya*, *Aloe vera*, *Haematococcus pluvialis*, *Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum*, *Echinacea purpurea*, *Grifola frondosa*, and *Glycine max* to contrast the OTC-toxicity exerted *in vitro* in human lymphocytes.

Our data suggested that the incubation with the mixture of these botanicals clearly reduced the OTC-induced INF-γ production in T cells. It is of relevance that the individual incubation with *Haematococcus pluvialis* or with *Glycine max* significantly reduced this cytokine production.

Such evidence may shed new light on the misunderstood scenario resulting from the increasing emergence of inflammatory diseases in humans, dogs, and cats [84–89]. Moreover, it has been suggested that tetracycline, in particular OTC, could take part in this scenario and could represent harmful compounds for human health and animals fed meat derived from intensive livestock [25–30, 33, 90].

In conclusion, this study could open an interesting approach regarding the use of anti-inflammatory and antioxidant botanicals in immune-mediated pathologies and in infectious diseases as well as to counteract the effect of several putative toxic substances present in food, such as the OTC, which can cause inflammatory disorders and diseases.

Ethical Approval

This study has been reviewed by Ethical Animal Care and Use Committee of the University of Naples Federico II and received formal Institutional approval (Protocol no. 2015/0071388) in accordance with local and national law, regulations, and guidelines (Circular no. 14 of September 25, 1996, and Italian civil code article 1175).

Consent

Informed consent from human donors was obtained in accordance with the Declaration of Helsinki, as approved within the study protocol by the Institutional Review Board at the Federico II University of Naples.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. This research was performed in collaboration with some scientists from the Division of Research and Development of SANYpet S.p.A. (as indicated in the authors' affiliations) according to scientific and ethical principles of the scientific community.

Authors' Contributions

Gianandrea Guidetti, Alessandro Di Cerbo, Angela Giovazzino, and Valentina Rubino contributed equally to this work.

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Oxytetracycline induces DNA damage and epigenetic changes: a possible risk for human and animal health?

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ABSTRACT

Background. Oxytetracycline (OTC), which is largely employed in zootechnical and veterinary practices to ensure wellness of farmed animals, is partially absorbed within the gastrointestinal tract depositing in several tissues. Therefore, the potential OTC toxicity is relevant when considering the putative risk derived by the entry and accumulation of such drug in human and pet food chain supply. Despite scientific literature highlights several OTC-dependent toxic effects on human and animal health, the molecular mechanisms of such toxicity are still poorly understood.

Methods. Here, we evaluated DNA damages and epigenetic alterations by quantitative reverse transcription polymerase chain reaction, quantitative polymerase chain reaction, chromatin immuno-precipitation and Western blot analysis.

Results. We observed that human peripheral blood mononuclear cells (PBMCs) expressed DNA damage features (activation of ATM and p53, phosphorylation of H2AX and modifications of histone H3 methylation of lysine K4 in the chromatin) after the *in vitro* exposure to OTC. These changes are linked to a robust inflammatory response indicated by an increased expression of Interferon (IFN)-γ and type 1 superoxide dismutase (SOD1).

Discussion. Our data reveal an unexpected biological *in vitro* activity of OTC able to modify DNA and chromatin in cultured human PBMC. In this regard, OTC presence in foods of animal origin could represent a potential risk for both the human and animal health.

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INTRODUCTION

The drug (4S,4aR,5S,5aR, 6S,12aS)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide, briefly oxytetracycline (OTC) is active towards a wide range of micro-organisms (*Nelson & Levy, 2011*), is efficiently absorbed in the duodenum forming complexes with metallic ions, is unstable at acid pH and its introduction along with food reduces its serum concentrations (*Palmieri, Di Cerbo & Laurino, 2014*). Moreover, such drug could accumulate within bone, skin, fat, tendons, muscles, liver and gastrointestinal tract (*Agwuh & MacGowan, 2006*).

OTC is commonly used in medicine and is one of the main antibiotics used in zootechnical and veterinary practices as feed supplement to ensure wellness of farmed animals (i.e., poultry, ovine, swine and livestock) (*Graham et al., 2014*; *Brüning et al., 2014*; *Di Cerbo et al., 2014*; *Odore et al., 2015*).

Several studies have investigated the potential toxicity of OTC ranging from teratogenic effects during pregnancy (*Czeizel & Rockenbauer, 2000*) to some effect on immune system (*Glette et al., 1984*; *Potts et al., 1983*; *Van den Bogert & Kroon, 1982*; *Myers, Farrell & Henderson, 1995*; *Di Cerbo et al., 2016*). Moreover, scientific literature suggested that the drug is able to inhibit or reduce catalase (*Chi, Liu & Zhang, 2010*) and affects avian cartilage degradation (*Peters et al., 2002*).

We recently demonstrated that OTC: (a) induces an *in vitro* inflammatory response characterized by T and non-T lymphocytes activation and Interferon (IFN)-γ release (*Di Cerbo et al., 2016*); (b) triggers the apoptosis of human and dog haematopoietic cells (*Di Cerbo et al., 2016*; *Odore et al., 2015*).

The potential OTC toxicity becomes more relevant when considering the potential risk derived by the eventuality of entry and accumulation of such drug in human and pet food with possible consequences on health (*Palmieri, Di Cerbo & Laurino, 2014*). In this regard, animal muscle, bone and fat are known to be the elective deposit for several antibiotics (*Palmieri, Di Cerbo & Laurino, 2014*; *Macy & Poon, 2009*) and are routinely employed for human and pet food production (*Palmieri, Di Cerbo & Laurino, 2014*).

In the light of the widespread use of OTC and considering the putative risk derived by the eventuality of entry and accumulation of such drug in human and pet food chain supply (*Graham et al., 2014*; *Brüning et al., 2014*; *Di Cerbo et al., 2014*; *Nelson & Levy, 2011*; *Palmieri, Di Cerbo & Laurino, 2014*), it is possible to speculate that the OTC accumulates in these edible tissues and that this occurrence represents the contact between the drug and the humans or companion animals (dogs and cats).

Here, we addressed the study over the relevance of some molecular mechanisms of drug toxicity and, specifically, on the genotoxic effect and epigenetic modifications potentially induced by OTC. This could be relevant since many of the effects observed could affect the gene expression and represent a potential risk for human and animal health.

MATERIALS & METHODS

Cells and incubation

Peripheral blood mononuclear cells (PBMCs) were obtained, as previously described (*Di Cerbo et al., 2016*). Briefly, we performed the centrifugation on Ficoll-Paque cushion (GE Healthcare, Uppsala Sweden) gradients of buffy coats obtained from six volunteer healthy donors. In order to inform the blood donors concerning the possibility to use minimal amount of their blood donation for scientific purpose, written informed consent (model n. 5526 of Azienda Ospedaliera Universitaria "FEDERICO II", Naples, Italy) was obtained from each donor at the time of venous peripheral blood donation performed at Blood Trasfusional Center of Azienda Ospedaliera Universitaria "FEDERICO II", Naples Italy, as established by Italian Law. All the experiments were performed anonymously, without any donor biographical reference. White blood cells have never been used to create a genome database.

To test the *in vitro* potential biochemical toxic role of OTC (Liquid Oxytetracycline 20% R, TreI, Reggio Emilia, Italy), the PBMCs (2.5×10^6/ml) were incubated in presence of RPMI 1,640 medium with 10% FCS (Invitrogen, Carlsbad, CA, USA) alone or with 2 μg/ml OTC (*Odore et al., 2015*; *Di Cerbo et al., 2016*) at 37 °C for different times (6 h, 12 h, 24 h).

RNA extraction and qRT-PCR and qPCR

Total RNA was extracted using TRI Reagent (T9424, Sigma-Aldrich, St Louis, MO, USA). cDNA was synthesized in a 20 μl reaction volume containing 1 μg of total RNA, in accordance to the life technology protocol (High-Capacity cDNA Reverse Transcription Kit 4368814; Applied Biosystem, Thermofisher Scientific, Foster City, CA, USA). The products were stored at −20 °C until use. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and quantitative polymerase chain reaction (qPCR) were performed three times in six replicates on a 7,500 Real Time PCR System (Applied Biosystems) using the SYBR Green-detection system (SYBR select Master Mix, 4473369, Applied Biosystem). The following primers were used: IFN-γ mRNA, 5′-TGGAAAGAGGAGAGTGACAGA-3′ and 5′-CTGTTTTAGCTGCTGGCGAC-3′; type 1 superoxide dismutase (SOD1) mRNA 5′-CTAGCGAGTTATGGCGACGA-3′ and 5′-GTCTCCAACATGCCTCTCTTCA-3′; 18S, 5′-GCGCTACACTGACTGGCTC-3′ and 5′-CATCCAATCGGTAGTAGCGAC-3′.

Chromatin Immuno-Precipitation (ChIP)

Cells were treated as indicated in *Cells and incubation* paragraph. The cells (~2.5×10^6 for each antibody) were crosslinked with a 1% formaldeyhyde/PBS solution for 10 min at room temperature, the reaction was stopped by the addition of glycine to a final concentration of 125 mM. Fixed cells were harvested and the pellet was resuspended in 1 ml of Lysis Buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% NP40) containing 1× protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland). The lysates were sonicated in order to have DNA fragments from 300 to 600 bp. An aliquot (1/10) of sheared chromatin was used as input DNA. Sonicated samples were processed according to the manufacturer's protocol of ChIP assay kit (Merck Millipore, Billerica, MA, USA). Samples were subjected to qPCR using the following primers: IFN-γ Promoter, 5′-GAA

CAATGTGCTGCACCTCC-3′ and 5′-CACAGGTGGGCATAATGGGT-3′; SOD1 Promoter, 5′-CATCATTTTGCCAATTTCGCGT-3′ and 5′-CGAGTGGCCGGGAATGACT-3′.

Real Time-qPCRs were performed using the SYBR Green-detection system (SYBR select Master Mix, 4473369; Applied Biosystem).

Western blot preparation and analysis

Aliquots of the cells collected for ChIP were used for western blot. Cells were washed twice with cold phosphate-buffered saline (PBS) and nuclei were extracted using 1 ml of Lysis Buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% NP40) containing 1× protease inhibitor cocktail. Nuclear lysates were obtained accordingly with Nuclear Fractionation Protocol (Abcam, Cambridge, UK). γH2AX was detected using part of the sonicated samples collected for ChIP. Lysates were cleared by centrifugation (13,000 rpm for 20 min). Protein concentrations were measured by Bio-Rad Protein Assay Dye Reagent Concentrate #500-0006. Equal amounts of cell extracts were then resolved by SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted using specific antibodies. Blots were detected using an ECL system (Lumilight Western Blotting Substrate, 12015200001, Roche).

Antibodies

Anti-DNMT1 ab87656 (Abcam, Cambridge, UK), -H3K4me2 ab32356 (Abcam), -H3K4me3 ab1012 (Abcam),—Total H3 ab1791 (Abcam), -Menin sc-0200 (Santa Cruz Biotechnology); phosphoATM ab81292 (Abcam), -phospho-H2AX (07164, Merck Millipore), MCM7 sc-9966 (Santa Cruz Biotechnology), Normal rabbit IgG sc-2027 (Santa Cruz Biotechnology), Normal mouse IgG sc-2025 (Santa Cruz Biotechnology) and -p53 ab1101 (Abcam).

Statistical analysis

Statistical significance between groups was determined using Student's *t* test.

RESULTS AND DISCUSSION

IFN-γ and SOD1 gene expression

We recently demonstrated the pro-inflammatory effect of OTC in causing both the IFN-γ secretion in T and non-T lymphocytes (*Di Cerbo et al., 2016*). Here, we evaluated the effect of drug treatment in the up-regulation of IFN-γ gene expression. Figure 1A shows that mRNA levels of IFN-γ robustly increased in PBMCs after 24 h of OTC incubation.

To investigate if OTC-mediated inflammatory condition could depend on oxidative stress, we evaluated whether the Cu–Zn Super Oxide Dismutase 1 (SOD1) could be increased after drug exposure. It is of note that one of the SOD1 is involved not only in oxidative metabolism but also in the T lymphocyte activation dependent on the accumulation of reactive oxygen species (*Terrazzano et al., 2014*). Our data (Fig. 1B) show that the mRNA levels of SOD1 increased from 12 to 24 h of OTC treatment.

The data reported in Fig. 1 showed that the enhancement of mRNA levels of IFN-γ occurred after 24 h of OTC-treatment, whereas the induction of SOD1 mRNA appeared already in 12 h. A possible explanation might be that SOD1 is a housekeeping gene (*Minc et*

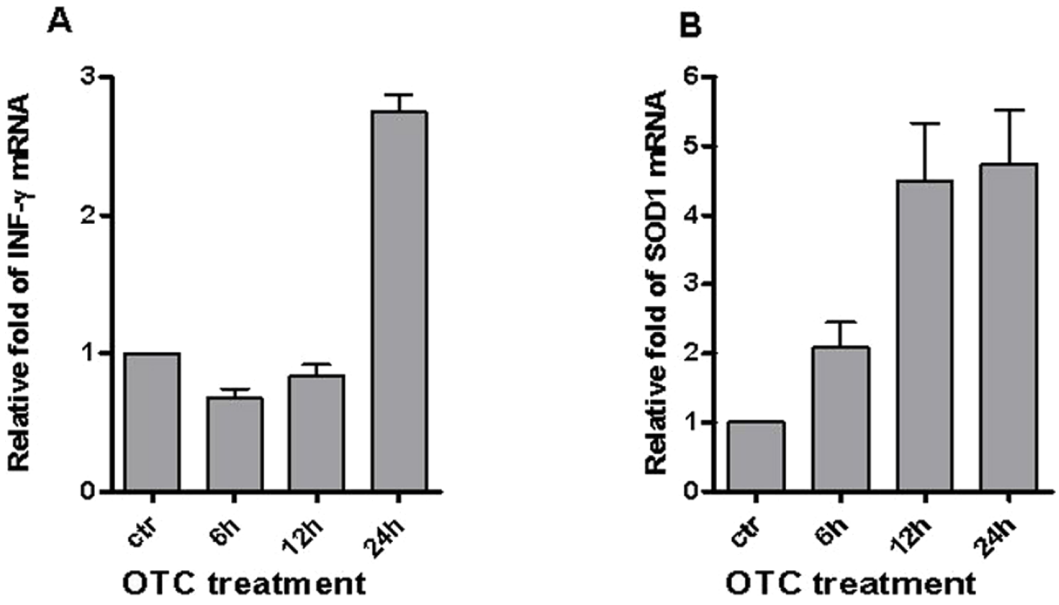

Figure 1 OTC induces IFN-γ and SOD1 mRNA. OTC significantly induces the increment of both IFN-γ and SOD1 mRNA. Total RNA was prepared from PBMC stimulated with OTC for 6, 12, 24 h, as indicated in 'Materials & Methods', and analyzed by qPCR with specific primers to IFN-γ (A) and SOD1 (B) mRNA normalized to 18S RNA levels. The statistical analysis derived from 2 experiments in triplicate ($n \geq 6$; Mean $\pm$ SD).

al., 1999) and its basal expression is usually higher than IFN-γ gene. Since the used *in vitro* model is based on freshly isolated PBMCs that are usually resistant to natural occurring apoptosis (*Miyawaki et al., 1992*), the increased SOD1 level after the drug incubation could be likely associated to the hypothesis of apoptosis induction upon chromatin and DNA damages (*Norbury & Zhivotovsky, 2004*; *Barbosa et al., 2010*).

These results suggest that the drug may affect some important cellular responses as the induction of a gene expression fostering the activation of previously observed immune response by T and non-T lymphocytes after *in vitro* OTC exposure (*Di Cerbo et al., 2016*).

OTC generates genotoxic damage

Since the OTC is able to induce apoptosis (*Odore et al., 2015*; *Di Cerbo et al., 2016*), we investigated on the potential ability of such drug in causing DNA damage and, in reason of that, in inducing apoptosis. In particular, we evaluated the presence of genotoxic markers after *in vitro* drug treatment of PBMCs. In this regard, it is worth noting the role for Ataxia Telangiectasia mutated protein (ATM) that is a serin/treonin kinase activated in response to the DNA double strand break to promote cell cycle arrest, DNA repair and, if necessary, the cell death by apoptosis (*Canman & Lim, 1998*; *Lee & Paull, 2007*).

As shown in Fig. 2A, the phosphorylated form of ATM (pATM) is clearly increased in human PBMCs after 6 h and 12 h of OTC incubation.

Furthermore, we investigated the levels of p53, as one of principal substrates of pATM and an important marker of DNA damage (*Sakaguchi et al., 1998*; *Williams & Schumacher, 2016*). Figure 2A indicates that p53 significantly increased after 12 h of drug exposure.

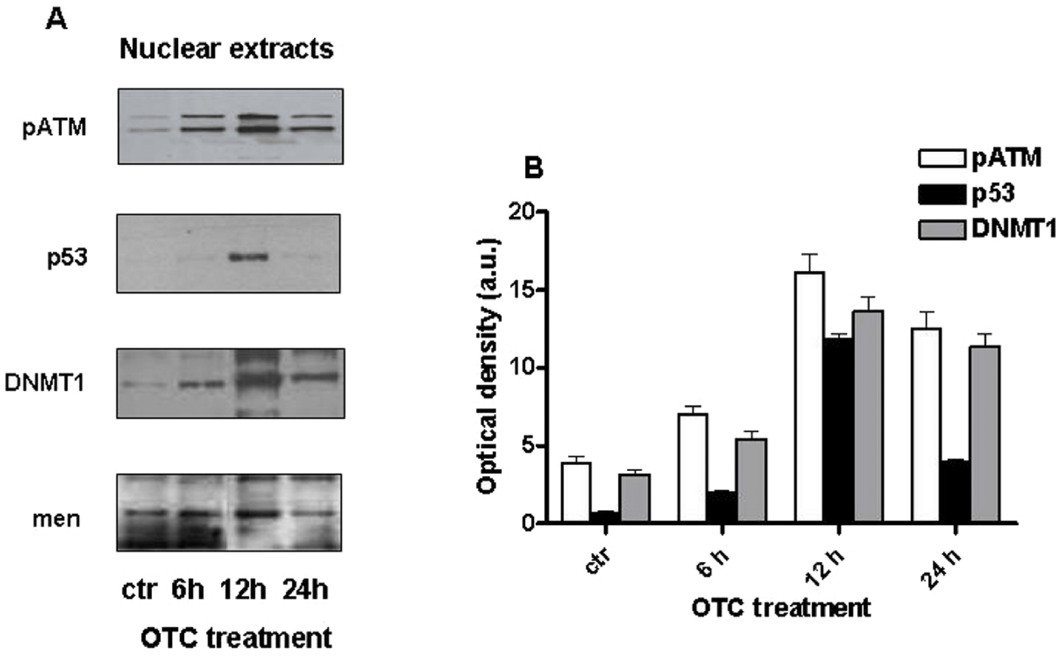

Figure 2 OTC induces genotoxic damage. Cells were treated with OTC for 6, 12 and 24 h and processed as indicated in 'Material and Methods'. (A) the western blot for pATM, p53 and DNMT1 was performed using nuclear extract. Menin is reported as loading control; (B) quantification of the western blots normalized to Menin levels. Values are reported as Optical density (arbitrary units = a.u.).

These observations suggest that some DNA damage may occur after OTC incubation.

One of the main epigenetic modifications involved in gene regulation is the DNA methylation (*Hamidi, Singh & Chen, 2015*). It is well known that DNA (cytosine-5)-methyltransferase 1 enzyme (DNMT1) is recruited to the chromatin, in response to the oxidative DNA damage, in order to inhibit gene transcription and to support DNA repair (*Ding et al., 2016*). It is of relevance that DNMT1 appears to increase after OTC treatment, following a similar kinetics of pATM and p53 (Fig. 2A).

Such evidence supports the idea that the enzymes could be recruited on the site of oxidative DNA damage occurred upon OTC incubation and cooperate each-other to induce chromatin modifications aimed to foster the DNA repair (*Morano et al., 2014*).

To better address the entity of DNA damage, we investigated the presence of DNA double strand break (DBS) markers. In particular, we evaluated the phosphorylated histone H2AX (γH2AX) (*Rogakou et al., 1998*; *Mah, El-Osta & Karagiannis, 2010*) by performing western blot analysis on chromatin samples. Figures 3A and 3B shows the significant increase of γH2AX with the highest peak from 12 to 24 h of drug treatment. Notably, the phosphorylated histone H2AX binds the regions of chromatin on the sites of DSB and DNA repair (*Mah, El-Osta & Karagiannis, 2010*). To better analyze the chromatin changes caused by the drug, we tested by ChIP assay the presence of γH2AX on the promoters of genes of our interest. We observed the accumulation of γH2AX at the site of promoter of IFN-γ gene (Fig. 3C). The presence of γH2AX was also enhanced at the level of SOD1 gene promoter after 24 h of drug incubation (Fig. 3D).

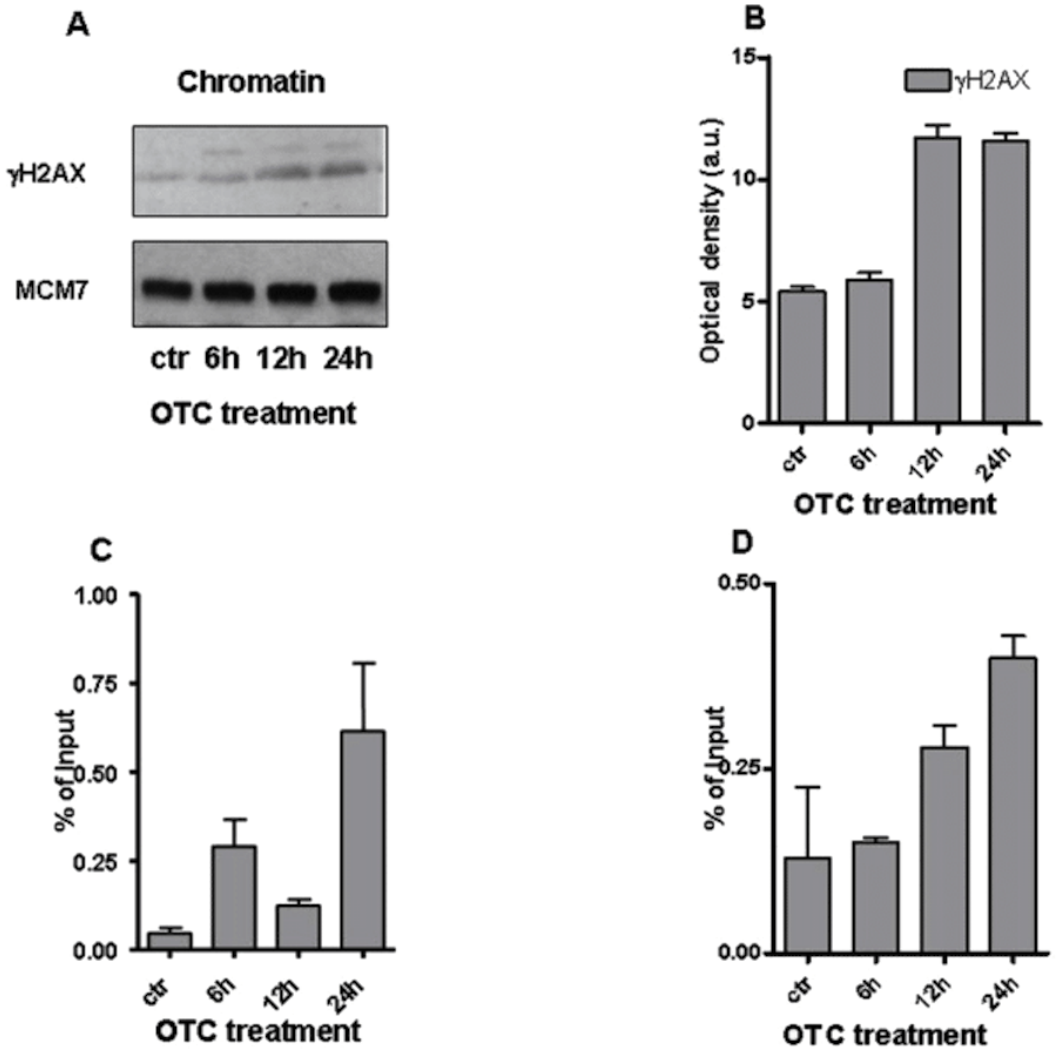

Figure 3 OTC and the chromatin changes. (A) The western blot for γH2AX performed on chromatin extracts. MCM7 is reported as loading control; (B) Quantification of γH2AX normalized to MCM7 levels. Values are reported as Optical density (arbitrary units = a.u.); qChIP analysis evideces that γH2AX accumulates on IFN-γ (C) and SOD1 (D) promoters. Cells were treated with OTC as indicated, crosslinked and sonicated. The statistical analysis derived from at least 2 experiments in triplicate ($n \geq 6$; Mean $\pm$ SD).

Therefore, our results strongly suggest the correlation between DNA damage occurrence and OTC administration. Moreover, the increased levels of IFN-γ and SOD1 mRNAs (Fig. 1) appear to be linked to the function of γH2AX, which cooperates with the induction of ATM mediated transcription (*Singh et al., 2015*).

Epigenetic changes

Histone modification represents an epigenetic mechanism that affects gene transcription by altering the chromatin structure and DNA accessibility. Histone methylation can be associated with the different status of chromatin (*Zhang, Cooper & Brockdorff, 2015*). Here, we evaluated if OTC treatment could be correlated to alterations of histone methylation. More specifically, we investigated on the methylation status of lysine 4 of Histone 3 (H3K4)

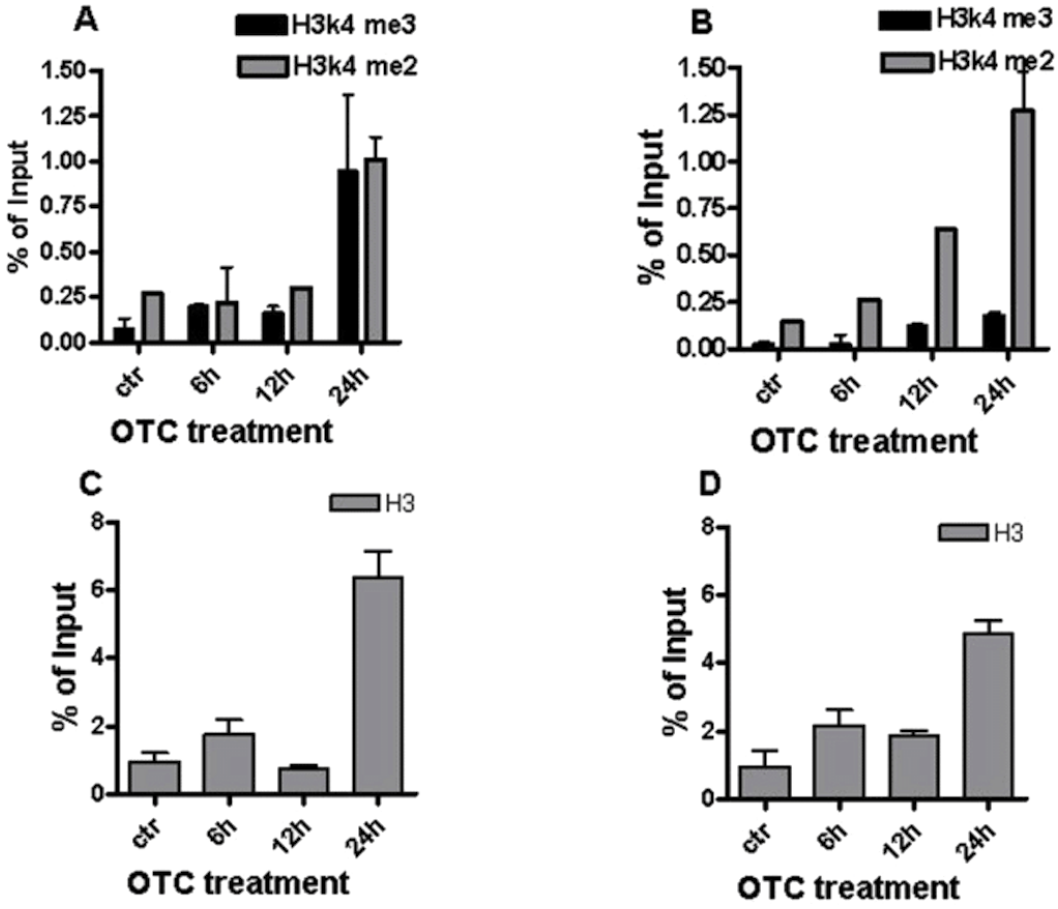

Figure 4 OTC and histone methylation. Methylation profile of histone H3K4 is induced by OTC on both the IFN-γ and SOD1 gene promoters. PMBC cells were exposed to OTC at the indicated times (0, 6, 12 and 24 h). qChIP was carried out using specific antibodies; (A) H3K4me3 and H3K4me2 occupancy at IFN-g promoter; (B): H3K4me2 and H3K4me3 occupancy at SOD1 promoter; (C) and (D); the TotalH3 occupancy at IFN-γ and SOD1 promoters respectively. The statistical analysis derived from at least 2 experiments in triplicate ($n \geq 6$; Mean $\pm$ SD).

that is implicated in the regulation of gene activation (*Barski et al., 2007*; *Ruthenburg, Allis & Wysocka, 2007*).

To this aim, we performed a ChIP for the promoter of the genes whose expression appeared to be modified by OTC. After 24 h of drug incubation, the increment of both the di-methylated (me2) and tri-methylated (me3) H3K4 is particularly evident for IFN-γ promoter (Fig. 4A), while the increase of H3K4 is more evident for the di-methylated form at the level of SOD1 promoter (Fig. 4B). These data are correlated with the observed activation of gene expression (*Bernstein et al., 2005*). Similar results are obtained from the analysis of total H3 histone levels (Figs. 4C and 4D).

Together, these data indicate that the OTC treatment can affect the status of chromatin.

CONCLUSION

Despite scientific literature that has been suggesting the potential toxicity of OTC (*Czeizel & Rockenbauer, 2000*; *Glette et al., 1984*; *Potts et al., 1983*; *Van den Bogert & Kroon, 1982*; *Myers, Farrell & Henderson, 1995*), the mechanisms of the toxic effect of such drug is still poor understood.

We recently demonstrated that OTC induces *in vitro* inflammatory response (*Di Cerbo et al., 2016*) and apoptosis (*Di Cerbo et al., 2016*; *Odore et al., 2015*). Therefore, this and other suggestions open an interesting scenario on the toxicity of OTC that requires a greater understanding over the nature of observed toxic effects.

This study emphasized the toxicity of OTC, investigating over the molecular mechanisms involved in human PBMC inflammatory response. It is of note that the drug promoted a robust inflammatory response as represented by the increasing of IFN-γ mRNA levels. This result reflects and confirms the previously observed increment of IFN-γ production in T and non-T lymphocytes (*Di Cerbo et al., 2016*). In addition, OTC significantly induced SOD1 mRNA in the same experimental condition and cellular model. This evidence extends our previous observations on apoptosis induction after OTC exposure (*Terrazzano et al., 2014*; *Odore et al., 2015*).

In addition, we observed that OTC induces genotoxic damage as well as such drug recruits some enzymes implicated in the delicate balance between cell death and survival. Indeed, our data evidenced the increased levels of pATM, p53 and DNMT1 after drug incubation. It is of note that p53 is a substrate of pATM and is crucial to the cell cycle arrest and/or to induce the cell death by apoptosis (*Norbury & Zhivotovsky, 2004*), while DNMT1 represents a specific enzyme involved in some epigenetic changes (*Hamidi, Singh & Chen, 2015*), associated with the DNA damage (*Rossetto et al., 2010*).

Moreover, we observed the activation of γH2AX, as a main DSB sensor protein, and suggested an epigenetic effect of OTC on the methylation status of H3K4 that is implicated in gene expression regulation (*Barski et al., 2007*; *Ruthenburg, Allis & Wysocka, 2007*). The increase of both me2 and me3 H3K4 occurred after OTC incubation and was evident for IFN-γ and SOD1 gene promoters.

Our data represent a preliminary step in the understanding of OTC toxicity, since the knowledge of the molecular mechanisms involved in the toxic effect may help in the generation of new drugs with reduced risk for human health.

In conclusion, it could be of great relevance to ascertain the possible acute and long term effects of OTC on human health. It is worth noting that the use of antibiotics for growth promotion is prohibited in Europe and it is considered a health hazard by WHO since 2006. The use of antibiotics in agriculture for non-therapeutic purposes is allowed in United States and Canada (*FaAOF, 2014*). Therefore, new regulations are urgently necessary to reduce antibiotic contaminants in foods as well as the antibiotic resistance phenomenon (*US Government Publishing Office, 2014*).

STUDY LIMITATIONS

Notably, the current study incurs some limitations that are not addressable without further researches. In this regard, our study did not perform chemical-pharmaceutical and pharmacological test to evaluate the molecular complexity and/or stability of the used OTC or to verify the possible presence of active sub-products generated during *in vitro* tests. In addition, this study did not address any chemical evaluation of the excipients (i.e., fillers, binders, dyes, flavorings, preservatives and other materials) present in the here used commercial liquid formulation of OTC drug employed in veterinary medicine. Therefore, further evaluations are required to complete the significance of OTC toxicity. In particular, the absence of *in vivo* experiments, able to confirm the *in vitro* observed OTC toxicity, represents the main relevant limitation. Therefore, clinical studies are required to ascertain the effect of the drug in inducing the inflammatory status in animals and/or in humans.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The authors received no funding for this work.

Competing Interests

None of the authors have financial or personal relationships with other people or organisations that could inappropriately influence or bias the content of the paper. This research was performed in collaboration with some scientists from the Division of Research and Development, Sanypet SpA, Padova, Italy (as indicated in the authors' affiliation) according to scientific and ethical principles of the scientific community. No financial funding was obtained from Sanypet Industry for this research study.

Author Contributions

- Adriana Gallo and Giuseppe Terrazzano conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Rosaria Landi conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables.
- Valentina Rubino conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Alessandro Di Cerbo, Sara Centenaro, Gianandrea Guidetti, Sergio Canello, Laura Cortese and Andrea Alessandrini contributed reagents/materials/analysis tools.
- Angela Giovazzino performed the experiments, contributed reagents/materials/analysis tools.
- Anna Teresa Palatucci performed the experiments, analyzed the data, prepared figures and/or tables.
- Giuseppina Ruggiero analyzed the data, wrote the paper.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The ethics committee approval is not required for this type of experimental test, as it is not a clinical trial *in vivo*, we do not use patients, but merely use peripheral blood leukocytes from healthy blood donors as volunteers who went at the Blood Bank of Federico II.

The experiments were only performed "*in vitro*" using peripheral blood mononuclear cells from these healthy donor's buffy coats, derived as discarded products upon preparation of medical blood components (red cells, platelets, plasma and its derivatives).

Italian Law (L.107/1990, L.219/2005, DL25/01/01 no. 25, DL20/12/07 no.261, DM 02/11/2015) allows the use of these "remnant" parts of blood donation to scientific purpose if the donors are informed and if they subscribe a written consent at the time of blood donation.

The informed consent (model n. 5526 of Azienda Ospedaliera Universitaria "FEDERICO II", Naples, Italy) has been already subscribed by the 6 donors at the time of blood donation, performed at Blood Trasfusional Center of Azienda Ospedaliera Universitaria "FEDERICO II", and steated the possibility to use some blood components also for scientific purposes. The *in vitro* experiments were performed fully anonymous. White blood cells have never been used to create a genome database.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as Data S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3236#supplemental-information.

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Research paper

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

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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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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.

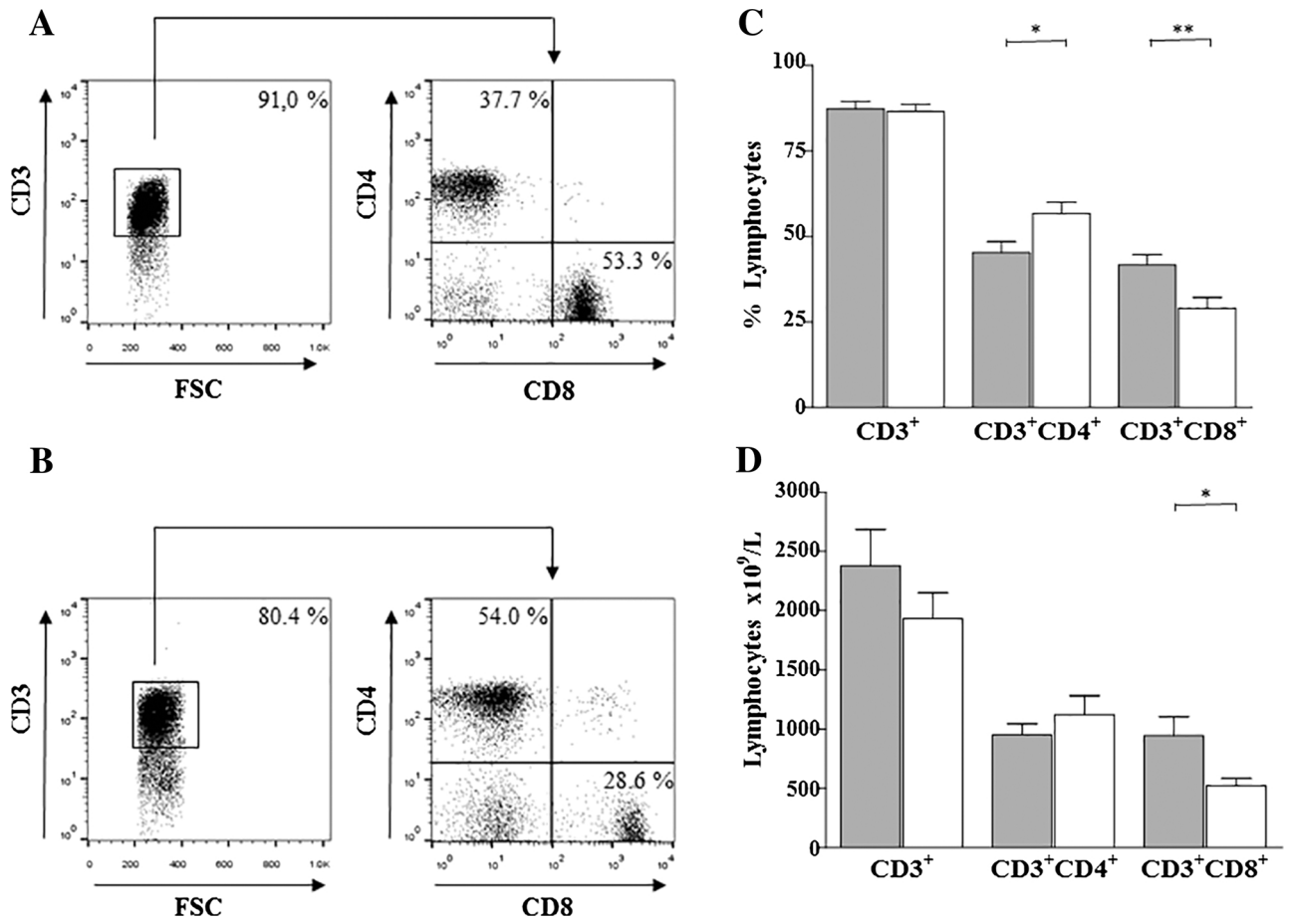

Fig. 1. Increased level of circulating $CD3^+CD8^+$ lymphocytes characterizes obese Labrador Retriever dogs. Panel A and B show flow cytometry analysis of one representative OB and CTR animal. As shown, expression of CD4 and CD8 co-receptors was analysed in the region of CD3 positive cells (identified 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

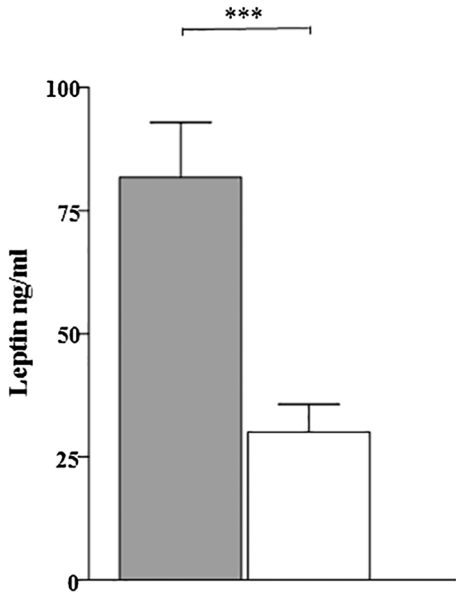

Fig. 2. Significant increase of leptin hormone in serum characterizes obese Labrador Retriever dogs as compared with the normal weight counterpart. As shown, significant difference ($p < 0.001$) in leptin serum concentration has been observed in obese (OB), as compared with control (CTR) dogs; grey and 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

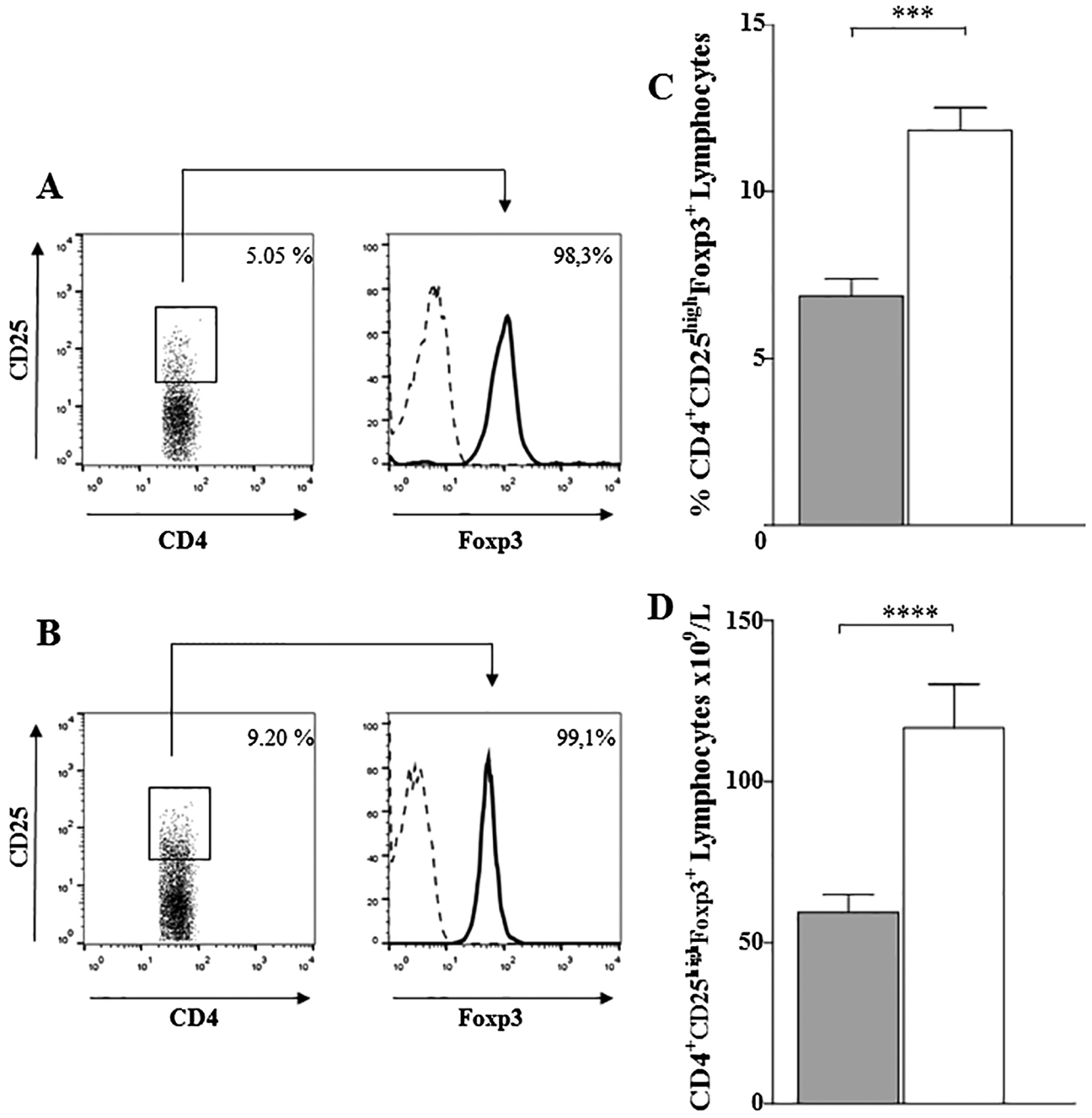

Fig. 3. Obese Labrador Retrievers show significant reduction of circulating regulatory T cells (Treg). Panel A and B show flow cytometry analysis of one representative OB and CTR animal. Expression of CD25 was analysed in the region of CD4 positive cells (identified in the CD3 region). Numbers indicate 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

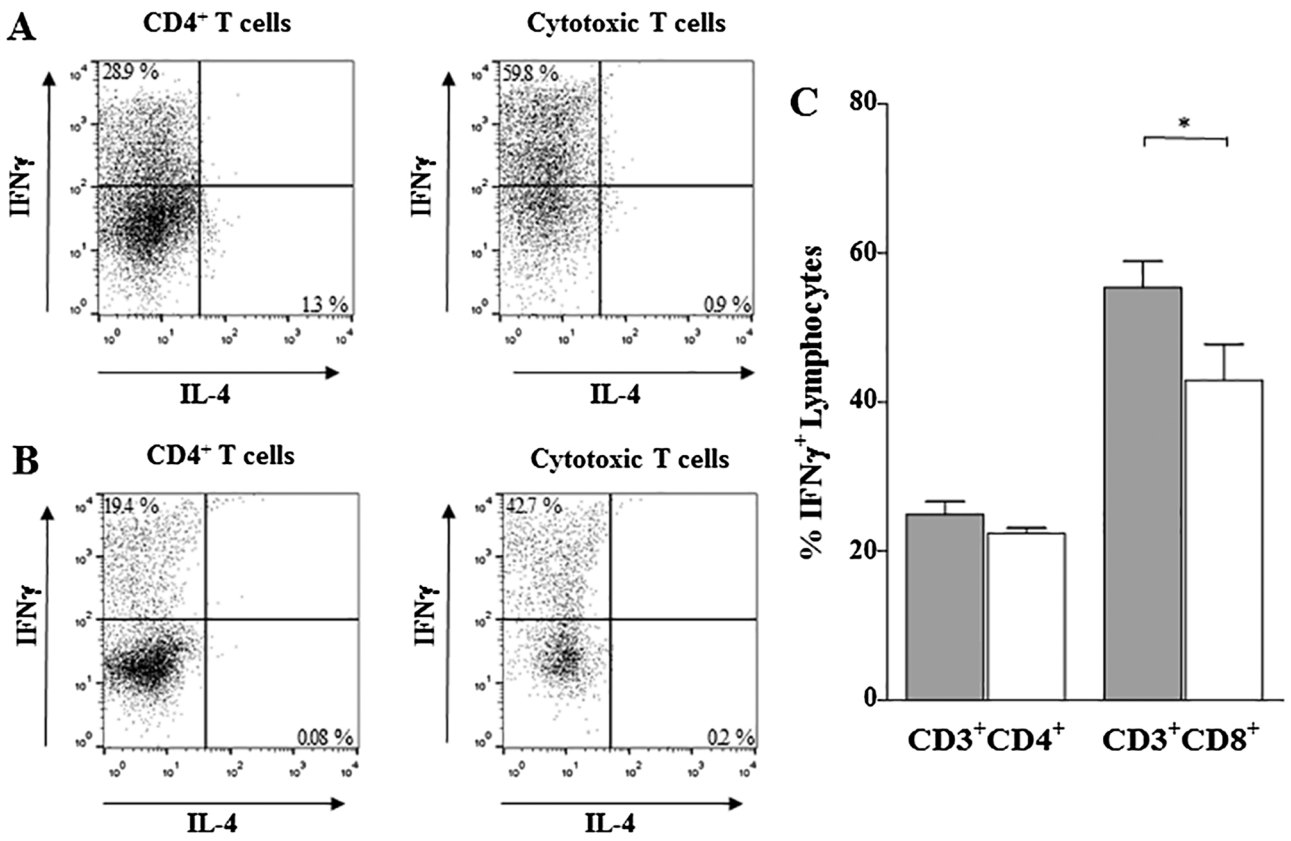

Fig. 4. Cytotoxic T cells of obese Labrador Retriever dogs were observed to produce increased level of Interferon-γ, as compared with the normal weight counterpart. Panel A and B show flow cytometry analysis of one representative OB and CTR animal. Intracellular expression of IFN-γ and IL-4 was analysed 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.

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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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Toxicological Implications and Inflammatory Response in Human Lymphocytes Challenged with Oxytetracycline

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ABSTRACT: Antibiotics are widely used in zoo technical and veterinary practices as feed supplementation to ensure wellness of farmed animals and livestock. Several evidences have been suggesting both the toxic role for tetracyclines, particularly for oxytetracycline (OTC). This potential toxicity appears of great relevance for human nutrition and for domestic animals. This study aimed to extend the evaluation of such toxicity. The biologic impact of the drug was assessed by evaluating the proinflammatory effect of OTC and their bone residues on cytokine secretion by in vitro human peripheral blood lymphocytes. Our results showed that both OTC and OTC-bone residues significantly induced the T lymphocyte and non-T cell secretion of interferon (IFN)-γ, as cytokine involved in inflammatory responses in humans as well as in animals. These results may suggest a possible implication for new potential human and animal health risks depending on the entry of tetracyclines in the food-processing chain. © 2015 The Authors Journal of Biochemical and Molecular Toxicology Published Wiley Periodicals, Inc. J. Biochem. Mol. Toxicol. 30:170–177, 2016; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.21775

KEYWORDS: **Oxytetracycline; Toxicity; Apoptosis; Inflammatory cytokine; food**

INTRODUCTION

The use of antibiotics in the agro-food industry is a relevant concern [1]. They have been still employed as growth promoters in livestock, aquaculture, and pesticides [1–3]. The topic is relevant considering the potential toxic risk derived by the entry/accumulation of antibiotics in animal feed and human food with consequences on health [4].

The use of antibiotics for growth promotion is prohibited in Europe, whereas the United States and Canada still allow use of antibiotics in agriculture for nontherapeutic purposes [5]. New regulations from the Food and Drug Administration (FDA) are endeavoring to reduce antibiotic contaminants in foods [6, 7]. Although allergic reactions are rarely related to antibiotics, meats and fruit induced by antibiotic residues have been reported in the literature [1, 8]. The occurrence of antibiotic toxic effects has negative consequences on the gastrointestinal tract, skin, central nervous system, and even accumulating in calcium-rich organs such as bones and teeth [9–11].

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Food intolerance has described in gym subjects due to intake of meat derived from administration of animals-fed tetracycline [12]. Several models have already been proposed to demonstrate cytotoxic effects of tetracyclines [13–15]. To the best of our knowledge, the possible interplay between tetracyclines, in particular the oxytetracycline (OTC), and immune system is still lacking and not sufficiently addressed to rule out the possible toxicity to human and animal health.

In this regard, the immune system acquires a new pivotal role in modulating toxicological mechanisms that could be triggered by tetracyclines derived by ingested food. Immunity, which has the fundamental role to protect and defend the organism from the disease [16–18], is also involved in the homeostasis and health maintenance against autoimmunity diseases and tumors [17]. The exacerbation of cytotoxic CD8+ T lymphocytes [18] and CD4+ T Helper 1 (T_H1) [19] responses were associated with inflammatory diseases and autoimmunity disorders [20]. T_H1 activity is mainly based on the production of interferon-gamma (IFN-γ, which optimizes the antimicrobial responses and fosters CD8+ T lymphocyte activity, and also appears to play a fundamental role in triggering autoimmune responses [21–29]. Natural killer (NK)-dependent secretion of IFN-γ is relevant to autoimmunity [30–33], allergy [34], and could have a pathogenetic role in gastrointestinal [35] and hematological disorders [36, 37]. T_H2 response is based on several cytokines including interleukin (IL)-4 that results in the activation of humoral immunity [21].

It is worth noting that several extrinsic factors, such as drugs and chemicals, can induce the development of autoimmune conditions [38–43].

Moreover, the use of these drugs to treat inflammatory conditions is still not conclusive and, in some way, controversial as well as the abuse of some veterinary drugs, including tetracyclines, which have a global impact on the environment that could be of great relevance [[44]–[50]].

OTC represents the main drug used to control gastrointestinal and respiratory diseases in broiler chickens [51], although its accumulation has been described in chicken edible tissues [52]. As a consequence, the European Union established the maximum residue level of OTC in poultry meat [53] to limit drug residues and to preserve health of final consumers that are mainly represented by domestic animals and humans.

Based on a previous study on the toxicity of OTC [54], we investigated the potential toxic effect of OTC in an in vitro human lymphocyte model. In particular, we addressed the potential induction of IFN-γ production caused by the in vitro exposure of human T and non-T lymphocytes to OTC or to chicken bone-derived residues.

MATERIALS AND METHODS

Cells

Peripheral blood samples from healthy donor volunteers were collected by vein puncture according to standard procedures and used within the 3 h from the collection. Informed consent was obtained in accordance with the Declaration of Helsinki, as approved within the study protocol by the Institutional Review Board at the Federico II University of Naples. Peripheral blood mononuclear cells (PBMC) were used as mixed population of T (CD3+) and non-T (CD3–) (the latter are mainly represented by NK cells) lymphocytes [17, 18]. Identification of cell subpopulations was performed by immune-fluorescence and flow cytometry (see paragraph 2.4, Monoclonal Antibodies, Flow Cytometry, Detection of Intracellular IFN-γ, and IL-4 Productions). PBMC were isolated by centrifugation on Lymphoprep (Nycomed Pharma) gradients, as described [35].

OTC and the Conditioned Cell Medium

To test the potential toxic role of OTC (Oxytetracycline 20%®, TreI, Reggio Emilia, Italy) and OTC bone residues, two different conditioned cell culture mediums (CCM) were used, as previously described [54]. Briefly, to obtain CCM, 10 mL of a RPMI 1640 cell culture medium was incubated and constantly shaken for 48 h at 37°C with 1 g of ground bone (sterilized by autoclaving at 121°C in a steam pressure of 2 atm for 10 min) from chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of treatments with OTC [54]. After incubation, the CCMs were recovered and filtered through 0.20 μm syringe filters (Sartorius Stedim Biotech, Goettingen, Germany) to remove any residual ground bone particles and microbial contamination.

Apoptosis Detection

Apoptosis detection was performed as previously described [54]. Briefly, OTC- CCM and C-CCM were used at the dilution of 1:4 with an absolute RPMI 1640 growth medium, and the resulting mixtures were incubated with 5×10^5 PBMC/mL for 10 or 48 h at 37°C and 5% CO_2 in a cell incubator (Thermo Scientific Heraeus). The effect of OTC alone was evaluated by incubating the drug (1 μg/ mL), as described above.

Apoptosis was assessed by staining of the cell membrane-exposed phosphatidylserine with

fluorescein isothiocyanate-conjugated (FITC) Annexin V, according to the manufacturer's instructions (Becton Dickinson PharMingen, San Jose, CA) and as previously described [55]. Samples were analyzed by means of flow cytometry, using a two laser-equipped FACSCalibur (Becton Dickinson PharMingen, San Jose, CA), and the CellQuest Analysis Software. The FACS analysis was based on the percentage of Annexin V-positive cells to have a measurement of the cells undergoing apoptosis.

Monoclonal Antibodies, Flow Cytometry, Detection of Intracellular IFN-γ, and IL-4 Productions

FITC, PE, Cychrome, and APC labeled mAbs against CD3, CD8, CD4, IFN-γ, IL-4, and isotype-matched controls (Becton Dickinson PharMingen, San Jose, CA) were used to identify the CD8+ T cytotoxic, CD4+ T_H lymphocytes, or CD3– non-T cells.

To analyze the production of IFN-γ and IL-4, purified PBMC were cultured overnight (10–12 h) in the presence of *phorbol*-12-myristate-13-acetate (PMA) and ionomycin (Sigma). To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/mL of Brefeldin-A (Sigma-Aldrich) as described [56].

Intracellular IFN-γ and IL-4 production was detected by using a triple staining technique and flow cytometry analysis. Briefly, after the incubation the culture was harvested, the cells were fixed and permeabilized by using a cytokine staining kit, following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Samples were analyzed by flow cytometry (see description in the *Apoptosis Detection* section).

Statistical Analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). All data are presented as the means ± standard error of the mean and were first checked for normality using the D'Agostino-Pearson normality test. The analysis pertaining to the proinflammatory effect was analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. $p < 0.05$ was considered significant. Statistical analysis was specifically performed to evidence differences within the pairs of comparison (OTC vs. ctr, OTC-CCM vs. ctr, C-CCM vs. ctr, as indicated in the Figures 1 and 3).

RESULTS

The Toxic Effect of OTC and Their Residues from Bone as Induction of Apoptotic Phenomenon in PBMC

According to previous data [56], the OTC was able to induce the apoptosis in PBMC after 48 h of incubation (Figure 1, panel A). A similar effect was obtained using the OTC-CCM, whereas no results were obtained with C-CCM (Figure 1).

Note that the incubation with OTC or with OTC-CCM did not exert significant apoptosis phenomenon in PBMC after an incubation of approximately 10–12 h (Figure 2).

These results significantly confirmed the toxicity of OTC and of their residues in bone (OTC-CCM) and also evidenced the difference between the early (10-12 h) and late (48 h) exposure to this drug. Such data allowed to identify the range of time (10-12 h), in which the cells do not undergo OTC-dependent apoptosis, to perform experiments of cytokine secretion (see the Materials and Methods section).

The Proinflammatory Toxic Effect of OTC and Their Residues from Bone as Significantly Increasing IFN-γ Production in T Lymphocytes as well as in Non-T Cells

In the light of the described observation (see the preceding paragraph), we evaluated the other possible alterations caused in human lymphocytes after the exposure to OTC [54]. With this aim, we focused on the IFN-γ production, as the main proinflammatory cytokine is able to foster the T_H1 and T cytotoxicity immune-responses as well as to be involved in several etiopathogenetic mechanisms on the basis of inflammatory-mediated disease [19].

Since the evaluation in vitro of IFN-γ production by T and non-T lymphocytes is usually performed in short time (8–18 h) to obtain an optimal functional cytokine secretion [56] and we demonstrated that after 10 h the apoptosis was not induced (Figure 1, panel B), we incubated human PBMC with OTC and OTC-CCM for 10 h to avoid this phenomenon. Indeed, the good viability of lymphocytes is crucial for the induction of cytokine production functions.

As shown, the incubation with OTC and OTC-CCM was able to significantly increase the IFN-γ production in CD4+ T_H cells (R1 in Figure 2 and panel A in Figure 3) and CD8+ lymphocytes (R2 in Figure 2 and panel B in Figure 3) as well as in non-T cells (R3 in Figure 2 and panel C in Figure 3). The cytokine was slightly detectable at the dilution 1:8 and 1:16 of OTC-CCM (data not shown), whereas

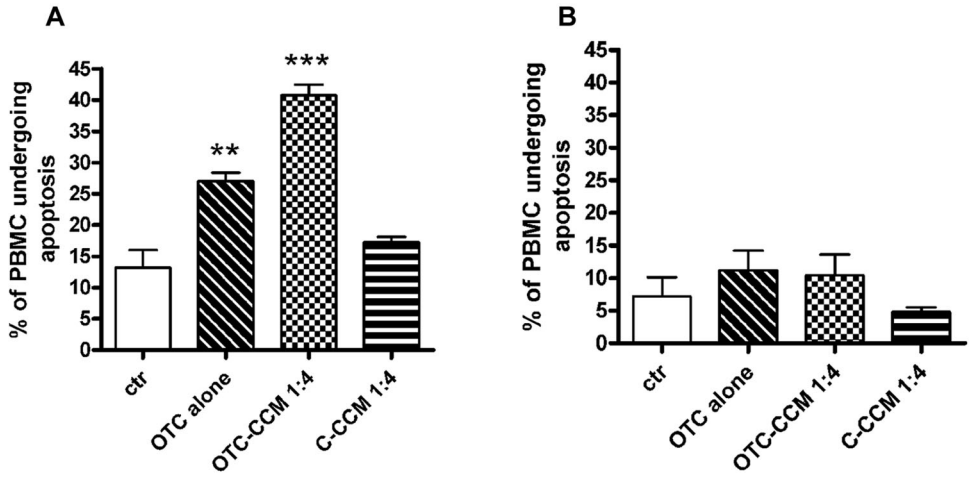

FIGURE 1. Apoptosis induction measured as a percentage of PBMC positive for the FITC-Annexin binding. The graph bar columns represent the mean values of the percentage of PBMC undergoing apoptosis in the performed experiments ($n = 4$). The different cell incubations and conditioned cell culture medium dilutions are indicated on the x-axis. The abbreviations indicate the growth medium with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of a treatment with OTC, a growth medium with the addition of 1 μg/ mL of OTC alone. The bar column depicted as "ctr" indicates the incubation in the growth medium with Annexin V staining, which has been used as a control of the apoptosis that occurs in the cells when in a culture without any other incubation is maintained. The statistical significance is indicated with asterisk(s): $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

the dilution of 1:2 appeared to induce high level of apoptosis [56].

In the same cytokine production test, the incubation of PBMC from 12 to 18–24 h resulted in a very poor cell viability (data not shown) likely dependent on the here described proapoptotic effect of OTC and on Brefeldin A exposure used to allow the cytokine intracellular retention for the measurement (see the Materials and Methods section)

It is worth noting that C-CCM incubation did not produce a cytokine increase, while appeared to reduce IFN-γ production. Although we did not investigated this phenomenon, we do not exclude that some substances present in the bone (i.e., cytokines derived from osteoblasts or fibroblasts) may have an inhibitory role on the cytokine secretion. However, the difference in effects between OTC-CCM and C-CCM highlights the specificity of OTC action since the used chickens were of the same type and the only difference was the OTC administration [54].

The basal IL-4 production was only slightly detectable in T and non-T lymphocytes, as expected in PBMC from healthy donors after exposure to PMA and ionomycin [56] and was not modulated after 10 h of OTC or CCM incubations (data not shown).

DISCUSSION

In this article, we suggest that an antibiotic, the OTC, is able to determine the in vitro toxic effects. Data acquire great relevance especially in light of the wide use of OTC in animal breeding.

The OTC or the OTC-conditioned culture medium, obtained with the incubation of ground bone from OTC-treated chickens, appeared to generate the in vitro toxic effect of cell death by apoptosis in human cells [56].

Here, we suggest that the toxicity of OTC may also be extended to the induction of a proinflammatory microenvironment potentially responsible for initiation of tissue inflammatory spreading [21] or of autoimmune diseases [59].

In this regard, besides the ability to induce mortality of both the T lymphocytes and non-T cells in an in vitro system for incubation with OTC for 48 h, the drug potently promotes the production of proinflammatory cytokines in the first 10–12 h of cell exposure. Specifically, human lymphocytes increase their production of IFN-γ when exposed to the OTC or to the conditioned culture media with the bone of chickens treated with such drug as usually happens in livestock common breeding [51]. In our in vitro model, both the innate (non-T cells that are mainly represented by NK lymphocytes) and acquired (CD8+ and CD4+ lymphocytes) immunity [17, 18] appeared to be involved in this process and to suffer the OTC-dependent toxicity.

In this context, it is known that IFN-γ represents the main cytokine involved in the immune response [19], as well as a crucial element in the onset of impaired tissue homeostasis conditions, typically related to autoimmunity or chronic inflammation [23–31].

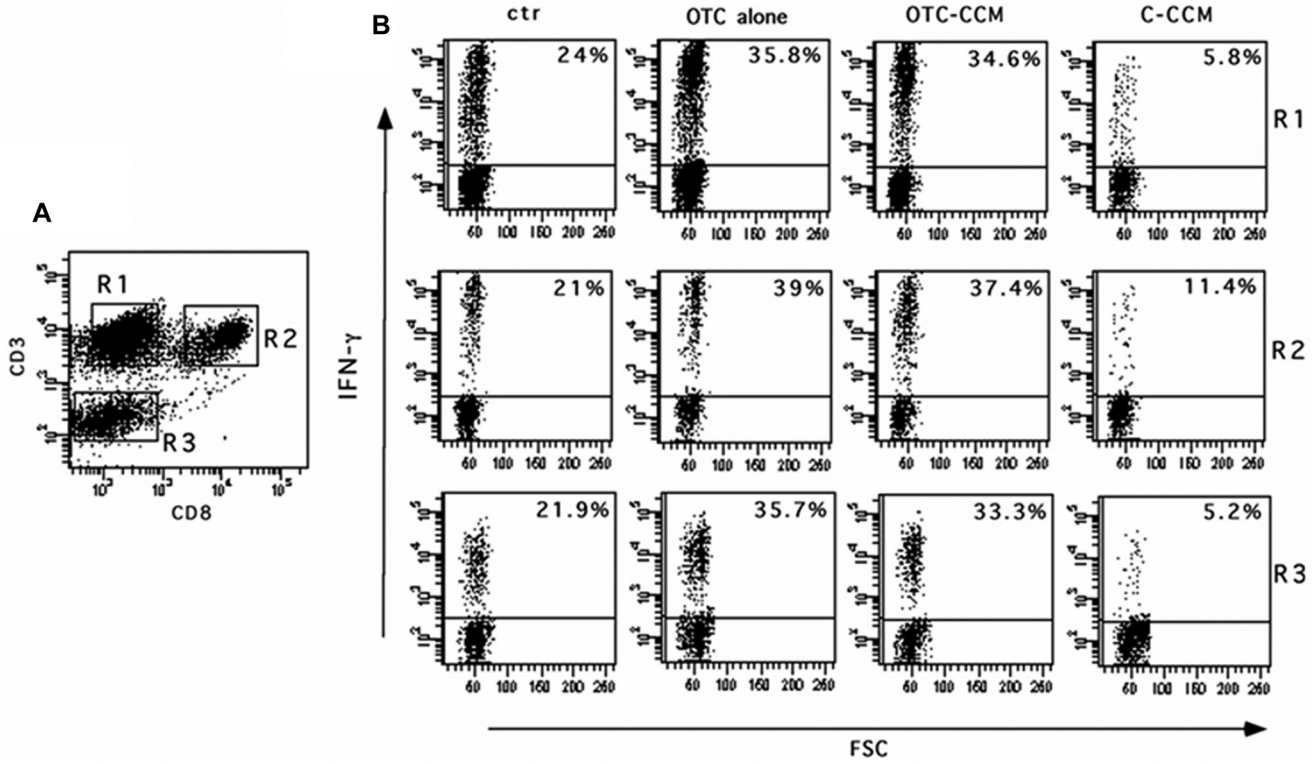

FIGURE 2. One representative experiment showing the IFN-γ production in CD4+ and CD8+ T lymphocytes and in non-T cells. Cytokine production was evaluated as the percentage of IFN-γ producing cells. *Panel A* refers to fluorescence gating strategy to identify the CD4+ T lymphocytes (CD3+ CD8–, cells in R1), CD8+ T lymphocytes (CD3+ CD8+ cells in R2) and the non-T cells (CD3– cells in R3). *B panels* represent the percentage of IFN-γ producing CD4 T (R1), CD8 T (R2), and non-T (R3) cells. The different cell incubations and conditioned cell culture medium dilutions are indicated on the top. The abbreviations indicate the growth medium with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of an OTC treatment, a growth medium with the addition of 1 μg/ mL of OTC alone. The condition indicates as "ctr" refers to basal IFN-γ production. All the cell cultures (ctr, OTC alone, OTC-CCM and C-CCM) were maintained in a growth medium added with PMA and ionomycin to induce cytokine production (see the Materials and Methods section).

A number of workers [46–52] have suggested that OTC would likely to represent a toxic compound and could be harmful to human health and animals that can eat meat derived from chikens by intensive livestock.

In addition, the induction of cell mortality could generate an altered tissue condition, as well as a relevant impact on tissue homeostasis [57, 58] and the emergence of autoimmune reactions [59–63].

Both pets and humans could take this antibiotic as a residue from meat or in meat-derived products and might likely suffer the OTC-dependent toxicity. In this respect, it is interesting that, over the past 20 years, there has been an exacerbation of the emergence of immune-mediated diseases (such as allergies, autoimmune reactions, and disorders of the gastrointestinal tract and the skin) in domestic animals [64–67] and humans [68, 69]. Moreover, it is surprising that the drastic increase of antibiotics resistance phenomena is partly due to the widespread and uncontrolled use of drugs in breeding [7, 70–74]. We previously correlated the use of specific meats to the occurrence of these pathologies in humans [12].

This unusual increase is probably dependent on a complex set of multifactor events related to new life habits of humans and pets, as well as to the increasingly introduction of industrialized diets. Hence, the needing to change the approach to livestock by promoting sustainable breeding avoiding overcrowding and by reducing antibiotics favoring the use of alternative treatments. Unfortunately, the use of several drugs can promote development of autoimmunity [38–43].

In conclusion, a special attention is probably needed on nutrition for large mass since it might expose humans and pets to increased risk of disease.

STUDY LIMITATIONS

Notably, this research has some study limitations. In this regard, the absence of in vivo experiments, able

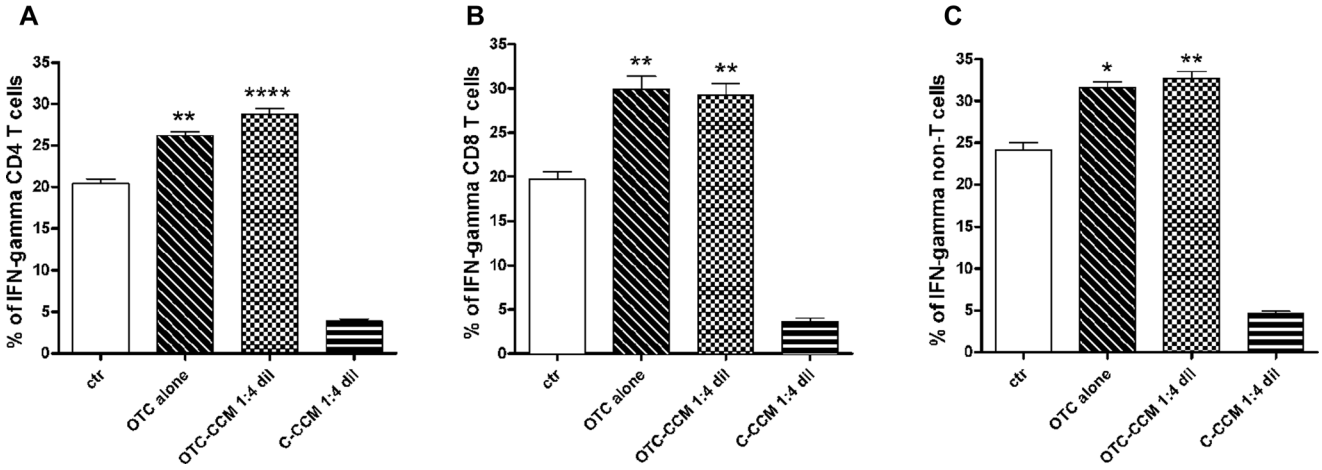

FIGURE 3. Statistical analysis of the all experiments ($n = 10$) showing the IFN-γ production in CD4+ and CD8+ T lymphocytes and in non-T cells. Cytokine production was evaluated as the percentage of IFN-γ producing cells. The bar column graphs represent the mean values of the percentage of IFN-γ producing cells. The different cell incubations and conditioned cell culture medium dilutions are indicated on the x axis. The abbreviations indicate the growth medium with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of an OTC treatment, a growth medium with the addition of 1 μg/ mL of OTC alone. The condition indicates as "ctr" refers to basal IFN-γ production. All the cell cultures (ctr, OTC alone, OTC-CCM and C-CCM) were maintained in a growth medium added with PMA and Ionomycin to induce cytokine production (see materials and methods). *Panels A, B,* and *C* show IFN-γ production in CD4+ T lymphocytes, CD8+ T lymphocytes and in non-T cells, respectively. The statistical significance is indicated with asterisk(s): *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

to verify the in vitro observed OTC toxicity, represents the main relevant limitation. Therefore, clinical studies are required to ascertain the in vivo effect of the drug in inducing the inflammatory status in animals and/or in humans.

In addition, the use of CCM obtained by incubation with bones from chickens reared in the presence of OTC did not directly demonstrate that the cell toxicity is due to bone's drug residues and did not ruled out that other substances could have a role.

CONFLICT OF INTERESTS

None of authors have financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper. This research was performed in collaboration with some scientists from the Division of Research and Development of Sanypet SpA and of GRAF S.p.A (as indicated in the author's affiliation) according to scientific and ethical principles of the scientific community. None financial funding was obtained from Sanypet Industry nor from GRAF Lab for this research study

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Clinical evaluation of a nutraceutical diet as an adjuvant to pharmacological treatment in dogs affected by Keratoconjunctivitis sicca

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Abstract

Background: Canine keratoconjunctivitis sicca (cKCS) is an inflammatory eye condition related to a deficiency in the tear aqueous fraction. Etiopathogenesis of such disease is substantially multifactorial, combining the individual genetic background with environmental factors that contribute to the process of immunological tolerance disruption and, as a consequence, to the emergence of autoimmunity disease. In this occurrence, it is of relevance the role of the physiological immune-dysregulation that results in immune-mediated processes at the basis of cKCS. Current therapies for this ocular disease rely on immunosuppressive treatments. Clinical response to treatment frequently varies from poor to good, depending on the clinical-pathological status of eyes at diagnosis and on individual response to therapy. In the light of the variability of clinical response to therapies, we evaluated the use of an anti-inflammatory/antioxidant nutraceutical diet with potential immune-modulating activity as a therapeutical adjuvant in cKCS pharmacological treatment. Such combination was administered to a cohort of dogs affected by cKCS in which the only immunosuppressive treatment resulted poorly responsive or ineffective in controlling the ocular symptoms.

Results: Fifty dogs of different breeds affected by immune-mediated cKCS were equally distributed and randomly assigned to receive either a standard diet (control, $n = 25$) or the nutraceutical diet (treatment group, $n = 25$) both combined with standard immunosuppressive therapy over a 60 days period. An overall significant improvement of all clinical parameters (tear production, conjunctival inflammation, corneal keratinization, corneal pigment density and mucus discharge) and the lack of food-related adverse reactions were observed in the treatment group ($p < 0.0001$).

Conclusions: Our results showed that the association of traditional immune-suppressive therapy with the antioxidant/anti-inflammatory properties of the nutraceutical diet resulted in a significant amelioration of clinical signs and symptoms in cKCS. The beneficial effects, likely due to the presence of supplemented nutraceuticals in the diet, appeared to specifically reduce the immune-mediated ocular symptoms in those cKCS-affected dogs that were poorly responsive or unresponsive to classical immunosuppressive drugs. These data suggest that metabolic changes could affect the immune response orchestration in a model of immune-mediated ocular disease, as represented by cKCS.

Keywords: Antioxidant and anti-inflammatory diet, Immune-mediated ocular disease, Keratoconjunctivitis sicca, Nutraceutical diet

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Background

Keratoconjunctivitis sicca, also defined as "dry eye disease" or Sjögren's syndrome in human [1], is a tear film disorder which causes inter-palpebral ocular surface damage and is associated with ocular discomfort [2, 3] both in humans and dogs [4, 5]. Canine keratoconjunctivitis sicca (cKCS) is an inflammatory eye condition which affects both cornea and conjunctiva and that is related to a deficiency in tear aqueous fraction [6]. The prevalence of such disease is estimated in about 4% when considering Schirmer test I (STT) values < 10 mm/min [7] reaching the 64% in male crossbred dogs between six to nine years of age [8]. Moreover, it is often an under-recognized and/or a sub-clinical condition [9] which, in some breeds, is preceded by an immune-mediated destruction of lachrymal glands [10, 11].

In this regard, the immune-mediated mechanisms of cKCS or of human, like the Sjögren's syndrome [1] induction are not clearly defined. Etiopathogenesis of such disease is substantially multifactorial, combining the individual genetic background with environmental factors that contribute to the process of immunological tolerance disruption and, as consequence, to the autoimmunity processes [12–14]. It is of relevance the role of the physiologic immune-dysregulation that results in the autoimmune process of cKCS and Sjögren's syndrome [12–15]. Notably, the T and B cell infiltration, the recruitment of dendritic cells, the up regulation of those molecules fostering the antigen presentation as well as the increased secretion of pro-inflammatory cytokines, such as interferon (IFN)-γ [16], in ocular tissues have been demonstrated to contribute to the inflammatory alterations of the lachrymal gland [17–19]. This process usually results in mucopurulent-like eye discharge, conjunctival hyperemia, keratitis, corneal pigmentation, neovascularization and blepharospasm in cKCS [20, 21].

Current therapies for this ocular disease rely on immune-suppressive treatments, represented by Cyclosporine A [22], glucorticoid [21], tacrolimus [23] and artificial tears in order to recover an adequate eye's lubrication [24]. Nevertheless, recognized complementary or alternative therapeutical approaches are represented by the cholinergic agents (pilocarpine) [25] and the surgical treatments (punctal occlusion, tarsorrhaphy, conjunctival flaps, contact lenses, superficial keratectomy, as well as parotid duct transposition) [26]. Clinical response to treatment frequently varies from poor to good, depending on the clinical-pathological status of eyes at diagnosis and on individual response to therapy [13]. Among other causes of cKCS traumas [27], congenital causes [28], distemper [29], radiation therapy [30, 31], neurological deficit [32], diabetes mellitus [33] and uncorrected prolapse of the nictitans gland [34] are of note. Intriguingly, majority of these aspects could correlate and contribute to both the determinism and exacerbation of inflammatory condition in ocular tissue.

In the light of the variability of clinical response to classical therapies, it could be useful the use of therapeutical adjuvants in cKCS management to improve the response to pharmacological treatment. Thus, we evaluated a combined therapeutical approach based on the classical drug administration and the use of an anti-inflammatory/antioxidant diet with potential immune-modulating activity. Such combination was administered to a cohort of cKCS dogs in which the only immune-suppressive treatment resulted poorly responsive or ineffective to control the ocular symptoms.

The nutraceutical diet used in this clinical evaluation consisted in a commercial mixed formula based on fish proteins, rice carbohydrates (whose carbohydrates percentage ranges from 75 up to 80, starch 65 to 70% with a beta-glucans quote of less than 0.1%), *Cucumis melo*, *Ascophyllum nodosum*, Astaxanthin (from *Hematococcus pluvialis*), *Aloe vera*, *Carica papaya*, *Punica granatum*, *Camellia sinensis*, *Polygonum cuspidatum*, *Curcuma longa*, *Piper nigrum*, zinc and a Omega3/6 ratio of 1:0.8), which already provided significant immunomodulating results, decreasing type 1 helper T lymphocyte (Th1) cells and increasing T regulatory (Treg) cells, in dogs affected by *Leishmania infantum* [35].

Cucumis melo (melon) shares some anti-oxidant and anti-inflammatory properties that involve the superoxide/peroxynitrite clearance and the modulation of macrophagal interleukin-10 production [36], while the immune-modulating activity is exerted by the induction of type 1 helper T lymphocyte (Th1) polarization [37].

The *Ascophyllum nodosum* activity is related to the presence of a sulfated-polysaccharide, ascophyllan, able to induce nitric oxide, tumor necrosis factor (TNF)-α and granulocyte colony-stimulating factor (GM-CSF) secretion in macrophages [38]. Astaxanthin, an orange-pinkish carotenoid, is known to act on polyunsaturated fatty acids oxidation [39], inflammatory responses modulation, and to promote eye's health in humans and animals [40]. This carotenoid induces lymphoblastogenesis and lymphocyte cytotoxicity in mice [41] as well as T-cell and B lymphocyte proliferation and natural killer cytotoxicity in humans [42]. Reduced production of Interleukin (IL)-1β, IL-6, TNF-α and IL-10 has been observed in vitro after the addition of *Aloe vera* (aloe) extracts to the culture of corneal cells [43]. The anti-inflammatory effect of *Carica papaya* (papaya) is related to an increase of regulatory T cells and a reduction of IFN-γ^+ $CD4^+$ T cells [44]. Reduction of IL-2 and IL-4 and enhancement of IL-12, interferon (IFN)-γ and TNF-α have been observed in blood mononuclear cells [45]. The seed oil and juice of *Punica granatum* (pomegranate) contains some flavonoids and anthocyanidins (delphinidin, cyaniding and pelargonidin)

with an antioxidant activity greater than green tea extract [46, 47]. Its antioxidant action is related to free radical scavenging by anthocyanidins [46] and to metal ions chelation [48]. A protective effects of *Punica granatum* on cardiovascular system has been correlated to angiotensin converting enzyme inhibition, blood pressure decrease [49] and endothelial nitric oxide syntase production [50]. *Punica granatum* also has been shown to inhibit cyclooxygenase, lipooxygenase [51] and IL-1β, modulate matrix metallo-proteinases in osteoarthritis, prevent collagen degradation [52], inhibit the p38-mitogen-activated protein kinase pathway and nuclear factor kappa (NF-kB) light-chain-enhancer in B cells [53, 54], and decrease malondialdehyde, TNF-*α*, IL-1β and IL-6 [55, 56].

The antioxidant effects of *Camellia sinensis* (green tea) are exerted through radicals scavenging and lipid-peroxidation inhibition [57] by flavonoids (catechin, epicatechin, epigallocatechin and gallate esters) [58]. In this context, epigallocatechin-3-gallate is known to inhibit UVB-mediated erythema, hydrogen peroxide production, leukocyte infiltration [59], matrix metallo-proteinases [60, 61], neutrophil chemotaxis [62], degradation of cartilage [63], TNF-α expression [64], neutrophil-mediated angiogenesis [62] and reduce the cyclooxygenase-2 and neutral endopeptidase activity [65]. *Polygonum cuspidatum* (japanese knotweed), a natural source of resveratrol, is endowed with anti-inflammatory and antioxidant activities [66, 67]. Resveratrol has been shown to directly act on TANK-binding kinase 1, an integral component in chronic inflammatory diseases [68], and on arteries by activating the nitric oxide/soluble guanylyl cyclase pathway [69]. Its anti-inflammatory effect is supposed to be regulated by estrogen receptor-α [70]. Moreover, certain resveratrol dimers (parthenocissin A, quadrangularin A and pallidol) exert free radical quenching and, selectively, single oxygen scavenging activity [71]. *Curcuma longa* (curcuma) induces powerful free radicals scavenging effect and anti-inflammatory activity [72, 73]. Curcumin, one of the constituents of such plant, reduces leukocyte adhesion and superoxide production, stimulates spontaneous apoptosis and inhibits IL-8 [74].

Moreover, a down regulation of Th1 cytokine response and of macrophagal nitric oxide production has also been observed [75]. The anti-inflammatory effect of curcumin involves the inhibition of NF-kB in activated B cells and the down-regulation of TNF-α and IL-6 [73] as well as the up-regulation of nuclear factor erythroid 2 activity [76], whose downstream proteins are involved in the protection mechanisms against oxidative stress [77]. *Piper nigrum* (pepper) commonly used in the treatment of flu, cold, rheumatism, pain, muscular aches, chills, exhaustion, fevers, is used as a useful nerve tonic also able to increase blood circulation and saliva production as well as to stimulate appetite and peristalsis [78]. It is also known to enhance the effectiveness and bioavailability of curcumin [79] by acting on membrane lipid dynamics in reason of the apolar nature of piperine, the main bioactive compound of *Piper nigrum*. Piperine has been shown to promote conformational changes of intestine enzymes [80] and significantly inhibit the expression of major histocompatibility complex class II, CD40 and CD86 in bone-marrow-derived dendritic cells as well as the production of TNF-α and IL-12 by the same cells [81]. In addition, piperine was proven to attenuate inflammatory processes by partially acting on pituitary adrenal axis [82], reduce high-fat diet-induced oxidative stress [83, 84] and enhance pancreatic activity [85]. The deficiency of zinc affects both innate and adaptive immunity [86]. This element is crucial for the balance between the different T-cell subsets and its deficiency was shown to decrease the production of Th1 cytokines (IFN-γ, IL-2 and TNF-α), whereas the Th2 response (IL-4, IL-6 and IL-10) is affected in a lesser extent [87]. While acute zinc deficiency seems to correlate with the decrease in innate and adaptive immunity, its chronic deficiency is known to increase pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) production influencing the outcome of several inflammatory diseases [88].

An optimal balance of the omega Omega 3/6 fatty acids ratio represents a fundamental requirement for tissue homeostasis recovering during inflammatory responses. The polyunsaturated fatty acids, usually found in fish oil (i.e., eicosapentaenoic acid and docosahexaenoic acids), are known to decrease proinflammatory cytokine production and to inhibit natural killer cell activity [89]. The gamma-linolenic acid has been demonstrated to exert an anti-inflammatory activity by suppressing IL-1β and TNF-α secretion by monocytes [90]. Additionally, eicosapentaenoic supplementation might foster the anti-inflammatory activity of gamma-linolenic acid by decreasing the synthesis of arachidonic acid and prostaglandin E2 [91].

Here, we evaluated the use of a commercially available nutraceutical diet as a therapeutical adjuvant in cKCS-affected dogs that were unresponsive to standard pharmacological therapies.

Methods

Experimental design, dogs and diets

This evaluation was designed as a randomized, placebo-controlled clinical one. Fifty client-owned dogs (19 females and 31 males) aged 6.5 ± 0.7 years [mean ± Standard Error of Mean] of different breeds (one poodle, two dachshund long hair, four dachshund smooth coat, four west highland white terrier, two yorkshire terrier, four maltese, one bulldog, two chinese crested dog, two chinese pug, eight shih tzu, four german shepherd, 10 mixed breed,

two chow chow, two cocker, two english setter) were enrolled in this evaluation. All dogs were previously evaluated by an Italian Animal Health Foundation certified panelist (Dr D. Giretto) to confirm the diagnosis of immune-mediated KCS. Inclusion criteria were the presence of blepharospasm, conjunctival inflammation, corneal keratinization, corneal pigmentation density, neovascularization, mucus discharge and a STT value < 10 mm/min. Exclusion criteria were the presence of correlated systemic diseases, neurological disease, traumatic and toxic keratoconjunctivitis, in order to better evaluate the clinical response to the immune-mediated cKCS, or general symptoms of intolerance/allergy to ingredients of the nutraceutical diet tested in this clinical evaluation. Moreover subjects affected by neurological cKCS were excluded.

Dogs were randomly and equally divided into two groups: 25 dogs fed a standard diet (SD group), as control group, and 25 fed an antioxidant/anti-inflammatory nutraceutical diet (ND group), as experimental group. Male and female dogs were equally represented in both groups. Regardless the type of diet, all dogs were treated over a 60 days period as follows: [0,03% Tacrolimus collyrium diluted into a benzalkonium chloride and methyl cellulose solution (Lacrimart, Fedel Farma S.r.l., Chieti, Italy) BID and 0,2% Hyalistil eye drops (artificial tears, S.I.F.I. S.p.A. Aci S. Antonio, Catania, Italy) five times a day] ([http://eng.forza10.com/immuno-active-755-2.html]).

The recommendations of the ARRIVE guidelines in animal research were consulted and considered [92].

In Table 1, we reported the background data of the dogs belonging to both groups along with their scores before starting the evaluation.

Both diets completely fulfil the recommendations for proteins, carbohydrates and fats in order to obtain a complete food for a daily ration in dog, as reported in Nutritional Guidelines for complete and complementary pet food for cats and dogs by The European Pet Food Industry Federation. Foods were in the form of kibbles industrially produced with extrusion technique. ND and SD foods reported similar analytical composition in nutrients (24% of crude protein, 12% of crude oils and fats, 3.7%, of crude fiber 5% of crude ash, 9% of moisture). Both diets had analogue recipes and included the same macro and micro nutrients including vitamins, trace elements and minerals. The two foods differed mainly from the presence of botanicals in ND food. ND food was composed by two mixed components: kibbles, included in the ideal percentage of 93-94% in weight, and cold-pressed tablets at the 6-7% in weight of complete food (European patent n. EP 2526781). Tablets were composed by 60-80% of protein hydrolyzed (fish and vegetable ones), 20-40% of minerals used as glidants and were added by therapeutical substances (*Ascophyllum nodosum, Cucumis melo, Carica papaya, Aloe vera, Astaxanthin from Haematococcus pluvialis, Curcuma longa, Camellia sinensis, Punica granatum, Piper nigrum, Poligonum spp, Echinacea purpurea, Grifola frondosa, Glycine max*, Omega 3 and Omega 6 unsaturated fatty acids from fish, as 1.60% and 1.25% of oil respectively).

The pet food used in SD group did not contain the above-mentioned active substances.

ND and SD dietary administration were administrated following a daily table recommendation (Table 2) and carefully adjusted during the trial to provide similar caloric animal food intake and to satisfy the nutritional requirement of adult dogs. In order to avoid any deficiency, the energy value of both complete food was calculated using the expression suggested by Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs and Nutrient requirements of dogs and cats, National research council of the National academies, (% crude protein x 3.5 + % crude fat x 8.5 + % NFE (Nitrogen-free extract) × 3.5). The correct dosage was calculated using another expression 110 kcal ME*kg $bw^{0.75}$ (Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs and Nutrient requirements of dogs and cats, National research council of the National accademies). The constant 110 is referred to the energy requested by a dog with normal physical activity. At the enrollment, each animal was weighed and the suggested daily ratio calculated. The Veterinarians clearly informed the owners about the correct dosage to be provided. Moreover the average of daily administered botanicals was calculated considering the ratio given to the dogs, related to the amount declared by the manufacturer. Table 3 highlights the average amount, in terms of mg/kg, of botanicals estimated according to the mean weight.

Ophthalmologic examination

Each dog was evaluated on day 0,15, 30, and 60 of the evaluation by an independent observer (SD, DG, CM).

Table 1 Background data of enrolled dogs

Group	Mean age (years ± SEM)	Mean weight (Kg ± SEM)	STT value (mm ± SEM)	Corneal pigment density score (0-3 ± SEM)	Conjunctival inflammation score (0-3 ± SEM)	Mucus discharge score (0-3 ± SEM)	Corneal keratinization score (0-2 ± SEM)
Control	6.03 ± 0.15	13.04 ± 1.12	4.3 ± 0.5	1.0 ± 0.1	2.1 ± 0.1	1.7 ± 0.1	1.5 ± 0.1
Treatment	6.1 ± 0.17	12.01 ± 1.17	4.7 ± 0.4	0.9 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	1.5 ± 0.1

Table 2 Daily table recommendation for diet

Weight (Kg)	Diet amount per day (g)
1 – 10	30 – 180
11 – 20	190 – 300
21 – 35	310 – 455
36 – 50	465 – 595

Each dog underwent a complete ophthalmological examination by three board-certified veterinary ophthalmologists (Dr. M.C. Muscolo and Dr. S. De Stefanis are board-certified by the D'Ophtalmologie ENV Alfor; Dr. D. Giretto is board-certified by Certificat d'Etudes Superieur en Ophtalmologie ENV Toulouse and is an Italian Animal Health Foundation board member).

Ophthalmic examinations included, slit-lamp biomicroscopy (Kowa Optimed Inc SL-14 Slit Lamp, Kowa Optimed, Europe Ltd, Berkshire, UK), funduscopic examination (Heine Omega 180 Binocular Indirect Ophthalmoscope, HEINE Optotechnik, Herrsching, Germany), applanation tonometry (Tono-Pen® Vet, Reichert Technologies, Depew, NY, USA) preceded by an ocular application of oxybuprocaine hydrochloride 0.4% (Novesina Novartis Farma S.p.A, Origgio (VA), Italy) in order to reduce the nuisanceand fluorescein dye staining (fluorescein 0.5% collyre unidose TVM, Laboratoires TVM, Lempdes, France) along with 0.9% physiologic rinsing solution (Eurospital S.p.A., Trieste, Italy).

Both eyes of each dog were photographed at each visit in the afternoon (3–6 pm) and clinical signs, such as corneal pigment density and corneal keratinization, were graded according to the scores proposed by Hendrix et al. [93], whereas conjunctival inflammation and mucus discharge were graded according to the scores proposed by Moore et al. [94].

- corneal pigment density (0-3): 0 = no pigment, 1 = iris easily visualized through the pigment, 2 = iris partially visualized through the pigment, 3 = iris not visible through the pigment);
- conjunctival inflammation (0-3): 0 = normal conjunctiva; 1 = mild hyperemia without chemosis; 2 = moderate hyperemia with mild chemosis; 3 = intense hyperemia with moderate to severe chemosis;
- mucus discharge (0 – 3): 0 = no visible mucus or clear mucus thread; 1 = scattered non-adherent mucopurulent strands; 2 = moderate adherent mucopurulent strands covering up to 25% of the cornea; and 3 = diffuse extensive adherent mucopurulent discharge covering 25% to 50% of the cornea;
- corneal keratinization (0-2): 0 = none, 1 = mild opacity, 2 = moderate opacity.

Enrolled dogs were treated by their owners at home by applying the pharmacological treatment as previously described and the diet administration approximately every 12 h.

Schirmer tear test

Schirmer tear test-1 (STT-1) is a routine examination which is performed by placing a standard test strip (Schirmer-Plus®, Gecis Ecoparc, Domaine de Villemorant, France) within the ventral conjunctival sac of each dog for 60 s. Tear production is then recorded in mm/min for each eye. STT-1 was performed on 100 eyes of dogs of several breeds.

Statistical analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the means ± standard error of the mean and were first checked for normality using the D'Agostino-Pearson normality test. Differences in Schirmer test, conjunctival inflammation, corneal keratinization, corneal pigmentation density and mucus discharge score between

Table 3 Average substances administer to dog depending on body weight (considering medium body weight)

Nutraceutical substances	Amount per kg of complete food		Dog weight 10 kg	11 kg	12 kg	13 kg
Ascophyllum nodosum	40000	mg/kg	7200	7600	8200	8600
Cucumis melo	300	mg/kg	54	57	61,5	64,5
Carica papaya	135	mg/kg	24,3	25,65	27,675	29,025
Aloe vera	135	mg/kg	24,3	25,65	27,675	29,025
Haematococcus pluvialis (astaxanthin)	49	mg/kg	8,82	9,31	10,045	10,535
Resveratrol (Poligonum Cuspidatum)	7	mg/kg	1,26	1,33	1,435	1,505
Zinc sulphate monohydrate	137	mg/kg	24,66	26,03	28,085	29,455
Curcuma longa	102	mg/kg	18,36	19,38	20,91	21,93
Camellia sinensis	70	mg/kg	12,6	13,3	14,35	15,05
Punica granatum	70	mg/kg	12,6	13,3	14,35	15,05
Piper nigrum	30	mg/kg	5,4	5,7	6,15	6,45

the two treatments at the end of treatment versus baseline for each eye were blindly analyzed by ADC using a two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Conjunctival inflammation, corneal keratinization, corneal pigmentation density and mucus discharge score between the two treatments at the end of treatment versus baseline for each eye were analyzed using a paired *t*-test. Veterinary ophthalmologists were not involved in the statistical analysis of the data.

Results and Discussion

Clinical evaluation of eyes in ND and SD group

Fifty dogs were enrolled in the trial: 25 dogs received the pharmacological treatment and a standard diet (SD Group), while 25 dogs received the pharmacological treatment plus an antioxidant/anti-inflammatory nutraceutical diet (ND Group).

An overall amount of 100 eyes was considered according to literature suggestions [95–97]. All dogs completed the 60-day evaluation period.

The overall improvement of eye's condition in two representative dogs of ND group at the day 0 of the trial (Fig. 1a, c) and at the end of the 60-days evaluation (Fig. 1b, d) is shown. In particular, our results highlight the clinical amelioration occurred in ND group (Fig. 1b, d) in terms of blepharospasm, ocular hyperemia, periocular swelling and ocular discharge that is strongly dependent on nutraceuticals administration since no effects were evident in SD group (Fig. 1e, h). In this regard, the comparative evaluation between the day 0 (Fig. 1e, g) and the end of 60-days (Fig. 1f, h) in two representative dogs of SD group showed none significant clinical amelioration. Indeed, blepharospasm, ocular hyperemia, periocular swelling and ocular discharge were still evident or, at least, poorly improved.

These results strongly pointed to a specific effect of nutraceuticals in inducing anti-inflammatory and immune-modulating outcomes in eyes of dogs belonging to ND group. Notably, the standard pharmacological treatment appeared to be substantially ineffective since no amelioration has been observed in dogs belonging to SD group. Therefore, the effect of nutraceuticals could be considered as highly fostering the clinical improvement during the pharmacological treatment in cKCS.

The eye's scores amelioration in cKCS dogs treated with ND

Figure 2 shows the eye's score intensity trend of each symptom of dogs belonging to SD and ND group.

Dogs conjunctival inflammation score significantly decreased from a baseline of 2.1 ± 0.1 to 0.6 ± 0.1 in the ND group, while no significant variation (from a score of 2.1 ± 0.1 to 1.9 ± 0.1) appeared in SD group (Fig. 2a–b).

In addition, corneal keratinization score resulted significantly decreased in ND group (from 1.5 ± 0.1 to 0.2 ± 0.1) and not in SD group (from 1.5 ± 0.1 to 1.4 ± 0.1)

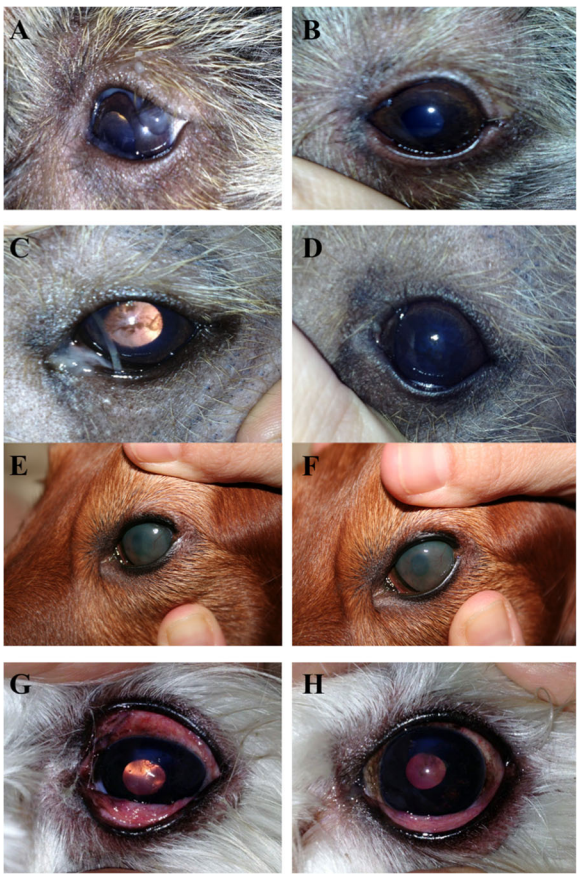

Fig. 1 Eyes improvement after 60-days treatment with 0,03% Tacrolimus collyrium BID and 0,2% Hyalistil eyewash plus the nutraceutical diet in ND group and with with 0,03% Tacrolimus collyrium BID and 0,2% Hyalistil eyewash plus the standard diet in SD group. **a**–**c** eye before treatment plus nutraceutical diet (time = 0), **b**–**d** eye at the end of treatment plus nutraceutical diet (time = 60). **e**–**g** eye before treatment plus standard diet (time = 0), **f**–**h** eye at the end of treatment plus standard diet (time = 60)

(Fig. 2c–d). Finally, corneal pigment density and mucus discharge resulted significantly decreased only in ND group, while no effects were evident in SD group. More in details, corneal pigment density scores decreased from a baseline value of 0.9 ± 0.1 to 0.2 ± 0.1 whereas mucus discharge scores decreased from 1.8 ± 0.1 to 0.3 ± 0.1 (Fig. 2e–h).

These results clearly suggest the role for ND in inducing the amelioration of eye's score testing in cKCS and that this occurrence appears independent on pharmacological treatment since drugs alone appeared ineffective, as evident in SD group.

As to STT-1 values, a significant increase was observed from a baseline value from 4.7 ± 0.4 mm to 10.7 ± 0.6 mm after the 60-days of treatment only in the dogs of ND group, while no significant improvement (STT-1 values from 4.3 ± 0.5 mm to 5.1 ± 0.5 mm) was evident in the dogs of SD Group at the end of the trial (Fig. 2i–l).

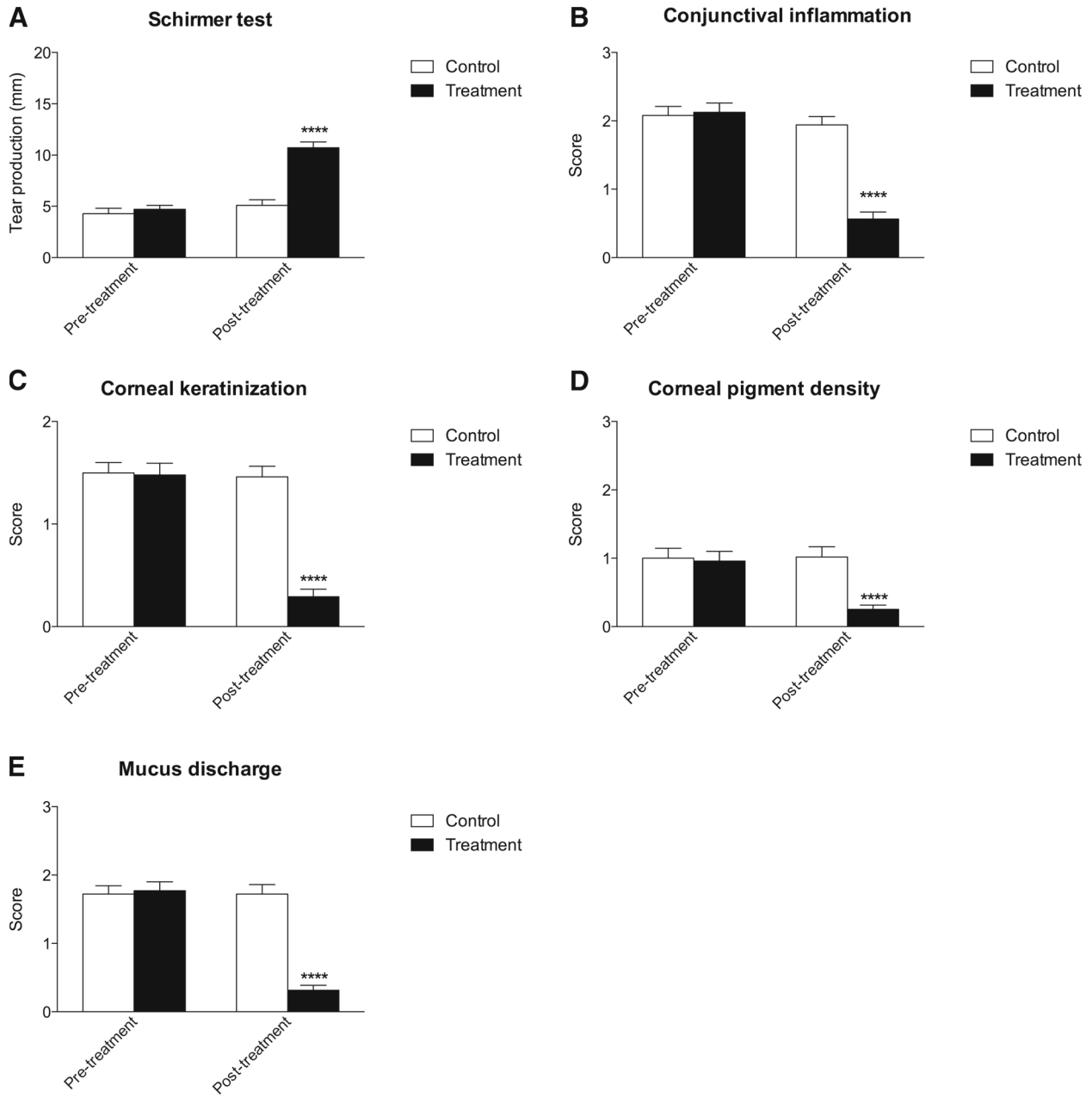

Fig. 2 a mean tear production (STT) in mm/min before and after 60 days treatment for ND and SD group, STT values resulted significantly increased (****$P < 0.0001$) in ND group at the end of treatment, **b** Mean conjunctival inflammation scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment; **c** mean corneal keratinization scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment; **d** mean corneal pigment density scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment; **e** mean mucus discharge scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment

These results evidenced the effectiveness of ND in increase the tear film in our cohort of sick dogs. It is reasonable that the anti-inflammatory effects of nutraceuticals could contribute to restore the physiological eye's tear production in cKCS.

The relapse/regression of cKCS symptoms in dependence of ND administration

After the 60 days of evaluation, dogs belonging to ND group interrupted the diet supplementation for 30 days, while continuing the pharmacological treatment.

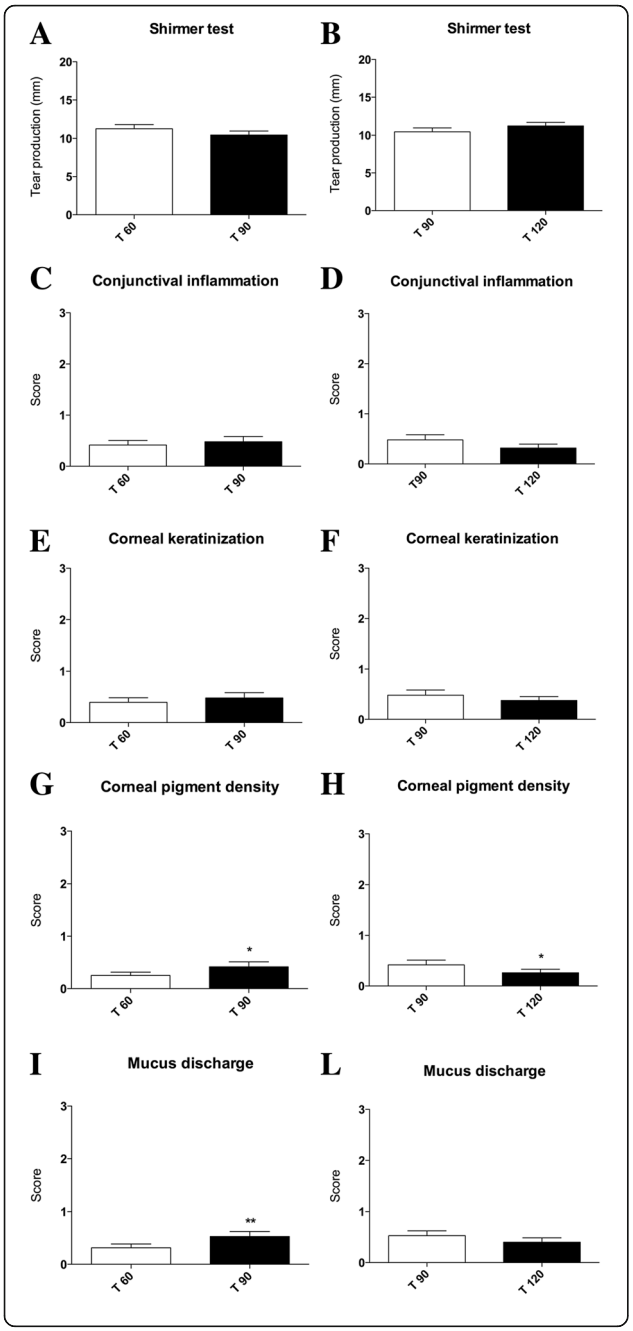

Fig. 3 Graphical schematization of clinical symptoms score trends after 30 days since treatment suspension and after 30 days since treatment resumption. **a** Mean conjunctival inflammation scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**b**) after 30 days since nutraceutical diet resumption (T 120); **c** mean corneal keratinization scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**d**) after 30 days since nutraceutical diet resumption (T 120); **e** mean corneal pigment density scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**f**) after 30 days since nutraceutical diet resumption (T 120), scores resulted significantly increased (*$P < 0.05$); **g** mean mucus discharge scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**h**) after 30 days since nutraceutical diet resumption (T 120), scores resulted significantly increased (**$P < 0.01$); **i** mean tear production (STT) in mm/min before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**l**) after 30 days since nutraceutical diet resumption (T 120)

It is worth noting that a rapid and intensive relapse of symptoms was observed after 15 days since ND suspension. All dogs were newly supplemented with the ND while continuing the pharmacological therapy for another 30 days. Intriguingly, an overall regression of symptoms was again observed after the reintroduction of ND (Fig. 3).

This occurrence clearly highlighted the specific effects of nutraceuticals as useful adjuvant in the treatment of cKCS-affected dogs, particularly for those animals poorly responsive or unresponsive to standard pharmacological therapy.

Conclusions

To the best of our knowledge, this clinical evaluation represents first study that proposed the use of a specific antioxidant/anti-inflammatory ND as an optimal combination of ingredients with synergistic effects able to potentially exert an immune-modulating activity in combination with standard pharmacological treatments in cKCS.

The nutraceutical approach appears to significantly increase the eye's tear production and to clinically ameliorate the conjunctival inflammation status as well as the corneal keratinization, corneal pigment density and mucus discharge in chronic cKCS dogs poorly responsive or unresponsive to immune-suppressive therapy.

The increased STT level in response to the proposed ND was in agreement with previously reported response to topical CsA and Tacrolimus [23, 98, 99]. Although we are unaware of the possible action mechanism of all ingredients, in particular for the phytotherapic extracts, we hypothesize that these substances and raw materials of the ND may exert a synergic action in the T-cell activation, possibly by preventing inflammatory gene transcription (IL-2, IL-3, IL-4, IFN- γ, TNF-α, GM-CSF, c-myc) [16, 100, 101].

Based on a possible mimicking action mechanism of all active substances with respect to CsA, we also hypothesized a reduced secretion of TNF-α by T cells. In this regard, TNF-α is known to increase mucin secretion from respiratory epithelial cells, thus it could possibly influencing the mucus production, corneal keratinization and conjunctival inflammation status [102, 103]. However, as observed by Hendrix et al. an overall significant improvement of clinical signs was not observed over time [93].

Intriguingly, our results seem to support the use of an anti-inflammatory/immune-modulating ND as an adjuvant to drug therapy in those cKCS dogs unresponsive to pharmacological treatment, in order to achieve analogue results of the responsive subjects (Moore et al., [94], Hendrix et al., [93]). Therefore, our investigation highlights the relevance of the possible administration of antioxidant/anti-inflammatory nutraceutical diet to cKCS dogs as useful adjuvant of immunosuppressive therapy.

The combination of a pharmacological treatment with a specific diet (Ocu-GLO Rx™) was also recently assessed by Williams et al. who successfully delayed the cataract formation in dogs with diabetes mellitus [104]. Specifically, the diet consisted in a mixture of a aldose reductase inhibitor, a glutathione regenerator alpha lipoic acid, grape seed extract, carotenoids, omega-3-fatty acids, and coenzyme Q10 which was provided to diabetic dogs as far as these developed lens opacification. Mean time without change in lens opacification was 278 ± 184 days with Ocu-GLO Rx™ and 77 ± 40 days in the placebo group.

In our treatement approach, the combination of several nutraceuticals, such as fish hydrolised proteins, rice carbohydrates, *Cucumis melo*, *Ascophyllum nodosum*, Astaxanthin, *Aloe vera*, *Carica papaya*, *Punica granatum*, *Camellia sinensis*, *Polygonum L.*, *Curcuma longa*, *Piper nigrum*, zinc and a omega3/6 polyunsaturated fatty acids (1:0.8 ratio), appears to exert beneficial immune-modulating effects on the clinical status of cKCS dogs. These data seams to confirm the action of nutraceutical diet on immune system modulation reducing Th1 and inproving TReg [35].

These plants and substances, widely used in traditional medicine, have been already shown to exert some intriguing antioxidant and anti-inflammatory activities in ocular tissues. In this regard, it is worth noting that *Camellia sinensis* extract was effective in conjunctival inflammation treatment [105] and *Curcuma longa* in several ocular diseases (chronic anterior uveitis, diabetic retinopathy, glaucoma, age-related macular degeneration and dry eye syndrome) [106, 107]. In addition, zinc was observed to reduce the progression of the age-related macular degeneration by the inhibition of the complement activation on retinal pigment epithelium cells [108] and omega 3 -6 fatty acids were closely correlated to development of vision and protection of eyes [109, 110].

The antioxidant/anti-inflammatory effects likely possessed by the mixture based on all these nutraceuticals in the diet supplementation seems to specifically reduce the immune-mediated ocular symptoms, particularly in those cKCS dogs that were poor responsive or unresponsive to classical immune-suppressive drugs.

In this regard, the pharmacological treatment alone was able to increase lachrymal production, while the increment was strongly higher and persistent when drugs were combined with the ND. Likewise, conjunctival inflammation was significantly reduced more in dogs belonging to ND group (receiving drugs in combination with nutraceutical supplemented diet) than in the SD group (receiving only the medical treatments). In addition, it is of relevance that corneal pigment density and mucus discharge were improved only in dogs belonging to the ND group. Finally, the occurrence of symptom relapsing, upon the suspension of nutraceutical diet, and of clinical amelioration, after its reintroduction, fosters the hypothesis of a possible therapeutical benefit of this nutraceutical diet in animal as well as in human ocular diseases [111, 112]

Taken in all, our results suggest that association of classical drug therapy with a nutraceutical diet with potential antioxidant/antiinflammatory and immune-modulating activities induce a significant amelioration of clinical signs and symptoms in keratoconjunctivitis sicca. Moreover, all symptoms appeared dependent on immune-mediated mechanisms. In this regard, the lachrymation impairment can be altered by an inflammatory condition of lachrymal gland and related ducts.

Therefore, it is reasonable to hypothesize that metabolic changes could affect immune response orchestration in a model of immune-mediated ocular disease, as represented by keratoconjunctivitis sicca, in dogs and, in a translational perspective, by Sjögren's syndrome in humans.

Study limitations

This research has some study limitations. For instance, neither the inflammatory cytokines present in the serum of dogs affected by KCS nor the percentage of regulatory T cells in the blood were evaluated. Ongoing experiments are characterizing the inflammatory cytokine release as well as the presence of Treg cells in peripheral blood. Moreover, preliminary results have evidenced that it is really hard to find in blood those alterations likely present in a well-defined peripheral tissue and body district, as represented by the eye.

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The contributions of the authors are as follows: SD, DG, MCM, SaC, SC, GG, GT participated in study design. SD, DG, MCM conducted the research. SD, DG, MCM, SaC, ADC, GT performed data interpretation. SD, DG, MGM. Sac, ADC and GT discussed the results and wrote the paper. SC and GG formulated the original idea. All authors read and approved the final manuscript.

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Competing interests
None of Authors has financial or personal relationships with other people or organizations and data interpretation was totally free from specific interests and the study has not been conditioned by any bias that could affect the results. This research was performed in collaboration with some scientists from the Division of Research and Development, Sanypet SpA, Padova, Italy (as indicated in the Author's affiliation) according to scientific and ethical principles of the scientific community. No financial funding was obtained from Sanypet Industry for this research study.

Consent for publication
Not applicable.

Ethics approval and and consent to participate
Operative procedures and animal care were performed in compliance with the national and international regulations (Italian regulation D.L. vo 116/1992 and European Union regulation 86/609/EC). The recommendations of CONSORT 2010 Statement in randomized controlled trials were also consulted and considered [113]. Written informed consent was obtained from the owners. A copy of the written consent is available for review by the Editor in Chief of this journal. Moreover, given that all procedures were part of routine care rather than an experimental intervention, an independent approval from an ethical committee was not necessary.

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