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A study of immune-tolerance control in the pathogenesis of Haematological disorders and Chronic-infection

Supervisor
Prof. Giuseppina Ruggiero

CANDIDATE
Dott.ssa Valentina Rubino

PhD COORDINATOR
Prof. Vittorio Enrico Avvedimento

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TABLE of CONTENTS

ABBREVIATIONS

AA: Aplastic Anemia
AIRE: Autoimmune Regulator
APC: Antigen Presenting Cells
BCR: B Cell Receptor
BM: Bone Marrow
CD: Cluster Differentiation
CL: Canine *Leishmaniosis*
CTL: Cytotoxic T Lymphocites
Foxp3: Forkhead box P3
GC: Granulopoietic Compartment
GPI: Glycosil-Phosphatidil-Inositol
HLA: Human Leukocyte Antigen
IFN-: Interferon-
IL-: Interleukin-
KIR: Killer-Cell Immunoglobulin-Like Receptor
LD: *Leishmania* infected Dogs
MAC: Membran Attack Complex
MDS: Myelodysplastic Syndromes
MHC: Major Histocompatibility Complex
NK: Natural Killer
NOD: Nucleotide Oligomerization Domain-Like Receptor
NOX: NADPH Oxidase
PB: Peripheral Blood
PBMCs: Peripheral Blood Mononuclear Cells
PMN: Polymorphonuclear Leukocytes
PNH: Paroxysmal Nocturnal Hemoglobinuria
PRR: Pattern Recognition Receptor
RAG: Recombinant Activator Gene
ROS: Reactive Oxygen Species
SOD: SuperOxide Dismutase
TCR: T Cell Receptor
TGF-: Transforming Growth Factor-
T_H: T Helper
TLRs: Toll Like Receptors
TNF-: Tumor Necrosis Factor-
Treg: Regulatory T cells
VL: Visceral *Leishmaniosis*

ABSTRACT

The main topic of this thesis was to evaluate the relevance of immune-tolerance dysregulation in the pathogenesis of some haematopoietic disorders and for the occurrence of immune pathological complications in chronic infection models.

In Paroxysmal Nocturnal Haemoglobinuria model, we described the occurrence of Treg reduction and NKTi increase in untreated PNH patients as well as the ability of the Complement blocking monoclonal antibody Eculizumab to selectively restore B and NKTi peripheral concentration without significant interference on NK and Treg numbers.

In MyeloDysplastic Syndromes model, the study focused on HLA-E and HLA-I molecules, key elements for NK and Cytotoxic T cell recognition. We consistently found that a subgroup of Low and Int-1 Risk MDS patients, categorised according with their altered immune profile, (low Treg level and high CD54 expression on CD8 T cell effectors in BM) showed significant decrease of HLA-I expression on peripheral PMN. This observation indicate the occurrence of CD8-dependent selection in BM. In addiction, the presence of polyclonal NK expansion bearing an activating receptor able to recognise HLA-E in BM has been observed to associate with the selection of dysplastic precursors lacking HLA-E expression.

In chronic infection model, as represented by natural infection by *Leishmania Infantum* in dogs we described a significant increase of CTL and T_H1 cells accompanied by reduction of Tregs. The possibility that such condition might be relevant for the occurrence of autoimmune platelet deficiency, frequently observed in Leishmania infected dogs has been hypothesised. In this model, pharmacological treatment associated with administration of a diet supplemented with nutraceuticals selected for their potential immune modulating properties and rich in essential fatty acids was observed to induce restoration of Treg and a progressive decrease of T_H1 CD4+ T cells. The possibility that such immune-modulating effects could affect the occurrence of immune-mediated platelet deficiency is currently under investigation.

In SOD1 model, we suggested that SOD-1 is part of the network of molecules involved in antigen-dependent T cell response. Indeed, we described that antigen-dependent activation of human T lymphocytes significantly increased extracellular SOD-1 levels in lymphocyte cultures. This effect was accompanied by the synthesis of SOD-1-specific mRNA and by the induction of microvesicle SOD-1 secretion. Moreover, confocal microscopy showed that antigen-dependent activation was able to modify SOD-1 intracellular localization in T cells, allowing association of the enzyme with the intracellular network of signalling molecules involved in TCR-dependent signal transduction processes. The possibility that such observation might indicate the involvement of peroxide/superoxide balance in fine tuning of TCR triggering, needs further investigation.

INTRODUCTION

The Immune System

The immune system is constituted by cells, tissues and organs and has the fundamental role to protect and defend the organism from the disease: this occurrence is usually defined as "immunity" and, in more complex point of view, such condition is not only related to the defence against infections, but also as ability to preserve the homeostasis and health in several non infectious disease, like tumours. Therefore, the immunity could be defined as the process leading to the preservation of the integrity of an individual from all those substances potentially dangerous for the organism, the antigens. The antigen may originate from the same organism (self) or from the external environment (non-self).

In order to consent the immunity status, the immune system exerts two principal functions in the different species: the humoral and the cellular responses. The humoral function is mediated by soluble substances (i.e. complement proteins, antibodies, natural compounds, etc), while the cellular response is based on the direct activity of immune cells (i.e. macrophage, poly-morpho-nucleated cells, B and T lymphocytes, Natural Killer cells, etc). Both responses have different characteristics and complexities between living species.

In this regard, the immune system can be classified into subsystems or phases: the innate and acquired (or adaptive) responses.

The innate immunity is present in vertebrates and in non-vertebrates and is a non-specific immune system that represents the first defence-line in the species and is based on cells and some mechanisms, mediated by soluble substances, that defend the plants and animals from infections. The cells of the innate response recognize and respond to pathogens by using several generic mechanisms, whose do not confer a long-lasting protective immunity to the host. The recognition of the infectious agents is mediated by several receptors encoded by genes that do not undergo any rearrangement to generate variants.

Adaptive immunity is present only in vertebrates and is a host defence related to several specific that specifically recognize the antigens.

These receptors are encoded by genes that undergo rearrangement to generate great diversity, and are expressed by the B and T lymphocytes. This rearrangement process is not driven by the infectious agent, but happens by genetic programming.

Previously thought to be crude and nonspecific, innate immunity is now know to involve a range of receptor families that recognize diverse microbial products. Importantly, these receptors program gene expression changes in target cells. The products of these genes drive inflammation and are also required for adaptive immunity. These protein include cytokines that promote activation of B and T cells. Other innate immune proteins have direct antimicrobial effects or act as opsonins, coating bacteria and promoting

phagocytosis. Innate immunity has therefore gone from being viewed as a process lacking sophistication, to a key element for both inflammation and adaptive immunity (Paul 2012).

The major principles of the immune response are:

- Elimination of many microbial agents through the non specific protective mechanisms of the innate immune system.
- Ability of the innate immune system to inform the cells of the adaptive counterpart about the appropriateness to make a response and the type of response to make.
- Capability of the cells of the adaptive immune system to display exquisitely specific recognition of foreign antigens and mobilize potent mechanisms for elimination of microbes bearing such antigens.
- Memory of its previous responses.
- Tolerance of self-antigens.

Innate Immune System

Powerful nonspecific defences prevent or limit infections by most potentially pathogenic microorganisms. The epithelium provides both a physical barrier to the entry of microbes and produces a variety of antimicrobial factors. Agents that penetrate the epithelium are met with macrophages and related cells possessing "microbial sensor" that recognize key molecules characteristic of many microbial agents. These "pattern recognition receptors" include several families of molecules, of which the most studied are the Toll-Like Receptors (TLRs) and the Nucleotide Oligomerization Domain-like receptors (NOD). Each TLR recognizes a distinct substance (or set of substances) associated with microbial agents; for example, TLR4 recognizes lipopolysaccharides, TLR3, double-stranded ribonucleic acid, and TLR9, unmethylated CpG-containing DNA. The interaction of a TLR with its ligand induces a series of intracellular signaling events, mainly represented by activation of the NF-kB system. Macrophage activation with enhancement of phagocytic activity and induction of antimicrobial system aid in the destruction of the pathogen. The induction of an inflammatory response as a result of the activation of the innate immune system recruits other cell type, including neutrophils, to the site (Paul 2012).
The main components of this response are:

- physical and chemical barriers, such as epithelia, mucosal membranes and antimicrobial substances they secrete.
- blood proteins such as the complement system, consisting of plasma proteins capable of promoting the killing of microbes and induce a inflammatory response .
- phagocytic cells such as neutrophils and macrophages.
- natural killer (NK) cells, a subpopulation that recognizes infected and/or damaged cells and kills them releasing perforin, a protein that creates a pore-channel in the target cells, and granzyme, which induce

apoptosis. NK cells also activate the microbial activity of macrophages by secreting interferon γ (IFN-γ).

Innate immunity, mainly due to the activity of NK lymphocytes, also contribute to maintenance of tissue integrity. Indeed, according to the "missing self" hypothesis, NK are able to recognize the genetically altered or damaged cells evaluating the expression of key molecules, related to the health status, such as the MHC Class I.

NK Lymphocytes

NK are a subpopulation of lymphocytes which represents 5-10% of circulating lymphocytes: are characterized by the ability to exercise cytotoxic functions against virus infected or transformed cells.

NK cells are larger than B and T lymphocytes and are characterized by intracytoplasmic azurophilic granules: for this reason are also called "large granular lymphocytes" (LGL). Phenotypically they constitutively express:

- CD16: low-affinity receptor for the Fc segment of IgG1 and IgG3 antibodies.
- CD56 and CD2: adhesion molecules.
- CD7: signal transduction molecule, also present in haematopoietic stem cells and thymocytes.

There is no antigen that characterizes only NK cells, such as the TCR for T lymphocytes or the BCR for the B lymphocytes, in fact, the $CD56^+$ antigen is also expressed by a number of cells like neurons, muscles and by a subset of T lymphocytes double positive for CD3 and CD56.

NK activity is governed by two main receptors families:

Activating receptors

- Ly49 (homodimers), relatively ancient, belonging to the C-type lectin family receptors; represent the receptor for classical (polymorphic) MHC I molecules.
- NCR (natural cytotoxicity receptors), upon stimulation, mediate NK killing and IFN-γ release.
- CD94: NKG2 (heterodimers), a C-type lectin family receptor, is conserved in both rodents and primates and identifies nonclassical (also nonpolymorphic) MHC I molecules such as HLA-E. Expression of HLA-E at the cell surface is dependent on the presence of nonamer peptide epitope derived from the signal sequence of classical MHC class I molecules, which is generated by the sequential action of signal peptide peptidase and the proteasome. Though indirect, this is a way to survey the levels of classical (polymorphic) HLA molecules.
- CD16 (FcγIIIA) playing a role in antibody-dependent cell-mediated cytotoxicity;

Inhibitory receptors

- Killer-cell immunoglobulin-like receptors (KIRs) belong to a multigene family of Ig-like extracellular domain receptors; they are present in nonhuman primates and are the main receptors for both

classical MHC I (HLA-A, HLA-B, HLA-C) and nonclassical HLA-G in primates. Some KIRs are specific for certain HLA subtypes. Most KIRs are inhibitory and dominant. Normal cells express MHC class I, so are recognised by KIR receptors and NK-dependent killing is inhibited.

- ILT or LIR (leukocyte inhibitory receptors); are recently discovered as members of the Ig receptor family.
- Ly49 (homodimers) have both activating and inhibitory isoforms. They are highly polymorphic on the population; Ly49s are receptor for classical (polymorphic) MHC I molecules.

NK effectors represent the guardians of tissue integrity. This condition is mainly associated with the expression, on the cell membrane, of high levels of MHC class I antigens, especially of non classical MHC molecules, as represented by HLA-E. MHC molecules, are mainly involved in antigen presentation to T lymphocytes and have been also associated with the control of transplant compatibility.

NK cells are able to recognize virus infected cells, that normally are opsonized with antibodies. The Fc portion of the antibodies (IgG1 and IgG3) that bind to antigens can be recognised by FcϒRIIIa(CD16) receptors expressed on NK cells, resulting in NK activation, release of cytolytic granules and consequent cell apoptosis. This mechanism, called Antibody Dependent Cell-mediated Citotoxicity (ADCC), is the major killing mechanism of some monoclonal antibodies, currently used for therapeutic purpose.

In addition to the cytotoxic activity, the NK cells are critical elements to establish a pro-inflammatory environment; indeed, they produce IFN-γ that activates macrophages and directs the response of T cells towards a T_H1 profile (Paul 2012).

Adaptive Immune System

The adaptive immune system, is mainly devoted to sterilize infectious environment. It also creates immunological memory after an initial response to a specific pathogen; memory response is usually rapid and efficient; it controls the pathogen before the establishment of a clinical disease. This process of acquired immunity is the basis of vaccination. Adaptive system includes both humoral immunity components (mediated by antibody produced by B lymphocytes) and cell-mediated immunity components (dependent on the T lymphocytes).

Acquired immunity is triggered in vertebrates when a pathogen evades the innate immune system and generates a threshold level of antigen. At this stage there is the recruitment, expansion and differentiation of high specific lymphocyte clones that modulate their effector activity sterilizing the infectious outbreak.

The major functions of the acquired immune system include:

- Recognition of specific "non-self" antigens.

- Generation of responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells.
- Development of immunological memory, in which pathogens are "remembered" through memory B and T cells.

Adaptive immunity is characterized by two functional categories, humoral and cell mediated response. B lymphocytes recognize *native* antigens; in this way are recruited, proliferate and differentiate into antibody-producing plasma cells.

T lymphocytes are not able to recognize *native* antigens; they recognize only peptides derived from antigen processing by Antigen Presenting Cells (APC) such as macrophages, B lymphocytes, dendritic cells, cells of Langerhans, and vascular endothelial cells. These peptides, exposed on APC in association with MHC molecules, are recognized specifically by the T Cells Receptor (TCR). To activate *naïve* T lymphocytes, are needed, in addition to the antigen recognition by TCR (I signal), also a series of signals sent by APC (II signal) only after binding of the pathogen with a TLR. Costimulatory molecules CD80/CD86 expressed by APC are recognized by CD28 molecule expressed by T lymphocytes. This interaction determines the activation of cytotoxic (CTL) or helper T lymphocytes (T_H).

MHC molecules are integral membrane proteins highly polymorphic, whose NH2-term extracellular region form a pocket in which is located the antigenic peptide. These molecules are divided into two classes: the MHC class I and MHC class II, which differ in structure and function and are recognized by different subpopulations of T cells. Indeed, the cytotoxic lymphocytes ($CD8^+$) recognize peptides expressed in association with MHC class I molecules; derived by processing of endogenous antigens, such as viral proteins, synthesized within the same cell or derived from bacteria and protozoa penetrated into the cell cytoplasm. Instead, the helper T cells ($CD4^+$) recognize, in association with MHC class II, exogenous antigens that are internalized by pinocytosis or endocytosis, processed and finally exposed as peptides on the APC. Different types of effector responses can be generated by $CD4^+$ helper T cells: T_H1, T_H2 and T_H17 are the most common. The T_H1 response is characterized by the production of Interferon-gamma (IFN-γ) which optimizes the bactericidal macrophages capability, induces the production of opsonizing (coating) and complement-fixing antibodies, favours the establishment of an optimal 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 immunity" (Paul 2012).

Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC) is a set of cell surface molecules encoded by a large gene family which controls recognition ability of adaptive compartment in all vertebrates. The major function of MHC is to bind peptide fragments derived from pathogens and display them on the cell

surface for recognition by the appropriate T-cell. In humans, the MHC is also called the Human Leukocyte Antigen (HLA).
The MHC gene family is present in the short arm of chromosome 6 and extends for 3500 kb thus occupying a large segment of DNA. The frequencies of recombination are around 4%. The MHC genes are highly polymorphic and have co-dominant expression: each individual expresses both alleles inherited from parents, allowing you to express the highest number of MHC polymorphisms available. Many different alleles exist in the different individuals inside a population.
The polymorphic regions in each allele are located in the region for peptide contact. Of all the peptides that could be displayed by MHC, only a subset will bind strongly enough to any given HLA allele, so by carrying two alleles for each gene, a much larger set of peptides can be presented.
On the other hand, inside a population, the presence of many different alleles ensures there will always be an individual with a specific MHC molecule able to load the correct peptide to recognize a specific microbe. The evolution of the MHC polymorphism ensures that a population will not succumb to a new pathogen or mutation, because at least some individuals will be able to develop an adequate immune response to win over the pathogen. The variations in the MHC molecules (responsible for the polymorphism) are the result of the inheritance of different MHC molecules and they are not induced by recombination, as it is the case for the antigen receptors.
MHC genes are divided into three subgroups: class I, class II, and class III. The latter encoding for molecules other than those which expose the peptide. The region of Class I contains the loci that encode for HLA Class I molecules classic (HLA-A, HLA-B, HLA-C) and non-classical (HLA-E, HLA-F, HLA-G, HLA-H, MIC-A), characterized by limited polymorphism. The HLA class II region is in turn divided into 3 loci HLA-DP, HLA-DQ, HLA-DR encoding the α and β chains of each molecule of Class II. This region contains genes encoding TAP (Transporter Associated with Antigen Processing), the ATP-dependent transporter of peptides, important for the correct biosynthesis of MHC class I molecules.
The two classes of molecules are expressed differently from the cells of the organism:

- MHC Class I on almost all nucleated cells
- MHC Class II on antigen-presenting cells, dendritic cells, macrophages and B cells; on thymic epithelial cells; on activated human T lymphocytes.

The MHC molecules expression is finely modulated during the immune response. Several cytokines, such as IFN-α, IFN-β and IFN-γ, induce the MHC I increase. The IFN-γ is able to increase the MHC Class II expression on macrophages and to induce of MHC class II on cell populations that normally do not express them. Cytokines increase expression of MHC molecules by gene transcription.

Antigen processing is an immunological process allowing antigen presentation to T lymphocytes. This process involves two distinct pathways for processing of endocellular and extracellular antigens. Both MHC class I and II are required to bind peptides to be stably expressed on a cell surface. MHC I antigen presentation typically involves the endogenous pathway of antigen processing, and MHC II antigen presentation involves the exogenous pathway of antigen processing.

Usually, old or defective endocellular proteins become ubiquitinated and marked for proteasome degradation and binding to Class I molecules; in virus infected cells, viral proteins gain such pathway allowing the immune system to recognize and kill the infected cells. Briefly, proteasome breaks the proteins into peptides around nine amino acids long (suitable for fitting within the peptide binding cleft of MHC class I molecules). Transporter associated with antigen processing (TAP) transports the peptides into the lumen of the rough endoplasmic reticulum (ER). Here, a series of chaperone proteins, including calnexin, calreticulin, ERp57 and Binding immunoglobulin protein (BiP) facilitates the proper folding of MHC class I and its association with β2 microglobulin. The partially folded MHC class I molecule then interacts with TAP via tapasin (the complete complex also contains calreticulin and Erp57). Once the peptide is transported into the ER lumen it binds to the cleft of the MHC Class I molecule, stabilizing the MHC and allowing it to be transported to the cell surface by the Golgi pathway.

The exogenous pathway is utilized by specialized antigen presenting cells (APC) to present peptides derived from proteins that the cell has endocytosed. The peptides are presented on MHC class II molecules. Proteins are endocytosed and degraded by acid-dependent proteases in endosomes.

The nascent MHC class II protein in the rough ER have peptide-binding cleft blocked by Ii (the invariant chain) to prevent it from binding cellular peptides or peptides from the endogenous pathway. The invariant chain also facilitates MHC class II export from the ER in a vesicle. This fuses with a late endosome containing the endocytosed, degraded proteins. The invariant chain is then broken down in stages, leaving only a small fragment called CLIP which still blocks the peptide binding cleft. An MHC class II-like structure, HLA-DM, removes CLIP and replaces it with a peptide from the endosome. The stable MHC class-II is then expressed on the cell surface (Paul 2012).

Immune Tolerance

The innate immune cells have a Pattern recognition receptors (PRRs), broadcast inherited with chromosomes, able to selectively recognize molecules expressed by pathogens (PAMPs). Adaptive cells express a clonal receptor (TCR and BCR), generated by processes of gene rearrangement, extremely variable, but substantially incapable of directly discriminate "self" from "non-self". Therefore, in order to maintain the "self tolerance", the immune system have a series of mechanisms to eliminate or inactivate self-

reactive clones. These processes occur both during lymphocyte development and in periphery (Alpdogan and van den Brink, 2012).

T cell development and (central tolerance)

Lymphoid precursors from the BM migrate to the thymus and then undergo differentiation under the control of multiple factors including Interleukin-7 (IL-7) and Notch-1. Thymocytes develop TCR β- and α- locus rearrangements and then start to express CD4 and CD8. This process is controlled by Recombinant Activator Gene (RAG) expression. After generation of a complete TCR all thymocytes, in order to avoid the occurrence of not functional (unable to bind MHC presented peptides) or dangerous self reactive clones, undergo positive and negative selection. Thus, the fate of each T cell precursor depends upon the ability of its newly rearranged TCR to properly interact with MHC molecules. Most MHC molecules display self-peptides and positive selection involves the recognition of the MHC-self peptide complex. More than 95% of thymocytes do not have any specificity to an MHC ligand and die by neglect. Only the small proportion of cells able to bind an MHC ligand with mild avidity will complete its maturation process.

Intra-thymic T cell tolerance occurs via deletion of auto-reactive T cell clones. If the TCR has high affinity to self-antigens (self MHC and peptide complex), this leads to the deletion of thymocytes by apoptosis. Thymocytes that express a low affinity TCR survive and continue to the next maturation step. In the last decade a new transcriptional regulator was discovered in the thymus, named Autoimmune Regulator (AIRE). AIRE is primarily expressed in medullary thymic epithelial cells. It promotes self-tolerance by inducing transcription of a wide array of tissue-specific antigens (TSAs) in the thymus and plays a critical role in negative selection. In the absence of functional AIRE, medullary TECs express a severely restricted array of self-antigens which results in severe autoimmune disease (Alpdogan and van den Brink, 2012).

Peripheral Immune Tolerance

Negative selection in the thymus effectively deletes thymocytes that have high affinity TCR to self-peptide-MHC complexes. Peripheral immune tolerance mechanisms are critical for controlling mature T cells with low/moderate affinity TCRs to self MHC/peptide complexes. Indeed, small amounts of T lymphocytes escape selection in the thymus; these can be eliminated in the periphery by deletion, induced anergy and/or suppression by other immunologically active cells (regulatory cells/suppressor cells) (Alpdogan and van den Brink, 2012).

Peripheral Deletion

Varying levels of antigenic stimulation of mature T cells in the periphery can result in T cell clonal deletion. Moreover, small amount of antigenic stimulation can induce T cell tolerance by partial down-regulation of T cell

receptors (TCR) on self-reactive $CD8^+$ cells (Alpdogan and van den Brink, 2012).

T cell Anergy and Costimulatory Signals

T cell activation requires two signals: i) TCR signal ii) costimulatory signal. T cells are not able to mount an immune response without a second costimulatory signal. CD28 is the main co-stimulatory receptor and has two ligands, B7.1 (CD80) and B7.2 (CD86), that are expressed on APCs (Figure 1A). CD28 signals are critical for T cell activation, proliferation and survival after T cell interaction with APCs. CD28 activation results in increased expression of cyclins and cyclin dependent kinases (cdk), downregulation of cdk inhibitor cdk27kip1 and upregulation of glucose metabolism through phosphoinositol 3-kinase (PI3K) and Akt activation. CD28 controls T cell survival by enhancing BCLXL expression in T cells, which prevents T cell death from apoptotic signals such as Fas activation or IL-2 withdrawal. Activation of T cells in the absence of CD28 results in an anergic state .

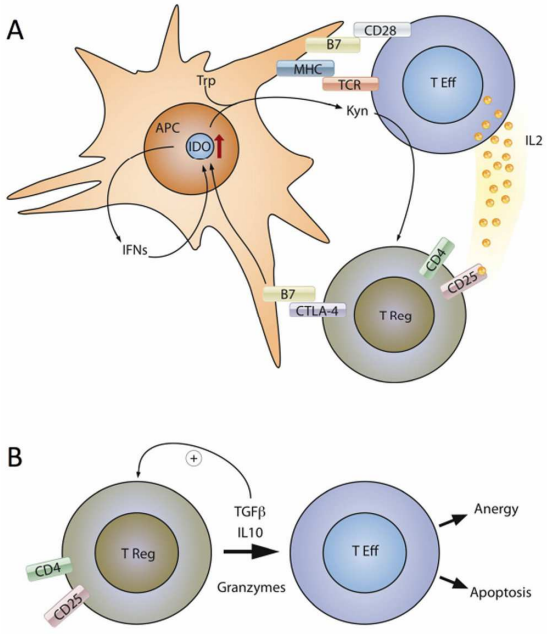

Figure 1. Main mechanisms of T regulatory cell function. Treg suppress effector T cell function through cell contact (Figure 1A) and inhibitory cytokine and granzyme secretion (Figure 1B). T Eff; effector T cells, IDO; indoleamine 2,3-dioxygenase, Trp; Tryptophan, Kyn; kynurenines, IFNs; interferons

Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and Inhibition of CD28 Costimulatory Signal

CTLA-4, a member of the immunoglobulin superfamily has a similar structure to CD28. Both molecules bind to CD80 and CD86 on antigen-presenting cells. CTLA-4 inhibits CD28 dependent T cell activation, cell cycle

progression and IL-2 production of T cells. Interestingly, CTLA-4 inhibition is more pronounced after initiation of T cell activation and anti-CTLA-4 antibody activity is optimized with continuous CD28 signaling, suggesting that upregulation of CTLA-4 is CD28 dependent. CTLA-4 has a significantly higher binding affinity for the B7 molecules than that of CD28 and acts as a competitive antagonist of CD28. This inhibitory function of CTLA-4 is mediated by its cytoplasmic tail (Alpdogan and van den Brink, 2012).

Programmed Death-1 (PD-1)/PD-1 Ligands

PD-1 (CD279) is a member of the CD28 family that is expressed on activated T, B, and myeloid cells. PD-1 ligands (PD-L1 and PD-L2) are expressed on T cells, B cells, antigen-presenting cells, endothelial cells, and tumor tissues. PD-L1 (CD274, B7-H1), a transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily plays an integral role in the regulation of immune tolerance and homeostasis. PD-1/PD-L1 interactions lead to inhibitory signals, and ligation of PD-1 occurs during autoimmunity, allergy, allograft rejection, antitumor immunity, and chronic virus infection. PD-1 is expressed on T cells after activation and delivers co-inhibitory signals via an immunoreceptor tyrosine-based switch motif in the cytoplasmic domain. The ability of PD-1 to block T cell activation requires receptor ligation, suggesting that co-localization of PD-1 with CD3 and/or CD28 may be necessary for inhibition of T cell activation. PD-1 signals interfere with CD28 mediated activation of phosphatidylinositol-3-kinase (PI3K) and subsequently inhibit interleukin-2 production resulting in an anergic state in T lymphocytes.

CD40/CD40L Pathway

CD40 and its ligand CD154 (CD40L) are members of the tumor necrosis factor (TNF)/TNF-receptor family. CD40 is constitutively expressed on APCs such as B cells, macrophages, dendritic cells, and thymic epithelium, but can also be found on endothelial cells and fibroblasts. CD154 (CD40L) is expressed on activated T cells and NK cells. Signaling through CD40/CD154 is critical for activation of B and dendritic cells. CD40 ligation leads to secretion of IL-1, TNF-α, and IL-12, and to endothelial cell secretion of monocyte chemotactic factors. Signaling downstream of the CD40 molecules is mediated by TRAFs and leads to the activation of NFκB, which results in the upregulation of MHC molecules and co-stimulatory molecules in B cells. CD154 is rapidly induced on $CD4^+$ and some $CD8^+$ T cells following T cell activation after stimulation with cognate antigen.

Suppression of immune responses: Regulatory Cells

The suppressive functions of T cells on the immune system has been known since the 1970s, but a clear definition of T regulatory cells was not achieved until the mid 90s. Regulatory T cells (Treg) play a major role in the development of tolerance by suppression of immune responses. Other

immunologically active cells can also suppress immune responses including NK-T cells, double negative ($CD4^-CD8^-$), $CD8^+CD28^-$ cells, and veto cells.

Regulatory T Cells

Regulatory T cells were first defined as $CD4^+CD25^+$ double positive cells with suppressive functions on immunological response in rodents. Approximately 5-10 % of peripheral $CD4^+$ cells express IL-2 receptors on their surface. The depletion of $CD4^+CD25^+$ cells results in development of autoimmune disease.

Normally, the thymus produces immunoregulatory $CD4^+CD25^+$ T cells that are anergic to TCR stimulation and are able to suppress proliferation of other T cells. They might have intermediate affinity to MHC/peptide resulting in an escape from negative selection in the thymus.

Naturally occurring Treg specifically express a transcription factor (Foxp3), which is the main inducer, regulator, and survival factor in Treg development and function. Only $CD4^+CD25^+$ single positive thymocytes exhibit expression of Foxp3 in the thymus. Naïve T cells in the periphery can also acquire Foxp3 expression and suppressive function in conditions such as chronic antigenic stimulation in the presence of transforming growth factor (TGF)-β1. TGF-β1 signaling is required for the maintenance of immune suppressive activity and expansion of regulatory T cells. Interestingly TGF-β1 deficient mice have a reduced number of peripheral Treg while preserving normal thymic Treg development. A defect in TGF-beta-mediated signaling in Treg is associated with a decrease in Foxp3 expression and suppressor activity. Other types of T regulatory cells can be induced in the periphery in the presence of interleukin-10 (IL-10), called Tr1 cells. Tr1 cells secrete IL-10 and TGF-β1 and their suppressive activities are independent of Foxp3 expression. Both naturally occurring Treg cells and Tr1 cells are hyporesponsive to stimulation of their TCR but can slowly proliferate in presence of IL-2. Recently, $CD8^+$ $Foxp3^+$ Treg have been shown to have a suppressive function in autoimmune disorders and after allergen immunotherapy as well as in GVHD.

Function of regulatory T cells

$CD4^+CD25^+$ T cells suppress the proliferation of $CD4^+$ as well as $CD8^+$ T cells, which requires direct cell contact with Treg. Survival and function of Treg is dependent on presence of IL-2; IL-2 deficient mice develop lymphoproliferation and severe autoimmune disease. Expression of IL-2R (CD25) on their surface and signaling through IL-2R is required for optimal T regulatory function. Treg are anergic after stimulation and therefore, IL-2 secretion from conventional T cells is critical for development of suppressive activity of Treg. The crosstalk between T cell effector and T regulatory cells is shown in Figure 1B. On the other hand $CD4^+CD25^+$ Treg can secrete IL-10 in vivo and autoimmunity can be suppressed by regulatory cells through IL-10 secretion. Treg interaction with CD80/CD86 through the CTLA-4 molecule may lead to suppression of effector T cells. Interaction of CTLA-4 expressing

Treg with APCs induces the activation of indoleamine 2,3-dioxygenase (IDO), which results in both a local deprivation of tryptophan and the production of inhibitory molecules known as kynurenines. CTLA-4 expressing $CD4^+CD25^+$ regulatory cells show a stronger suppressive activity than their CTLA-4 counterparts. Moreover, blocking CTLA-4 on Treg can abrogate the suppression of T effectors. CD28-deficient $CD25^+$ $CD4^+$ T cells can also suppress activation of normal T cells, indicating that CD28 is not required as a costimulatory molecule for activation of the regulatory T cells. Treg may also act as cytotoxic T cells that expresses granzyme A after activation and are able to kill activated $CD4^+$ and $CD8^+$ T cells by a perforin dependent mechanism (Alpdogan and van den Brink, 2012).

NKT cells

These are T cells that recognize highly hydrophobic antigens associated with CD1d, a non polymorphic molecule belonging to the conserved family of MHC class I-like CD1 genes. NKT cells secrete high levels of IL-4 and IFN-γ and their prompt cytokine secretion has been suggested as a key element in pursuing specific T cell cytokine profile (Alpdogan and van den Brink, 2012).
In mice and humans, NKT cells can express a restricted TCR repertoire with an invariant TCR alpha and beta chain, V apha 14 and V alpha 24, respectively. Recent reports suggest that NKT cells might promote immune tolerance through an interaction with Treg, by increasing expression of negative costimulatory molecules (like PD-1) on Treg, which is mostly dependent on enhanced IL-4 secretion by NKT cells. Their involvement in citotoxic mechanisms has been also suggested (Alpdogan and van den Brink, 2012).

Immune system is involved in tissue homeostasis control. In order to manage damage recognition and tolerance maintenance, multiple mechanisms have been observed to regulate immune activation processes. Perturbation of such complex network might be associated with autoimmune reactions and/or damage of tissue homeostasis maintenance.

Bone Marrow failure syndromes

Syndromes of Bone marrow failure are a heterogeneous group of disorders characterized by alterations of the haematopoietic stem cells. These alterations deeply compromise the physiological haematopoiesis.
These syndromes may have a primary ethiopatho-genesis that is dependent on genetic alterations intrinsic to haematopoietic stem cell and due to inherited germ-line mutations. However, majority of the bone marrow failure, are acquired. In this context, the occurrence of damages of the bone marrow stem cell, progressively alters its ability to generate a physiological haematopoiesis (Figure 2). These disorders are characterized by the inappropriate production of peripheral blood cells. The stem cell defect could affect single

haematopoietic cell lineage or involve more lineages generating a pan-cytopenia.
Several experimental evidences indicates that immune-mediated processes may be relevant in fostering the bone marrow failure. In particular, the abil**i**ty to escape immune-mediated attacks could be a determining factor in the selection of pathologic haematopoietic precursors, as frequently observed in bone marrow failure conditions. (Maciejewski et al. 2007; Luzzatto et al. 1997; Barrett 2004); syndromes of the bone marrow failure most representative are: aplastic anemia (AA) due to widespread bone marrow failure; Paroxysmal Nocturnal Hemoglobinuria (PNH) and Myelodysplastic Syndromes (MDS) characterized instead by the emergence and dominance of mutated or dysplastic haematopoietic clones.

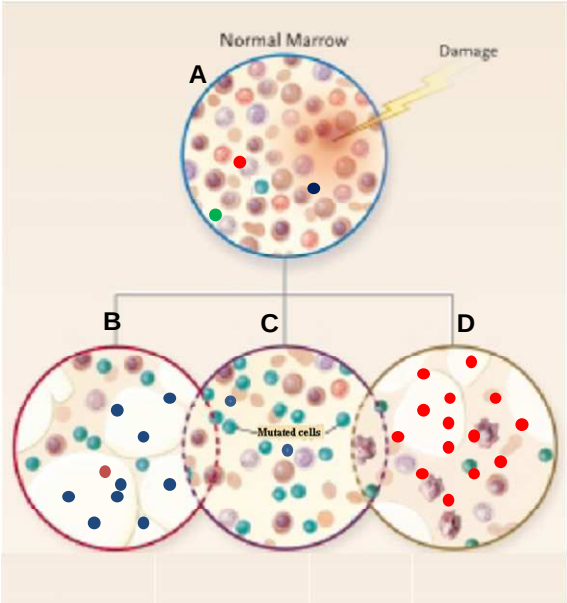

Figure 2. Healthy BM (A) hosts a number of precursors mutated (indicated by circular elements green, red, blue) that normally are not used in the processes physiological polyclonal haematopoietic. The immune-mediated damage at precursors healthy brings out the pathological haematopoiesis (mutated) able to resist the attack immune . In the three cases mentioned (B-D) emerge precursors characterized by different genetic defects (red, green or blue) , based on the ability of specific resistance to auto-immune damage occurred .

Paroxysmal Nocturnal Hemoglobinuria (PNH)

Paroxysmal Nocturnal Haemoglobinuria (PNH) is a complex acquired haematological disorder characterised by the emergence of a haematopoietic precursor bearing somatic mutations of the phosphatidyl-inositol glycan-A (PIG-A) gene (Rotoli and Luzzatto 1989; Luzzatto and Bessler 1996; Bessler et al. 1994; Takeda et al. 1993; Kinoshita et al. 1995).
This gene codifies for the glycosil-phosphatidil-inositol (GPI) structure and the cells carrying these PIG-A mutations lack the expression of proteins needing this molecule for their membrane anchoring (Miyata et al. 1993; Rosse and

Ware 1995). Defective clonal haematopoiesis develops trough several lineages, along with a residual normal haematopoiesis thus accounting for the mixed (GPI^+ and GPI^-) phenotype commonly present in the peripheral blood of PNH patients (Rotoli and Luzzatto 1989; Bessler et al. 1994; Rosse 1993; Hillmen et al. 1995).

PNH is characterized by clinical manifestations such as severe haemolytic anemia, thrombophilia and cytopenia, with increased susceptibility to infections (Rotoli and Luzzatto 1989; Rosse and Ware 1995; Rosse 1992 ; Ortolan et al. 2006). The disease is associated with serious complications usually represented by thrombosis involving hepatic, abdominal and brain vessels. Mortality is highly frequent (Luzzatto and Bessler 1996; Luzzatto 2006).

The lack of the GPI-linked molecules CD55 (DAF) and CD59 (MIRL), involved in the protection of red cells from the lytic attack of activated complement fractions (Wilcox et al. 1991), causes a chronic haemolysis with capricious exacerbations. Platelet surface activation by small amounts of complement and urokinase plasminogen activator receptor (uPAR) deficiency (Gralnick et al. 1995; Hall et al. 2002) might explain thrombophylia. However, the origin of the underlying bone marrow failure is still unknown (Rotoli and Luzzatto 1989; Luzzatto et al. 1997; Karadimitris et al. 2000; Risitano et al. 2002).

GPI-defective clones have been identified in healthy individuals (Rosti et al. 1997; Araten 1999; Karadimitris et al. 2000) and Pig-a KO chimeric mice are unable to mimic a PNH syndrome (Risitano et al. 2002; Greenberg et al. 2002). These observations strongly suggest that an isolated PIG-A mutation is insufficient to account for the clonal dominance of the GPI-defective clone and for the development of PNH. The possibility that some extrinsic selective pressure could favour the preferential expansion of the GPI-defective clone has been hypothesised (Luzzatto et al. 1997; Karadimitris et al. 2000; Guidetti et al. 2004). In this context, the absence of GPI-linked proteins might provide the GPI-defective clone with a survival advantage in a background that impairs normal haematopoieis. A relationship between PNH and Aplastic Anaemia (AA), whose autoimmune origin is generally accepted, has been reported, (Rotoli 1998; Luzzatto 2000) while other and our data suggest the critical involvement of immune–mediated mechanisms for the clonal dominance of the GPI-defective compartment (Terrazzano et al. 2005),

PNH onset may be characterized by fatigue, pallor and occasional emission of dark urine in the morning, while thrombosis, infections and bone marrow failure are the clinical features of advanced disease.

Therapy is based on blood transfusion to correct anemia. Anticoagulant drugs are frequently used to control the thrombophilia. Highly hypoplastic disease is treated with immuno-suppressive approach and bone marrow transplantation. Clinical management of PNH has been completely revolutionized by the

introduction of drugs to suppress the activity of the complement (Hillmen 2006).
The complement system is an essential element in the defense against infectious agents. It is constituted by several circulating proteins and cell membrane ligands, each other interacting and leading to the microrganism lysis and to the optimization of the inflammatory processes. The bactericidal function of complement is expressed by the formation of a peculiar "pore-channel", namely *membrane attack complex* or MAC, at the surface of cell membrane of the target: it generates the colloid-osmotic collapsing of the target cell , and, therefore, the cell destruction (Ricklin D et al. 2010).

Complement activation is a *cascade* sequentially activating the various complementary components that circulate in an inactive form. There are three distinct pathways of complement activation: the *classical* pathway, which is stimulated by the binding of antibodies to the pathogen surface;, the *lectin* pathway and the *alternative* pathway. Pathways converge in the formation of an enzyme, the *C3 convertase*, which cleaves the complement component C3 into C3b and C3a. Several proteins on normal host cells inhibit complement activation and this event minimizes the harmful effect that the complement could have against self (cells, tissues and organs). The CD55 and CD59 proteins are involved in regulating complement activation. They are linked to cell membrane *via* a GPI anchor. Therefore, CD55 and CD59 are defective in the cells derived from haematopoietic stem cell, carrying a mutation inactivating the PIG-A gene.
The two pathways, *classical* and *alternative*, convey to the formation of two different complexes, C4b-C2b and C3b-Bb, but with an identical function: to cleave C3 into two fragments, C3a and C3b, and to bind to C3b constituting the *C5 convertase*. *C5 convertase* cleave the C5 into two fragments, C5a which remains in solution by carrying out important functions pro-inflammatory and C5b that binds to the cell membrane. The remaining components of the complement cascade (C6, C7, C8, C9) devoid of enzymatics activity, bind to the cell membrane set in the lipid layer of the cell wall. The last component of the cascade, the C9, has the ability to polymerize at the point where it is inserted by forming pores in the cell membrane of about 100A°. Through the pores produced by the polymerization of C9, the water and the ions have free access to the inside of the cell by determining first the colloid-osmotic collapsing and then lysis. The molecule GPI-linked CD59 or MIRL is able to regulate the formation of MAC.

Traditionally, the complement system has been considered as a first line of defense against microorganisms, able to contain rapidly growth, in order to give time adaptive immunity to act appropriately, sterilizing the infectious outbreak. The current acquisitions highlighted that the complement is able to orchestrate the immune response as a whole, extending its activities far beyond the simple elimination of the pathogen. Indeed, the complement

system participates to several process as, the antigen-antibody complexes elimination , angiogenic processes regulation , the haematopoietic precursors mobilization, the lipid metabolism modulation as well as the modulation of the adaptive response both humoral and cell-mediated.
A new therapeutic approach for patients with PNH involves the use of the humanized monoclonal antibody Eculizumab, produced by recombinant DNA technology. Eculizumab is an anti-C5 and therefore blocks the cleavage of C5 into C5a and C5b, preventing the formation of the terminal complex C5-9 on GPI^- cells devoid of CD55 and CD59, insensitive to complement-dependent lysis.
Considering its large size is neither eliminated by renal filtration or urine, but it breaks down into small peptides and amino acids by lysosomal enzymes.
In the evaluation of the effectiveness, several clinical studies have shown that this therapy reduces or eliminates the need for transfusion and determines a significant improvement in quality of life in PNH patients (Risitano et al. 2010).
Given the extremely broad role that the complement plays in the regulation of physiological status, a study of the effects of a therapeutic block of complement is of great importance.
Complement functions are not limited to amplification of inflammatory process, it also acts in the apoptotic cells removal, in inhibiting fibrinolysis, in regulating B and T lymphocytes interactions and differentiation (Ricklin et al. 2010).

In this context, we examined the effect of Eculizumab treatment on innate and adaptive immune effectors in a cohort of 34 PNH patients also investigating whether complement inhibition might interfere with the selection/expansion of the GPI-defective haematopoiesis. (Alfinito et al. 2012)

Myelodysplastic syndromes (MDS)

Myelodysplastic Syndromes (MDS) are clonal haematopoietic disorders characterised by ineffective haematopoiesis accompanied by expansion/dominance of dysplastic haematopoietic clones and frequent development of Acute Myeloid Leukaemia (Heaney and Golde 1999). They are a heterogeneous group of diseases mainly characterised by an active but ineffective, often clonal haematopoiesis. Peripheral blood cytopenias combined with dysplastic changes in a hyper-cellular bone marrow represent the hallmarks of MDS (Corey et al. 2007). In this regard, there is a very significant clinical variability in MDS.

The presence of normal polyclonal precursors able to maturate *in vitro* is accompanied by the emergence of the dysplastic population. Aetiology of MDS is still unclear. Genetic changes have been suggested to cause stem cell alteration with perturbation of the multistep processes involved in the control of cell proliferation, differentiation and apoptosis induction.

Moreover the growth advantage of the dysplastic, often clonal haematopoiesis, could be hypothesised to be also dependent on damaging mechanisms preferentially affecting the normal counterpart in the bone marrow microenvironment.

Stem cell defects, cellular and cytokine-mediated stromal lesions and immune-mediated mechanisms have been suggested to be involved in the pathogenesis of MDS and in their clinical evolution. Multiple parameters like chromosomal changes, bone marrow blast cells number and the presence of multiple cytopenias appear to be useful in predicting the survival and transformation rate in MDS patients.

The possible relevance of immune-dependent mechanisms for the selection/shaping of the dysplastic stem precursor in MDS needs to be addressed.

A number of data have been suggesting that immune-dependent mechanisms are relevant for the selection, expansion and dominance of dysplastic clone/s, at least in a subgroup of MDS patients (Stern et al. 2007; Sloand et al. 2008; Ruggiero et al. 2009). Aberrantly activated, likely auto-reactive, oligoclonal T cell populations have been suggested to suppress polyclonal haematopoiesis by direct damage of stem progenitors as well as by releasing cytokines (IFN-γ, TNF-α) and other factors inducing extensive apoptotic response in stem cells and progenitors. Thus, it is possible to hypothesise that, in such group of patients, a defect in tolerance control could be relevant in the selection of pathological clones and in fostering their leukaemia progression.

Several trials of immune-suppressive therapy have been performed in the last ten years, based on the evidence concerning the involvement of immune-mediated events in MDS pathogenesis. Majority of them employed anti-T lymphocyte sera (ATG) with or without Cyclosporine A. Response rate varied widely (0-66%), rarely exceeding 30%. Younger age, Low Risk group of IPSS classification and presence of the HLA-DR15 allele were proposed as useful criteria to predict clinical response to immune-suppression in MDS (Sloand et al. 2008). Identification of the mechanisms underlying the immune-mediated MDS pathogenesis is expected to be of critical relevance to propose new biological targets for innovative immune modulating approaches in MDS.

Characteristics, Classification criteria and Therapeutic approach in MDS

In order to classify MDS patients the French American British Group (FAB) has suggested criteria that takes account of the different morphological features found in the periphery and the BM. This group proposed the division into five subgroups of the disease: refractory anemia with ring sideroblasts (RARS); refractory anemia with excess blasts (RAEB; 5-20% marrow blasts); refractory anemia with excess blasts in transformation/RAEB-t; 21-30% marrow blasts); chronic myelomonocytic leukemia (CMML).

The grading of MDS is defined by the WHO classification 2008 that includes five groups nosographical also characterized by a different prognosis: refractory cytopenia with unilineage dysplasia (RCUD); refractory anemia

with ring sideroblasts (RARS); refractory cytopenia with multilineage dysplasia (RCMD) with or without ring sideroblasts; refractory anemia with excess blasts (RAEB 1-2; 5-20% marrow blasts); myelodyplastic syndromes-unclassified (MDS-U); MDS associated with isolated del(5q).
A proper assessment of the probability of leukemic evolution can be obtained by calculating the IPSS which is a "prognostic score" based on three variables (number of marrow blasts, cytogenetic abnormalities, number of cytopenias); this assessment allows splitting the MDS in four risk categories (low, int-1, int-2, high) (Malcovati et al. 2007; Garcia-Manero 2012). Recently a more detailed classification of recurrent cytogenetic abnormalities in MDS has led to the redefinition of "prognostic score" (RIPSS); currently it recognizes five categories of risk (very low, low, intermediate, high, very high) in which are also better defined the "cut off" for the evaluation of cytopenias.

The therapy is based on the index of risk and considers two groups of patients: those with low risk (low and Int1) and those at high risk (Int2 and high). In the first group the therapeutic aim is the correction of cytopenias: the increase of the cell number is achieved through the use of erythropoietin and when you lose the effect of the cytokine by regular transfusion therapy; currently the use of growth factors for granulocytes and platelets is not yet allowed, thus it is recommended a careful prophylaxis against infectious for neutropenia and appropriate supportive treatment for thrombocytopenia. Patients with 5q-syndrome represent a separate category because they show good response to lenalidomide, an immune-modulating agent. Immuno-suppressive therapy with anti-lymphocyte serum and/or cyclosporine is reserved for those cases where it is most obvious he occurrence of a autoimmune noxa (young patients with MDS hypoplastic, HLA-DR15 positive). In patients in the high risk group the therapeutic goal is the elimination or containment of the dysplastic clone; therapies are: the allogeneic bone marrow transplantation (only in patients under the age of sixty years, with family donor), methylating agents and cell growth inhibitors (Fenaux et al. 2009).

Pathogenic mechanisms in PNH and MDS: the immune-mediated hypothesis

Different data suggest that common pathogenic mechanisms can underlie hematopoietic disorders characterized by emergence and dominance of defective or dysplastic clones, as PNH and MDS.
In this regard, the pathogenic role of an immune-mediated attack directed against autologous hematopoietic stem cells and/or bone marrow mesenchymal elements has been suggested. This attack, resulting in destruction of physiological polyclonal haematopoiesis, could exercise a significant selective pressure for the emergence of resistant clones, also fostering their leukaemia progression. The research team I joined in order to develop my PhD project has been investigating such hypothesis.

Main results obtained by our research Team: the Hematopoiesis disorder model

Regarding the PNH model, data obtained by our group (Terrazzano et al. 2005) have shown that clonal expansion of GPI-defective precursors is accompanied by an altered GPI+ T lymphocyte counterpart. Indeed, the GPI+ T lymphocytes show clear signs of hyperactivity with significant functional persistence of CD154 after TCR triggering. Such an attitude, already observed in autoimmune diseases, as Systemic Lupus Erythematosus (Koshy et al. 1996) suggests that dysregulation of immune-mediated mechanisms, able to control the self-reactive response, is relevant for the expansion and dominance of the GPI- defective clones in PNH (Terrazzano et al. 2005).

These observations were further confirmed by the study of HLA gene expression, widely considered as an important factor for susceptibility to auto-immune diseases (Spurkland and Sollid 2006). The hypothesis is that certain HLA alleles are able to preferentially bind peptides derived from autologous proteins, allowing their T cell recognition. In this context, our studies (Lombardi et al. 2008) have shown, in PNH patients, a significant increase in the frequency of alleles of class I A*0201 ($p<0.05$), B*1402 ($p<0.001$) and Cw*0802 ($p<0.005$), the alleles of class II DRB1*1501 ($p<0.01$) and DRB1*01 ($p<0.05$) and an increase of more than four times of the frequency of the Class I haplotype B*1402, Cw*0802 ($p<0.0005$), and fifteen times of the ancestral Mediterranean haplotype A*33, B*1402, Cw*0802, DRB1*0102, DQB1*0501 ($p<0.005$). These data suggest the presence of multiple associations that engage both T helper cells (association with class II alleles DRB1*1501 and DRB1*01), as well as cytotoxic T cell presentation (association with alleles of class I A*33, B*1402, Cw*0802).

NK cells are critical components of innate immune response. The possibility that NK cells might directly participate in PNH pathogenesis has been proposed, but it is still controversial. Indeed, it was suggested that the inability by GPI^- cells to express (GPI-linked) stress-related molecules might protect the PNH progenitors from NK effectors recognition, raising the question of the mechanisms underlying the interdependence of T and NK-mediated selection of GPI-defective clone(s) (Luzzatto et al. 1997; Karadimitris et al. 2000; Gargiulo et al. 2007; Terrazzano et al. 2005; Poggi et al. 2005).

NK activity is dependent on dynamic balance between activating and inhibiting signals. A key component of such regulatory network is represented by KIR. KIR genes are highly polymorphic, with individual genes exhibiting allelic variability and individual haplotypes differing in gene content. Combination of KIR and HLA genes has been associated with autoimmunity, viral infections, reproductive failure and cancer.

A number of data indicate that NK cells can drive, shape and regulate adaptive immune compartment. In a study (Cosentini et al. 2012) done by our group has been investigated the frequency of KIR genes and of their known human leukocyte antigen (HLA) ligands in 53 PNH Italian patients (almost

the half of all the Italian PNH patient population). The results showed an increased frequency of genotypes characterized by ≤2 activating KIR and by the presence of an inhibitory/activating gene ratio ≥3.5. In addition, a better match between KIR-3DL1 and its ligand HLA-Bw4 has been found. These genotypes may be associated with a lower NK-dependent recognition of self molecules related to stress. This is conceivable with the hypothesis that increased availability of specific molecular targets, not eliminated by NK cells, for T cell effectors could be involved in the pathogenesis of PNH.

In MDS model, the study also focused on the hypothesis that immune-mediated mechanisms may be pathogenetically relevant to the expansion and dominance of dysplastic clones being MDS. For this purpose, were recruited 50 MDS patients, classified according to WHO and IPSS criteria in Low Risk (N = 27, Int-1 Risk (N = 16, Int-2-High Risk (N = 7), and 12 healthy donors. The study allowed us to:
A. To identify a single clinical case, in which dysregulation of the NK response was related to the selection and progression of a myeloid dysplastic clone characterized by a severe deficiency in the expression of the molecule HLA-E, a critical element in regulating the cytotoxic NK response (Ruggiero et al. 2009) In this patient, NK effectors, which expressed high levels of the receptor CD94/NKG2C (an activating receptor recognizing HLA-E) were able to lyse PMNs, but not autologous monocytes (both defective for HLA-E) due to highe expression of HLA Class I molecules on dysplastic monocytes.

B. To identify subgroups of MDS patients, in stage Low Risk and Int-1 Risk, in which a peculiar immune profile was correlated with the intensity of bone marrow impairment of polyclonal physiological erythroid compartment (Alfinito et al. 2010). Indeed, the analysis of the patient cohort allowed the identification of a subgroup of MDS patients characterized by low Treg levels and high CD54 expression on the $CD8^+$ T population in Bone Marrw (BM). In these patients the pathogeneic relevance of immune-mediated mechanisms might be inferred. In the later stages of the disease, the leukemic progression was observed to result in an increase of Treg cells in BM, likely able to inhibit anti-tumor cytotoxic response. In this context, the measurement of the levels of Treg in BM could represent, in groups Low Risk and Int-1 Risk patients, a useful addition to the pathogenetic classification of the patients. Moreover, the possibility that the criteria described by us can contribute to the selection of sub-groups of patients likely susceptible to immune-modulating therapies represents an intriguing experimental hypothesis.

Infectious Disease Model: *Leishmania infantum* natural infection and immune profile of infected host

Canine Leishmaniosis (CL) is a zoonotic disease potentially fatal to humans and very common in dogs that are the main reservoir of infection for humans. CL is caused by the protozoan parasite *Leishmania infantum* in the

Mediterranean area (Baneth et al. 2008) and is usually transmitted by blood-feeding phlebotomine. Several immune responses and clinical manifestations have been described in CL (Ciaramella et al. 1997). In this regard, clinical appearance and evolution of leishmaniosis appear to be the consequence of complex interactions between the parasite and both the genetic and immunological backgrounds of host animals (Baneth et al. 2008; Maia and Campino, 2011).

Several studies pointed out that lymphocyte level in sick dogs is decreased but returns to normal values after treatment (Moreno et al., 1999; Guarga et al.,2002; Guerra et al., 2009); an increased number of $CD4^+$T cells in dogs with low parasitism (Reis et al., 2006) has been also referred. Furthermore, the number of $CD4^+$ T cells in peripheral blood was observed to be similar in dogs with leishmaniasis and in healthy dogs while there was no correlation between the clinical status or response to therapy and $CD4^+$ T cell counts (Miranda et al. 2007). The results obtained highlight the complexity of the immune response to L. *infantum* infection.

Control of the immune response in human and murine leishmaniasis has been observed to largely depend on the production of interleukin-10 (IL-10), which can come from several different cell types (Kaye and Scott, 2011). In human visceral leishmaniasis (VL) reports on the frequency and function of Tregs are not conclusive. Recently, Maurya et al. (2010) reported that active CL is not associated with increased frequencies of peripheral Foxp3 Treg or accumulation at the site of infection. In veterinary medicine, Tregs have been observed to significantly increase in dog tumour models (Biller et al., 2007; Houriuchi et al., 2009; O'Neill et al., 2009; Rissetto et al., 2010). Few studies addressed the level of regulatory cytokines interleukin-10 (IL-10) and the transforming growth factor beta (TGF-β) (Strauss-Ayali et al., 2007; Boggiatto et al., 2010) in CL. The study of Treg involvement in the regulation of dog immune-response against Leishmania needs further investigation. Indeed, IL-10 producing regulatory T cell subsets have been described to be relevant in the early phase of visceral leishmaniasis in mouse model of *L. infantum* infection (Rodrigues et al., 2009), while a reduction in lymphnode Treg was described to significantly correlate with immune protection consequent to Leishmune vaccination (de Lima et al., 2010).

Different treatment protocols and prognoses have been suggested for the clinical stages of CL (Solano-Gallego et al., 2009). The combination of N-methylglucamine antimoniate (75–100 mg/kg/SID or 40-75 mg/kg/BID) for 4–8 weeks, S.C. with allopurinol (10 mg/kg/BID) is considered as the most effective therapy and constitutes the first line protocol against the disease. Therapy with allopurinol alone should be continued for 6 months (Mirò et al., 2009) or for one year, as currently recommended (Solano-Gallego et al., 2009; Torres et al., 2011; Paradies et al., 2012). The association of Miltefosine with allopurinol and Amphotericin B represents the second and the third lines in CL therapy (Solano-Gallego et al., 2009). Clinical response varies from poor to good, depending on the animal's overall initial clinical status and on its

individual response to therapy. Dogs with renal insufficiency could manifest a lower recovery rate in comparison to the animals without kidney impairment or with mild proteinuria. Therapy with anti-leishmaniosis drugs often leads to clinical recover (Noli and Auxilia, 2005), although treated dogs may continue to harbour the parasite (Ribeiro et al., 2008; Mirò, Galvez, Fraile, Descalzo, & Molina, 2011). Majority of sick dogs obtain clinical amelioration within the first month of therapy (Pennisi et al., 2005). The clinical parameters to be monitored during treatment depend on the individual abnormalities (Solano-Gallego et al., 2009).

The critical relevance of host immune response in CL outcome has been largely demonstrated (Baneth, Koutinas, Solano-Gallego, Bourdeau, & Ferrer, 2008; Reis et al., 2009; Solano-Gallego et al., 2009; Alexandre-Pires et al., 2010; Coura-Vital et al, 2011). The involvement of Tregs is of critical relevance in the presence of infectious agents usually associated with chronic diseases, as in CL. Occurrence of Treg modulation represents an useful mechanism to optimise immune response against the infection, since Treg regulate the recruitment and activation of immune effectors (Sakaguchi, 2005). During CL, Treg activity could hamper inflammatory responses required for infection clearance. This occurrence exacerbates the risk that the unbridled parasite growth could lead to a severe disease. However, Treg recruitment is necessary to prevent the onset of severe immune-mediated mechanisms in infected tissues, particularly for the presence of autoimmune processes highly frequent in CL (Kharazmi et al. 1982; Ferrer, 1992; Terrazzano et al., 2006; Solano-Gallego et al., 2009; Cortese et al., 2009 and 2011).

It is worth noting that the unbalanced diet and malnutrition represent primary causes of immune suppression and has been demonstrated to be a major risk factor for the development of visceral leishmaniosis in human and animal models (Anstead et al., 2001; Malafaia, 2009; Carrillo et al., 2014; Mengesha et al., 2014). Energetic/metabolic status has been described to regulate immune response and immune tolerance in human and animal models (Matarese et al. 2005; Gerriets & Rathmell, 2012). Furthermore, the pathways that control immune cell function and metabolism are intimately linked and this relationship might intriguingly provide new strategies to modulate immune functions in several infectious diseases.

In this regard, the role of some biological principles (mainly derived from plants), referred as “nutraceuticals”, in modulating the immune system homeostasis (Andlauer & Furst, 2002) needs to be investigated. In this context, *Cucumis melo*, *Aloe vera*, *Punica granatum*, *Piper nigrum*, *Camellia sinensis*, *Ascophyllum nodosum*, *Grifola frondosa*, *Glycine max*, *Echinacea purpurea*, *Poligonum spp*, *Carica papaya* and *Curcuma longa* have been described to mediate several immune-modulating effects (Mujumdar et al. 1990; Barak et al. 2002; Inoue et al. 2002; Kodama et al. 2002; Barrett 2003; Lee et al. 2008; Butt and Sultan, 2009; Dhasarathan et al. 2010; Liu et al. 2010; Buttle et al. 2011; Kim et al. 2011; Sakarkar and Deshmukh 2011; Halder et al. 2012; Kim et al. 2013; Senthikumara et al. 2013). Anti-oxidant properties have been described

for *Cucumis melo*, *Carica papaya* and *Curcuma longa*, astaxanthin from *Haematococcus pluvialis* as well as for poly-unsaturated fatty acids derived from fish oil. *Piper nigrum*, polyphenols from *Camellia sinensis*, β-glucans from *Grifola frondosa* and *Glicine max* extract have been associated with the modulation of inflammatory pathways (Devasagayam and Sainins 2002; Vouldoukis et al. 2004a,b; Galli and Calder, 2009; Barros et al. 2012). Punicalagin, the most important active substance contained in the fruit of *Punica granatum*, exerts an immune suppressant activity (Lee et al. 2008). *Poligonum cuspidatum* extract, source of Resveratrol, in humans induced a significant reduction in the generation of reactive-oxygen species and can suppress plasma concentrations of pro inflammatory factors like TNF-α, IL-6, and C-reactive protein (Ghanim et al. 2010).

In order to investigate the immune regulatory networks involved in CL control, we focused the immune profile of dogs naturally infected by *L. infantum*. In particular, lymphocyte T cell subsets, peripheral Treg levels and the presence of pro-inflammatory T cells have been assessed, in a cohort of 45 Leishmania infected dogs (LD), compared with 30 sex/age matched healthy animals, by using immune fluorescence and flow cytometry detection. In this study, Leishmaniotic dogs have been categorised according to their clinical-pathological status and their serological anti specific-*Leishmania* antibody titer.

Subsequently, we focused on the potential immune-modulating activities of specific nutraceutical pet food containing anti-inflammatory and anti-oxidant nutrients, described above. In this study, was analyzed the immune profile of a cohort of 40 Leishmania infected dogs treated with conventional therapy associated with standard diet (20 animals) or a diet enriched with essential fatty acids and nutraceuticals with described immuno-stimulatory/antioxidants characteristics, mainly derived from medicinal plants (20 animals). A cohort of 20 healthy animals age/sex matched with the infected dogs has been also included in the study as control population. (manuscript submitted for publication).

T lymphocyte activation and Super Oxide Dismutase (SOD)1 involvement: ROS and adaptive immune response regulation

T lymphocytes play a critical role in the orchestration of both the immune response and immune tolerance. Their activation is a complex phenomenon in which intracellular signals mediated by the TCR engagement are integrated by accessory signals able to finely tune antigen-dependent triggering. In this context, TCR-mediated signalling is a critical event for proper channelling the immune response and to obtain pathogen control and self-tolerance (Fraser I D and Germain R N 2009). Several studies have suggested that the production of small amounts of Reactive Oxygen Species (ROS) characterizes many receptor systems of human cells. In this context, TCR-dependent T cells activation has been associated with the production of ROS. This phenomenon involves several enzymatic sources as the mitochondrial respiratory chain

(Sena et al. 2013), lipooxygenases, NADPH oxidase NOX2 and DUOX1 (Los et al. 1995; Jackson et al. 2004).

TCR stimulation generates both H_2O_2 and superoxide anion and antioxidant enzymes specific for H_2O_2 enhance and/or prolong TCR-dependent ERK activation (Jackson et al. 2004; Devadas et al. 2002). ROS are highly reactive chemical species with a very short life; for example H_2O_2 (1 minute half-life). They are constituted by an atom with an unpaired electron which makes the extremely reactive radical, as capable of binding to other radicals or subtract an electron to other neighboring molecules. ROS include superoxide oxygen (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical and peroxides. Generally ROS production in mammalian cells is associated with the presence of metabolic products or apoptotic signals. In some cells of the innate immunity ROS produced by NADPH oxidase (NOX), are used in the defense against pathogens.

Recent studies have shown that the ROS generation is relevant for a series of physiological systems, interactions receptor/ligand such as TGF-β (Thannickal et al. 2000), insulin (Mahadev et al. 2001), angiotensin (Ushio-Fukai et al. 1998) and EGF (Bae et al. 1997). ROS formation has proved essential to optimize the signal transduction pathways by acting as a second messenger regulating several crucial cellular responses, as protein kinase activation, gene expression and cell proliferation/apoptosis rate (Burdon 1996).

Ros are able to cause cellular damage of various nature; in fact, may act on the fatty acids, on proteins and nucleic acids. Oxidative damage is controlled by a series of enzymatic mechanisms *scavenger* capable of interfering with the mechanisms of the cascade of reactions activated by the radicals:

- **superoxide dismutase** which converts superoxide anion into hydrogen peroxide and molecular oxygen
- **catalase** which converts the hydrogen peroxide into water and molecular oxygen
- **glutathione peroxidase** which eliminates peroxides at the expense of glutathione.

Superoxide dismutase molecules belongs to a large family of isoenzymes oxido-reductases. The type of metal which binds SOD identifies the different enzymatic forms. The form is constituted by the enzyme cytosolic SOD bound to copper (Cu) and zinc (Zn) and is known as SOD1. SOD-1 is a homodimer of molecular weight 32,500 Da, in which the sub-units are linked via hydrophobic interactions and electrostatic while the two cofactors are linked to de side chains. The Mn SOD (SOD-2) is localized at the level of the mitochondrial matrix; tetrameric Cu,Zn SOD (SOD-3) is localized extracellularly.

ROS production has been observed to be involved in the physiological regulation of antigen-dependent signal transduction. At light of this, the availability of SOD-1, that is able to modulate cytosolic superoxide and peroxide level could behave as a possible adjustment for specific targeting of proliferation and/or apoptosis in immune effectors.

SOD-1 may be relased in vitro by fibroblasts, hepatocytes (Mondola et al. 1996), human neuroblastoma cells (Mondola et al. 1998) and Sertoli cells (Mruk et al. 1998). The extracellular release of such enzyme is related to specific stress conditions (Ookawara et al. 1998). ER/Golgi involvement in SOD-1 secretion has been described (Turner et al. 2005; Urushitani et al. 2006; 2008), while it is unclear how this cytosolic protein con be targeted into the ER/Golgi network.

Studies conducted by our research group showed that SOD1 plays a key role in mediating the response to oxidative stress in primary lymphoid organs such as the thymus, where were detected significant levels of SOD-1 especially in medullary area, critical for the selection of T lymphocyte repertoire (Cimini et al. 2002). In the study of 2002 was also detected the ability of human epithelial thymic cells of releasing the SOD1 by a mechanism of active secretion, in response to mild stress represented by the deprivation of nutrients. Further, the group has focused on the possibility that SOD-1 may be involved in paracrine control of oxidative stress in other human biological systems particularly susceptible to oxidative damage, such as neurons. In the work of 2003, it has been characterized SOD-1 role in the neuronal secretion mechanism, using the human neuroblastoma cell line SK-N-BE. The data obtained have revealed that the SOD-1 is secreted by a microvesicular pathway sensitive to pharmacological block from Brefeldin-A, 2-deoxy-D-glucose and sodium azide. Thus, the SOD-1 secretion could represent a significant phenomenon for the neuronal maintenance, probably able to influence the excitability of cells extremely sensitive to perturbations (Mondola et al. 2003).

In order to highlight a possible paracrine role of SOD-1, (Mondola et al. 2004) the ability of the enzyme to directly interfere with human neuroblastoma cell line SK-N-BE has been analysed. Data indicate that SOD-1 may interact specifically with the membrane of SK-N-BE cells, mobilizing intracellular calcium and protein kinase C (PKC). The SOD-1 activity seems to depend to phospholipase C (PLC) pathway, since the effects are sensitive to block by U73122, a specific inhibitor of PLC. Some experimental data indicate that SOD-1 is not constitutively expressed: in fact, in the cell line SK-N-BE derived from neuroblastoma, it is possible to observe the increase of the intracellular SOD-1, after exposure to oxidative stress.

Taken in all, these results suggest the involvement of SOD-1 in the regulation of complex biological events. The possibility that the enzyme might be involved in multiple regulatory pathways involving adaptive immune response and its functional effectiveness needs to be investigated in different physiological and pathological models.

AIMS

The aim of this work is to investigate whether immune-tolerance dysregulation might be involved in the pathogenesis of haematopoiesis disorders and in the immune-pathological effects consequent to chronic natural infections. The mechanisms underlying such involvement and the possibility to specifically modulate such effects by targeting immune-metabolic pathways and ROS production has been also addressed.

The first model allowed us to identify some immune-mediated mechanisms underlying emergence, expansion and dominance of pathological haematopoietic precursors in PNH and MDS patients.

In this model we are proposing that specific alterations of immune profile in BM could identify a subgroup of MDS patients in which an immune-mediated pathogenesis of the disease might be inferred. Indeed, low Treg levels and high expression of CD54 on CD8 effectors in BM has been consistently associated with the selection of dysplastic precursors expressing low level of MHC Class I molecules, the key recognition element for cytotoxic T cells.

In addiction, in selected patients the expansion of NK effectors expressing high levels of NKG2C receptors has been associated with the selection of dysplastic precursor lacking HLA-E expression.

The above described studies indicate the key role of Treg population for immune tolerance control in MDS model as well the possible involvement of polyclonal NK expansion in some specific conditions.

A number of studies has been suggesting that mTOR (mammalian Target of Rapamicine), a key sensor of nutrient availability in the extra-cellular microenvironment, plays a major role in orchestrating immune cell differentiation and haematopoietic Proliferation/Quiescence transition. A specific involvement of mTOR-dependent mechanisms in the expansion of Treg population has been also largely demonstrated. (Battaglia et al. 2012; Procaccini et al. 2010). The possibility to specifically target such pathways by metabolic (diet) and/or pharmacological approaches has been also addressed taking advantage from a chronic infection model represented by *Leishmania Infantum* natural infection in dogs, usually complicated by the occurrence of autoimmune platelet deficiency.

The immune response regulation includes the ability to integrate signals. This integration is physiologically exerted by the availability of accessory molecules capable of finely tune the plasticity of T lymphocytes response. Superoxide dismutase (SOD) belongs to a family of isoenzymes involved in the scavenging of oxygen radicals to hydrogen peroxide (H_2O_2) and molecular oxygen. TCR stimulation has been observed to generate balanced production of H_2O_2 and superoxide anion. In this context, the possible involvement of cytosolic SOD-1 in intracellular signalling network regulating TCR-dependent immune response in both physiological and pathological settings (Terrazzano, Rubino et al. 2014) might be hypothesized. The possibility that SOD1 targeting might allow specific modulation of T cell response needs further investigation.

MATERIALS AND METHODS

Paroxysmal Nocturnal Haemoglobinuria (PNH)

Patients

The study was conducted in 34 Italian PNH patients. Diagnosis was made according to International PNH Interest Group Criteria (Parker et al. 2005). All patients showed primary haemolytic PNH with a large GPI-defective granulocyte population (CD66b− and/or CD59− granulocytes >50%). No patients showing bone marrow failure with emergence of PNH clones (i.e. Aplastic Anaemia/PNH syndrome or Hypoplastic PNH) were enrolled in the study (Brodsky 2008). Notably, some of the patients were on Eculizumab treatment for more than 48 months. Biological samples were collected by venipuncture 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. Patients were studied at diagnosis, before any treatment; 17 of them were retested after at least six months of Eculizumab therapy; 14 patients were tested at least three times before and after Eculizumab treatment. None of PNH patients received immune-suppressive therapies or other treatments potentially interfering with immune responses.

Immunofluorescence and flow cytometry

Blood samples from patients and controls were analyzed by immunofluorescence and flow cytometry by using a two laser equipped FACScalibur apparatus and the CellQuest analysis software (Becton Dickinson). FITC, PE, Cychrome and APC labelled mAbs against CD3, CD4, CD25, FoxP3, CD45, CD8, CD16, CD56, NKTi, CD19, CD20, CD45 and isotype-matched controls were purchased from Becton Dickinson PharMingen, San Jose, CA. All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using FSC and SSC parameters, as well as CD45 labelling. We choose to evaluate T, B, NK, NKTi and Treg populations.

Statistical analysis

Statistical evaluation of data, by using software InStat 3.0 (GraphPad Software Inc., San Diego, CA, USA), has been performed by means of the Mann-Whitney test. Comparisons in the paired samples were evaluated by means of the Wilcoxon matched-pairs signed-rank test. Two-sided p values of less than 0.05 were considered to indicate statistical significance and were not adjusted for multiple testing.

MyeloDysplastic Syndromes (MDS)

Patients and controls

We examined whole BM and peripheral blood (PB) specimens of 30 MDS patients belonging to Low Risk and Int-1 Risk groups. BM and PB sample

collection, haematological investigation, cytogenetics were performed according to the WHO recommendations and IPSS score (Greenberg F et al. 1997; Malcovati L et al. 2005). 17 patients belonged to Low Risk Group (4 refractory anaemia, RA, 8 refractory cytopenia with multilineage dysplasia, RCMD, 2 refractory neutropenia, RN, 3 refractory thrombocytopenia, RT), and 13 to Intermediate-1 (Int-1) Risk Group (2 RA, 8 RCMD, 3 RN). BM and PB samples from MDS patients were obtained during routine diagnostic procedures. Informed consent was obtained from each individual patient. Local Ethical Committee approved the study. None of the patients has been receiving medical treatments that could have an impact on their immune condition. Patients were devoid of immune-mediated diseases and acute or chronic viral infections to avoid any interference on immuneregulatory mechanisms. A group of 26 healthy blood donors were recruited in the study as controls. All the MDS patients were clustered in two sub-groups, according to their BM Treg levels and CD54 expression on BM CTL, as described (Alfinito F et al. 2010). Briefly, 14 MDS patients showing a Treg percentage in BM $\geq$ 2% of the lymphocyte population or a ratio <10 between the mean intensity fluorescence (MIF) for CD54 on BM CD8 T cells and the control MIF value, obtained after staining of the same cell population with the isotype control mAb, were considered with normal immune profile (NIP). At variance, 16 MDS patients showing a Treg percentage in BM<2% of the lymphocyte population or a ratio$\geq$10 between the mean intensity fluorescence (MIF) for CD54 on BM CD8 T cells and the control MIF value obtained after staining of the same cell population with the isotype control mAb, were considered with altered immune profile (AIP).

mAb, immunofluorescence, and flow cytometry

FITC, PE, Cychrome and APC labelled mAb against CD3, CD4, CD8, CD56, CD19, CD10, CD25, CD45, CD54 and isotype-matched controls were purchased from BD PharMingen (San Jose, CA). Biotin labelled HLA-E mAb clone MEM-E/07, FITC labelled MHC-I mAb clone W6/32 and PE labelled avidin, were purchased from Sigma–Aldrich, Milan, Italy. To analyse Foxp3 expression, intracellular staining was performed by anti-human Foxp3 kit (eBioscience San Diego, USA), following the manufacturer's instructions. Treg subset was identified as the high CD25 expressing CD4+CD3+ population appearing as a tail, with a slightly but reproducible lower CD4 expression, distinct from the major CD4 population containing both the CD25 low and negative cells (Alfinito F. et al., 2010). These cells were observed to express Foxp3 at a percentage >98%, as described (Alfinito F. et al., 2010). All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using forward scatter (FSC) and Side Scatter (SSC) parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by a two laser equipped FACScalibur apparatus and CellQuest software (Becton Dickinson).

Statistical analysis

BM and PB values were compared in the paired samples by means of the Wilcox on matched-pairs signed-rank test. Comparisons between MDS patients and controls were performed by Mann–Whitney test. The corrected p value (p_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

Case report

An 85-year-old man presented with thrombocytopenia, whose first record was referred to few months before. Hb was 12.9 g/dL, MCV 67fl, white blood cell count 4.4 X 10^9/L with 0.15 X 10^9/L neutrophils, 0.096 X 10^9/L monocytes, and 0.166 X 10^9/L lymphocytes. PMN appeared hypogranular and monocytes highly dysmorphic. Platelet count was 80 X 10^9/L. BM aspirate was characterized by good cellularity with erythroid and granulocytic dysplasia, numerous micromegakaryocyte and 5% of blast cells. Patient received diagnosis of refractory cytopenia with multilineage dysplasia according to WHO classification (Mufti GJ et al. 2004). Microcytosis was due to beta thalassemia, and no organomegaly was present. Patient showed a normal karyotype (46,XY). Patient has been not receiving medical treatment, which may impact his immune response. Informed consent was obtained from the patient and the controls.

mAb, immune fluorescence and flow cytometry

Anti-CD3, -CD8, -CD4, -CD54, -CD25, -CD56, -CD14, -CD45, -CD33,-CD127, -p140 (Q66 clone) and isotypematched labeled controls mAbs (Becton-Dickinson, Los Angeles, CA, USA), anti-HLA-I (W6/32 clone) mAb (SIGMA, Milan, Italy), anti-HLA-E mAb (MEM-E/08 clone) (Immunological Sciences, Rome, Italy); anti-CD158e1/e2 (p58.1/2), -CD244 (2B4), -CD335 (NKp46), -CD337 (NKp30), anti-NKG2A (Z199), -Vb14, -Vb12, -Vb7.2, -Vb20, -Vb18, -Vb7.1, -Vb22, -Vb13.2, -Vb1, -Vb17, -Vb5.3, -Vb5.1 -Vb23, -Vb4, -Vb2, -Vb13.1, -Vb5.2, -Vb8, -Vb9, -Vb11, -Vb3, -Vb13.6, -Vb21.3F, -Vb16 mAbs (Beckman-Coulter, Paris, France), and -NKG2C (134591 clone) mAb (R&D System, Minneapolis, MN, USA). Flow cytometry was performed by FACSCalibur and CellQuest analysis software (Becton Dickinson), as described (Ruggiero G et al. 2009; Alfinito F et al. 2010; Terrazzano G et al. 2013). Three sex- and age-matched healthy donors were used as controls.

Statistical analysis

Statistical analysis for p calculation was performed by using Student's t-test. Results were considered significant with a p value < 0.05.

L. infantum.chronic **natural infection in dog model**

First study

Animals

Forty-five dogs naturally infected by *L. infantum* (20 males and 25 females, 5-9 years old) from the Campania region (South Italy) were enrolled with the

owner's consent. Ten dogs were pure breed, while 35 were mongrels. Also, 30 healthy dogs (12 males, 18 females, 5–7 years old) with no clinical signs of CL, which were also being negative for serological, parasitological and molecular examinations, were considered as healthy control group.
This work has been reviewed by Ethical Animal Care and Use Committee of the University of Naples Federico II and received institutional approval (Prot. 2011/0043885).

Clinical parameters and diagnostic procedure

For all dogs, the history (none of dogs received a specific treatment for CL) and the clinical examination were performed. Leishmania infected dogs were classified according to (Solano-Gallego et al. 2009). The LD cohort has been categorised in two groups: LD-A dogs (n =23), presenting neither clinical signs on physical examination nor clinical-pathological abnormalities by routine laboratory tests (CBC, biochemical profile and urinalysis) but with a confirmed *L. infantum* infection and LD-S dogs (n=22) with clinical leishmaniasis, presenting clinical signs and/or clinical-pathological abnormalities and a confirmed L. infantum infection. Diagnosis of CL has been confirmed by detection of amastigotes in stained cytological smears of aspirates from lymph nodes or bone marrow, serologically by a positive indirect fluorescent antibody test (IFAT ≥1:80) and by a polymerase chain reaction (PCR). Animals presenting IFAT titers ≥1:80 and positive molecular diagnosis were included in the study as infected animals.

The relevance of humoral immune response on susceptibility/resistance mechanism during ongoing canine visceral leishmaniasis has been recognized throughout ex vivo and in vitro investigations (Oliva G et al. 2006; Reis AB et al. 2006). Thus, in order to analyse the immune profile in Leishmania infected animals showing different degree of specific immune activation, Leishmania infected dogs were further sub-classified as Low IFAT titer animals (LD-L) when showing a IFAT titer 1:80–1:160 or High IFAT Title animals (LD-H) when showing a IFAT titer >1:160.

Animals presenting neither clinical signs on physical examination nor clinical-pathological abnormalities by routine laboratory tests, and with IFAT negative (≤1:40) and negative PCR, were considered non-infected and included as healthy control group. Occurrence of other infectious diseases was always excluded in all dogs. No Ehrlichia canis nor Anaplasma phagocytophilum morulae, Babesia canis trophozoites and microfilariae were observed in peripheral blood smears. These infections were also excluded using IFAT and/or PCR. Finally, Dirofilaria immitis infection was ruled out using the Snap Canine Combo Heartworm Antigen Antibody Test (IDEXX).

Blood sample collection

Ten millilitres of peripheral blood was collected from the jugular vein into tubes containing ethylene diamine tetraacetic acid (EDTA). A complete cell blood count was performed in each sample within 30 min from the collection

by using a semi-automatic cell counter (Genius S; SEAC Radom Group, Florence, Italy). All samples were maintained at room temperature up to 5–6 h prior to processing. In addition, serum aliquots were obtained from all the dogs enrolled for the study of their biochemical profile and serological examination.
Serological diagnosis Detection of anti-Leishmania IgG antibodies was performed by an in-house IFAT assay using *L. infantum* promastigotes (WHO reference strain MHOM/TN/1980/IPT-1) as antigen and following the protocol recommended by the Office International des Epizooties (Gradoni L and Gramiccia M, 2008). Samples were classified as positive if promastigote cytoplasmatic or membrane fluorescence was observed at a serum dilution of 1:80 or higher.

Molecular diagnosis

To perform a nested-PCR assay for Leishmania spp. DNA was obtained from bone marrow. In 4 animals, due to lack of bone marrow specimens, lymph nodes aspirates and peripheral blood were used as DNA source for nested-PCR assay.

Monoclonal antibodies, immunofluorescence and flow cytometry

Peripheral blood from each dog was employed as whole blood sample to evaluate the number of CD3+CD4+, CD3+CD8+ and CD4+- Foxp3+ T cells by immune-fluorescence technique and flow cytometry analysis. Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Cy-chrome and Allophycocyanin (APC) labelled monoclonal antibodies (mAb) against dog CD3, CD4, CD8, and isotype-matched controls were purchased from Serotec Ltd, London, UK. Intracellular detection of Foxp3 was performed using a cross-reactive, directly conjugated murine Foxp3 antibody (Clone FJK-16s, eBioscience, San Diego, CA) and the permeabilisation buffer provided by the detection Kit (Foxp3 Staining Set, eBioscience), as described (Biller B.J. et al., 2007). Foxp3 staining was carried out on Peripheral Blood Mononuclear Cells (PBMC) purified by Ficoll (Sigma–Aldrich, St. Louis, MO) density gradient centrifugation, as described (Biller BJ et al. 2007). CD8+ and CD4+ T cell subsets were always identified by a combination of canine specific anti-CD3 together with anti-CD4 or anti-CD8 mAbs. A typical phenotype analysis strategy is shown in Figure 8.

To analyze the production of IFN-γ and IL-4, purified PBMC were cultured overnight (ON) in presence of Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin, all purchased from Sigma–Aldrich. This approach has been widely described as useful in the study of established cytokine profile in human and animal models (Terrazzano G et al. 2005; Papadogiannakis EI et al. 2009; Olsen I and Sollid LM, 2013). Intracellular staining with the mAbs recognising dog IFN-γ, IL-4 or isotype-matched controls (Serotec) was performed by a fixing/permeabilization kit (Caltag, Burlingame, CA), following the manufacturer's instructions. To avoid extracellular cytokine export, the cultures were incubated in the presence of 5 µg/ml of Brefeldin-A (Sigma–

Aldrich), as described (Terrazzano G et al. 2005; Papadogiannakis EI et al. 2009).

All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using Forward Scatter (FSC) and Side Scatter (SSC) parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by using a two laser equipped FACScalibur apparatus and the Cell Quest analysis software (Becton Dickinson, Mountain View, CA).

Statistical analysis

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

Second study

The diets.

Two groups of dogs (see next paragraph) were fed with two industrial dry pet foods. Both diets were based on the same receipt and completely fulfil the recommendations for protein, carbohydrate and fat 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). In particular, the two foods reported similar analytical composition in nutrients (24% of crude protein, 12% of crude oils and fats, 3.7%, of crude fibre 5% of crude ash, 9% of moisture) and, as a consequence, similar Metabolised Energy (ME) of 3.477 kcal/kg corresponding to 14.6 MJ/kg. Both foods are commercial and in the form of kibbles industrially produced with extrusion technique. The potential immune-modulating diet 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). Overall nutrient profile of the product was obtained by the sum of a first nutrient profile of the kibbles, for feeding purpose, and a second nutrient profile of the tablets for both nutrient and therapeutic purposes. Tablets were composed by 60-80% of protein hydrolisated (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 un-saturated fatty acids from fish oil). The dry pet food used as control diet did not contain the above-mentioned active substances.

The standard and the nutraceutical supplemented dietary administration were carefully adjusted to provide similar caloric animal food intake and to satisfy the nutritional requirement of adult dogs. In this regard, the food was administered twice a day according to the specific requirements for the nutrient amount. To guarantee the maintenance energy requirement, food dosage was established using a daily dietary table based on the equation 130 kcal ME/kg of Body Weight$^{0.75}$ as recommended by the National Research Council Committee

on Animal Nutrition, USA. The coefficient used referred to a moderate activity. The two commercial products used in this trial completely respect the nutritional guidelines established by European Pet Food Industry Federation to provide all nutrients necessary for a canine diet.

Animals and study design

Forty dogs naturally infected by *L. infantum* (20 males and 20 females, 5-9 years old) from the Campania region (South Italy), which is a CL endemic area, were enrolled with the owner consent. Ten dogs were pure breed (4 German Shepherds, 4 Rottweilers and 2 Labrador Retrievers), while 30 were mongrels (15 females and 15 males, between 20 and 35 kg in weight). The study was performed on household dogs under the biweekly supervision of the veterinaries from the Department of Veterinary Medicine and Animal Productions. Animals were allocated to two groups. Group 1 was composed of 20 dogs treated with meglumine antimoniate (50 mg/kg, subcutaneous, twice daily, for 1 month), allopurinol (10 mg/kg, oral, twice daily, for 6 months) and fed with standard diet. Group 2 included 20 dogs subjected to the same therapeutic treatment combined with the administration of a diet with potential immune-modulating activity (see previous paragraph). Animals were equally distributed for breed, sex, weight and clinical signs in the two groups.
Immune profile evaluation was performed at diagnosis (T0) and after three (T3), six (T6) and twelve (T12) months of trial in all the animals. As controls, 20 healthy dogs (12 males, 18 females, 5-7 years old) were enrolled. The full blood count, total proteins, albumin/globulin ratio, urea and creatinine value determinations and immune profile analysis were evaluated at T0, T3, T6 and T12. In addition, at T0 and T12, both indirect fluorescence antibody test (IFAT) and sternal bone marrow aspirate for Leishmania DNA detection by n-PCR were performed.

Clinical evaluation of dogs

History and clinical examination were performed with the accuracy of guideline criteria for CL diagnosis and classified according to Solano-Gallego et al. (2009). No dog received a specific treatment for CL before the enrolment. Dogs were monitored for clinical signs correlated to other illnesses potentially occurring during the trial. Clinical recovery was evaluated at T3, T6 and T12 and was based on the reduction/disappearance of clinical signs listed in the inclusion criteria. At the beginning of the study (T0) and during the follow-up (T3, T6 and T12), the dogs were evaluated for their weight by five-point body condition score (BCS). Enrolled dogs were of medium body weight of 27 ± 2 kg while the BCS, which assesses the nutritional status, ranged from 2.75 to 2.90.

Monoclonal antibodies, immunofluorescence and flow cytometry

See above in the same session referred to the first study.

Statistical analysis

Statistical analysis was performed by One-way Analysis of Variance (ANOVA) Tukey-Kramer Test for Multiple Comparisons using GraphPad Prism Software (GraphPad Prism Inc, San Diego, CA, USA). Results were considered significant at $p<0.05$.

T lymphocyte activation and SOD1 involvement

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 healthy donors, after informed consent, by centrifugation of peripheral blood on Ficoll-Paque cushion (GE Healthcare, Uppsala, Sweden) gradient. T cells have been isolated from PBMC by using a negative isolation kit (Invitrogen Corporation, Carlsbad, CA, USA) and following the manufacturer's instructions. PBMC or T cells (1×10^6/ml) were cultured in 96 well flat-bottomed plates (Falcon) in RPMI 1640 medium with 2% FCS (Invitrogen, Carlsbad, CA, USA). TCR triggering was performed by anti-CD3 mAb (Becton Dickinson, Mountain View, CA, USA) at 5 ng/ml or by using anti-CD3/anti-CD28 beads (Invitrogen), at 0.3 bead/cell. This activation strategy has been largely demonstrated to mimic antigen-dependent T cell triggering. To analyze TCR-dependent SOD-1 T cell export, distinct experiments were performed in the presence of Brefeldin-A, (BFA) at 5 μg/ml or of 1 mM methylamine, all purchased from Sigma-Aldrich (Milan, Italy), as described (Mondola P et al. 2003). Cell viability was evaluated by using PropidiumIodide (PI) (Sigma-Aldrich) labeling and flow cytometry detection (Mondola P et al. 2003) as well as by analyzing lactate dehydrogenase (LDH) activity in culture supernatants by using the Roche Molecular Biochemical LDH kit (Mannheim, Germany). Written informed consent (model n. 5526 of Azienda Ospedaliera Universitaria "FEDERICO II") was obtained from each donor at the time of venous peripheral blood donation. All the experiments done by using blood donations were performed and analyzed anonymously, without any biographical reference to donors.

ELISA

The quantitative detection of human SOD-1 in medium of cultured PBMC was carried out using the Bender Med System kit (Bender Med System Diagnostic, Vienna, Austria), as described (Mondola P et al. 2003). Results were always normalized for total protein content of the tested sample. SOD-1 ELISA detection has been always performed on culture supernatants immediately frozen at −80 °C. Protein concentrations were determined according to the method of Lowry et al. 1951 using BSA, as standard.

RNA preparation, semi-quantitative RT-PCR and DNA sequencing.

Analysis of SOD-1 specific RNA has been performed, as described (Russo M et al. 2011). Briefly, total RNA was extracted with High Pure RNA isolation kit (Roche Italia, Milano, Italia), according to the manufacturer's instructions. Traces of contaminated DNA were removed with DNAse I treatment.

Quantification was achieved in a single reaction by using the housekeeping β-actin gene as internal standard. To rule out genomic DNA contamination we performed a negative control that contained RNA instead of cDNA. The signal intensities of PCR products were separated on a 1.2% agarose gel and were visualized by ethidium bromide staining. The products' signal intensitieswere determined by computerized densitometric analysis using Fotoplot software. The expression of SOD-1 was normalized to β-actin mRNA levels. To check the specificity of the amplified products, DNA bands were eluted from the gel and purified; sequence analysis was determined by the Big Dye Terminator Cycle Sequencing method (ABI-PRISM Sequencer 310 Perkin-Elmer).

Microvesicle isolation and western blotting for SOD-1 detection

To purify the membrane microvesicle-containing fraction, supernatants were collected immediately after culture and treated, as described (Thery C et al. 2006). Briefly, they were sequentially centrifuged at 500 g for 15 min to remove cellular debris and again at 10,000 g for 20 min. The obtained supernatant was collected and further centrifuged at 100,000 g for 2 h. The resulting pelletwas then collected and considered to represent the enriched membrane vesicle fraction. Western blotting analysis of the purified material was performed as previously described (Mondola P et al. 2003). Comparative analysis of SOD-1 was performed by using 40 μg of total proteins.

Immunofluorescence and flow cytometry analysis

Intracellular SOD-1 contentwas evaluated anti-SOD-1mAb and FITC labeled anti-mouse IgG secondary antiserum (Sigma-Aldrich) staining of permeabilized cells and immunofluorescence technique. A commercial fixing/permeabilization kit, purchased from Becton Dickinson was always employed, following the manufacturer's instructions. For the analysis of SOD-1 content in distinct cell subsets and to evaluate T cell activation after TCR triggering, co-staining with FITC, PerCP or APC labelled anti-CD3, anti-CD45 and anti-CD69 mAb was performed. Labeled antibodies and isotype-matched controls were purchased from Becton Dickinson. T cell staining and activation were performed by anti-CD3 mAb recognizing different CD3 epitopes. Cell death was always less than 5% as evaluated by using PI (Sigma-Aldrich) staining. Immunofluorescence, flow cytometry and data analysis were performed by using a two laser equipped Becton–Dickinson FACSCalibur flow cytometer and the Cell Quest analysis software.

Fluorescence microscopy

PBMC or purified T cells (0.5×10^6) were adhered to polylysine coated glass slides for 16–18 h at 37 °C. When indicated, the above populations were stimulated with anti-CD3 mAb CLB-CD3/4E at 1:100 ascites dilution or antiCD3/anti-CD28 beads (at 0.3 bead/cell) and 1 mM NAC (Karlsson H et al. 2011). Cells were incubated at 37 °C and immediately fixed with 3%

paraformaldehyde solution. Fixed cells were incubated with FITC labelled anti-CD3 and anti-human Cu,Zn SOD-1 rabbit antibody (Santa Cruz Biotechnology, CA, USA) for 45 min in a humidified chamber, washed three times with PBS and incubated with Alexa Fluor 594- conjugated goat anti-rabbit secondary antibody (Molecular Probes,
Life Technologies) for additional 45 min at 37 °C in the same conditions. After 3 washes with PBS the glass slides were mounted using a 50% solution of glycerol in PBS and examined with a Zeiss LSM 510 confocal microscope with a 63× oil immersion objective (N.A. 1,4) at room temperature. Pictures were taken from selected fields of control and treated samples.

Cell to cell aggregate evaluation

To evaluate cell aggregation, PBMCs were cultured in 96 wells flat bottomed microtiter plates (Falcon) in the presence of Medium, anti-CD3 and 1 mM NAC (Sigma-Aldrich), as indicated. This NAC concentration was demonstrated in preliminary experiments to completely block ROS formation, as described (Orci L et al. 1991). Contrast phase microscopy analysis was performed with a Leitz DIAVERT microscope with a 10× objective at room temperature. Pictures were taken from selected fields by using a digital Nikon Coolpix Camera. NAC treatment was unable to significantly affect T cell viability and proliferation, as evaluated by PI staining after 1 to 5 h of culture and 3H thymidine incorporation after 72 h of culture. Quantification has been performed by counting the number of cell aggregates (identified by the presence of at least 8 clustered cells) in the cell culture of 1 × 105 PBMC plated on the flat bottomed microtiter wells.

Statistical analysis

Statistical evaluation of data, by InStat 3.0 software (GraphPad Software Inc., San Diego, California, USA), has been performed by means of the Mann-Whitney test or Paired t test, as indicated. Two-sided p values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Immune tolerance in bone marrow failure syndromes.

Paroxysmal Nocturnal Haemoglobinuria (PNH)

PNH was our first research model to study the impact of immune tolerance-dysregulation in bone marrow failure syndromes.
In particular, we analysed the effects of the Eculizumab treatments on innate and adaptive immune effectors in a cohort of 34 PNH patients (Alfinito et al. 2012).
To investigate whether complement inhibition might interfere with the selection/expansion of the GPI-defective haematopoiesis, comparative analysis of immuno-phenotypic profile was performed among PNH patients before (PNH BT) and during (PNH OT) Eculizumab treatment. In Figure 3, a decrease in levels of B cells, NK cells and Treg accompanied by an increase in lymphocytes NKTi was observed in PNH patients BT compared to controls. Eculizumab therapy was able to selectively restore B and NKTi peripheral concentration without significant interference on NK and Treg numbers.

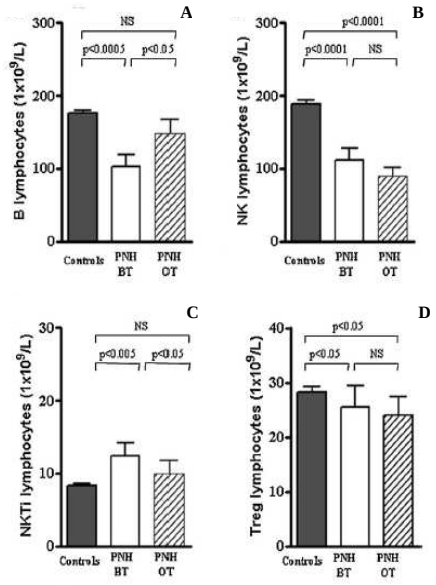

Figure 3. Analysis of B, NK, NKTi and Treg lymphocytes in PNH patients before and on Eculizumab treatment. As indicated, panels A-D refer comparative analysis of B, NK, NKTi and Treg lymphocytes in PNH patients before (BT) and on (OT) at least six months of Eculizumab treatment. The analysis has been performed by considering the values obtained in the same patients before and after Eculizumab therapy. For the patients, each value represents a mean obtained in at least three independent evaluations before and after treatment. Statistical analysis has been performed by Wilcoxon matched-pairs signed-rank test.

MyeloDysplastic Syndromes (MDS)

In a subset of MDS patients (Low/Int-1 risk) with low levels of Treg and high expression of CD54 on cytotoxic T lymphocytes ($CD8^+$) (CTL) in BM, in which the involvement of immune-mediated mechanisms in the pathogenesis of the espansion of the dysplastic precursor might be inferred, were analyzed the expression of HLA-I and HLA-E, as key elements for the regulation of $CD8^+$ T cells and NK effectors (Terrazzano et al. 2013).

Therefore, we evaluated the HLA-E expression on Granulopoietic compartment (GC) versus PMN in peripheral blood (PB) in paired samples. As shown in Panel A of the Figure 4, HLA-E levels on peripheral PMN are significantly higher than GC in BM, while no significant differences have been observed between peripheral PMN from patients and healthy controls (not shown).

In the light of this result, we hypothesize that the higher HLA-E characterises mature PMN effectors in the peripheral blood. Such higher HLA-E expression could be the result of a specific up-regulation of the molecule on circulating PMN or it could be dependent on a preferential selection of a BM precursor showing higher HLA-E expression. To investigate on this issue, we also analysed the staining profile of HLA-E on GC in BM. As showed in panel B of the Figure 4, two distinct populations with different HLA-E expression levels are present in the GC of BM.

Since HLA-E molecules are believed to preferentially regulate NK-dependent cytotoxicity, these data suggest the role for NK cells in the selection of PMN in all MDS patients regardless the occurrence of an altered immune profile.

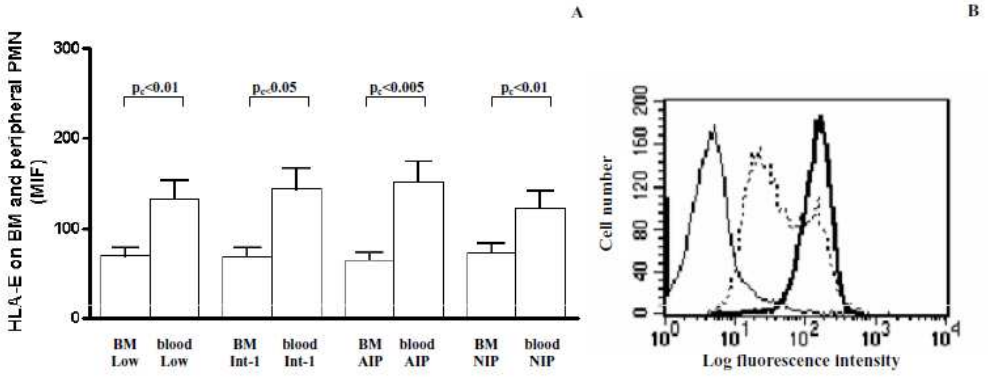

Figure 4. HLA-E expression on PB and BM cells. (A) Mean of immunofluorescence (MIF) graph for HLA-E on BM GC and on PB PMN in Low, Int-1, AIP and NIP patients. A p value < 0.05 has been considered significant and (B) staning profiles for HLA-E on BM GC as compared with peripheral PMN in one representative MDS patient: BM GC profile (dotted line), PB PMN (bold plain line) and isotype control (plain line).

A significant decrease of HLA-I on peripheral PMN, when compared to BM PMN, was observed in Low as well as in Int-1 Risk MDS patients

(p_c<0.01 and p_c<0.05, respectively) (Panel A of the Figure 5). If the patients were analysed according to their BM immune profile (AIP= with altered immune profile and NAIP= without altered immune profile), only the patients with an altered immune profile showed a significant decrease of HLA-I expression (p_c<0.001) on peripheral PMN. It is worth noting that a tendency to present lower HLA-I levels was observed also when the AIP MDS sub-group was compared to the healthy donors (panel B of Figure 5).

Intriguingly, lower MHC-I expression in peripheral PMN seems to be strongly correlated with the occurrence of an altered BM immune profile in MDS patients.

To ascertain if the presence of PMN with lower HLA-I expression in PB is dependent on down-modulation mechanisms occurring in the periphery or it is the result of immune-selection pressure favouring BM precursors carrying the lower HLA-I levels, we evaluated the cell distribution of GC in the BM and of PMN in PB. In this regards, two distinct populations with different HLA-I levels can be identified in GC of BM: the peripheral PMN seem to belong to the GC subset bearing the lower HLA-I expression (Figure 5, panel C). This feature significantly characterised the AIP subgroup (Figure 5, panel A). Such observations point to the role of CD8-dependent immune-mediated selection mechanisms occurring in BM. Indeed, PMN cells with lower HLA-I could be unable to present unknown self-antigens and, in reason of this mechanism, to become "targets" for autoreactive CD8 T lymphocytes. Indeed, the PMN cell maturation of effectors bearing lower HLA –I expression seems to be favoured in AIP subgroup, likely showing a deranged T cell response.

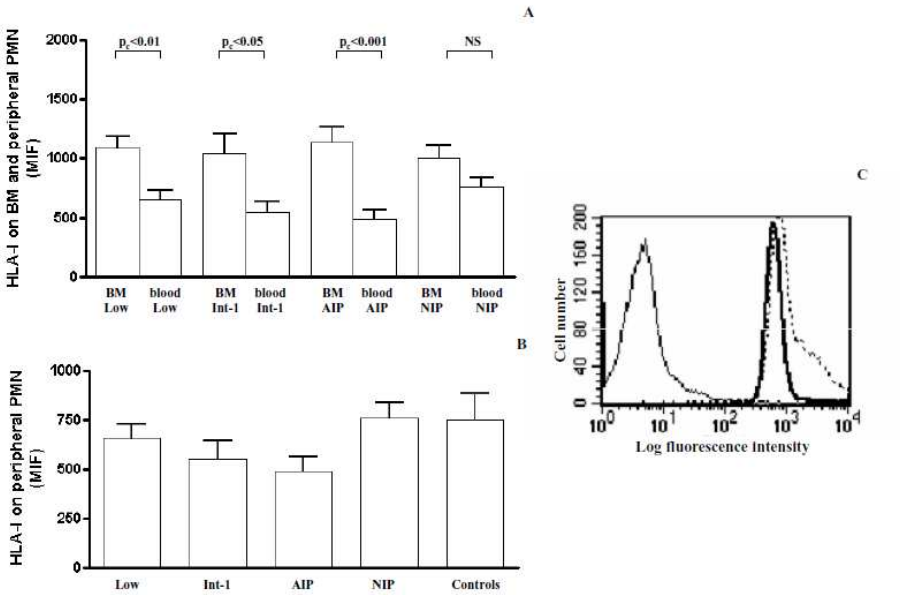

Figure 5. HLA-I on PB and BM cells. (A) MIF graph for HLA-I expression on BM GC and PB PMN in Low, Int-1 AIP and NIP patients. A p value < 0.05 has been considered significant, (B) HLA-I levels on PB PMN of patients and controls and (C) staining profile for HLA-I in

one representative AIP patient: BM GC (dotted line), PB PMN (bold plain line) and isotype control (plain line).

To investigate on the occurrence of immune-mediated selection in BM of MDS patients, we compared the percentage of HLA-I negative PMN cells in PB between healthy controls and MDS patients. A significant increase of HLA-I negative PMN has been observed in Int-1 patients if compared with the Low Risk Group (p_c<0.05) and the healthy donors (p_c<0.005) (Figure 3). When MDS patients were grouped according with their BM immune profile, the AIP group showed significant higher percentage of HLA-I defective PMN cells in comparisons with the NIP counterpart (p_c<0.05) or healthy donors (p_c<0.05).

Such evidence, together with the observation of a lower expression of MHC-I on PMN in PB than on GC in BM of AIP patients (Figure 6), suggests the involvement of immune-mediated, HLA-I dependent mechanisms in the selection of PMN cells in AIP subgroup of MDS patients, characterised by low Treg and increased CD54 expression on CTL.

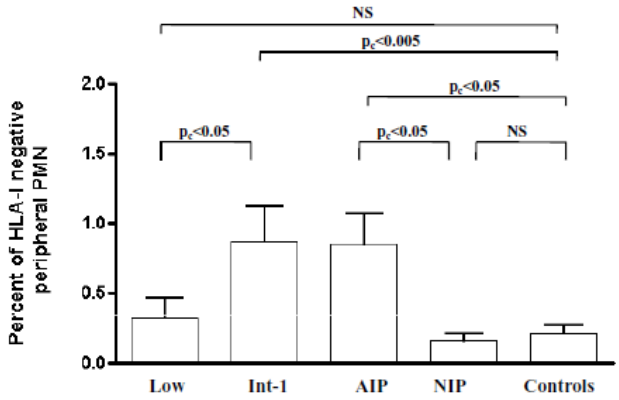

Figure 6. HLA-I negative PMN in PB of MDS patients. Percentage of HLA-I negative PMN in PB of Low, Int-1, IP and NIP groups of MDS patients as well as in healthy donors. A p value <0.05 has been considered significant.

In the 2013 an intriguing case of MDS patient carrying CD14(+) CD56(+) monocytes in bone marrow (BM), in the presence of a defective human leukocyte antigen (HLA)-E expression on peripheral blood (PB) cells and of natural killer (NK) cell expansion in PB and BM, was by us characterized. The defective HLA-E expression and the NK expansion were proposed to be relevant for the pathogenesis of myelodysplasia in those patients showing $CD14^+$ $CD56^+$ monocytes in BM (Terrazzano et al. 2013)

In particular MDS patient showed a dysplastic monocytosis, characterized by the following features:

1. a significant expansion of $CD14^+$ monocytes co-expressing the CD56 in BM (more than 30% of monocytes) than in periphery (2% of PB monocytes);

2. a Treg percentage and CD54 expression on both the $CD4^+$ and $CD8^+$ T Lymphocytes, similar between BM and PB, and to healthy donors; the Natural Killer expansion (more than 35% of lymphoid cell in BM and PB compartment) carried several NK receptors (CD335, CD337, CD244, CD158e1/e2, CD158/p70, CD94/NKG2A, CD94/NKG2C), suggesting that NK cells are polyclonal;
3. a TCR repertoire of T lymphocytes that appears to be polyclonal in BM and PB, without TCR-Vβ skewing (TCR polyclonal repertoire) of one or more T clones.

Therefore, the patient showed dysplastic monocytosis (data not shown) and an extensive defect of HLA-E expression in both the monocytes and PMN in PB.

The two PB populations evidenced only a very low staining for HLA-E (Figure 7A).

In contrast, the monocytes and PMN are positive for HLA-E in BM (Figure 7B). Intriguingly, the BM monocytes appear to express HLA-E as large distribution (see pick 2 in Figure 7B) and at higher level if compared to PMN (Figure 7B).

In this regard, monocytes are divided into two subpopulations (Figure 7C): one expresses at lower intensity the HLA-E and is $CD14^+CD56^-$, while the second one shows higher level of the molecule and is $CD14^+CD56^+$.

In addition, the expression of HLA-I on lymphocytes, monocytes, and PMN was similar between BM and PB (Figure 7D). The percentage of $CD14^+$ monocytes co-expressing the CD56 was expanded in BM (more than 30% of monocytes) than in periphery (<2% of PB monocytes) (data not shown).

Natural killer expansion (more than 35% of lymphoid cell in BM and PB compartment) carried several NK receptors (CD335, CD337, CD244, CD158e1/e2, CD158/p70, CD94/NKG2A, CD94/NKG2C), suggesting that NK cells are polyclonal (data not shown).

The BM and PB NK cells were highly positive for two HLA-E receptors; in particular, the $CD3^-CD56^+$ cells expressing CD94/NKG2A were the 37% and CD94/NKG2C the 43% (data not shown). Treg percentage and CD54 expression on both the $CD4^+$ and $CD8^+$ T Lymphocytes were similar to BM and PB, and the percentages were comparable to healthy donors (data not shown). Finally, the TCR repertoire of T lymphocytes appeared to be polyclonal in BM and PB, without TCR-Vβ skewing of one or more T clones.

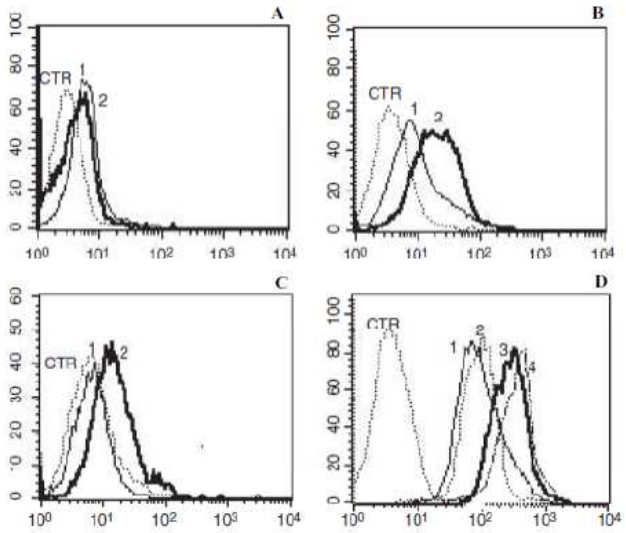

Figure 5. Flow cytometry analysis. Panel A: surface expression of human leukocyte antigen (HLA)-E on polymorphonuclear cells (PMN) (1) and Monocytes (2) in peripheral blood (PB); Panel B: surface expression of HLA-E on PMN (1) and Monocytes (2) in bone marrow (BM); Panel C: surface expression of HLA-E on CD14+ CD56- (1) and CD14+CD56+ (2) Monocytes in bone marrow; Panel D: surface expression of HLA-I on PMN from BM (1) and PB (2) and on Monocytes from BM (3) and PB (4). The staining for Isotype mAb control is reported in all the panels (CTR). PB and BM PMN and monocyte populations were gated by FSC and SSC parameters and by CD45, CD14, CD33, CD56 labelling.

Immune tolerance in chronic infectious diseases

The aim of the study has been to investigate the immune profile of dogs naturally infected by *L. infantum*. To address the immune regulatory networks involved in Canine Leishmaniosis, lymphocyte T cell subsets, peripheral Treg levels and the presence of pro-inflammatory T cells have been assessed, in a cohort of 45 *Leishmania* infected dogs (LD), compared with 30 sex/age matched healthy animals, by using immune fluorescence and flow cytometry detection. Leishmaniotic dogs have been categorised according to their clinical-pathological status and their serological specific anti-*Leishmania* antibody titer.

Figure 8 shows the analysis strategy of immune phenotype performed to identify the helper ($CD4^+$ $CD3^+$), the cytotoxic ($CD8^+$ $CD3^+$) T lymphocytes, as well as the level of theTreg sub-population.

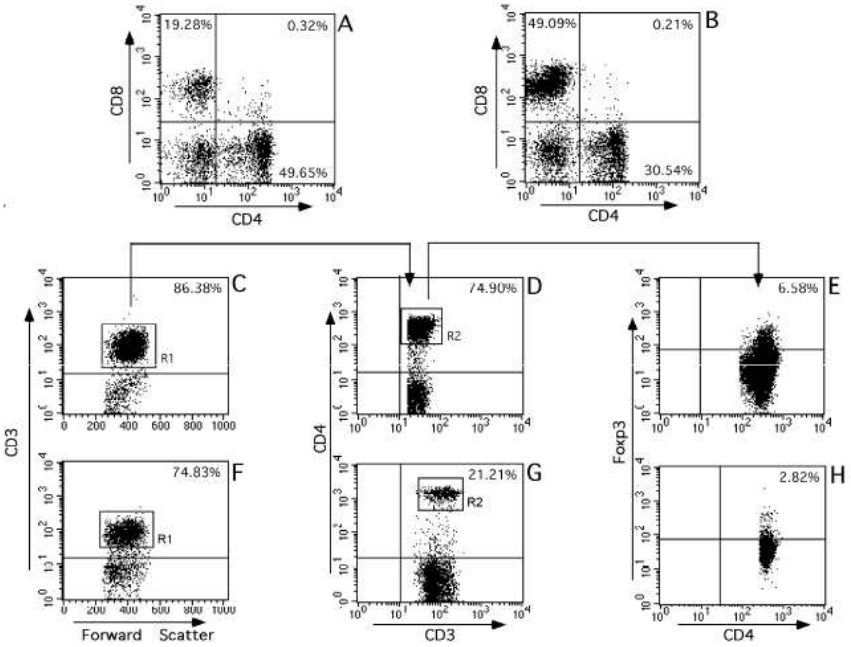

Figure 8. Flow cytometry analysis strategy for phenotype studies. Panel A and B refer typical staining profiles for CD4+ T and CD8+ T cells obtained in a healthy control and a Leishmania infected animal, respectively; C–E Panels show the analysis strategy employed to identify CD4+Foxp3+ Treg subset in PBMC sample obtained from a control dog. As shown, T cell region (defined by CD3 staining as R1) has been employed to identify the CD4+ T cell subset (R2); in this region the presence of Foxp3 transcription factor allowed the identification of the CD4+Foxp3+ Treg subset. D–H Panels show a typical analysis for CD4+Foxp3+ Treg subset in a Leishmania infected dog.

As shown in Figure 9A, Leishmania infected dogs (LD) are characterised by significant increase ($p<0.0001$) of the percentage of cytotoxic T cell effectors ($CD8^+CD3^+$) compared with healthy controls (67.47 ± 1.85 in LD vs 44.2 ± 2.5 in healthy controls). This observation has been confirmed by the significant decrease of the percentage (31.94 ± 2.05 in LD vs 55.76 ± 2.36 in controls; $p<0.0001$) of $CD4^+CD3^+$ T cell subset (Figure 9C). No significant differences have been observed considering the occurrence of a High versus Low IFAT titer (LD-H vs LD-L) as well as by the presence of a symptomatic versus an asymptomatic disease (LD-S vs LD-A). Panels B and D of the Figure 9 show the comparison of the cell count obtained in the different animal groups. As shown, the significant increase in $CD8^+CD3^+$ T cells (1224 ± 135 in LD vs 366.9 ± 17 in controls; $p<0.0001$) is confirmed in all the disease subgroups in comparison with healthy control animals (Figure 9B). The data on $CD4^+CD3^+$ T cells showed a significant decrease (564.5 ± 55.6 in LD vs 731 ± 26.19 in controls; $p<0.05$) of this cell subset in comparison with controls in all the LD animals, except the LD-A sub-group (Figure 9D) in which no significant differences have been observed with controls (701 ± 59 in LD-A vs 731 ± 26.19 in healthy animals). Notably, a slight no significant increase in the

number of $CD3^+$ lymphocytes have been observed in the Leishmania infected animal in comparison with controls (data not shown).

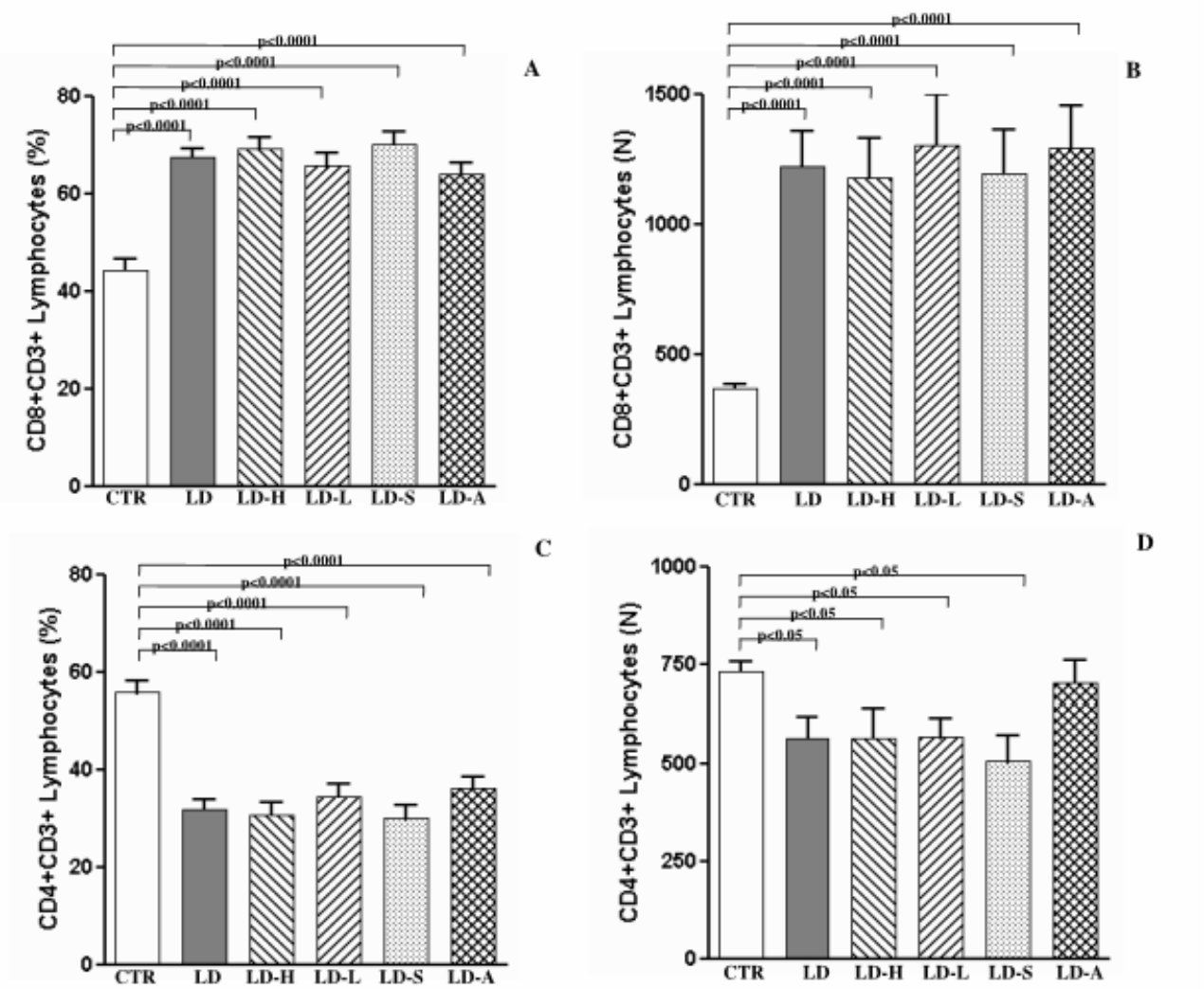

Figure 9. Significant increase of CD8+ T lymphocyte subset characterises Leishmania infected dogs. Panel A and B refer percentage and count (106/L) of CD8+CD3+ lymphocytes in peripheral blood of healthy animals (CTR; white column); Leishmania infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross lined column); Panel C and D refer percentage and count (106/L) of CD4+CD3+ lymphocytes in peripheral blood of the dogs categorised as previously indicated. Statistical analysis has been performed by using Mann–Whitney test. P values are indicated only for significant comparisons.

Figure 10 shows that a significant difference (0.53 ± 0.04 in LD vs 1.83 ± 0.15 in controls; $p<0.0001$) in the $CD4^+/CD8^+$ ratio characterises our cohort of Leishmania infected animals. This observation has been confirmed in all the sub-groups of Leishmania infected animals, regardless of the IFAT titer or the occurrence of a symptomatic versus asymptomatic disease.

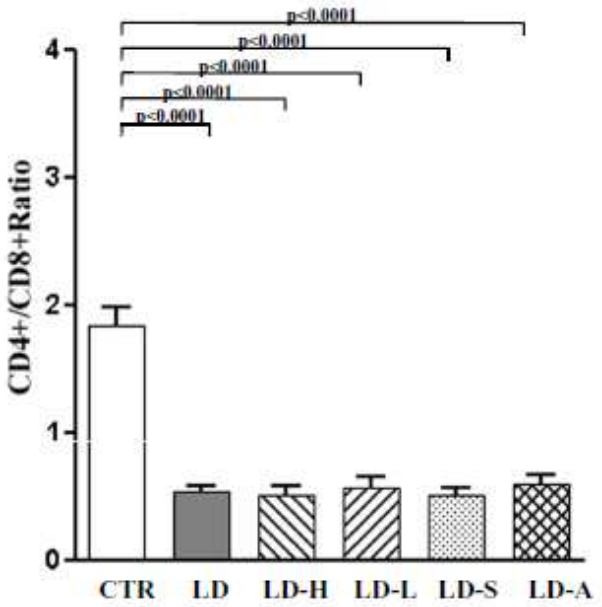

Figure 10. CD4+/CD8+ ratio is significantly reduced in Leishmania infected dogs. Referred values have been observed in peripheral blood of healthy animals (CTR; white column); Leishmania infected dogs (LD; grey column); infected animals with an IFAT titer >1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Statistical analysis has been performed by using Mann– Whitney test. P values are indicated only for significant comparisons.

Figure 11 refers to the comparative analysis of Treg level in the peripheral blood of the pathological cohort and control group. As shown, significant reduction of the percentage of Treg (Fig. 11A) in the peripheral blood of the Leishmania infected cohort has been observed (5.39 ± 0.72 in LD vs 7.17 ± 0.63 in controls; $p<0.05$). As shown, the reduced Treg percentage significantly correlates with the occurrence of a high IFAT titer (4.35 ± 0.95 in LD-H vs 7.17 ± 0.63 in controls; $p<0.01$). Notably, when the Treg count was analysed in the pathological cohort versus controls (Fig. 11B), the difference appeared more consistent, regardless of the pathological subgroup considered (12.74 ± 1.59 in LD vs 27.79 ± 1.94 in controls; $p<0.0001$; 13.28 ± 2.3 in LD-H; $p < 0.001$; 11.80 ± 1.2 in LD-L; $p<0.0001$; 12.78 ± 2.16 in LD-S; $p<0.0005$; 12.77 ± 1.18 in LD-A; $p<0.0001$). Unlike $CD4^+$ Treg that are largely defined by the expression of Foxp3, Foxp3 expression in $CD8^+$ clones was not correlated with their suppressive activity (Hu et al., 2012, 2013). In this study, we checked for Foxp3 expression $CD8^+$ effectors, but very faint expression was observed in both controls and Leishmania infected dogs (data not shown).

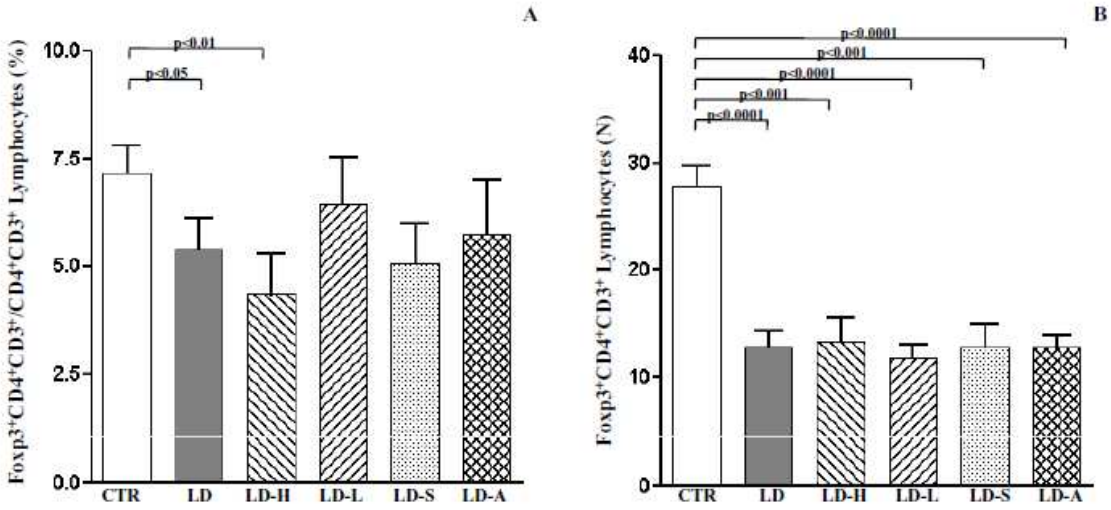

Figure 11. Significant reduction of percentage and Treg count have been observed in Leishmania infected dogs. Panel A and B refer percentage and count (10^6/L) of CD4+CD3+Foxp3+ (Treg) cells in healthy animals (CTR; white column); Leishmania infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Statistical analysis has been performed by using Mann–Whitney test. P values are indicated only for significant comparisons.

The cytokine profile has been largely described as relevant for Leishmania spreading control (Chamizo et al., 2005; Carillo and Moreno, 2009; Boggiatto et al., 2010). In order to assess whether the immunological profile by us described (high $CD8^+CD3^+$ T lymphocytes and reduced Treg cells), might be related with the induction of a specific cytokine profile in T lymphocytes, we analysed the IFN-γ and IL-4 production in T lymphocytes of LD animals as compared with healthy controls by immunofluorescence and flow cytometry detection. The analysis has been performed after an ON culture of PBMC, isolated from the peripheral blood of the animals, with Medium or PMA plus Ionomycin. This experimental approach is expected to allow the analysis ex vivo of the cytokine profile acquired by the T cells in vivo (Terrazzano et al., 2005; Olsen and Sollid, 2013). As shown in Figure 12, the percentage of T_H1 T cells (specifically producing IFN-γ and negative for IL-4) in LD animals is significantly increased as compared with controls ($p<0.05$) regardless of the grouping criteria used. No significant difference between the controls and the infected cohort was observed in the basal production of IFN-γ, detected in Medium cultures.

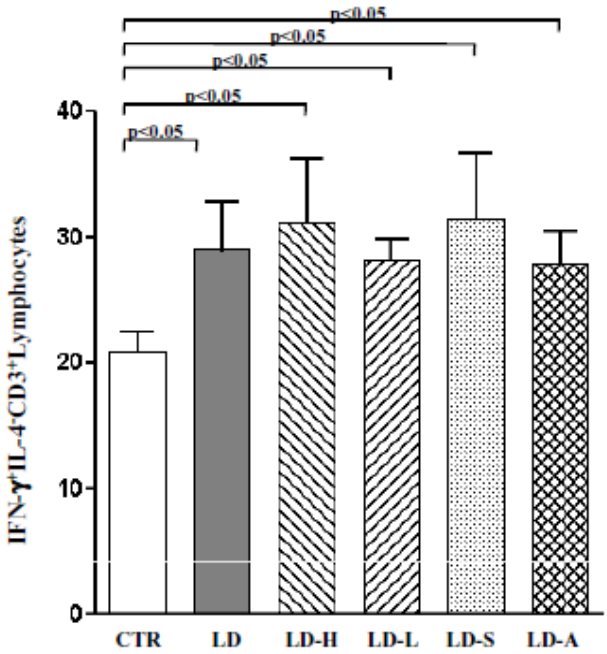

Figure 12. Significant increase of IFN-γ+IL-4-CD3+ T lymphocytes characterises Leishmania infected dogs. Referred values have been observed in peripheral blood of healthy animals (CTR; white column); Leishmania infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L; left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Statistical analysis has been performed by using Mann–Whitney test. P values are indicated only for significant comparisons.

The same model of natural chronic infectious disease, CL, was used to study whether administration of a specific pet food, supplemented with nutrients selected for their putative immune-modulating properties and rich in essential fatty acids, could modulate immunological profile of CL infected dogs. Thus, Lymphocytes T cell subset, peripheral Treg levels and the presence of pro-inflammatory T cells were evaluated at diagnosis and along a one year follow up in Leishmania infected dogs, submitted to standard anti-Leishmania pharmacological treatment alone (Group 1) or associated with the above specified diet (Group 2). To investigate whether diet administration during pharmacological anti-Leishmania treatment cold affect immunological profile of CL animals, analysis of $CD4^+/CD8^+$ ratio was performed.
We analysed CD4/CD8 ratio in dogs along the 12-months of follow-up. As indicated in Figure 13, we confirmed the significant decrease of $CD4^+/CD8^+$ ratio at T0 in CL dogs when compared to healthy animals, as by us already described (Cortese et al., 2013). The observed increase of $CD8^+$ T cells explains the decrease of $CD4^+/CD8^+$ ratio. This alteration remained substantially unmodified along the follow up in both Group 1 and Group 2.

Indeed, sick dogs maintained a significant reduction of $CD4^+/CD8^+$ ratio in comparison to control animals at all the time points.
Moreover, when the analysis specifically focused on $CD4^+/CD8^+$ ratio in the Group 1 and Group 2, significant differences between the groups were observed along the follow-up. As shown, an increase of $CD4^+/CD8^+$ ratio was revealed in Group 1 at T3 (0.81±0.09; $p<0.001$), while at T6 (0.58±0.08) and at T12 (0.31±0.02) the values substantially resembled to those observed at T0 (0.43±0.05). At variance, the trend of increase in $CD4^+/CD8^+$ ratio at T3 (0.73±0.08 versus 0.46±0.04) was maintained at T6 (0.71±0.08) and T12 (0.74±0.09) in Group 2. Notably, these values remained steadily and significantly lower than in healthy dogs (Figure 13).

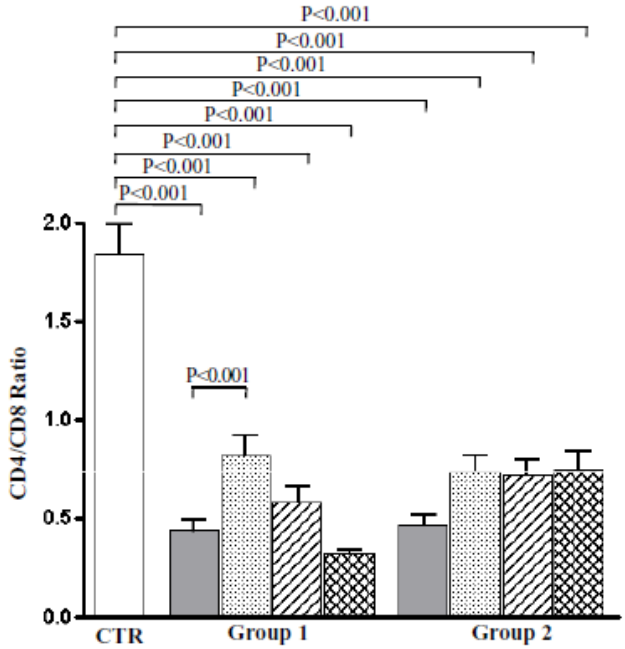

Figure 13. The significant CD4/CD8 ratio reduction, observed in *Leishmania* sick dogs as compared with controls, is maintained in a one year follow up in both Group 1 and Group 2 dogs, regardless the association of the pharmacological treatment with an immune-modulating diet. Values indicate the CD4/CD8 ratio observed in healthy animals (white column), in sick dogs at T0 (grey column), T3 (dotted column), T6 (right striped column) and T12 (crossing line column) in Group 1 and Group 2 animals, as indicated. Results were considered significant at $p<0.05$.

As shown in Figure 14, a significant reduction in the percentage of Treg of all sick dogs was observed at T0. This result confirms our previous observation on Treg levels in CL (Cortese et al., 2013). A slight recovery of Treg percentage was observed only at T3 in Group 1, while this effect disappeared at T6 and T12. In contrast, it is worth noting that percentage of Treg became similar to healthy animals in Group 2 at T3, T6 and T12 (Figure 14). Indeed, the

differences between the Treg percentages between healthy animals and Group 2 dogs substantially disappeared.

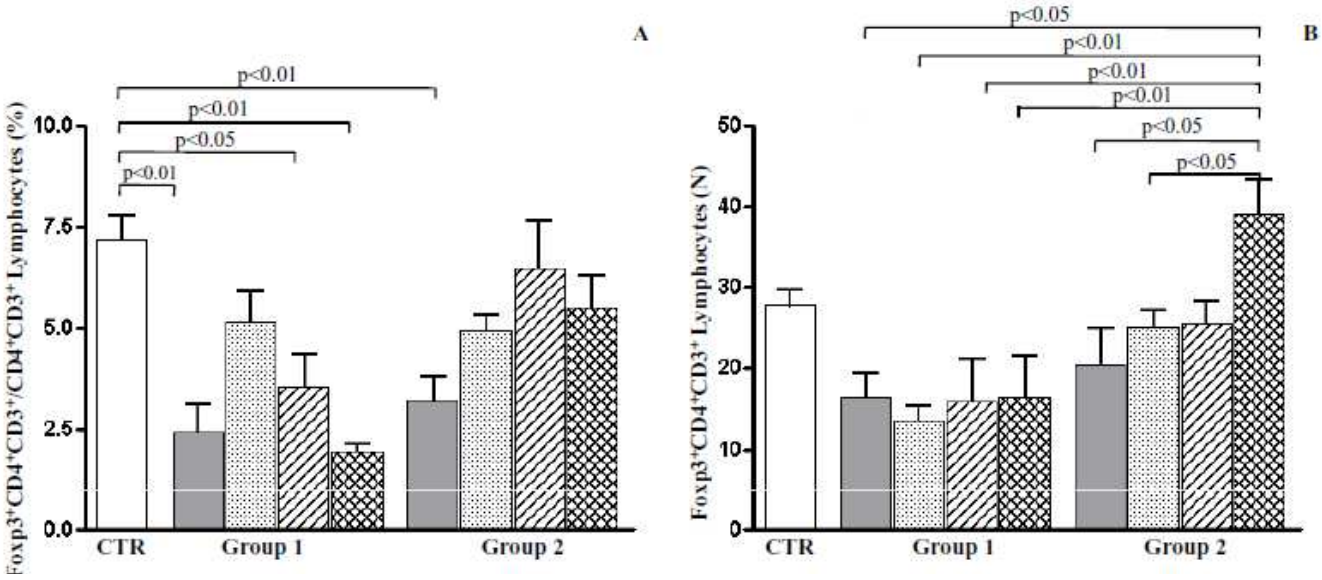

Figure 14. Association of the pharmacological treatment with an immune-modulating diet restores Treg percentage and count in *Leishmania* sick dogs. Panel A and B show the percentage and count (x10-6/L) of Foxp3+CD4+CD3+ 18 (Treg) cells in healthy animals (white column) in sick dogs at T0 (grey column), T3 (dotted column), T6 (right striped column) and T12 (crossing line column) in Group 1 and Group 2 animals, as indicated. Results were considered significant at $p<0.05$.

This result strongly suggests that the occurrence of a quite stable recovery of Treg was correlated to the immune-modulating diet administration. Similar results were obtained considering Treg number (data not shown).

In addition, anti-Leishmania treatment alone was unable to modify Treg level in CL. At variance, the combination of drug with the potential immune-modulating diet seems to be associated with a significant increase of Treg population that reaches normal values in Group 2.

Moreover, we asked if anti-Leishmania therapy alone and/or associated with diet modification could affect T_H1 activity in CL. As shown in Figure 15, sick dogs showed a significant increase of T_H1 cells at T0 as compared with control animals, regardless the group assignment. This result is in accordance with our previous data on CL (Cortese et al., 2013). The comparative analysis of sick dogs with healthy controls revealed the occurrence of a significant decrease of Th1 cells from T3 to T12 in Group 1, although a trend of increase was observed at T6 and T12. At variance, Group 2 animals showed a progressive decrease of T_H1 cells, whose levels became similar to healthy controls at T6 and T12.

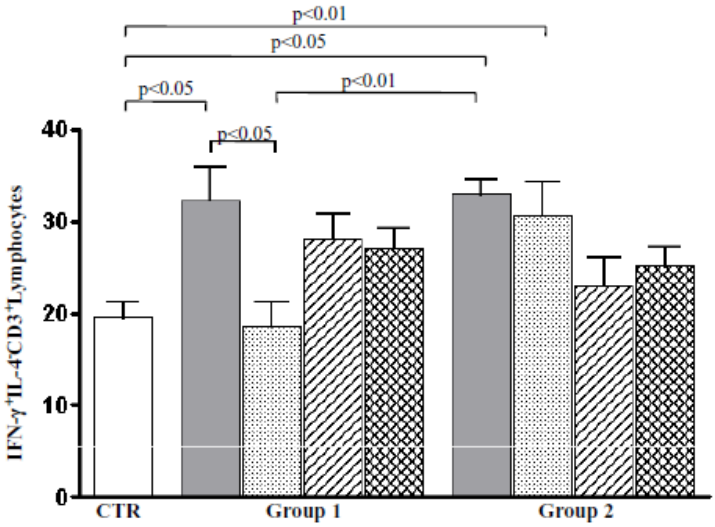

Figure 15. Association of the pharmacological treatment with an immune-modulating diet significantly modulates the presence of IFN-γ+ IL-4-CD3+ T lymphocytes in *Leishmania* sick dogs in a one year follow up. Values indicated the percentage of IFN- γ + IL-4-CD3+ T lymphocytes in healthy dogs (white column), sick dogs at T0 (grey column), T3 (dotted column), T6 (right striped column) and T12 (crossing line column) in Group 1 and Group 2 animals, as indicated. Results were considered significant at $p<0.05$.

T cell activation and SOD1-dependent ROS modulation

The models described above, frame two pathological conditions in which on the one hand we have an inflammatory microenvironment that favours the mechanisms of selection of pathological hematopoietic clones (PHN and MDS), on the other the same inflammatory condition is exploited by the physiological system in order to control the infection in dogs naturally infected with *Leishmania infantum*. In both cases the cells of adaptive immunity play a fundamental role. Thus, we focused on the role of oxidative balance regulation in TCR-dependent T cell activation, as a possible tool to modulate antigen-dependent T cell activation in physiological and pathological conditions.

Reactive oxygen species (ROS) behave as second messengers in signal transduction for a series of receptor/ligand interactions. A major regulatory role is played by hydrogen peroxide (H_2O_2), more stable and able to freely diffuse through cell membranes. Copper-zinc superoxide dismutase (CuZn-SOD)-1 is a cytosolic enzyme involved in scavenging oxygen radicals to H_2O_2 and molecular oxygen, thus representing a major cytosolic source of peroxides. Previous studies suggested that superoxide anion and H_2O_2 generation are involved in T cell receptor (TCR)-dependent signalling. To investigate whether cytosolic SOD-1 might be part of the molecular network involved in TCR triggering, the SOD-1 intracellular level and localization, as well as SOD-1 microvesicle secretion, was analysed in TCR-triggered human T lymphocytes.

To investigate whether antigen-dependent T cell activation induces SOD-1 production and extracellular secretion, we measured the release of this enzyme in supernatants of PBMC cultured from 15 min to 18 h in presence of Medium

alone or with anti-CD3 mAb, that induces the TCR triggering mimicking antigen-dependent activation of T lymphocytes. As shown in Figure 16A, human PBMC cultures secreted small amount of SOD-1 that remained substantially stable from 15 min till to 18 h of culture. Anti-CD3 treatment slightly, but significantly ($p<0.05$), increased such basal secretion. The increment was detectable after 4 h of culture and reached the highest level after 18 h of activation. This effect was independent on cell damage, always evaluated by PI labeling (Figure 16B) and significantly correlated with TCR dependent activation, as revealed by the up-regulation of the activation molecule CD69 (Figure 16C). Therefore, TCR-triggering was associated with the induction of SOD-1 secretion in human lymphocytes.

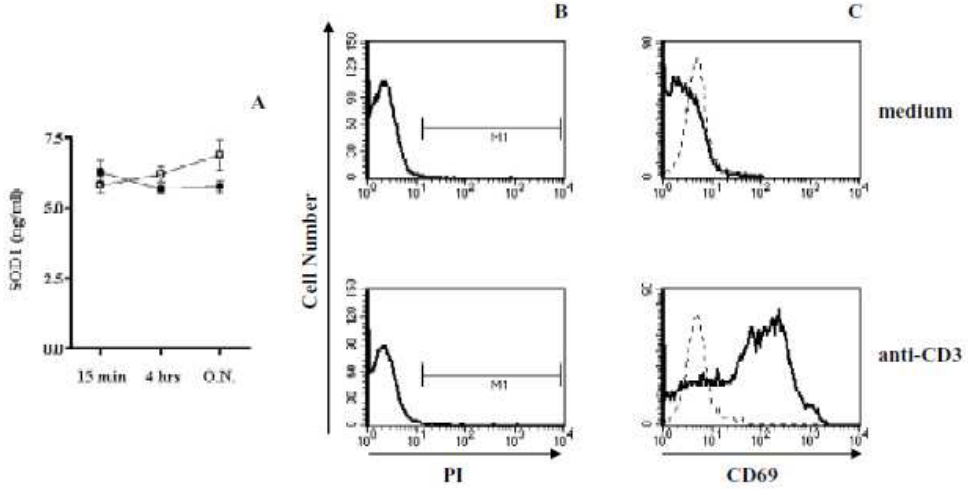

Figure 16. SOD-1 concentration in anti-CD3 triggered cultures of human lymphocytes. (A) SOD-1 amount in supernatants of PBMC cultured in the presence ofMedium(black squares) or anti-CD3 (white squares). SOD-1 concentrations were analyzed in undiluted samples by using ELISA assay; results were normalized for total protein content of the tested sample. Each point refers mean value obtained in five independent experiments; error bars indicate SEM. * indicates the occurrence of statistically significant ($p<0.05$) higher SOD-1 concentration in anti-CD3 treated cultures. (B and C) PI and CD69 labeling of PBMC cultured O.N.with Medium or anti-CD3, as indicated. Results refer to one of five independent experiments. As shown (B), no significant differences have been observed in PI staining profiles of PBMC cultured withMediumand anti-CD3; (C) CD69 staining profile (bold line) of PBMCculturedwithMediumor anti-CD3, as indicated; broken line indicates isotype control; as shown, activated PBMCs were characterized by significant increase of the activation molecule CD69.

To ascertain whether such secretionwas sustained by up-regulation of SOD-1 gene transcription, we analyzed SOD-1 specific mRNA. As shown in Figure 17, an increase of more than 70% of SOD-1 specific mRNA has been observed in the cultures treated with anti-CD3 mAb as compared with resting cells. Therefore, antigen-dependent T cell activation induced SOD-1 secretion that was sustained by the increase of SOD-1 transcription in the whole PBMC population.

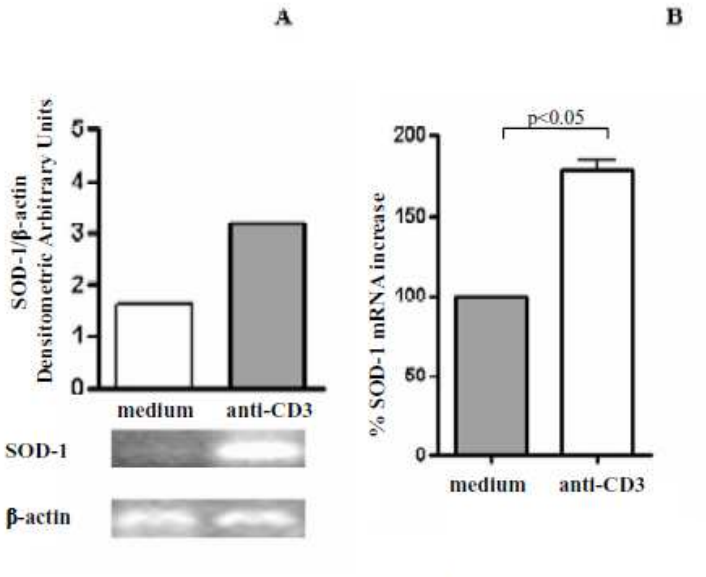

Figure 17. Anti-CD3 treatment induces significant increase of SOD-1mRNA in human lymphocytes. (A) Densitometric arbitrary units ratio between SOD-1 and beta actin in Medium and anti-CD3 treated cultures. Results refer one of five independent experiments. mRNA was measured by RT-PCR, as detailed in Material and methods section. (B) Comparative analysis of SOD-1 mRNA percent increase in all the five experiments performed. For each experiment mRNA amount in Medium cultured PBMC was considered the reference value (100) for calculation of percent increase in the anti-CD3 treated culture. As shown, a mean increase of more than 70% of SOD-1 specific mRNA has been observed in anti-CD3 treated PBMC. Error bars indicate SEM. Statistical analysis has been performed by using Paired t test.

To evaluate whether SOD-1 export might be part of micro-vesicle production upon TCR triggering, we purified the micro-vesicle fraction from the supernatants of 18-hour cultures of PBMC and purified T cells. TCR triggering of PBMC was performed with anti-CD3mAb. In order to mimic costimulatory signals, usually mediated by accessory cells, a combination of anti-CD3/anti CD28 beads was used to fully activate purified T cells.

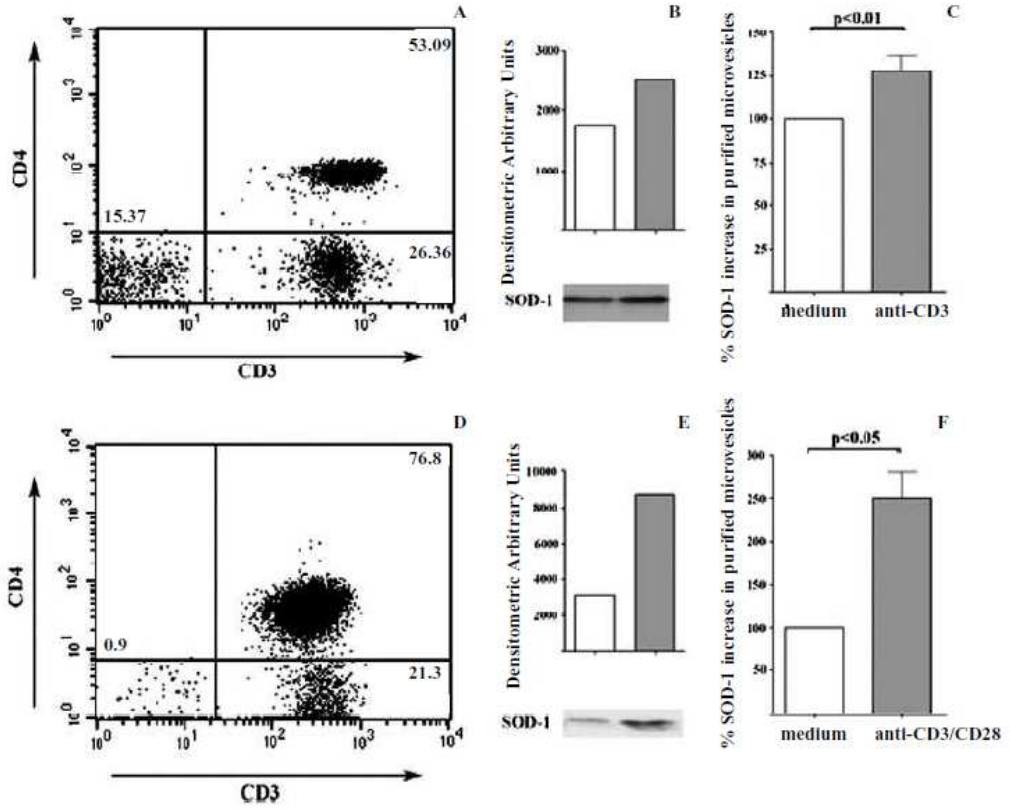

Figure 18. TCR-dependent T cell activation increases SOD-1 containing microvesicle secretion by human T lymphocytes. (A) Flow cytometry analysis of PBMC population. As shown, T lymphocytes (CD3+ cells) represent less than 85% of the total population; (B)western blot of enriched membrane vesicle fractions, isolated fromculture supernatants ofMedium(white column) and anti-CD3 treated PBMC (gray column), as detailed inMaterial and methods section. Densitometric analysis shows increased presence of SOD-1 in TCR triggered PBMC. Results refer one representative experiment of the six performed; (C) Analysis of percent increase of SOD-1 containing microvesicle in supernatants derived from anti-CD3 treated PBMC in six experiments. For each experiment, SOD-1 amount in Medium cultured PBMC was considered the reference value (100) for calculation of percent increase in the anti-CD3 treated culture. As shown, amean increase of 25% was observed in themicrovesicle enriched fraction obtained from the supernatants of anti-CD3 treated PBMC. (D) Flow cytometry analysis of a typical purified T cell population isolated by using negative selection strategy, as indicated in theMaterial and methods section. As shown, T lymphocytes (CD3+ cells) representmore than 98% of the total population; (E)western blot of enrichedmembrane vesicle fractions, isolated fromculture supernatants of Medium(white column) and anti-CD3/antiCD28 treated T cell cultures (gray column), as detailed inMaterial andmethods section. Densitometric analysis shows increased presence of SOD-1 in the sample obtained fromTCR triggered T cells. Results refer one representative experiment of the three performed; (F) analysis of percent increase of SOD-1 containing microvesicle in supernatants derived from anti-CD3/anti-CD28 treated T lymphocytes in three experiments. For each experiment, SOD-1 amount in Medium cultured T cells was considered the reference value (100) for calculation of percent increase in the anti-CD3/ anti-CD28 treated cultures. As shown, an increase of more than 120% was observed in the microvesicle enriched fraction obtained from the supernatants of anti-CD3/anti-CD28 treated purified T cells. Error bars indicate SEM.

Western blot comparative analysis of SOD-1 has been always performed on aliquots of enriched microvesicle fractions containing 40 μg of total proteins. Statistical analysis has been performed by using Paired t test.

Figure 18 shows western blotting analysis of SOD-1 in the enriched membrane vesicle fractions. Figure 18A–B reports one representative experiment, while Figure 18C refers the analysis of data obtained in all the six experiments performed in PBMC cultures. The comparison revealed the occurrence of a mean 25% increase of SOD-1 content in the enriched membrane vesicle fraction obtained from anti-CD3 treated lymphocytes ($p<0.01$). As shown, in the enriched membrane vesicle fraction isolated from anti-CD3/anti-CD28 treated T cells, a more consistent increase of SOD-1 content was observed (Figure 18D–F). Indeed, a mean increase >120% of SOD-1 content was evidenced in three experiments performed with purified T lymphocytes ($p<0.05$).

To investigate whether other cell populations, present in PBMC, contribute to SOD-1 production and secretion in response to TCR-triggering, we analyzed intra-cellular SOD-1 levels in the T cell subset and in the "non-T" counterpart in a mixed context. This evaluation was performed by the combination of immune fluorescence and flow cytometry detection, to preserve the biological complexity of antigen-dependent T cell response and allow specific detection of SOD-1 in the T cell subset and in "non-T" population (Figure 19A). As shown (Figure 19B), very low amount of intracellular SOD-1 was observed in all the "resting" lymphocytes (T and non-T cells). After TCR-triggering, the up-regulation of SOD-1 intracellular level was observed only in T lymphocytes if compared to non-T cells (Region 1 versus Region 2 in Figure 19C).

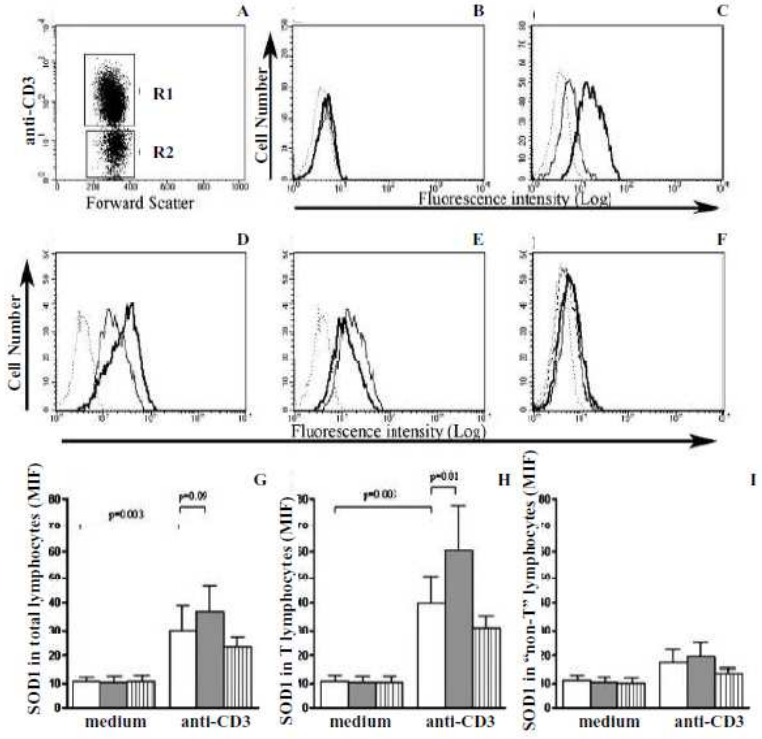

Figure 19. Significant increase of intracellular SOD-1 and BFA-dependent SOD-1 export can be specifically demonstrated in anti-CD3 triggered human T cells. (A) Gating criteria for the identification of the "T cell subset" (R1) and of the "non-T population" (R2) in a PBMC culture; (B) SOD-1 staining profile of T cells (bold line) and "non-T population" (plain line) in O.N. Medium cultured PBMC. Dotted lines show the isotype control. As shown, similar, very low amount of intracellular SOD-1 characterizes both population. (C) SOD-1 staining profile of T cells (bold line) and "non-T population" (plain line) in anti-CD3 cultured PBMC. Dotted lines showthe isotype control. As shown, specific increase of SOD-1 intracellular content can be observed in the T cell subset (bold line) as compared with the "non-T" counterpart (plain line); (D) SOD-1 staining in T cell population (R1) culturedwith anti-CD3 alone (plain line) or in the presence of BFA (bold line). Dotted lines show the isotype control. As shown, anti-CD3/BFA co-culture increases intracellular SOD-1 content in T cell population. (E) SOD-1 staining in T cell population (R1) cultured with anti-CD3 alone (plain line) or in the presence of methylamine (bold line). Dotted lines show the isotype control. As shown, no significant changes in SOD-1 intracellular levels can be observed in anti-CD3/methylamine co-cultures. (F) SOD-1 staining profiles of "non-T population" (R2) in anti-CD3 cultures (plain line), in BFA/anti-CD3 co-cultures (bold line) or in methylamine/anti-CD3 cultures (broken line). Dotted lines show the isotype control. As shown, no significant changes in SOD-1 intracellular content have been observed. Results refer one of 4 independent experiments. (G–I) Column graphic reports the means of SOD-1 fluorescence intensities observed in 4 independent experiments in the whole PBMCpopulation (G), in the T cells (H) and in the "non-T" population (I).White columns indicate SOD-1 levels in cells, cultured as indicated; gray columns indicate SOD-1 in BFA co-treated cultures; striped columns refer SOD-1 in methylamine co-treated cultures.

To investigate the pathway involved in SOD-1 secretion, we analyzed intracellular SOD-1 retention in T cells in the presence of BFA (Figure 19D) or methylamine (Figure 19E) described to block ER/Golgi intracellular network and cell endocytosis, respectively (Russo M. et al., 2011; Thery C. et al., 2006). In this regard, BFA but not methylamine treatment induced significant increase of the enzyme content in T cells. No significant changes in intracellular levels of SOD-1 were observed in "non-T" cell population, in the same experimental conditions (Figure 19F). Notably, none extracellular SOD-1 increase was detected by ELISA in anti-CD3 cultures incubated with BFA (data not shown). Figure 19G–I reports the statistical comparisons of SOD-1 intracellular amount in PBMC (Figure 19G), in T cells (Figure 19H) and "non-T" cells (Figure 19I), as evaluated by considering the mean fluorescence intensity (MFI) values obtained in all the 4 experiments performed. As shown, no significant changes in SOD-1 levels were observed in the absence of TCR triggering. Thus, SOD-1 amountwas strictly dependent on antigen mediated T cell activation. Indeed, anti-CD3 treatment significantly increased SOD-1 intracellular level ($p<0.005$) in thewhole lymphocyte population (Figure 19G). This effect specifically characterized the T cell subset ($p<0.005$; Figure 19H), while no differences were observed in the "non-T" population (Figure 19I). Moreover, the block of ER/Golgi network, mediated by BFA treatment, was observed to mediate significant ($p<0.05$) intra-cellular SOD-1 retention only in T lymphocytes (Figure 19H). Similar results have been obtained by anti-CD3/anti-CD28 triggering of purified T cells (data not shown). As control, intracellular

accumulation of Interferon-gamma was specifically detected in TCR triggered cultures treated with BFA (not shown).

We analyzed SOD-1 and TCR cellular localization by confocal microscopy after 2 min of culture in the presence of Medium alone or with anti-CD3. Figure 20 shows TCR and SOD-1 co-staining after 2 min of culture with Medium alone (Figure 20A) or anti-CD3 (Figure 20B and C). As expected, the homogeneous surface TCR distribution observed in resting T cells (Figure 20A) was completely changed by anti-CD3 triggering (Figure 20B and C). Indeed, significant TCR clustering (Figure 20B and C) characterized activated T cells. SOD-1 staining revealed a quite homogeneous intracellular distribution of the enzyme in resting T cells; staining profiles also confirmed the presence of SOD-1 at very low levels in human T lymphocytes (see Figure 19). Notably, confocal microscopy revealed that TCR triggering was able to induce a clustered distribution of SOD-1 enzyme (Figure 20B and C). Merged images clearly showed that TCR clusters have been
recruiting intracellular SOD-1, whose localization strictly reflected TCR distribution (Figure 20B and C). TCR/SOD-1 co-localization disappeared 20 min after anti-CD3 treatment (not shown). To preserve the physiological complexity, we chose to perform the analysis in PBMC population as a whole and we identified T cells by labelling with specific antibodies. In this model, TCR triggering is allowed by the physiological cross talk between T cells and autologous antigen presenting cells (APC). Notably, we never observed a TCR clustering decoupled from SOD-1 co-localization. No significant changes in SOD-1 intracellular localization were observed in “non-T” population after anti-CD3 triggering (not shown). Notably, SOD-1/TCR intracellular co-clustering was observed also in anti-CD3/anti-CD28 triggered purified T cells (not shown).

To investigate whether SOD-1/TCR co-localization in anti-CD3 activated T cells is dependent on ROS bioavailability, we performed experiments in the presence of the ROS scavenger NAC at 1 mM concentration. In this condition, TCR clustering was significantly reduced (from 70 to 95% in NAC/anti-CD3 co-cultures). As shown, NAC significantly inhibited both TCR and SOD-1 clustered localization (Figure 20D). Indeed, TCR was homogeneously distributed on cell membrane, similarly to what was observed in resting condition (Figure 20A). SOD-1 co-staining in anti-CD3/NAC treated lymphocytes also resembled basal images with the presence of small areas of faint cytosolic accumulation (Figure 20D). Merged images revealed a clear-cut distinct distribution of TCR and SOD-1 in anti-CD3/NAC treated T cells. Thus, ROS availability significantly affected activation-dependent TCR/SOD-1 re-localization in human T cells.

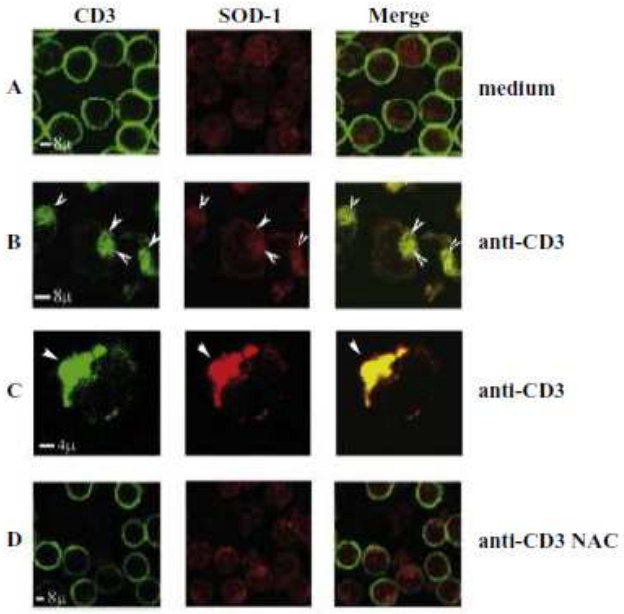

Figure 20. Anti-CD3 triggering induces ROS-dependent TCR and SOD-1 co-clustering in activated lymphocytes. (A) Confocal microscopy image of CD3 (green) and SOD-1 (red) in resting T cells. A homogeneous, distinct, membrane and intracellular distribution of the TCR and SOD-1 can be appreciated. (B and C) After 2 min of anti-CD3 treatment a clustered distribution of TCR can be observed (white arrows). SOD-1 distribution becomes strongly clustered and resembles that of TCR (white arrows). This cell re-localization is better showed in panel C where a single cell has been focused. Merged images show that TCR clusters recruit intracellular SOD-1, whose localization strictly reflects TCR distribution (white arrows). (D) anti-CD3/NAC co-incubation induces a homogeneous TCR surface distribution. SOD-1 localization also resembles basal images,with the presence of small areas of faint cytosolic accumulation. Merged images reveal a clear-cut distinct distribution of TCR and SOD1.

To ascertain whether ROS availability might also affect the cell-to-cell aggregation dependent on TCR triggering, we analyzed the effect of NAC incubation on early cell clustering (usually detectable after 45 min of incubation with anti-CD3). As shown in Figure 21, anti-CD3-sitimulation was able to induce cell aggregation after 1 h of treatment (Figure 21C). Such effect became more evident after 3 h of incubation (Figure 21G). Anti-CD3/NAC co-treatment severely impaired anti-CD3 induced cell clustering after 1 h of incubation (Figure 21D). Quantification has been performed by direct counting of cell aggregates, identified by the presence of at least 8 clustered cells, in the microtiter wells. Comparative analysis showed a cell aggregate inhibition of 83.64 ± 1.62 in anti-CD3/NAC co-treated cultures in 6 independent experiments; ($p<0.05$). This inhibition was transient and progressively decreased, likely mirroring the ROS scavenging activity of NAC. The inhibiting effect of NAC co-incubation completely disappeared after 6 h of anti-CD3/NAC cotreatment. NAC co-incubation was unable to mediate significant effects on cell viability and proliferation (not shown).

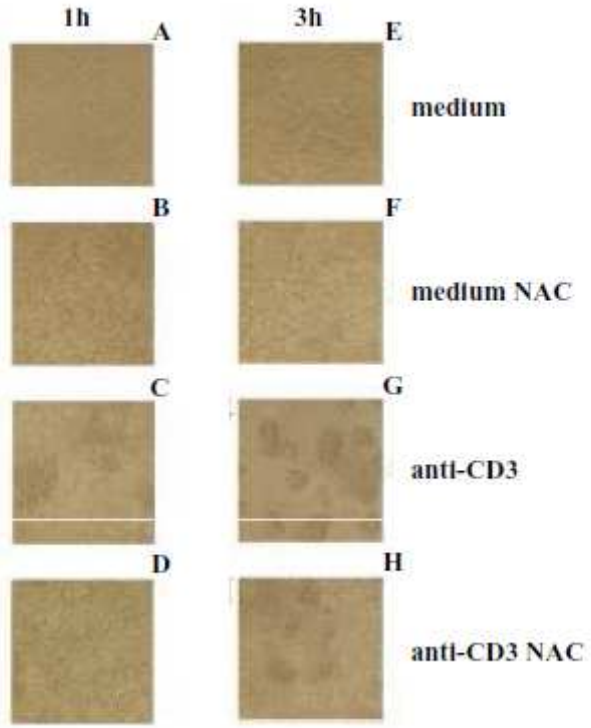

Figure21. NAC treatment inhibits early activation-induced aggregation of TCR triggered lymphocytes. Contrast phasemicroscopy images showing 1 and 3 hour cultures of PBMC incubated with Medium (A and E), NAC (B and F), anti-CD3 (C and G) or anti-CD3 and NAC (D and H). As shown, significant cell aggregation is observed in anti-CD3 triggered cells. Lymphocyte clustering is significantly inhibited in the anti-CD3/NAC co-treated cultures; (D) after 1 h of incubation. Quantification is performed by counting cell aggregates, identified by the presence of at least 8 clustered cells. Comparative analysis (not show) shows a 83.64 ± 1.62 inhibition of cell aggregates in anti-CD3/NAC co-treated cultures in 6 independent experiments; (p b 0.05). (H) After a 3 hour period the inhibiting effect of NAC treatment is observed to be lowered (55.35 ± 2.23 inhibition of cell aggregates in 6 independent experiments). Quite normal clustering of anti-CD3 treated PBMC is observed at longer culture time in anti-CD3/NAC co-treated cells (not shown). Results show one representative experiment of the six performed.

DISCUSSION and CONCLUSIONS

Paroxysmal Nocturnal Haemoglobinuria (PNH)

In the first work, we describe the occurrence of Treg reduction and NKTi increase in untreated PNH patients as well as the ability of Eculizumab therapy to selectively restore B and NKTi peripheral concentration without significant interference on NK and Treg numbers. These results confirm previous other and our data (Schubert et al. 1990; Alfinito et al. 1996; Richards et al. 1998) on the reduced number of B and NK cells in PNH also proposing that some of the immune alterations have to be considered as dependent on complement derangement. Indeed, the ability of Eculizumab to modify some of the alterations described in untreated patients, suggests their dependence on complement-mediated pathway/s.

The correction of complement hyper activation is unable to revert the immune-mediated mechanisms involved in the selection and dominance of PNH precursors (Luzzatto et al. 1997; Karadimitris et al. 2000; Luzzatto 2006). Indeed the size of PNH clone remains substantially unmodified in Eculizumab treated patients. The enhanced complement activation observed in PNH patients is due to absence of the GPI-linked CD55 and CD59 molecules cells and likely accompanied by an altered bio-availability of C3 and C5 activation fragments (Luzzatto et al. 2010). The blockade of C5 by Eculizumab (Hillmen et al. 2006; Schubert et al. 2008; Luzzatto et al. 2010) acts on this process, probably modifying the availability of complement fragments.

Interaction of C3 and C5 fragments with their cognate receptors on adaptive immune cells has been described to induce pleiotropic effector functions, (Carroll 2004b; Le Friec and Kemper 2009; Ricklin et al. 2010). Complement fragments regulate B cell proliferation, activation and trafficking (Carroll 2004a; Ottonello et al. 1999; Roozendaal and Carroll 2007) and finely tune T cell cytokine patterns (Longhi et al. 2006). In addition, C3 and C5 fragments also interfere with the stromal cell-derived factor-1 (SDF-1)/CXCR4 axis, crucial for BM homing regulation (Reca et al. 2003). Finally, NK and Treg alterations seem not to be influenced by Eculizumab, thus suggesting their independence on complement related pathways. Since similar alterations have been described in Aplastic Anaemia and Myelodysplastic Syndromes (Kiladjian et al. 2006; Kotsianidis et al. 2009; Alfinito et al. 2010), we can argue their involvement in the immune-mediated pathogenesis of BM failure disease that underlies PNH.

Taken in all our data suggest that the unbalanced complement activation on the surface of GPI-defective cells mediates significant alteration of immune effectors in PNH patients without significant interference with the immune-mediated mechanisms underlying the selection/expansion of GPI-defective haematopoiesis. Eculizumab treatment is able not only to correct the effect of Complement hyper-activation on red cells, but is also able to normalize complement-dependent alteration of immunological targets. This

observation suggests that changes in the immune-profile could be of some relevance for the amelioration of immune response in the PNH patients on Eculizumab treatment.

MyeloDysplastic Syndromes (MDS)

In the hypothesis that an altered immune profile might underlie the occurrence of CTL-dependent immune/mediated selection of the dysplastic compartment in MDS, the study focused on HLA-E and HLA-I molecules, as major elements in regulating NK and CTL recognition, respectively (Ruggiero et al. 2009; Lanier 2005; Terrazzano et al. 2007; Zinkernagel and Doherty 1974; Germain 1994; Ploegh 1998; Seliger et al. 2006).

Thus the analysis focused on granulopoietic population, as the more suitable compartment for studying BM selection processes. Our immune-based sub classification of MDS patients (Alfinito et al. 2010) is dependent on the occurrence of lower Treg percentage, increased BM recruitment of CTL and high CD54 expression on CD8 T cells in a sub-group of Low/Int-1 Risk patients. Low/Int-1 Risk MDS patients bearing the altered immune profile are characterized by both the decrease in HLA-I expression and the increased presence of HLA-I defective cells on peripheral PMN (Alfinito et al. 2010). In this context, the occurrence of immune-mediated selection processes has been suggested by the presence of BM precursors characterised by different expression levels of HLA-E as well as HLA-I in MDS patients.

We consistently found that the expression of HLA-E is lower in BM GC than in PB PMN population. Since HLA-E is the main inhibiting element for NK recognition, we hypothesise that NK could kill the lower HLA-E-expressing PMN precursors in BM and it could consent the migration into PB only of those cells expressing HLA-E at higher level. The expression of NK ligands during BM maturation (Nowbakht et al. 2005) and the presence of defects in both HLA-E and CD94/NKG2 molecule, the main HLA-E-NK receptor,have been described, in some haematopoietic diseases (Ruggiero et al. 2009; Majumder et al. 2006; Warren et al. 2003). Therefore a perturbation of such regulatory networks might generate the maturation of dysplastic PMN with significant modulation of HLA-E surface expression (Ruggiero et al. 2009).

Our study refers that both Low and Int-1 Risk MDS patients show significant decrease of HLA-I expression on peripheral PMN if compared with the BM counterpart. Notably, when patients were categorised according with their immune profile, only AIP patients significantly maintain a lower expression of MHC-I on peripheral PMN cells than in BM GC. This condition is conceivable with the hypothesis that a subpopulation of BM-PMN-precursors, characterised by high level of MHC-I molecules, could behave as preferential target for a deranged autoreactive T cell response. Indeed, CTL are expected to be less sensitive to PMN precursors expressing low level of MHC-I. These cells represent the only population leaving the BM as peripheral PMN in AIP MDS patients.

HLA-I absence has been associated with escape mechanisms to CTL recognition (Zinkernagel and Doherty 1974; Germain 1994; Ploegh 1998; Seliger et al. 2006). The recognition of neo-antigens expressed by dysplastic cell clones and an altered self-tolerance in BM (Sloand and Rezvani 2008; Barrett 2004; Sloand et al. 2008 might account for our observations. The study evidences a significant increase of PMN lacking HLA-I in Int-1 Risk MDS patients. Such evidence could point to CTL-mediated mechanisms acting in those patients with a more advanced MDS disease stage. Notably, when patients were categorised according with their immune profile, the increase of peripheral PMN lacking HLA-I expression was preferentially observed in AIP patients if compared with the NIP counterpart.

The immune-based classification criteria, by us proposed, seem to identify a subgroup of Low/Int-1 patients in whom CTL selection mechanisms are patho-genetically relevant.

The HLA-E and HLA molecules are part of the biological mechanisms involved in the selection of BM precursors. Such immune-dependent selection processes might become pathogenetically relevant in patients in which the immunological activation is deranged (AIP subgroup of our patients).

These results propose that the reduced presence of Treg in BM (Alfinito et al. 2010) might be unable to efficiently suppress autoreactive T clones that preferentially recognise self-antigens on PMN precursors expressing higher level of MHC-I molecules. The deranged immune response could be enhanced by the inflammatory BM microenvironment, described in MDS (Zeng et al. 2006). In this context, the cytotoxic activity of NK effectors that could recognise and kill (Lanier 20005) the PMN precursors expressing low level of HLA-E/MHC-I (Terrazzano et al. 2007) is not to exclude.

A model for immune-mediated selection is hypothesised in Figure 22. Ideally, the immune-dependent signals, allowing the maturation and migration of BM precursors in PB, might depend on expression of optimal levels of both HLA-E and HLA-I molecules that regulate NK and CTL recognition. These mechanisms could preferentially affect the selection of GC in MDS patients with a deranged adaptive response in BM (AIP group).

In support of our hypothesis are at least two evidences: (a) PB PMN cells of all patients in AIP group express higher HLA-E and lower HLA-I molecules (corresponding to B cell type in Figure 22); (b) we found a greater percentage of MHC-I negative PMN (the C cell type in our model) in Int-1, where the MDS progression is more advanced in respect to the Low Risk. Notably, when the patients have been categorised according to their immune-profile, this feature characterised the AIP subgroup.

This and our previous investigations over the immune alterations in MDS (Ruggiero et al. 2009; Alfinito et al. 2010) suggest the introduction of immune-based criteria in the classification of MDS patients. In particular, we aimed to better characterise those patients in which the immune/mediated mechanisms could be part of MDS pathogenesis. In this regard, it could be of some relevance the correlation between the expression of MHC-I/HLA-E

molecules and the selection of PMN dysplastic clones in MDS. Therefore, the expression of the activating and inhibitory receptors on NK effectors and their interplay with classical and non classical MHC-I molecules on haematological cell target need further investigation to better understand the molecular mechanism underlying the emergence of dysplastic clones in MDS.

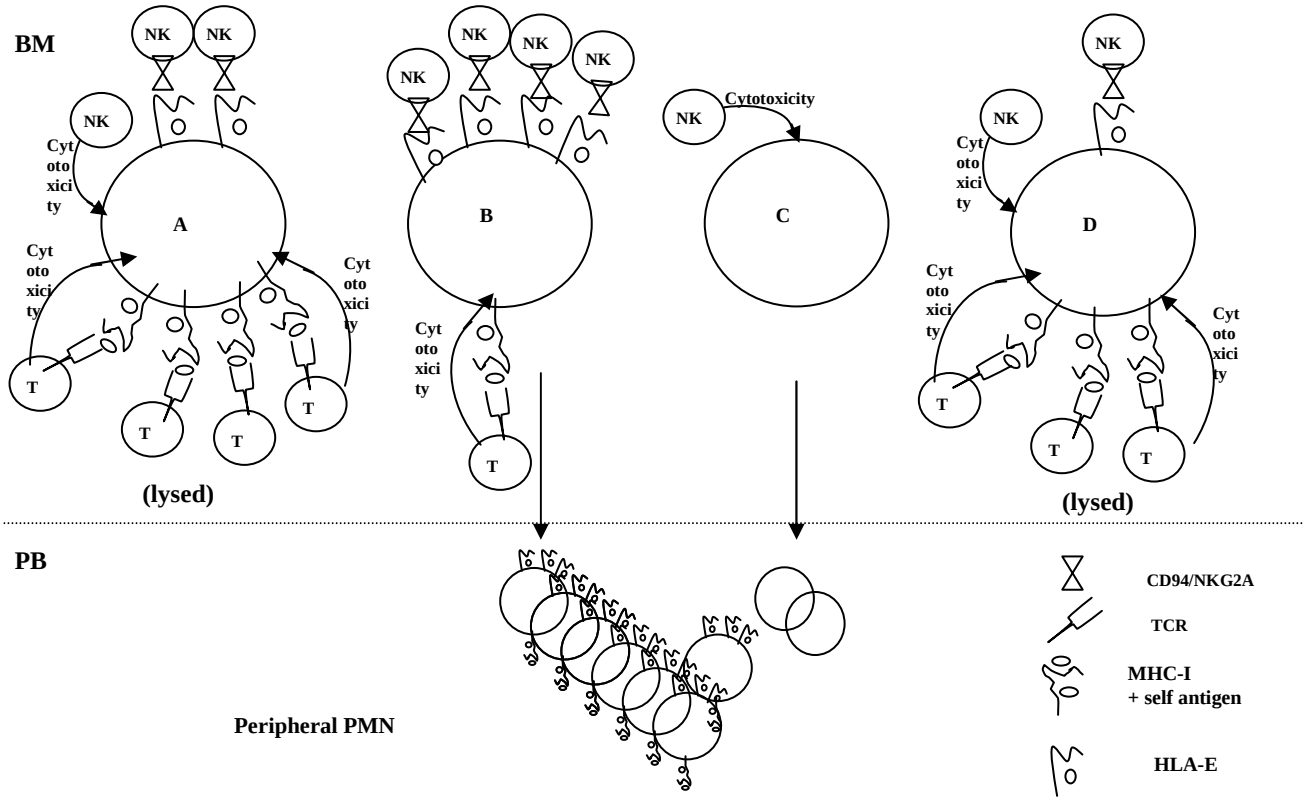

Figure 22 . Involvement of NK and CTL in the selection of dysplastic clones. BM polyclonal haematopoiesis (upper panel) with at least four GC cell types (from A to D). A and D cell types express high level of HLA-I and represent more susceptible targets for deranged CTL recognising self-antigens (indicated as T in the model). B and C cell types express low level the HLA-I or completely lack these molecules and are spared by CTL. NK effectors recognise the presence of HLA-E molecules by CD94/NKG2A receptor. This mechanism likely mediates the clearing of C and D cell types. In the presence of a deranged CTL response (AIP patients), the outcome of these interactions is the result of the rescue and the preferential migration/expansion in PB (lower panel in the model) of the B cell type expressing low HLA-I and high HLA-E. These conditions spare the B cell type from both NK and CTL lysis. The dominance of a CTL-dependent selection pressure might account the higher presence of D cell type in AIP group.

A special case of MDS patient shows thrombocytopenia, monocytosis and expansion of polyclonal NK cells, and normal karyotype. These features are accompanied by a defect of HLA-E expression on PMN and monocytes in PB. Notably, despite the similarities of this MDS case with the other by us observed for the likely involvement of HLA-I and HLA-E in fostering the myelodysplasia (Terrazzano et al. 2013), this patient seems to be substantially

unframed with the immunological criteria that we previously proposed (Alfinito et al. 2010). Indeed, the patient reveals no significant variation in the percentage of Treg or of expression of CD54 when compared with healthy controls. Nevertheless, the patient shows an intriguing analogy with previous patient (Ruggiero et al. 2009) for the presence of dysplastic monocytosis, mainly represented by $CD14^{+}CD56^{+}HLA\text{-}E^{+}$ cells in BM, and for the expansion of $CD94/NKG2C^{+}$ NK cells. These characteristics suggest that BM NK expansion, with increased expression of CD94/NKG2C, generates a selective pressure on hematopoietic compartments. In this regard, patient $CD94/NKG2C^{+}$ NK cells could have exerted an efficiently killing of $HLA\text{-}E^{+}$ myeloid precursors, thus targeting $HLAE^{+}$ monocytes and $HLA\text{-}E^{+}$ PMN in BM and sparing those cells carrying the lowest HLA-E levels. Indeed, the dysplastic monocytes are mainly $HLAE^{+}$ $CD14^{+}CD56^{+}$ in BM and become $HLA\text{-}E^{-}CD14^{+}CD56^{-}$ in PB likely due to the action of NK selection. These evidences suggest that the action of NK in the selective pressure was exerted on the monocyte and PMN populations that were observed totally defective for HLA-E expression in PB. We could not rule out that other receptor/ligand pathways could have a role in regulating NK activity in this patient.

The expression of HLA-I appears to be not involved in the selection by NK in this patient, as the levels of these molecules on PMN and monocytes are similar between PB and BM. In addition, the TCR repertoire appears to be unskewed, and such result could formally exclude antigen specific T-cell response in the patient, at least at the time of our observation.

Taken together, the current study and the others by us published (Ruggiero et al. 2009; Terrazzano et al. 2013; Alfinito et al. 2010) suggest that the emergence and dominance of dysplastic clones could be inferred and/or sustained in some MDS patients also by NK activity alone and/or as contribute, together with other immune-mediated mechanisms. The relevance for the expression of HLA-E and HLA-I on hematopoietic cells could be largely different, and it can range from etiopathogenetic role to some physiopathological aspects. This consideration makes sense when one considers the balance between anti-tumor immune response and the possible detrimental implication of immune editing in cancer (Dunn et al. 2004). Likewise, MDS condition is identifiable as the consequence of several clonal cytogenetic abnormalities conferring proliferative and surviving advantage to those stem cells generating dysplastic clones (Mufti 2004; Greenberg et al. 1997). In this context, it has been proposed that inflammatory BM microenvironment and an altered immune response could not only represent huge protective reactions of the individual from disease, but also favourable conditions for the emergence, expansion, and dominance of dysplastic clones in a subgroup of MDS patients (Mufti 2004; Greenberg et al. 1997; Malcovati et al. 2005; Maciejewski 2007; Sloand and Rezvani 2008; Ruggiero et al. 2009; Terrazzano et al. 2013; Alfinito et al. 2010). The plain significance of this mechanism in counteracting, causing, sustaining, or accompanying the

MDS is not definitively established, but it appears to be of great relevance to better understand the role as 'Janus Bifront' for immune response in MDS.

***Leishmania infantum* infection**

In our report we describe a significant increase of $CD8^+CD3^+$ T lymphocytes and T_H1 T cells ($IFN\text{-}\gamma^+IL\text{-}4^-CD3^+$ lymphocytes) accompanied by a significant reduction of Tregs lymphocytes in a cohort of Leishmania infected dogs regardless of the IFAT titer or the occurrence of clinical symptomatic disease.

Reduced percentage of $CD4^+$ T cells has been described in CL, suggesting that CD8-dependent IFN-γ production and lysis of infected- macrophages can mediate protective immunity during the disease (Guarga et al. 2000). Increased number of $CD8^+$ T effectors has been referred to preferentially characterise asymptomatic animals (Reis et al. 2006, 2009). In addition, higher counts of T lymphocytes, mainly due to increased $CD8^+$ T lymphocytes and accompanied by a decreased $CD4^+/CD8^+$ ratio, were described to characterise asymptomatic animals with positive serology and positive molecular results (Coura-Vital et al. 2011). Furthermore, the relevance of variations in the percentage of $CD3^+$ and $CD4^+$ T cells in mildly and severely infected dogs has been highlighted (Miranda et al. 2007) while prolonged allopurinol monotherapy was observed to improve the number of circulating $CD4^+$ T cells, but not to restore their number to within the normal range (Papadogiannakis et al. 2010).

Our results indicate that increase of $CD8^+$ T cells, in presence of normal levels of T lymphocytes can be observed in CL, regardless of the IFAT titer or the presence of clinical signs of disease. Such observations confirm the relevance for $CD8^+$ T lymphocytes in the control of intracellular-parasites (Guarga et al. 2000; Reis et al. 2006, 2009; Miranda et al. 2007; Coura-Vital et al. 2011).

A complex network of peripheral mechanisms, co-evolved to prevent or dampen immune-mediated diseases, usually accounts for the regulation of the activation, expansion and recruitment of T cell effectors in the infected microenvironment. Regulatory systems include mechanisms intrinsic to the antigen activation and T cell differentiation as well as those mediated by regulatory suppressor immune-populations, as represented by the Treg subset. The involvement of such regulatory network is of critical relevance in the presence of infectious agents usually associated with chronic diseases, as represented by Leishmania. To investigate on such issue, we focused the complex network of regulatory/effector T cell populations in a cohort of 45 Leishmania infected dogs, categorised according to their IFAT titer and the occurrence of a symptomatic versus asymptomatic disease. In order to analyse T cell behaviour, maintaining the biological complexity of the immune-regulatory networks, we chose to analyse T cells in the whole blood samples or in the bulk PBMC preparations, by using immune fluorescence and multi parametric flow cytometry detection. Our data consistently indicate $CD8^+$ T effector cells as a key effector population in CL. Thus the critical role for adaptive immunity in clearing intracellular parasites (Reis et al. 2006, 2009)

has been confirmed in our model. Moreover, our results indicate that in active CL an inflammatory response, characterised by increased levels of $CD8^+$ T cells and T_H1 T cells, might be sustained by a decrease of Treg. Thus, parasite persistence could maintain adaptive cytotoxic and pro-inflammatory response by reducing Treg-dependent mechanisms. Several observations, particularly on the murine cutaneous leishmaniasis model, demonstrated that T_H1 and T_H2 responses via their signatory cytokines, IFN-γ, and IL-4 respectively, were counter-regulatory. However, later studies using gene-deficient mice have questioned the precise role of the T_H2 response and IL-4 in regulating the T_H1 response. Depending on the parasite strain/species/host model studied the T_H2 response could either promote infection or at best be irrelevant to disease progression (Alexander and Brombacher, 2012). The presence of reduced Tregs represents a key mechanism to optimise T cell activation and effector functions in the presence of persistent infection. Tregs have been described to modulate recruitment and activation of immune effectors (Sakaguchi, 2005). Thus, in active CL, Treg could hamper inflammatory responses required for infection clearance, in such way exacerbating the risk that the unbridled parasite growth could lead to severe disease. On the contrary, Treg recruitment in infected tissues might prevent the onset of severe immune-mediated pathology. Such intriguing scenario could underlie immune-mediated pathology, likely accounting for the presence of immune-mediated auto-immune processes, very common in this parasitic disease (Kharazmi et al. 1982; Ferrer 1992; Terrazzano et al. 2006; Solano-Gallego et al. 2009; Cortese et al. 2009, 2011). These data may provide new insights into the pathogenesis of immune-mediated alterations associated with CL. Further studies are in progress in order to more specifically address such issue.

In the second report, we describe that a diet supplemented with nutraceuticals selected for their potential immune modulating properties and rich in essential fatty acids is able to induce an immune-modulation in dog chronically infected by *L. infantum* undergoing 12 months of treatment with standard pharmacological therapy. Moreover, this study addressed if the administration of specific nutrients could also improve the clinical response to the standard treatment in a model of chronic spontaneous infection, as represented by CL.

We observed that the significant reduction of Treg subset, by us previously associated with the occurrence of chronic CL (Cortese et al. 2013), was significantly restored by an immune-modulating diet. Such modification was maintained in a one-year follow up. Notably, Group 2 dogs also showed a progressive decrease of, T_H1 $CD4^+$ T cells, whose levels became similar to healthy dogs at T6 and T12.

Therefore, anti-Leishmania treatment combined with the administration of a specific nutraceutical pet food supplemented by nutrients selected for their potential immune-modulating properties and rich in essential fatty acid has been associated with significant changes in immune profile of sick dogs.

The effect of nutraceutical diet appeared to be specific for Treg, since other immune cells were unaffected. Indeed, CL pharmacological treatment alone or in the presence of specific immune modulating nutrient supplementation did not alter the CD4/CD8 ratio and did not affect the increase in $CD8^+$ T cell effectors in sick dogs along the follow up. Notably, it is conceivable that the maintenance of a high percentage of T cytotoxic effectors could foster an effective immune response against the parasite and it is mainly correlated to the persistence of this chronic infection in the animals. Moreover, the increase in Treg percentage could have a role in reducing the immune-mediated damages to the tissues frequently associated with CL. In this regard, both the clinical outcome of the disease and the occurrence of immune-pathological complications have been largely associated with the anti-Leishmania immune response orchestration: i.e. murine cutaneous leishmaniosis model demonstrated that T_H1 and T_H2 responses are in counter regulatory dependency (Alexander and Brombacher, 2012). It is of note that the pharmacological-treated dogs fed by a potential immune-modulating diet recovered the clinical condition in a higher percentage if compared with those animals maintained with standard diet (65% vs. 50%) at the end of the study. In addition, the dogs fed by immune-modulating diet showed a significant increase in platelet number all along the study. Intriguingly, our data also indicate that pharmacological anti-Leishmania treatment induced significant improvement of clinical conditions (for biochemical and haematological evaluation) in all CL animals, regardless the type of diet administration.

Our data evidenced as the pharmacological treatment alone was unable to induce long lasting changes in pro-inflammatory response and to modulate Treg in sick dogs, while the combination with immune-modulating supplemented diet was associated with a significant restoration of Treg level. To this regard, it is of note that the nutraceuticals used in this study were previously suggested as anti-oxidants and immune-modulating substances to reach the physiological status in several models of disease in human (Barak et al, 2002; Ghanim et al, 2010) and animals (Dhasarathan et al, 2010; Buttle et al, 2011; Halder et al, 2012; Barros et al, 2012; Kim et al, 2013).

Our study suggested a role for nutraceuticals as food implementation able to modulate the immune response. In particular, the nutraceuticals used in this study appear to correlate with the decrease of T_H1 cells and with the increase of Treg in CL sick dogs. It is of some relevance that these effects seem to be not associated with fully changes in clinical outcome of infection in leishmaniosis, but the increase of Treg could have a putative role in reducing the immune-pathological injury resulting from CL disease.

In this context, the possibility that the occurrence of a mild inflammatory context accompanied by increased Treg level could ameliorate immune-mediated pathological effects, as the immune-mediated thrombocytopenia (Cortese et al. 2009), appears to be of some interest and it needs to be further addressed.

Finally, this study opens an interesting scenario on the role for metabolism and nutraceuticals in modifying the immune response in dogs and, as a perspective, in humans.

Physiological model: T lymphocyte activation and SOD1 involvement

This study revealed that SOD-1 is part of the network of molecules involved in antigen-dependent T cell response. SOD-1 was recruited by antigen triggered TCR and its intracellular content was specifically upregulated in human T cells after 16–18 h of anti-CD3 incubation. Moreover, SOD-1 was secreted by a BFA-dependent microvesicle pathway by TCR triggered T cells. These effects have been observed maintaining the biological complexity of antigen-dependent T cell response and confirmed in purified T cells activated by anti-CD3/anti-CD28 beads.

We showed that extra-cellular SOD-1 is increased in PBMC cultures after anti-CD3 treatment. This effect was accompanied by both the induction of SOD-1 mRNA and increase of SOD-1 containing microvesicles in culture supernatants. Moreover, we identified the T cell population as the specific target for SOD-1 induction and extracellular export. Therefore, TCR-dependent activation behaves as a triggering element for SOD-1 production and secretion by human T cells.

SOD-1 production is induced in neuroblastoma SK-N-BE cells after oxidative stress (Mondola et al. 1998; Mondola et al. 2003). Moreover, other and our data showed that cytosolic SOD-1 is secreted by many cell lines carrying out a paracrine modulatory role (Mondola et al. 1998; Mruk et al. 1998; Mondola et al. 2003) and SOD-1 extracellular export was by us described in primary lymphoid organs (Cimini et al. 2002). Induction of extra-cellular export of SOD-1 after TCR-triggering proposes a more complex physiological involvement of such enzyme in T cell activation. In this study, we described that a BFA-dependent secretion mechanism (Orci et al. 1991) characterized SOD-1 micro-vesicle intercellular trafficking upon antigen-dependent immune response. No effect has been observed in the presence of methylamine that impairs cell endocytosis (Maxfield et al. 1979). Thus, a major involvement of endocytic recycling pathways might be excluded. Such mechanism, previously described in neuronal model (Mondola et al. 1998; Mruk et al. 1998, Ookawara et al. 1998; Turner et al. 2005; Kikuchi et al. 2006; Mondola et al. 2003), represents an intriguing issue for further investigations.

SOD-1 is a cytosolic protein lacking signal peptide and consequently considered to be excluded from ER translocation. The small amount of wild type SOD-1 detected in ER–Golgi apparatus (Urushitani et al. 2008), does not support the direct involvement of this organelles in SOD-1 secretion. Moreover, the possible interference of BFA, a classical inhibitor of ER–Golgi dependent protein secretion, in vesicular pathways not directly involving ER–Golgi apparatus cannot be excluded. In this context, our data propose that SOD-1 could be part of the micro-vesicle-dependent pathways functioning as secondary messenger between immune cells (Mathivanan et al. 2010; Thery et

al. 2009; Blanchard et al. 2002). A number of data indicate that exogenously added H_2O_2 induced signals in the absence of ligands, whereas catalase is able to inhibit such effect (Bae et al. 2000; Konishi et al. 1997; Sundaresan et al. 1995). Moreover, the observations that production of catalase characterizes many pathogens (Wang et al. 2006) and that viral infection modulates H_2O_2 production (Peterhans 1997) confirm the multiple roles played by extracellular H_2O_2 in the activation processes of lymphocytes (Kraaij et al. 2010).

We consistently found that antigen-dependent T cell triggering mediated changes in the intra-cellular localization of SOD-1 that was observed to co-localize with clustered TCR. This event was dependent on ROS availability since it was impaired by NAC co-treatment.

ROS production is an essential component in signalling cascades that mediate actin cytoskeleton rearrangements. Small G protein Rac, a key element in the network assembly of actin in lamellipodia (Ridley and Hall, 1992; Hall 1998; Heo 2011) participates in activation dependent ROS production by different cell types (Lambeth 2004; Bedard and Krause 2007). Moreover, Rac-mediated ROS production results in the downmodulation of Rho activity thus regulating cellular morphology and migratory behavior (Nimnual et al. 2003). SOD-1 associates with Rac-1 regulated NADPH oxidase complexes in different mouse tissues and cell lines (Harraz et al. 2008). In this context, massive ROS scavenging is expected to disrupt ROS-dependent regulation of cell contractility and motility. This event could account for the TCR/SOD-1 intracellular redistribution (Figure 20). The impairment of early cell aggregation in presence of anti-CD3/NAC co-treatment (Figure 21) strongly supports such hypothesis. Compelling evidences indicate that ROS, together with their essential role in innate antimicrobial defence (Fang 2004), are critically involved in the regulation of antigen-dependent response of adaptive immune effectors (Devadas et al. 2002; Kwon et al. 2003; Lee et al. 2007; Purushothaman and Sarin 2009; Richards and Clark 2009; Nadeau et al. 2012). Exposure of T cells to oxidant agents, such as pervanadate or H_2O_2, induces and/or enhances TCR signaling during T cell activation (Secrist et al. 1993; Cenciarelli et al. 1996; Hehner et al. 2000). TCR-dependent signaling generates both superoxide anion and H2O2 that selectively regulate antigen-dependent proliferation and Fas ligand expression by T effectors (Devadas et al. 2002). An oxidative signal implies its tight regulation and transient character. Thus, in the presence of multiple intracellular ROS sources (Sena et al. 2013; Los et al. 1995; Jackson et al. 2004; Kwon et al. 2010), the involvement of multiple anti oxidant mechanisms in fine tuning of antigen-dependent T cell response can be hypothesized. Indeed, Mn-SOD/SOD-2 a major mitochondrial antioxidative enzyme has been consistently associated with T cell activation (Kamiński et al. 2012) and a role of catalase, glutathione and thioredoxin has been also proposed (Kesarwani et al. 2013).

In the models of ROS generation upon stimulation of receptors it has been shown that H_2O_2 is the relevant oxidant species that regulate signaling (Rhee et al. 2000; Reth 2002; Rhee 2006). Notably, H_2O_2 has a short half-life in the

reducing environment of the cytosol, and it acts close to its site of production. Thus, an important aspect of ligand dependent TCR activation might be the rapid translocation of receptors to a source of H_2O_2 or, vice versa, the clustering of such a source to the receptor. To this regard, our data strongly support the hypothesis that SOD-1 intracellular localization in antigen-triggered T cells could provide H_2O_2 generation in the cell compartment specifically involved in tuning antigen-dependent signals. A number of data suggested the role for H_2O_2 as key modulator of protein phosphorylation on either serine–threonine and tyrosine residues (Fetrow et al. 1999). Indeed, all protein tyrosine phosphatases (PTPs) contain an essential cysteine residue in the signature active enzyme site motif that has been demonstrated to be target of specific H_2O_2 oxidation.

The H_2O_2-mediated inhibition of PTP activity is expected to result in a shift of protein tyrosine kinases toward protein phosphorylation. The involvement of SOD-1 in such regulatory pathways has been suggested (Juarez et al. 2008). Our data on SOD-1 intracellular re-localization upon TCR-triggering suggests that SOD-1 could directly modulate kinase/phosphatase activity related to proximal TCR signaling. The evidence (Devadas et al. 2002) that anti-CD3 induced ERK phosphorylation requires H_2O_2 but is independent on superoxide anion, strongly supports such hypothesis. Thus, subcellular compartmentalization of H_2O_2 generating enzymes (like SOD-1) could represent a relevant element in achieving the superoxide/peroxide balance required to optimize antigen-dependent T cell response.

Taken in all, our data suggest that SOD-1 is part of the molecular network involved in antigen-dependent T cell response. At the best of our knowledge, this is the first observation revealing a relationship between SOD-1 secretion/intracellular re-localization and the antigen dependent T cell activation. Further studies are needed to investigate on the involvement of SOD-1 in the regulation of TCR signalling cascades as well as in the functional cross talk between immune effectors.

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Eculizumab treatment modifies the immune profile of PNH patients

Fiorella Alfinito[a,1], Giuseppina Ruggiero[b,*,1], Michela Sica[c], Annapoorani Udhayachandran[b], Valentina Rubino[b], Roberta Della Pepa[a], Anna T. Palatucci[b], Mariangela Annunziatella[b], Rosario Notaro[c], Antonio M. Risitano[a], Giuseppe Terrazzano[b,d]

[a] Department of Biochemistry and Biomedical Technologies, University of Naples Federico II, Italy
[b] Department of Cellular and Molecular Biology and Pathology "L. Califano", University of Naples Federico II, Italy
[c] CORE Research Lab, Istituto Toscano Tumori, Florence, Italy
[d] Department of Chemistry, University of Basilicata, Italy

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ABSTRACT

Paroxysmal Nocturnal Haemoglobinuria (PNH) is due to pathological expansion of a stem progenitor bearing a somatic mutation of *PIG-A* gene involved in the biosynthesis of the glycosyl-phosphatidyl-inositol (GPI) anchor. Numerous data suggest a role for immune-mediated mechanisms in the selection/expansion of GPI-defective clone. Haemolytic anaemia in PNH is dependent on the effect of complement against GPI-defective red cells. Eculizumab, an anti-C5 monoclonal antibody, is dramatically effective in controlling haemolysis and thrombosis, in reducing fatigue and in improving quality of life of patients. However, this therapy presents new challenges that need to be properly faced.

Here, we report the decrease in B, Natural Killer (NK) and regulatory T cells (Treg), an altered cytokine profile of invariant-NKT cells (NKTi) and the increasing of C-X-C chemokine receptor type 4 (CXCR4) receptor in PNH patients before the Eculizumab therapy. Treatment significantly affects some of these alterations: after Eculizumab, the number of B lymphocytes, the cytokine secretion of NKTi and CXCR4 expression on CD8 T cells became similar to healthy donors. No effects were observed on NK and Treg. The amplitude of the GPI-defective compartment remained unchanged.

Introduction

PNH is a rare acquired clonal disorder of haematopoiesis with an estimated prevalence in the western population around 1–5 cases per million. It is characterized by haemolytic anaemia, thrombophilia and cytopenia resulting in serious life-threatening complications and early mortality (Luzzatto 2006). PNH is due to the expansion of a stem progenitor bearing a somatic mutation of the *PIG-A* gene in a context of a bone marrow failure condition. Since the *PIG-A* gene is required for the synthesis of the GPI anchor, the *PIG-A*-defective cells do not bear those molecules needing for GPI to be expressed on cell surface. The absence of the GPI-linked complement inhibitor CD55 and CD59 molecules on red cells explains complement-mediated haemolysis in PNH patients. Platelet surface activation by small amounts of complement, urokinase plasminogen receptor (uPAR) deficiency and the release of free haemoglobin by chronic haemolysis might explain thrombophilia, while the origin of the underlying bone marrow failure is still unknown (Luzzatto 2006). The effect of the complement dysregulation on GPI-defective cells on the immune effectors of PNH patients needs to be investigated.

GPI defective clones have been identified in healthy controls and PIG-A KO murine models are unable to fully mimic a PNH syndrome. These observations strongly suggest that PIG-A mutation is insufficient to account for the dominance and development of PNH clones and that some extrinsic selective pressure might favour the preferential expansion of the GPI-defective compartment. Several evidences suggest the involvement of T-cell-dependent autoimmune mechanisms in the expansion of GPI-defective progenitors in PNH patients (Luzzatto et al. 1997; Karadimitris et al. 2000; Luzzatto 2006). The possibility that NK cells might directly participate in PNH pathogenesis has been also proposed (Hanaoka et al. 2006).

Therapeutic approach with Eculizumab, a humanized anti-C5 monoclonal antibody, is able to inhibit the terminal complement activation, successfully reducing intravascular haemolysis as well as thrombosis in PNH. Several clinical trials have demonstrated that

Abbreviations: BM, Bone Marrow; CXCR4, C-X-C chemokine receptor type 4; GPI, glycosyl-phosphatidyl-inositol; IFN-γ, interferon-gamma; IL-4, interleukin-4; IL-17, interleukin-17; NKTi, invariant-NKT cells; mAb, monoclonal antibody; NK, Natural Killer; PNH, Paroxysmal Nocturnal Haemoglobinuria; Treg, regulatory T cells; SDF-1, stromal cell-derived factor-1.

* Corresponding author. Tel.: +39 0817463311.
E-mail address: giruggie@unina.it (G. Ruggiero).

[1] These authors contributed equally.

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Table 1
Patient clinical and haematological characteristics.

Parameters [PNH patients ($N = 34$)]	Mean ± SD
Male/female ratio	21/13
Mean age (years)	45.62 ± 16
Haemoglobin (g/dL)	9.83 ± 1.48
White blood cell count ($\times 10^9$/L)	3.95 ± 0.78
Neutrophil count ($\times 10^9$/L)	2.37 ± 0.69
Lymphocyte count ($\times 10^9$/L)	1.4 ± 0.6
Platelet count ($\times 10^9$/L)	141.6 ± 39
Reticulocyte count (%)	4.2 ± 2.4
Neutrophil CD66b$^-$ (%)	80.87 ± 17.2

such therapy reduces or eliminates transfusion requirement, significantly improving the quality of life in PNH patients (Hillmen et al. 2006; Schubert et al. 2008).

Eculizumab mAb binds to human C5 complement protein preventing its cleavage in C5a and C5b and the subsequent formation of the membrane attack complex. The potential consequences of complement inhibition are of concern, since the complement system has evolved as a key part of innate immune system and it has a relevant role in modifying both the innate and adaptive immune response (Carroll 2004a; Le Friec and Kemper 2009; Ricklin et al. 2010).

Eculizumab treatment could represent an useful tool for the study of complement-dependent immune-alterations in PNH patients.

Here, we examined the effect of Eculizumab treatment on innate and adaptive immune effectors in a cohort of 34 PNH patients. Moreover, we investigated whether complement inhibition might interfere with the selection/expansion of the GPI-defective haematopoiesis.

Materials and methods

Patients

The study was conducted in 34 Italian PNH patients. Clinical characteristics are reported in Table 1. Diagnosis was made according to International PNH Interest Group Criteria (Parker et al. 2005). All patients showed primary haemolytic PNH with a large GPI-defective granulocyte population (CD66b$^-$ and/or CD59$^-$ granulocytes >50%). No patients showing bone marrow failure with emergence of PNH clones (i.e. Aplastic Anaemia/PNH syndrome or Hypoplastic PNH) were enrolled in the study (Brodsky 2008). Notably, some of the patients were on Eculizumab treatment for more than 48 months. Biological samples were collected by venipuncture 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. Patients were studied at diagnosis, before any treatment; 17 of them were retested after at least six months of Eculizumab therapy; 14 patients were tested at least three times before and after Eculizumab treatment. None of PNH patients received immune-suppressive therapies or other treatments potentially interfering with immune responses.

Immunofluorescence and flow cytometry

Blood samples from patients and controls were analyzed by immunofluorescence and flow cytometry by using a two laser equipped *FACScalibur* apparatus and the *CellQuest* analysis software (Becton Dickinson). FITC, PE, Cychrome and APC labelled mAbs against CD3, CD4, CD25, FoxP3, CD45, CD8, CD16, CD56, NKTi, CD19, CD20, CD45, Interferon (IFN)-γ, Interleukin (IL)-4 and isotype-matched controls were purchased from Becton Dickinson PharMingen, San Jose, CA.

To analyze the production of IFN-γ and IL-4, purified peripheral blood mononuclear cells were cultured overnight in presence of PMA and ionomycin. Intracellular staining with the specific mAbs was performed by a fixing/permeabilization kit (Caltag, Burlingame, CA), following manufacturer's instructions. To avoid extracellular cytokine export, the cultures were incubated in the presence of 5 μg/mL of Brefeldin-A (Sigma–Aldrich, St. Louis, MO), as described (Terrazzano et al. 2005).

All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using FSC and SSC parameters, as well as CD45 labelling. We choose to evaluate T, B, NK, NKTi and Treg populations. Cytokine profile (IFN-γ, IL-4, IL-17) was analyzed in NKTi effectors, as major players in driving cytokine polarization. In order to evaluate bone marrow (BM) homing of such effectors we also evaluated their expression of CXCR4 (Pelus 2008).

Statistical analysis

Statistical evaluation of data, by using software *InStat 3.0* (GraphPad Software Inc., San Diego, CA, USA), has been performed by means of the Mann–Whitney test. Comparisons in the paired samples were evaluated by means of the Wilcoxon matched-pairs signed-rank test. Two-sided P values of less than 0.05 were considered to indicate statistical significance and were not adjusted for multiple testing.

Results

Fig. 1 shows the immune profile of our cohort of PNH patients. A significant reduction of B lymphocytes, NK cells and Treg with an increased number of NKTi effectors were found (Fig. 1A–D). As shown, Eculizumab treatment significantly modified B and NKTi levels in patients, restoring normal values (Fig. 1A and C). No effect was observed on the NK and Treg subsets (Fig. 1B and D). Using Wilcoxon matched-pairs signed-rank test has always performed comparison of values before and on Eculizumab therapy in the same patients. Each reported value represents a mean of at least three longitudinal evaluations obtained at a six month interval before and after Eculizumab treatment. Panel E indicate the B and NK cell number observed in two exemplificative patients along the study. No significant differences have been observed in the peripheral white cell count of the patients before and on Eculizumab treatment (not shown). Percentages confirmed observations on absolute numbers for B, NK and NKTi effectors. No significant difference in the percentage of Treg cells was observed between controls and patients, before or after Eculizumab therapy (data not shown). The ability of Treg to inhibit T lymphocytes proliferation and IFN-γ production was normal before and on therapy (data not shown).

Fig. 2 reports the analysis of cytokine production profile of NKTi effectors. As shown, NKTi cytokine secretion profile was characterized by a significant decrease of IFN-γ and IL-17 and by an increase of IL-4 production in the PNH cohort. On Eculizumab therapy, the cytokine patterns became comparable with normal controls (Fig. 2A–C).

Fig. 3 reports the analysis of the surface expression of CXCR4 molecule on NKTi, NK and CD8 T cells. As shown, significantly higher CXCR4 expression than healthy donors characterized all these lymphocyte subsets from PNH patients. Eculizumab treatment normalized CXCR4 expression on CD8 T cells, while no significant effect was observed on NKTi and NK subsets (Fig. 3A–D). The amplitude of the GPI-defective compartment remained unchanged in all the patients enrolled in the study (not shown).

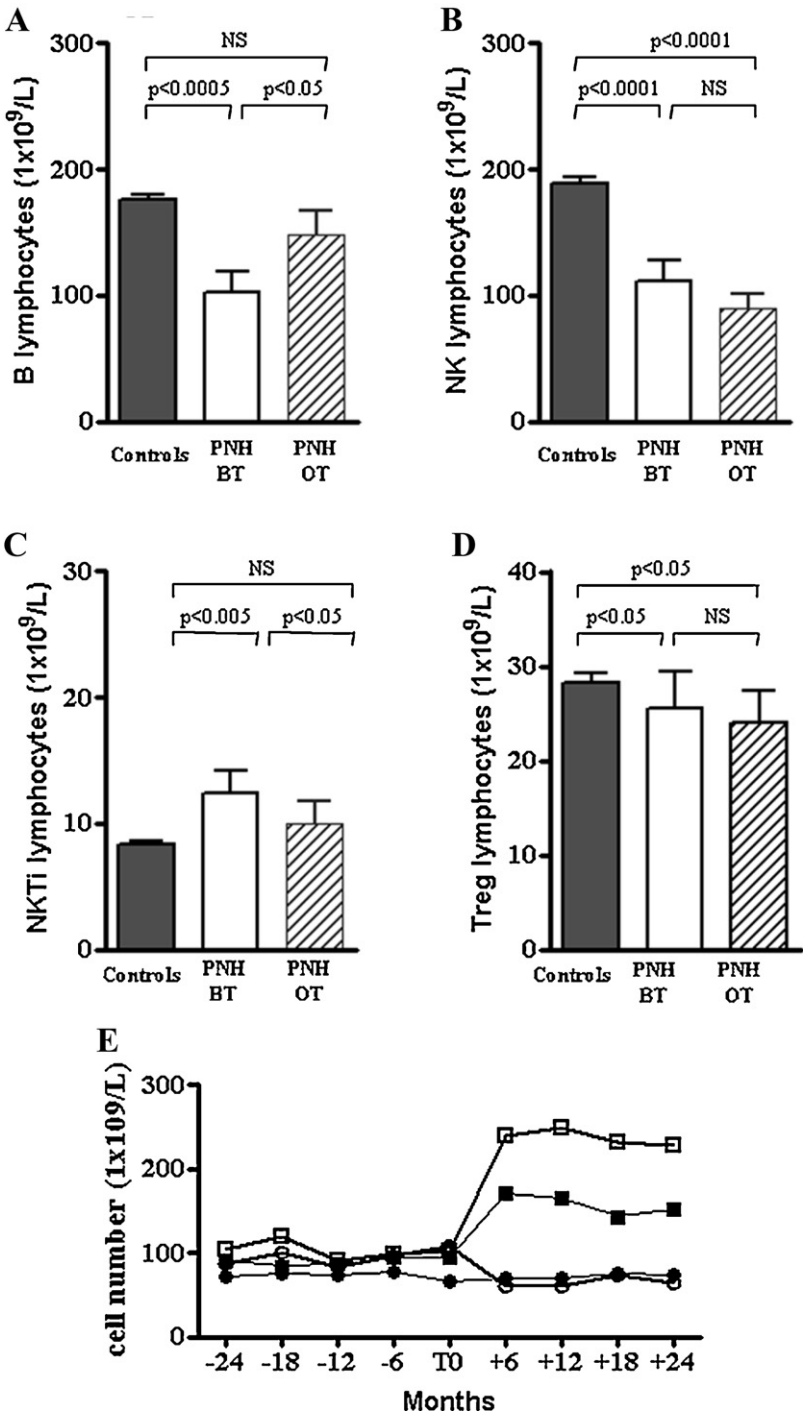

Fig. 1. Analysis of B, NK, NKTi and Treg lymphocytes in PNH patients before and on Eculizumab treatment. As indicated, panels A–D refer comparative analysis of B, NK, NKTi and Treg lymphocytes in PNH patients before (BT) and on (OT) at least six months of Eculizumab treatment. The analysis has been performed by considering the values obtained in the same patients before and after Eculizumab therapy. For the patients, each value represents a mean obtained in at least three independent evaluations before and after treatment. Statistical analysis has been performed by Wilcoxon matched-pairs signed-rank test. Panel E shows longitudinal evaluation of B (squares) and NK (circles) lymphocytes in two representative patients (full and empty symbols, respectively) along the study. T_0 indicates the beginning of Eculizumab treatment.

Discussion

Here, we describe for the first time the occurrence of Treg reduction and NKTi increase in untreated PNH patients as well as the ability of Eculizumab therapy to selectively restore B and NKTi peripheral concentration without significant interference on NK and Treg numbers. These results confirm previous other and our data (Schubert et al. 1990; Alfinito et al. 1996; Richards et al. 1998) on the reduced number of B and NK cells in PNH.

Since Eculizumab modifies some of the alterations here described in untreated patients, we hypothesize their dependence on complement-mediated pathway/s. Thus, the lack of the GPI-linked complement regulatory proteins on the surface of the

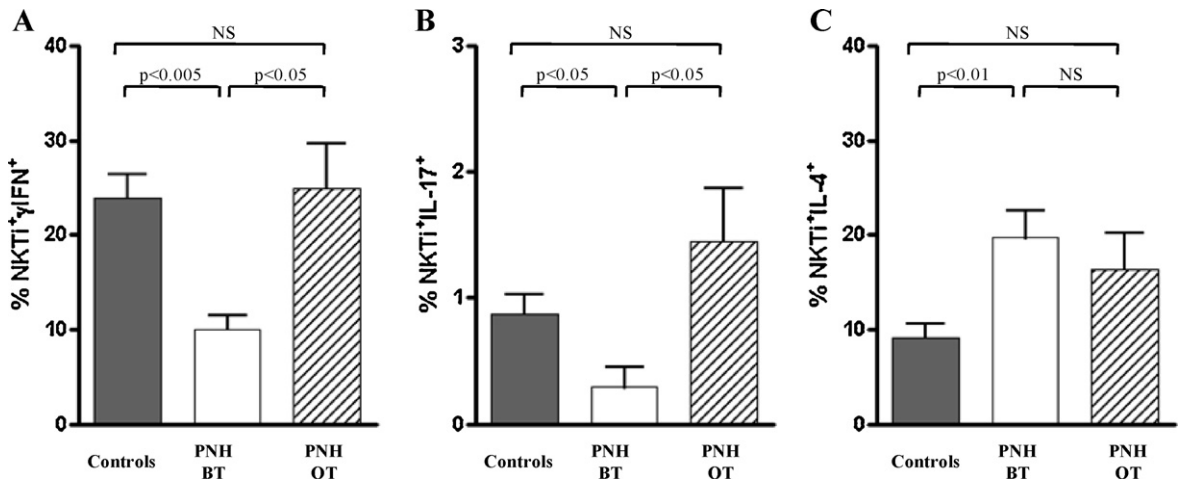

Fig. 2. Analysis of cytokine profile of NKTi cells in PNH patients before and on Eculizumab treatment. As indicated, panels A–C represent γ-IFN, IL-17 and IL-4 production in NKTi cells obtained from PNH patients before (BT) and on (OT) Eculizumab therapy. The analysis has been performed by immune-fluorescence and Flow Cytometry detection after a 16–18 h culture in the presence of PMA and Ionomycin. For each sample, a minimum of 5000 events in the region of NKTi lymphocytes has been acquired. Statistical analysis has been performed by Mann–Whitney test. For five of the patients, the results have been confirmed in at least three independent experiments.

GPI-defective cells is able to mediate significant quantitative alterations of B and NKTi effectors.

It is worth noting that the size of PNH clone remains substantially unmodified in Eculizumab treated patients. This evidence suggests that the correction of complement hyper activation is unable to revert the immune-mediated mechanisms involved in the selection and dominance of PNH precursors (Luzzatto et al. 1997; Karadimitris et al. 2000; Luzzatto 2006).

The absence of the GPI-linked CD55 and CD59 molecules in PNH cells has been largely observed to mediate enhanced complement

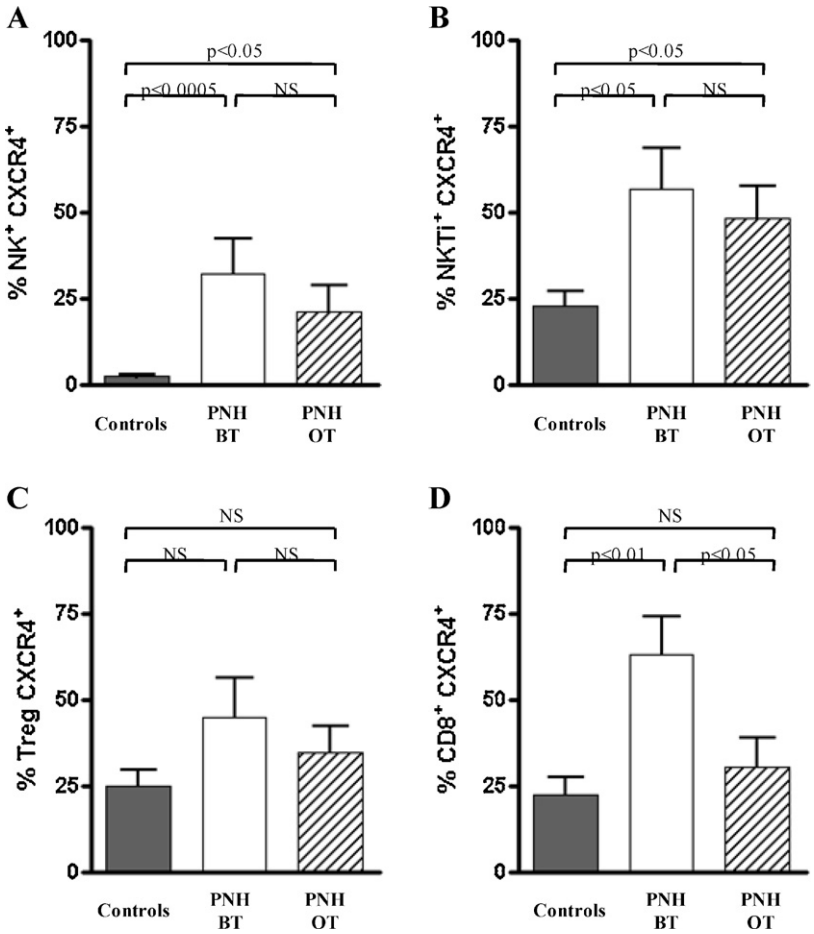

Fig. 3. Analysis of CXCR4 expression on NK, NKTi, Treg and CD8 T cells of PNH patients before and on Eculizumab treatment. As indicated, panels A–D represent CXCR4 expression on NK, NKTi, Treg and CD8 T lymphocytes obtained from PNH patients before (BT) and on (OT) Eculizumab therapy. Statistical analysis has been performed by Mann–Whitney test. For five of the patients the results have been confirmed in at least three independent experiments.

activation, likely accompanied by an altered bio-availability of C3 and C5 activation fragments (Luzzatto et al. 2010). The blockade of C5 by Eculizumab (Hillmen et al. 2006; Schubert et al. 2008; Luzzatto et al. 2010) acts on this process, probably modifying the availability of complement fragments. Such altered availability of C3 and C5 activation fragments could play a major role in mediating the effects here described.

Interaction of C3 and C5 fragments with their cognate receptors on adaptive immune cells has been described to induce pleiotropic effector functions, thus translating the danger information into defined T and B cell responses (Carroll 2004b; Le Friec and Kemper 2009; Ricklin et al. 2010). Complement fragments regulate B cell proliferation, activation and trafficking (Carroll 2004a; Ottonello et al. 1999; Roozendaal and Carroll 2007) and finely tune T cell cytokine patterns (Longhi et al. 2006). In addition, C3 and C5 fragments also interfere with the stromal cell-derived factor-1 (SDF-1)/CXCR4 axis, crucial for BM homing regulation (Reca et al. 2003).

In PNH patients, we observed a decreased IFN-γ and IL-17 production with increased IL-4 secretion by peripheral NKTi cells. Eculizumab treatment modifies this cytokine profile by increasing IFN-γ and IL-17 and lowering IL-4. These data, according with previous observations (Longhi et al. 2006; Liu et al. 2005; Heeger et al. 2005), suggest a role for complement fragments in polarizing T cell responses. Moreover, mouse model defective for CD55 (Daf $1^{-/-}$ mice) showed as an altered production of IFN-γ by T cell is strongly dependent on functional complement system: this alteration is reverted when Daf $1^{-/-}$ mice are backcrossed with complement-deficient mice ($C3^{-/-}$ mice) or treated *in vivo* with a neutralizing anti-C5 antibody (Longhi et al. 2006; Liu et al. 2005; Heeger et al. 2005).

In addition, deacylated complement activated fractions could interfere with PNH cytokine profile trough enhanced metabolic activity (Cianflone et al. 2003).

Here, we report that CXCR4, the molecule mainly responsible for BM recruitment, was significantly higher in untreated PNH patient than controls on NKTi, CD8 and NK cells. Eculizumab treatment normalized CXCR4 expression on CD8 T cells; these changes could be mediated by C3 and C5 cleavage fragments that have been observed to perturb leukocyte BM retention acting on the SDF-1/CXCR4 axis (Reca et al. 2003). In addition, the modified cytokine profile observed after Eculizumab could be involved in modulating CXCR4 expression on T cells since IFN-γ has been observed to inhibit CXCR4 expression (Shirazi and Pitha 1998), while IL-4 can induce opposite effects (Jourdan et al. 2000).

Moreover, the ability of Eculizumab treatment to restore NKTi peripheral levels suggests the involvement of still unknown complement-dependent mechanism/s for this alteration.

Finally, NK and Treg alterations seem not to be influenced by Eculizumab, thus suggesting their independence on Complement related pathways. Since similar alterations have been described in Aplastic Anaemia and Myelodysplastic Syndromes (Kiladjian et al. 2006; Kotsianidis et al. 2009; Alfinito et al. 2010), we can argue their involvement in the immune-mediated pathogenesis of BM failure disease that underlies PNH.

Taken in all our data suggest that the unbalanced complement activation on the surface of GPI-defective cells mediates significant alteration of immune effectors in PNH patients without significant interference with the immune-mediated mechanisms underlying the selection/expansion of GPI-defective haematopoiesis. Eculizumab treatment is able not only to correct the effect of Complement hyper-activation on red cells, but is also able to normalize complement-dependent alteration of immunological targets. This observation suggests that changes in the immune-profile could be of some relevance for the amelioration of immune response in the PNH patients on Eculizumab treatment. The effects, here described, could get new insight into the investigation of PNH pathogenesis and into the pleiotropic effects of Eculizumab therapy.

Authors' contributions

M.S., A.U., V.R., A.T.P. and M.A. performed experiments and analyzed the data; A.F., A.M.R., R.N. and R.D.P. provided the patient clinical management; F.A., G.R., M.S., A.M.R. R.N. and G.T. contributed to the design of the study and to the conception of the experimental work. A.F., G.R. and G.T. planned and coordinated the research and wrote the paper. All authors reviewed the manuscript and approved the final version to be submitted.

Competing interests

Dr. R. Notaro and Dr. A.M. Risitano received until 2010 lecture fees and grant support from Alexion Pharmaceuticals. The authors declare no other potential conflict of interest relevant to this article.

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HLA-E and HLA class I molecules on bone marrow and peripheral blood polymorphonuclear cells of myelodysplatic patients

Giuseppe Terrazzano[a,b,*], Fiorella Alfinito[c], Anna Teresa Palatucci[b], Valentina Rubino[b], Roberta Della Pepa[c], Angela Giovazzino[b], Giuseppina Ruggiero[b]

[a] Dipartimento di Scienze, Università della Basilicata, Potenza, Italy
[b] Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli "Federico II", Napoli, Italy
[c] Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli "Federico II", Napoli, Italy

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ABSTRACT

Relevance of immune-dysregulation for emergence, dominance and progression of dysplastic clones in myelodysplastic syndromes (MDS) was suggested, but valuable or predictive criteria on this involvement are lacking.

We previously reported that reduced T-regulatory cells (Treg) and high CD54 expression on T cell identify a sub-group of patients in whom an immune-pathogenesis might be inferred.

Here, we suggest the occurrence of immune-selection of dysplastic clones in a subgroup of MDS patients, with reduced HLA-I and HLA-E on PMN, and propose that an altered immune profile might represent a valuable criterion to classify Low/Int-1 patients on the basis of immune-pathogenesis of MDS.

1. Introduction

MDS are haematological clonal disorders characterised by ineffective haematopoiesis, with a large degree of cytopenia and an incremental risk of leukaemia progression [1].

Emergence of the dysplastic clones in MDS is believed to represent the consequence of several clonal cytogenetic abnormalities of bone marrow (BM) stem cells. More recently, it has been proposed that inflammatory microenvironment and an altered immune response in BM could be relevant for the emergence, expansion and dominance of dysplastic clones in a sub-group of MDS patients [2–6]. Dysregulation of immune response has been also suggested in the pathogenesis of BM failure occurring in Aplastic Anaemia (AA) and Paroxysmal Nocturnal Hemoglobinuria (PNH) [4,7,8]. In support of immune-mediated pathogenesis is also the efficacy of immune-suppressive therapies in MDS [5,9,10], even if valuable criteria to predict clinical response to such therapies are still lacking.

Leukaemia risk represents one of the major criterion to classify MDS patients according to International Prognostic Scoring System (IPSS) [11], but several reports indicates the need for a better stratification of MDS patients, especially in Low Risk group [12] and a revised IPSS score (IPSS-R) is expected to be published [13].

The identification of the sub-groups of MDS patients in which immune-mediated mechanism could be involved in the selection of dysplastic progenitors could be of some relevance to improve MDS clinical management.

A complex scenario of immune-mediated mechanisms may contribute to the altered haematopoiesis in MDS: i.e., the impairment of erythroid and granulocytic progenitors caused by $CD8^+$ T lymphocytes [2], the evidence of oligoclonal $CD8^+$ T cells in BM [4], the presence of cytotoxic T cell (CTL) and Natural Killer (NK) effectors able to recognise and kill BM precursors [6,14], the occurrence of immune-tolerance dysregulation [3,5] and the presence of a pro-inflammatory cytokine microenvironment in BM [15].

The expression of NK ligands during BM maturation suggests the involvement of NK in the control of haematopoiesis [16].

A major role for Human Leucocyte Antigen (HLA)-E, a non-classical HLA molecule, in regulating NK activity was largely demonstrated [17]. HLA-E is expressed by a variety of immune and non-immune cells [6,18,19] and its defects have been described in some haematopoietic diseases [6,20]. Moreover, an altered expression of the HLA-E receptors CD94/NKG2 was associated to chronic NK lymphocytosis, LGL disorders and to the selection of dysplastic progenitors in MDS [6,21,22].

* Corresponding author at: Dipartimento di Scienze, Università della Basilicata, Via N. Sauro, 85, 85100 Potenza, Italy. Tel.: +39 0817463059; fax: +39 0817463252.
E-mail address: terrazza@unina.it (G. Terrazzano).

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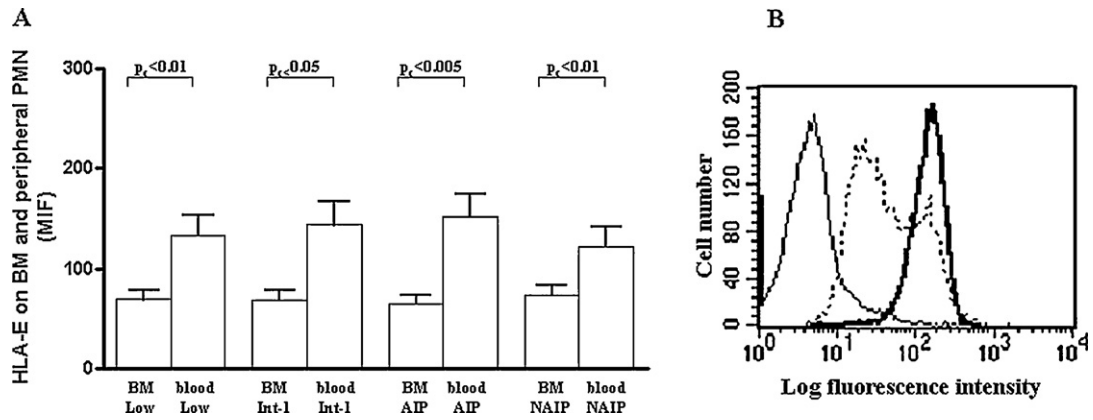

Fig. 1. HLA-E expression on PB and BM cells. (A) Mean of immunofluorescence (MIF) graph for HLA-E on BM GC and on PB PMN in Low, Int-1, AIP and NIP patients. A p value < 0.05 has been considered significant and (B) staning profiles for HLA-E on BM GC as compared with peripheral PMN in one representative MDS patient: BM GC profile (dotted line), PB PMN (bold plain line) and isotype control (plain line).

Alteration of Regulatory T cells (Treg) was described in MDS [23–25]. In this context, the observed increasing percentage of Treg in the High Risk patients might favour the leukaemia progression by suppressing the immune response against dysplastic clones. Treg defects could promote the immune-mediated clearing of healthy BM precursors by auto-reactive T cell effectors in the Low/Int-1 Risk group [23–25].

We evidenced [25] that low levels of Treg and high CD54 expression on CTL in BM identify a sub-group of Low/Int-1 risk MDS patients in whom immune-mediated mechanisms are likely involved in the emergence/dominance of the dysplastic precursors.

HLA-I represents the restriction element for CTL recognition in physiological and pathological conditions [26,27]. Defective HLA-I expression was described as a major immune escape mechanism from CTL recognition for virus infected and transformed cells [28,29].

Here, we investigated if the above grouping criteria (low Treg and high CD54 expression on CTL in BM) could identify a subgroup of MDS patients in whom immune-mediated/CD8-dependent mechanisms could play a role in the emergence/expansion of dysplastic precursors. To analyse the selection mechanisms operating in dysplastic BM, we focused on PMN and their BM precursors investigating on the expression level of HLA-E and HLA-I, as major regulating elements for NK and CTL effectors in Low/Int-1 MDS patients.

2. Design and methods

2.1. Patients and controls

We examined whole BM and peripheral blood (PB) specimens of 30 MDS patients belonging to Low Risk and Int-1 Risk groups. BM and PB sample collection, haematological investigation, cytogenetics were performed according to the WHO recommendations and IPSS score [11,12]. 17 patients belonged to Low Risk Group (4 refractory anaemia, RA, 8 refractory cytopenia with multilineage dysplasia, RCMD, 2 refractory neutropenia, RN, 3 refractory thrombocytopenia, RT), and 13 to Intermediate-1 (Int-1) Risk Group (2 RA, 8 RCMD, 3 RN). BM and PB samples from MDS patients were obtained during routine diagnostic procedures. Informed consent was obtained from each individual patient. Local Ethical Committee approved the study.

None of the patients has been receiving medical treatments that could have an impact on their immune condition. Patients were devoid of immune-mediated diseases and acute or chronic viral infections to avoid any interference on immune-regulatory mechanisms. A group of 26 healthy blood donors were recruited in the study as controls.

All the MDS patients were clustered in two sub-groups, according to their BM Treg levels and CD54 expression on BM CTL, as described [25]. Briefly, 14 MDS patients showing a Treg percentage in BM ≥ 2% of the lymphocyte population or a ratio <10 between the mean intensity fluorescence (MIF) for CD54 on BM CD8 T cells and the control MIF value, obtained after staining of the same cell population with the isotype control mAb, were considered with normal immune profile (NIP). At variance, 16 MDS patients showing a Treg percentage in BM < 2% of the lymphocyte population or a ratio ≥10 between the mean intensity fluorescence (MIF) for CD54 on BM CD8 T cells and the control MIF value obtained after staining of the same cell population with the isotype control mAb, were considered with altered immune profile (AIP).

2.2. mAb, immunofluorescence, and flow cytometry

FITC, PE, Cychrome and APC labelled mAb against CD3, CD4, CD8, CD56, CD19, CD10, CD25, CD45, CD54 and isotype-matched controls were purchased from BD PharMingen (San Jose, CA). Biotin labelled HLA-E mAb clone MEM-E/07, FITC labelled MHC-I mAb clone W6/32 and PE labelled avidin, were purchased from Sigma–Aldrich, Milan, Italy. To analyse Foxp3 expression, intracellular staining was performed by anti-human Foxp3 kit (eBioscience San Diego, USA), following the manufacturer's instructions. Treg subset was identified as the high CD25 expressing $CD4^+CD3^+$ population appearing as a tail, with a slightly but reproducible lower CD4 expression, distinct from the major CD4 population containing both the CD25 low and negative cells [25]. These cells were observed to express Foxp3 at a percentage >98%, as described [25]. All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using forward scatter (FSC) and Side Scatter (SSC) parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by a two laser equipped FACScalibur apparatus and CellQuest software (Becton Dickinson).

2.3. Statistical analysis

BM and PB values were compared in the paired samples by means of the Wilcox on matched-pairs signed-rank test. Comparisons between MDS patients and controls were performed by Mann–Whitney test. The corrected p value (p_c) was calculated by applying Bonferroni adjustment for multiple comparisons.

3. Results

3.1. HLA E molecule on peripheral PMN of Low and Int-1 risk MDS patients is significantly increased as compared with GC in BM, regardless the immune-profile

To analyse whether immune-dependent mechanisms could affect the selection of dysplastic compartment in our cohort of Low, Int-1 risk MDS patients categorised according to their immune profile [25], we evaluated the HLA-E expression on BM granulopoietic compartment (GC) versus PB PMN on paired samples. As shown in Panel A of Fig. 1, HLA-E levels on peripheral PMN are significantly higher than on BM GC. No significant differences have been observed between peripheral PMN from patients and healthy controls or if the patients were grouped according with their immune profile in BM (AIP vs NIP) and with their clinical classification (RA, RMCD, RN, RT) (see Section 2.1) (data not shown).

Higher HLA-E expression on PB PMN could be dependent on a specific up-regulation of the molecule on circulating PMN or it could derive from a preferential selection of a BM precursor showing higher HLA-E expression. Thus, we analysed the staining profile

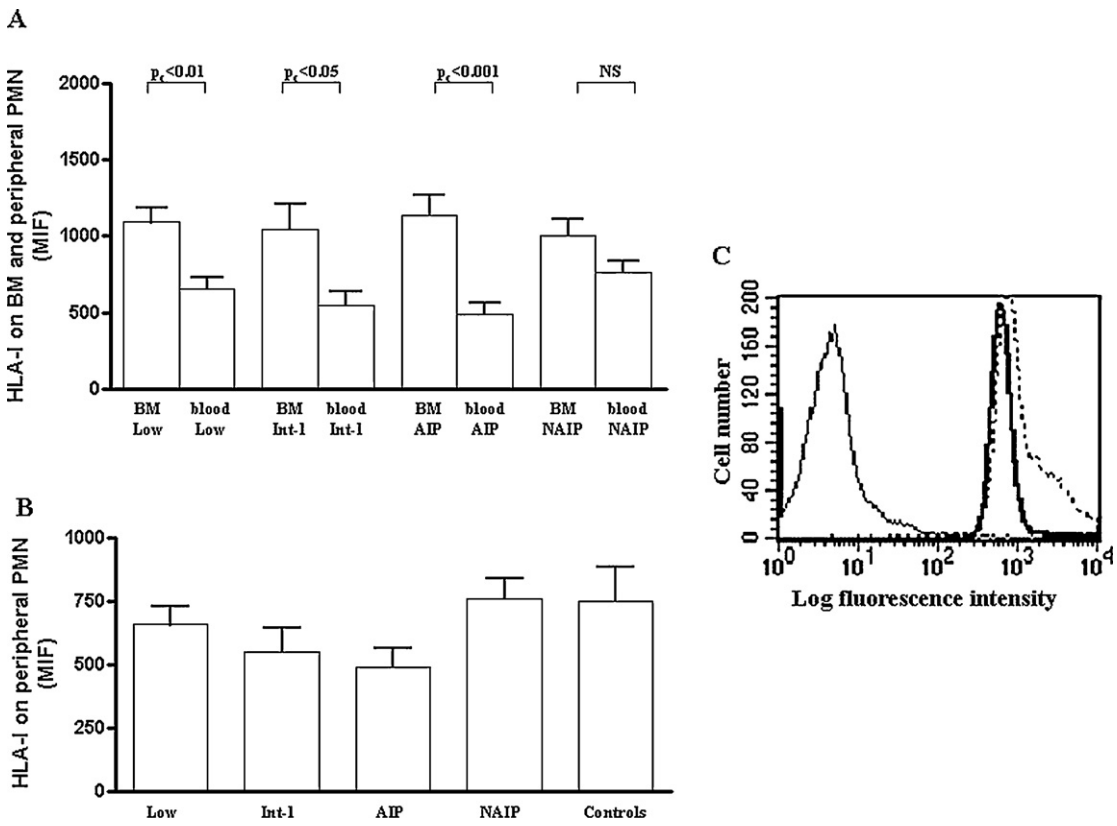

Fig. 2. HLA-I on PB and BM cells. (A) MIF graph for HLA-I expression on BM GC and PB PMN in Low, Int-1 AIP and NIP patients. A p value < 0.05 has been considered significant, (B) HLA-I levels on PB PMN of patients and controls and (C) staining profile for HLA-I in one representative AIP patient: BM GC (dotted line), PB PMN (bold plain line) and isotype control (plain line).

of HLA-E on BM GC. As showed in Fig. 1B, two distinct populations with different HLA-E expression levels are present in the BM GC. Therefore, peripheral PMN seems to belong to the BM GC subset with higher HLA-E expression.

Since HLA-E molecules are believed to preferentially regulate NK-cytotoxicity [17–19], these data suggest the involvement of NK effectors in the selection of PMN in our cohort of MDS patients.

3.2. Occurrence of an altered immune profile in BM is associated with significant decrease of HLA-I expression on peripheral PMN in Low and Int-1 Risk MDS patients

Fig. 2A evidences the decrease of HLA-I on peripheral PMN, as compared with the BM GC, in both Low and Int-1 Risk MDS patients ($p_c < 0.01$ and $p_c < 0.05$, respectively). Notably, when the patients were grouped according with their immune profile in BM (AIP vs NIP, see Section 2), only AIP group demonstrated significant decrease of HLA-I expression ($p_c < 0.001$) on PB PMN. Notably, a tendency towards the presence of lower HLA-I was observed by comparing AIP sub-group with controls (Fig. 2B).

Intriguingly, lower MHC-I expression in peripheral PMN seems to be strongly correlated with the occurrence of an altered BM immune profile in MDS patients.

To investigate whether the presence of PB PMN expressing lower HLA-I is dependent on down-modulation mechanisms occurring in the periphery or it is the result of immune-selection pressure favouring BM precursors bearing lower HLA-I levels, we analysed the cell distribution in the cytometry staining profiles of BM GC and of PB PMN. As shown in Fig. 2C, two distinct populations with different HLA-I levels can be identified in BM GC: the PB PMN seem to belong to the GC subset bearing lower HLA-I expression. This feature significantly characterised the AIP subgroup (Fig. 2A).

These observations point to the role for CTL-mediated selection mechanisms occurring in BM. Indeed, the maturation of PMN cells in which the HLA-I expression is likely unable to effectively present one (or more) unknown self-antigen to autoreactive CTL seems to be favoured in AIP subgroup showing a deranged T cell response.

3.3. MDS patients with altered immune profile in BM show significant increase of HLA-I negative peripheral PMN

To investigate on the occurrence of immune-mediated selection in BM of MDS patients, we compared the percentage of HLA-I negative PMN cells in PB of healthy controls with that observed in our MDS cohort. As shown in Fig. 3, significant increase of HLA-I

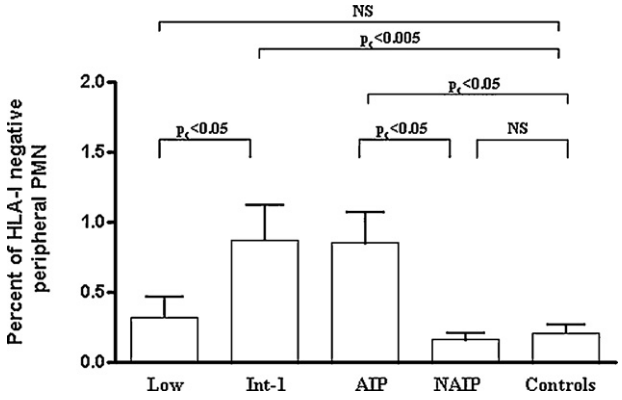

Fig. 3. HLA-I negative PMN in PB of MDS patients. Percentage of HLA-I negative PMN in PB of Low, Int-1, IP and NIP groups of MDS patients as well as in healthy donors. A p value < 0.05 has been considered significant.

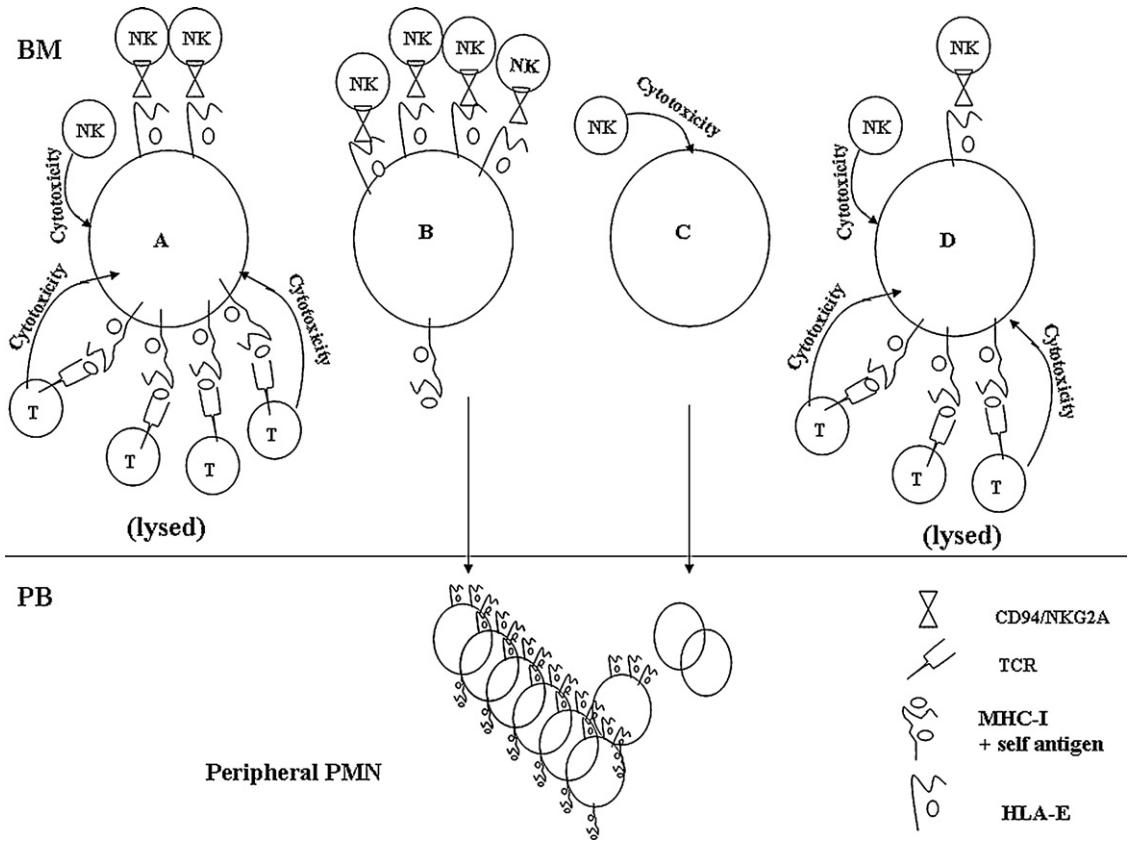

Fig. 4. Involvement of NK and CTL in the selection of dysplastic clones. BM polyclonal haematopoiesis (upper panel) with at least four GC cell types (from A to D). A and D cell types express high level of HLA-I and represent more susceptible targets for deranged CTL recognising self-antigens (indicated as T in the model). B and C cell types express low level the HLA-I or completely lack these molecules and are spared by CTL. NK effectors recognise the presence of HLA-E molecules by CD94/NKG2A receptor. This mechanism likely mediates the clearing of C and D cell types. In the presence of a deranged CTL response (AIP patients), the outcome of these interactions is the result of the rescue and the preferential migration/expansion in PB (lower panel in the model) of the B cell type expressing low HLA-I and high HLA-E. These conditions spare the B cell type from both NK and CTL lysis. The dominance of a CTL-dependent selection pressure might account the higher presence of D cell type in AIP group.

negative PMN was observed in Int-1 patients if compared with the Low Risk Group ($p_c < 0.05$) and the healthy donors ($p_c < 0.005$). When MDS patients were grouped according with their BM immune profile, AIP group showed significant higher percentage of HLA-I defective PMN cells in comparisons with the NIP counterpart ($p_c < 0.05$) or healthy donors ($p_c < 0.05$).

Such evidence, together with the observation of a lower expression of HLA-I on PB PMN than on BM GC of AIP patients (Fig. 2), suggest the involvement of immune-mediated, HLA-I dependent mechanisms in the selection of PMN cells in AIP subgroup, characterised by low Treg and increased CD54 expression on CTL.

4. Discussion

Our immune-based sub classification of MDS patients [25] is dependent on the occurrence of lower Treg percentage, increased BM recruitment of CTL and high CD54 expression on CD8 T cells in sub-group of Low/Int-1 Risk patients.

This study focused on the hypothesis that this altered immune profile might underlie the occurrence of CTL-dependent immune-mediated selection of the dysplastic compartment in MDS. To this aim, we investigated on HLA-E and HLA-I molecules, as major elements in regulating NK and CTL recognition, respectively [6,17,18,26–29].

The analysis focused on granulopoietic population, as the more suitable compartment for studying BM selection processes.

Our data indicate that both the decrease in HLA-I expression and the increased presence of HLA-I defective cells preferentially characterise the peripheral PMN of Low/Int-1 Risk MDS patients bearing the altered immune profile, by us described [25].

BM precursors are characterised by different expression levels of HLA-E as well as HLA-I in MDS patients. Our data indicate that the recognition of both molecules could be of some relevance in the selection mechanism for mature PMN in MDS.

We consistently found that the expression of HLA-E is lower in BM GC than in PB PMN population. Since HLA-E is the main inhibiting element for NK recognition, we hypothesise that NK could kill the lower HLA-E-expressing PMN precursors in BM and it could consent the migration into PB only of those cells expressing HLA-E at higher level. In support of this speculation is the evidence over the expression of NK ligands during BM maturation [16] and the presence of defects in both HLA-E and CD94/NKG2 molecule, the main HLA-E-NK receptor, in some haematopoietic diseases [6,20,22].

Therefore, HLA-E dependent/NK-mediated mechanisms could likely be part of physiological PMN maturation processes. In addition, a perturbation of such regulatory networks might generate the maturation of dysplastic PMN with significant modulation of HLA-E surface expression [6].

This study refers that both Low and Int-1 Risk MDS patients show significant decrease of HLA-I expression on peripheral PMN if compared with the BM counterpart. Notably, when patients were categorised according with their immune profile (see Section 2.1), only AIP patients significantly maintain a lower expression of MHC-I on peripheral PMN cells than in BM GC. This condition is conceivable with the hypothesis that a subpopulation of

BM-PMN-precursors, characterised by high level of MHC-I molecules, could behave as preferential target for a deranged autoreactive T cell response. Indeed, CTL are expected to be less sensitive to PMN precursors expressing low level of MHC-I. These cells represent the only population leaving the BM as peripheral PMN in AIP MDS patients.

HLA-I absence has been associated with escape mechanisms to CTL recognition [26–29]. Therefore, the presence of HLA-I defective PMN suggests a correlation with the occurrence of CTL-dependent immune-selection processes.

Our study evidences a significant increase of PMN lacking HLA-I in Int-1 Risk MDS patients. Such evidence could point to CTL-mediated mechanisms acting in those patients with a more advanced MDS disease stage. The recognition of neo-antigens expressed by dysplastic cell clones and an altered self-tolerance in BM [5,30,31] might account for our observations. Notably, when patients were categorised according with their immune profile, the increase of peripheral PMN lacking HLA-I expression was preferentially observed in AIP patients if compared with the NIP counterpart.

The immune-based classification criteria, by us proposed, seem to identify a subgroup of Low/Int-1 patients in whom CTL selection mechanisms are pathogenetically relevant.

Our data are conceivable with the idea that HLA-E and HLA-I molecules are part of the biological mechanisms involved in the selection of BM precursors. Such immune-dependent selection processes might become pathogenetically relevant in patients in which the immunological activation is deranged (AIP subgroup of our patients). In this context, it is likely that the observed reduced presence of Treg in BM [25] might be unable to efficiently suppress autoreactive T clones that preferentially recognise self-antigens on PMN precursors expressing higher level of MHC-I molecules. This deranged immune response could be enhanced by the inflammatory BM microenvironment, described in MDS [15]. Another component of such complex scenario is the cytotoxic activity of NK effectors that could recognise and kill [17] the PMN precursors expressing low level of HLA-E/MHC-I [18].

A model for immune-mediated selection is hypothesised in Fig. 4. Ideally, the immune-dependent signals, allowing the maturation and migration of BM precursors in PB, might depend on expression of optimal levels of both HLA-E and HLA-I molecules that regulate NK and CTL recognition. These mechanisms could preferentially affect the selection of GC in MDS patients with a deranged adaptive response in BM (AIP group).

In support of our hypothesis are at least two evidences: (a) PB PMN cells of all patients in AIP group express higher HLA-E and lower HLA-I molecules (corresponding to B cell type in Fig. 4); (b) we found a greater percentage of MHC-I negative PMN (the C cell type in our model) in Int-1, where the MDS progression is more advanced in respect to the Low Risk. Notably, when the patients have been categorised according to their immune-profile, this feature characterised the AIP subgroup.

This and our previous investigations over the immune alterations in MDS [6,25] suggest the introduction of immune-based criteria in the classification of MDS patients. In particular, we aimed to better characterise those patients in which the immune-mediated mechanisms could be part of MDS pathogenesis. In this regard, it could be of some relevance the correlation between the expression of MHC-I/HLA-E molecules and the selection of PMN dysplastic clones in MDS. Therfore, the expression of the activating and inhibitory receptors on NK effectors and their interplay with classical and non classical MHC-I molecules on haematological cell target need further investigation to better understand the molecular mechanism on the basis of the emergence of dysplastic clones in MDS.

The better understanding of these aspects could provide useful tools for the clinical management of MDS.

Conflict of interest statement

The authors declare no competing financial interests.

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Author's contributions: FA coordinated the clinical management of patients; RDP participated in the clinical management of the patients; ATP, VR, RDP, AG participated in the design, interpretation and analysis of data. FA, GT and GR planned, directed and coordinated the research and wrote the paper. GT and FA equally contributed to the study.

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CASE REPORT

Natural killer expansion, human leukocyte antigens-E expression and CD14$^+$ CD56$^+$ monocytes in a myelodysplastic syndrome patient

Giuseppe Terrazzano[1,2], Valentina Rubino[2], Anna Teresa Palatucci[2], Angela Giovazzino[2], Mariangela Annunziatella[2], Orsola Vitagliano[3], Fiorella Alfinito[3,*], Giuseppina Ruggiero[2,*]

[1]Department of Science, University of Basilicata, Potenza; [2]Department of Translational Medical Sciences, University of Naples "Federico II", Naples; [3]Department of Clinical Medicine and Surgery, University of Naples "Federico II", Naples, Italy

Abstract

Myelodysplastic syndromes (MDS) are clonal disorders characterized by ineffective hematopoiesis and possible evolution to acute leukemia. Occurrence of stem cell defects and of immune-mediated mechanisms was evidenced as relevant for pathophysiology of MDS. Here, we described one case of MDS patient carrying CD14$^+$CD56$^+$ monocytes in bone marrow (BM), in the presence of a defective human leukocyte antigen (HLA)-E expression on peripheral blood (PB) cells and of natural killer (NK) cell expansion in PB and BM. The defective HLA-E expression and the NK expansion are proposed to be relevant for the pathogenesis of myelodysplasia in those patients showing CD14$^+$CD56$^+$ monocytes in BM.

Key words myelodysplastic syndromes; immune-mediated etiopathogenesis; natural killer; human leukocyte antigens-E; CD94/NKG2 receptors

Correspondence Dr. Giuseppe Terrazzano, Department of Science, University of Basilicata, Potenza, Italy. Tel: +39 081 7463059; Fax: +39 081 7463252; e-mail: terrazza@unina.it

*FA and GR equally contributed.

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Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of stem cell characterized by dysplasia, ineffective hematopoiesis, cytopenia, and frequent progression to acute myeloid leukemia (AML) (1). The International Prognostic Scoring System considers some clinical variables (i.e., blast percentage, cytopenia, and genetic abnormalities) to define four patient groups of risk to develop AML: low-, intermediate-1-, intermediate-2-, and high-risk patients (2). The needing of new grouping criteria to better characterize the patients and to improve MDS clinical management is recognized (3).

The pathophysiology of the bone marrow (BM) failure is complex, and MDS are more than a single disease entity. The occurrence of intrinsic stem cell defects and of immune-mediated mechanisms has been largely suggested (1–8).

Our previous data evidenced the relevance for immune dysregulation in MDS (7). On the different levels of BM regulatory T cells (Treg), we identified two subgroups of low-risk MDS patients. Intriguingly, only the subgroup with lower Treg percentage showed a BM CD8 lymphocytes recruitment. In addition, the different levels of the activation marker CD54 on BM CD8 lymphocytes revealed two subgroups of intermediate-1-risk patients. Notably, BM recruitment of CD8 lymphocytes in the low-risk group and/or the presence of high CD54 expression on BM CD8 cells in intermediate-1 patients are associated with more pronounced dyserythropoiesis and erythropoietin treatment. In addition, we suggested the occurrence of immune selection of dysplastic clones in such subgroup of MDS patients. Indeed, reduced human leukocyte antigens (HLA)-I and increased

HLA-E on peripheral polymorphonuclear cells (PMN) when compared with the BM counterpart were observed. We proposed that an altered immune profile might represent a valuable criterion to grouping low/intermediate-1 patients on the basis of immune pathogenesis of MDS (8).

These and other desirable criteria (3) could be useful to extend and improve the stratification of MDS patients.

In 2009, we characterized an MDS patient showing neutropenia, $CD14^+CD56^+$ monocytosis, and polyclonal expansion of natural killer (NK) expressing CD94/NKG2A and CD94/NKG2C receptors (6). In this patient, we proposed that the observed defective HLA-E expression on myeloid lineages, the constitutive presence of CD178/Fas-L on $CD14^+CD56^+$ monocytes, and the expansion of $CD94/NKG2A^+$ $CD94/NKG2C^+$ NK cells could be relevant for the pathogenesis of MDS.

Expression of ligands for NK receptors during myeloid maturation (9) highlights the NK involvement in the hematopoietic homeostasis. Literature suggested the role for IFN-γ secretion by large granular lymphocytes (LGL), belonging to T or NK lineages, in hematopoietic diseases (10).

Natural killer cells are crucial for the establishment of innate immunity and in the complex regulation of all the immune responses (11). They express two families of HLA-recognizing receptors: the killer immunoglobulin-like receptors and the lectin-like receptors, as CD94/NKG2A and CD94/NKG2C (12, 13). Altered expression of CD94/NKG2 receptors was correlated with chronic NK lymphocytosis and with other LGL disorders (10, 14, 15). HLA-E, a non-classical major histocompatibility complex class I molecule, is expressed by several immune and non-immune cells in healthy and pathological conditions (16, 17). The involvement of HLA-E in hematopoietic diseases has been proposed (6, 8, 10, 18). HLA-E mainly regulates NK functions (11–13). In this regard, CD94/NKG2A generates an inhibitory signal after the recognition of HLA-E, while CD94/NKG2C binds the molecule and induces the activation of NK cells (11–13). The balance between positive and negative signals, generated by these and other receptors, orchestrates the NK activities.

$CD56^+CD14^+$ cells is a minor and poorly characterized monocyte population, usually representing 0.2–3.5% of total peripheral mononuclear cells (19). They produce a variety of cytokines, present antigens, and could mediate regulatory functions.

Here, we describe a peculiar case of MDS patient showing thrombocytopenia in peripheral blood (PB), $CD14^+CD56^+$ monocytosis in BM, and polyclonal expansion of NK expressing CD94/NKG2A and CD94/NKG2C. In addition, we observed a defective HLA-E expression in the PB myeloid lineages. We speculate that the expansion of CD94/NKG2A+ CD94/NKG2C+ NK cells is relevant for the emergence/dominance of dysplastic clone(s) in those patients showing $CD14^+CD56^+$ monocytosis in BM.

Patient and methods

Case report

An 85-year-old man presented with thrombocytopenia, whose first record was referred to few months before. Hb was 12.9 g/dL, MCV 67fl, white blood cell count 4.4×10^9/L with 0.15×10^9/L neutrophils, 0.096×10^9/L monocytes, and 0.166×10^9/L lymphocytes. PMN appeared hypogranular and monocytes highly dysmorphic. Platelet count was 80×10^9/L.

BM aspirate was characterized by good cellularity with erythroid and granulocytic dysplasia, numerous micromegakaryocyte and 5% of blast cells. Patient received diagnosis of refractory cytopenia with multilineage dysplasia according to WHO classification (1). Microcytosis was due to beta thalassemia, and no organomegaly was present.

Patient showed a normal karyotype (46,XY).

Patient has been not receiving medical treatment, which may impact his immune response. Informed consent was obtained from the patient and the controls.

mAb, immune fluorescence and flow cytometry

Anti-CD3, -CD8, -CD4, -CD54, -CD25, -CD56, -CD14, -CD45, -CD33, -CD127, -p140 (Q66 clone) and isotype-matched labeled controls mAbs (Becton-Dickinson, Los Angeles, CA, USA), anti-HLA-I (W6/32 clone) mAb (SIGMA, Milan, Italy), anti-HLA-E mAb (MEM-E/08 clone) (Immunological Sciences, Rome, Italy); anti-CD158e1/e2 (p58.1/2), -CD244 (2B4), -CD335 (NKp46), -CD337 (NKp30), anti-NKG2A (Z199), -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 mAbs (Beckman-Coulter, Paris, France), and -NKG2C (134591 clone) mAb (R&D System, Minneapolis, MN, USA).

Flow cytometry was performed by FACSCalibur and CellQuest analysis software (Becton Dickinson), as described (6–8).

Three sex- and age-matched healthy donors were used as controls.

Statistical analysis

Statistical analysis for P calculation was performed by using Student's t-test. Results were considered significant with a P value ≤ 0.05.

Results

The patient shows dysplastic monocytosis (data not shown) and an extensive defect of HLA-E expression in both the monocytes and PMN in PB; indeed, the two populations

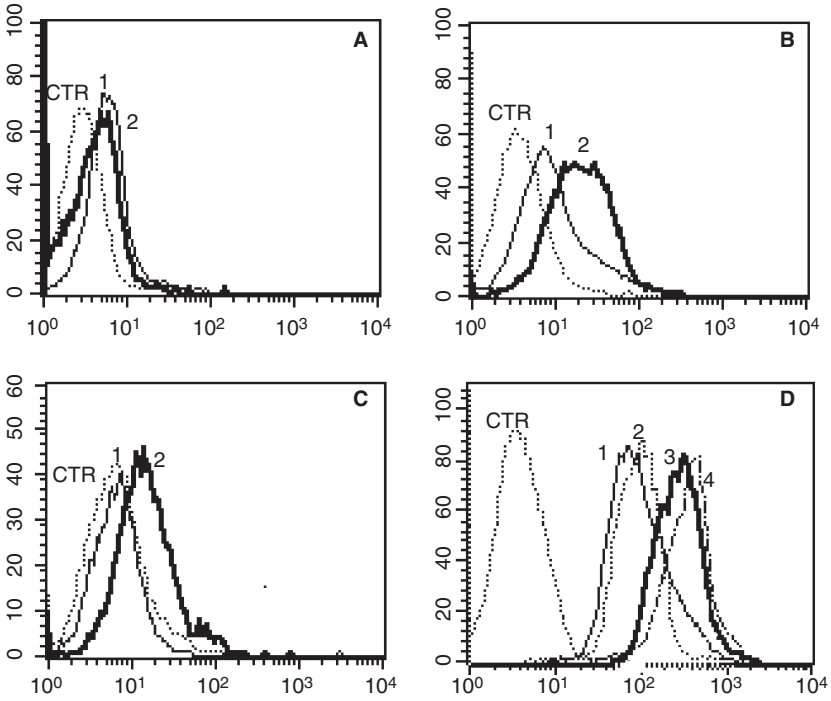

Figure 1 Flow cytometry analysis. Panel A: surface expression of human leukocyte antigen (HLA)-E on polymorphonuclear cells (PMN) (1) and Monocytes (2) in peripheral blood (PB); Panel B: surface expression of HLA-E on PMN (1) and Monocytes (2) in bone marrow (BM); Panel C: surface expression of HLA-E on $CD14^+$ $CD56^-$ (1) and $CD14^+CD56^+$ (2) Monocytes in bone marrow; Panel D: surface expression of HLA-I on PMN from BM (1) and PB (2) and on Monocytes from BM (3) and PB (4). The staining for Isotype mAb control is reported in all the panels (CTR). PB and BM PMN and monocyte populations were gated by FSC and SSC parameters and by CD45, CD14, CD33, CD56 labelling.

evidenced only a very low staining for HLA-E (Fig. 1A). In contrast, the monocytes and PMN are positive for HLA-E in BM (Fig. 1B). Intriguingly, the BM monocytes appear to express HLA-E as large distribution (see pick 2 in Fig. 1B) and at higher level if compared to PMN (Fig. 1B). In this regard, monocytes are divided into two subpopulations (Fig. 1C): one expresses at lower intensity the HLA-E and is $CD14^+CD56^-$, while the second one shows higher level of the molecule and is $CD14^+CD56^+$.

In addition, the expression of HLA-I on lymphocytes, monocytes, and PMN is similar between BM and PB (Fig. 1D).

The percentage of $CD14^+$ monocytes co-expressing the CD56 is significantly expanded in BM (more than 30% of monocytes) than in periphery (<2% of PB monocytes) (data not shown).

Natural killer expansion (more than 35% of lymphoid cell in BM and PB compartment) carried several NK receptors (CD335, CD337, CD244, CD158e1/e2, CD158/p70, CD94/NKG2A, CD94/NKG2C), suggesting that NK cells are frankly polyclonal (data not shown).

Intriguingly, the BM and PB NK cells are highly positive for two HLA-E receptors; in particular, the $CD3^-CD56^+$ cells expressing CD94/NKG2A are the 37% and CD94/NKG2C the 43% (data not shown).

Treg percentage and CD54 expression on both the $CD4^+$ and $CD8^+$ T Lymphocytes are similar to BM and PB, and the percentages are comparable to healthy donors (data not shown). Finally, the TCR repertoire of T lymphocytes appears to be polyclonal in BM and PB, without TCR-Vβ skewing of one or more T clones that, if present, could indicate a significant antigen specific T response in BM (data not shown).

Discussion

The patient here described shows thrombocytopenia, monocytosis and expansion of polyclonal NK cells, and normal karyotype. These features are accompanied by a defect of HLA-E expression on PMN and monocytes in PB.

Notably, despite the similarities of this MDS case with the other by us observed patients for the likely involvement of HLA-I and HLA-E in fostering the myelodysplasia (8), this patient seems to be substantially unframed with the immunological criteria that we previously published (7). Indeed, the patient reveals no significant variation in the percentage of Treg or of expression of CD54 when compared with healthy controls. Nevertheless, the patient shows an intriguing analogy with previous patient (6) for the presence of dysplastic monocytosis, mainly represented by $CD14^+CD56^+$HLA-E^+ cells in BM, and for the expansion of CD94/$NKG2C^+$ NK cells.

Here, we suggest that BM NK expansion, with increased expression of CD94/NKG2C, generates a selective pressure

on hematopoietic compartments. In this regard, patient CD94/NKG2C$^+$ NK cells could have exerted an efficiently killing of HLA-E$^+$ myeloid precursors, thus targeting HLA-E$^+$ monocytes and HLA-E$^+$ PMN in BM and sparing those cells carrying the lowest HLA-E levels. Indeed, it is worth noting that the dysplastic monocytes are mainly HLA-E$^+$CD14$^+$CD56$^+$ in BM and become HLA-E$^-$CD14$^+$CD56$^-$ in PB likely due to the action of NK selection.

Moreover, the expression of HLA-I appears to be not involved in the selection by NK in this patient, as the levels of these molecules on PMN and monocytes are similar between PB and BM.

These evidences suggest that the action of NK took place as a sort of 'customs' that allowed to pass only certain cells with certain characteristics from the BM toward the periphery. In reason of this NK selective pressure, the monocyte and PMN populations have been totally defective for HLA-E expression in PB.

We could not rule out that other receptor/ligand pathways could have a role in regulating NK activity in this patient. In addition, the TCR repertoire appears to be unskewed, and such result could formally exclude antigen specific T-cell response in the patient, at least at the time of our observation.

Taken together, the current study and the others by us published (6–8) suggest that the emergence and dominance of dysplastic clones could be inferred and/or sustained in some MDS patients also by NK activity alone and/or as contribute, together with other immune-mediated mechanisms. The relevance for the expression of HLA-E and HLA-I on hematopoietic cells could be largely different, and it can range from etiopathogenetic role to some physiopathological aspects. This consideration makes sense when one considers the balance between anti-tumor immune response and the possible detrimental implication of immune editing in cancer (20). Likewise, MDS condition is identifiable as the consequence of several clonal cytogenetic abnormalities conferring proliferative and surviving advantage to those stem cells generating dysplastic clones (1, 2). In this context, it has been proposed that inflammatory BM microenvironment and an altered immune response could not only represent huge protective reactions of the individual from disease, but also favorable conditions for the emergence, expansion, and dominance of dysplastic clones in a subgroup of MDS patients (1–8).

The plain significance of this mechanism in counteracting, causing, sustaining, or accompanying the MDS is not definitively established, but it appears to be of great relevance to better understand the role as 'Janus Bifront' for immune response in MDS.

In conclusion, this speculation could open new insight into the etiopathogenesis of MDS, and it could offer useful tools to the clinical decision making and for a more accurate stratification of those patients in whom the hypothesis of immune-mediated mechanisms is conceivable.

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Author contributions

FA coordinated the clinical management; OV participated in the clinical management; ATP, VR, and AG participated in the design, interpretation, and analysis of data. FA, GT, and GR planned, directed, and coordinated the research and revised the manuscript.

Disclosure

The authors declare no competing financial interests.

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Regulatory T cells, Cytotoxic T lymphocytes and a T_H1 cytokine profile in dogs naturally infected by *Leishmania infantum*

Laura Cortese [a], Mariangela Annunziatella [b], Anna Teresa Palatucci [b], Valentina Rubino [b], Diego Piantedosi [a,*], Antonio Di Loria [c], Giuseppina Ruggiero [b], Paolo Ciaramella [a], Giuseppe Terrazzano [b,d]

[a] Department of Veterinary Medicine and Animal Productions, Division of Internal Medicine, University of Naples Federico II, Via Delpino, 1, 80137 Naples, Italy
[b] Department of Translational Medical Sciences, University of Naples Federico II, Via Pansini, 5, 80131 Naples, Italy
[c] Department of Health Science, University of Catanzaro "Magna Graecia", Viale Europa, Località Germaneto, 88100 Catanzaro, Italy
[d] Department of Science, University of Basilicata, Via Sauro, 85, 8100 Potenza, Italy

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Flow cytometry

ABSTRACT

Canine leishmaniasis caused by the protozoan parasite *Leishmania infantum* is a chronic systemic disease endemic in Mediterranean basin. The aim of the study is to investigate the immune profile of dogs naturally infected by *Leishmania infantum*. In order to address such issue, $CD4^+$ and $CD8^+$ lymphocyte T cell subsets, peripheral $CD4^+CD3^+Foxp3^+$ (Treg) levels and the presence of pro-inflammatory T cells have been assessed, in 45 infected dogs and in 30 healthy animals, by using immunofluorescence and flow cytometry detection. Animals were categorised according to their clinical-pathological status and their antibody titer at diagnosis. Results showing a significant increase of $CD8^+CD3^+$ T lymphocytes, a reduced percentage of the T regulatory $CD4^+CD3^+Foxp3^+$ subset and a significant increase of T_H1 cells, characterise the infected dogs, regardless of their antibody titer or the occurrence of clinical symptomatic disease. These data may provide new insights into the pathogenesis of immune-mediated alterations associated with canine leishmaniasis.

1. Introduction

Canine leishmaniasis (CL), caused by the protozoan *Leishmania infantum* and transmitted by the bite of phlebotomine sand fly vectors, is a severe zoonosis potentially fatal to humans and dogs, which comprise the main reservoir of infection to humans. CL is endemic in more than 70 countries in the world. It is present in regions of southern Europe, Africa, Asia, South America and Central America and has been reported also in United States of America (Petersen and Barr, 2009). A broad range of immune responses and clinical manifestations has been described in CL. In fact, clinical appearance and evolution of leishmaniasis is the consequence of complex interactions between the parasite and the genetic and immunological background of the host (Baneth et al., 2008; Maia and Campino, 2012). *Leishmania* infection in dogs may be manifested as a subclinical infection, a self-limiting disease, or a non-self-limiting and severe illness (Bottero et al., 2006). In dogs, the two opposite extremes of this clinical spectrum are characterized by a protective immunity that is mediated by $CD4^+$ T cells by the release of interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor (TNF-α) that induce macrophage anti-*Leishmania* activity, and a disease susceptibility that is associated with the production of a marked humoral non-protective immune response and a reduced or depressed cell mediated immunity with a mixed T helper 1(T_H1) and T helper 2 (T_H2) cytokines response (Alvar et al., 2004; Baneth et al., 2008). Within this spectrum, clinical signs of disease can range from a mild dermatitis and alopecia, associated with specific cellular immunity and low humoral responses (Ordeix et al., 2005) to a severe disease, characterized by renal damage with glomerulonephritis due to immune complex deposition, associated with a massive humoral response and high parasite loads (Costa et al., 2003). In addition, asymptomatic visceral leishmaniasis has been consistently associated with the induction of cellular immunity in endemic areas (Baneth et al., 2008; Reis et al., 2009; Solano-Gallego et al., 2009; Alexandre-Pires et al., 2010; Coura-Vital et al., 2011).

The characterization of the ongoing immune response in animals infected by *L. infantum* has been described to be relevant to establish a prognosis and to predict a clinical response to treatment (Papadogiannakis et al., 2010). Several studies pointed out that lymphocyte level in sick dogs is decreased but returns to normal values after treatment (Moreno et al., 1999; Guarga et al., 2002; Guerra et al., 2009); an increased number of $CD4^+$T cells in dogs with low parasitism (Reis et al., 2006) has been also referred. Furthermore, the number of $CD4^+$ T cells in peripheral blood was

* Corresponding author. Tel.: +39 0812536004; fax: +39 0812536008.
E-mail address: dapiante@unina.it (D. Piantedosi).

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observed to be similar in dogs with leishmaniasis and in healthy dogs while there was no correlation between the clinical status or response to therapy and $CD4^+$ T cell counts (Miranda et al., 2007). The results obtained highlight the complexity of the immune response to *L. infantum* infection. Indeed, while low levels of circulating B cells and monocytes have been observed to behave as important markers of severe CL, the number of $CD4^+$ T lymphocytes cannot be used alone as a prognostic marker (Miranda et al., 2007; Alexandre-Pires et al., 2010). Increased levels of $CD8^+$T lymphocytes have been referred as a mayor phenotypic feature of asymptomatic disease (Reis et al., 2006; 2009).Treatment and clinical recovery have been associated with improved and/or normalized levels of T cells, $CD4^+$ subset, $CD4^+/CD8^+$ ratio and antigen-dependent proliferation in CL (Moreno et al., 1999; Guarga et al., 2000, 2002; Papadogiannakis et al., 2010).The preferential association of a pro-inflammatory microenvironment with a favourable outcome of the disease has been largely suggested in CL (Chamizo et al., 2005). Moreover, the analysis of cytokine production in peripheral blood revealed some discrepancy (Maia and Campino, 2012).

The occurrence in CL of immune-mediated pathological processes that have been observed to be involved in the induction of polyarthritis, uveitis, glomerulonephritis, (Kharazmi et al., 1982; Ferrer, 1992; Ciaramella et al., 1997; Koutinas et al., 1999; Costa et al., 2003; Solano-Gallego et al., 2009), as well as of antibody-depend platelet abnormalities (Terrazzano et al., 2006; Cortese et al., 2009, 2011), suggest the relevance of immune response control in this infection. Control of the immune response in human and murine leishmaniasis has been observed to largely depend on the production of interleukin-10 (IL-10), which can come from several different cell types (Kaye and Scott, 2011). A key immune regulatory mechanism is represented by the $CD4^+CD25^+Foxp3^+$ T (Tregs) subset (Sakaguchi, 2005). Tregs are able to control the immune effector cells in terms of clonal expansion, differentiation, cytokine pattern and tissue migration during immune response. It is relevant that Tregs control T cell priming in lymphoid organs but they are also able to inhibit immune response in peripheral tissues (Miller et al. 2004; Huehn and Hamman, 2005; Sakaguchi, 2005). The role of Tregs in preventing harmful autoimmune responses and their dynamic involvement in the modulation of immune-response against infectious agents (Nylén and Sacks, 2007; Belkaid and Tarbell, 2009) and transformed cells (Curiel et al., 2004) has been consistently described. In human visceral leishmaniasis (VL) reports on the frequency and function of Tregs are not conclusive. Recently, Maurya et al. (2010) reported that active VL is not associated with increased frequencies of peripheral Foxp3 Treg or accumulation at the site of infection.

In veterinary medicine, Tregs have been observed to significantly increase in dog tumour models (Biller et al., 2007; Houriuchi et al., 2009; O'Neill et al., 2009; Rissetto et al., 2010). Few studies addressed the level of regulatory cytokines interleukin-10 (IL-10) and the transforming growth factor beta (TGF-β) (Strauss-Ayali et al., 2007; Boggiatto et al., 2010) in CL. The study of Treg involvement in the regulation of dog immune-response against *Leishmania* needs further investigation. Indeed, IL-10 producing regulatory T cell subsets have been described to be relevant in the early phase of visceral leishmaniasis in mouse model of *L. infantum* infection (Rodrigues et al., 2009), while a reduction in lymph-node Treg was described to significantly correlate with immune protection consequent to Leishmune vaccination (de Lima et al., 2010).

The aim of the present study is to investigate the immune profile of dogs naturally infected by *L. infantum*. In order to address the immune regulatory networks involved in CL, lymphocyte T cell subsets, peripheral Treg levels and the presence of pro-inflammatory T cells have been assessed, in a cohort of 45 *Leishmania* infected dogs (LD), compared with 30 sex/age paired healthy animals, by using immune fluorescence and flow cytometry detection. Leishmaniotic dogs have been categorised according to their clinical-pathological status and their serological anti specific-*Leishmania* antibody titer.

2. Materials and methods

2.1. Animals

Forty-five dogs naturally infected by *L. infantum* (20 males and 25 females, 5–9 years old) from the Campania region (South Italy) were enrolled with the owner's consent. Ten dogs were pure breed, while 35 were mongrels. Also, 30 healthy dogs (12 males, 18 females, 5–7 years old) with no clinical signs of CL, which were also being negative for serological, parasitological and molecular examinations, were considered as healthy control group.

This work has been reviewed by Ethical Animal Care and Use Committee of the University of Naples Federico II and received institutional approval (Prot. 2011/0043885).

2.2. Clinical parameters and diagnostic procedure

For all dogs, the history (none of dogs received a specific treatment for CL) and the clinical examination were performed. *Leishmania* infected dogs were classified according to Solano-Gallego et al. (2009). The LD cohort has been categorised in two groups: LD-A dogs ($n = 23$), presenting neither clinical signs on physical examination nor clinical-pathological abnormalities by routine laboratory tests (CBC, biochemical profile and urinalysis) but with a confirmed *L. infantum* infection and LD-S dogs ($n = 22$) with clinical leishmaniasis, presenting clinical signs and/or clinical-pathological abnormalities and a confirmed *L. infantum* infection (Table 1). Diagnosis of CL has been confirmed by detection of amastigotes in stained cytological smears of aspirates from lymph nodes or bone marrow, serologically by a positive indirect fluorescent antibody test (IFAT $\geqslant$ 1:80) and by a polymerase chain reaction (PCR). Animals presenting IFAT titers $\geqslant$ 1:80 and positive molecular diagnosis were included in the study as infected animals. The relevance of humoral immune response on susceptibility/resistance mechanism during ongoing canine visceral leishmaniasis has been recognized throughout ex vivo and in vitro investigations (Oliva et al., 2006; Reis et al., 2006). Thus, in order to analyse the immune profile in *Leishmania* infected animals showing different degree of specific immune activation, *Leishmania* infected dogs were further sub-classified as Low IFAT titer animals (LD-L) when showing a IFAT titer 1:80–1:160 or High IFAT Title animals (LD-H) when showing a IFAT titer >1:160.

Animals presenting neither clinical signs on physical examination nor clinical-pathological abnormalities by routine laboratory tests, and with IFAT negative ($\leqslant$1:40) and negative PCR, were considered non-infected and included as healthy control group.

Occurrence of other infectious diseases was always excluded in all dogs. No *Ehrlichia canis* nor *Anaplasma phagocytophilum* morulae, *Babesia canis* trophozoites and microfilariae were observed in peripheral blood smears. These infections were also excluded using IFAT and/or PCR. Finally, *Dirofilaria immitis* infection was ruled out using the Snap Canine Combo Heartworm Antigen Antibody Test (IDEXX).

2.3. Blood sample collection

Ten millilitres of peripheral blood was collected from the jugular vein into tubes containing ethylene diamine tetraacetic acid (EDTA). A complete cell blood count was performed in each sample within 30 min from the collection by using a semi-automatic cell counter (Genius S; SEAC Radom Group, Florence, Italy). All samples

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Table 1
Results of diagnostic tests and clinical and laboratory findings of 45 *Leishmania* infected dogs.

LD – A dogs	IFAT	PCR	Cyt	CS	HT%	PLT $10^3/\mu L$	UREA mg/dL	CREATININE mg/dL	TPg/dL	A/G
1	1/160	■	■		40	470	28	0.6	7.3	0.8
2	1/320	■	ND		38	223	26	0.5	6.6	1.0
3	1/320	■	■		44	195	33	1.3	6.7	1.2
4	1/160	■	■		40	337	24	0.6	7.2	0.9
5	1/320	■	■		38	258	49	1.4	6.8	1.0
6	1/160	■	N		41	452	29	0.7	6.6	0.6
7	1/160	■	N		37	350	26	1.2	5.8	1.0
8	1/160	■	ND		42	364	44	1.1	7.3	1.2
9	1/160	■	N		38	433	46	0.9	7.4	0.7
10	1/160	■	N		46	267	36	1.2	6.9	1.3
11	1/80	■	■		45	482	34	0.7	7.4	0.9
12	1/160	■	■		37	400	41	0.5	6.0	1.0
13	1/80	■	ND		49	353	43	1.1	7.3	0.9
14	1/320	■*	N		40	450	17	0.8	7.0	0.8
15	1/640	■	■		38	180	24	1.0	5.5	1.4
16	1/160	■	N		39	181	49	1.0	7.3	0.9
17	1/640	■	ND		38	236	21	0.8	6.9	1.1
18	1/80	■	N		39	241	39	1.0	5.2	1.0
19	1/80	■	N		42	231	23	0.5	6.8	1.2
20	1/80	■	N		40	221	27	0.6	7.4	1.0
21	1/80	■	ND		39	253	48	1.0	7.2	0.9
22	1/80	■	N		50	177	34	0.6	7.0	1.5
23	1/80	■	■		45	268	25	0.7	7.6	0.8
LD – S dogs										
1	1/1280	■	■	■	34	123	21	0.6	6.6	1.0
2	1/1280	■	■	■	36	280	45	1.2	9.0	0.5
3	1/160	■	■	■	35	100	55	1.4	9.2	0.5
4	1/1280	■*	ND		48	311	57	0.7	8.7	1.0
5	1/2560	■	■	■	47	107	65	0.7	8.1	0.7
6	1/320	■	■		38	130	22	0.9	7.1	0.8
7	1/320	■	■	■	45	268	27	1.0	9.4	0.5
8	1/80	■	■		35	275	32	1.0	9.6	0.5
9	1/10240	■	■	■	33	425	35	0.9	9.4	0.5
10	1/80	■	■	■	40	255	40	0.6	8.1	0.5
11	1/640	■	ND	■	36	410	50	0.5	6.0	1.0
12	1/1280	■	■	■	33	216	156	3.2	8.7	0.5
13	1/1280	■	■	■	45	378	28	0.9	9.4	0.5
14	1/640	■	■	■	57	450	31	0.6	9.1	0.5
15	1/320	■	ND	■	42	337	34	0.8	9.0	0.5
16	1/2560	■	■	■	31	277	37	0.9	8.9	0.4
17	1/2560	■*	■	■	33	200	56	0.8	10.3	0.3
18	1/10240	■	■	■	23	58	49	0.6	12.7	0.3
19	1/1280	■	N	■	41	148	61	1.5	6.1	0.3
20	1/160	■	■	■	41	308	38	0.8	10.2	0.4
21	1/80	■	■	■	34	96	21	0.7	8.0	0.7
22	1/320	■*	N	■	43	384	41	1.0	7.2	0.7

LD – A dogs, *Leishmania* asymptomatic dogs; LD – S dogs, *Leishmania* symptomatic dogs; IFAT, indirect fluorescent antibody test; PCR, polymerase chain reaction on bone marrow (lymph node and peripheral blood *, 4 dogs); Cyt, lymph nodes or bone marrow cytology; CS, Clinical signs. The squares indicate the positivity for each specific test and the presence of any clinical sign (alopecia, dermatitis, skin ulcers, ocular lesions, conjunctivitis, onychogryphosis, weight loss and lymphadenopathy); ND, not determined; N, negative; HT, hematocrit (anemia: HT value < 37 %); PLT platelet count (thrombocytopenia: platelets count < 150 $10^3/\mu L$); UREA, increase of urea > 50 mg/dL; CREATININE, increase of creatinine > 1.5 mg/dL; TP, total protein (increase of total proteins >7.7 g/dL); A/G, albumin/globulin ratio (hyperglobulinemia: A/G < 0.6).

were maintained at room temperature up to 5–6 h prior to processing. In addition, serum aliquots were obtained from all the dogs enrolled for the study of their biochemical profile and serological examination.

2.4. Serological diagnosis

Detection of anti-*Leishmania* IgG antibodies was performed by an in-house IFAT assay using *L. infantum* promastigotes (WHO reference strain MHOM/TN/1980/IPT-1) as antigen and following the protocol recommended by the Office International des Epizooties (Gradoni and Gramiccia, 2008). Samples were classified as positive if promastigote cytoplasmatic or membrane fluorescence was observed at a serum dilution of 1:80 or higher.

2.5. Molecular diagnosis

To perform a nested-PCR assay for *Leishmania spp.* DNA was obtained from bone marrow. In 4 animals, due to lack of bone marrow specimens, lymph nodes aspirates and peripheral blood were used as DNA source for nested-PCR assay. Briefly, the first amplification was carried out in a 50 μl reaction containing 10 μL DNA and 40 μL PCR Master Mix (Promega) with 50 pmol of the kinetoplastid-specific primers R221 and R332 of the small-subunit rRNA gene (van Eys et al., 1992). For the second amplification, 3 μL of the first PCR product were added to 47 μL of PCR Master Mix (Promega) containing 50 pmol of the *Leishmania*-specific primers R223 and R333 of the same gene (van Eys et al., 1992). The nested-PCR product was 358 bp. For the *E. canis* nested-PCR assay, DNA was extracted from peripheral blood, bone marrow and/or lymph nodal aspirate material. Briefly, the first PCR was performed in a 25 μL reaction containing 5 μL of DNA template with 12.5 pmol of primer set of universal fD1 and *Ehrlichia* genus-specific EHR16SR. These primers can amplify the ehrlichial 760 bps partial sequence of the 16SrRNA gene (Inokuma et al., 2001). For the second amplification, 5 μL of the first PCR product were added to 20 μL of PCR

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Master Mix containing 12.5 pmol of the *Ehrlichia*-specific primers CANIS and GA1UR of the same gene (Inokuma et al., 2001). The nested-PCR product was 409 bps. The amplification products were analysed by 1.5% agarose gel and visualized under UV light.

2.6. Monoclonal antibodies, immunofluorescence and flow cytometry

Peripheral blood from each dog was employed as whole blood sample to evaluate the number of $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD4^+$-$Foxp3^+$ T cells by immune-fluorescence technique and flow cytometry analysis. Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Cy-chrome and Allophycocyanin (APC) labelled monoclonal antibodies (mAb) against dog CD3, CD4, CD8, and isotype-matched controls were purchased from Serotec Ltd, London, UK. Intracellular detection of Foxp3 was performed using a cross-reactive, directly conjugated murine Foxp3 antibody (Clone FJK-16s, eBioscience, San Diego, CA) and the permeabilisation buffer provided by the detection Kit (Foxp3 Staining Set, eBioscience), as described (Biller et al., 2007). Foxp3 staining was carried out on Peripheral Blood Mononuclear Cells (PBMC) purified by Ficoll (Sigma–Aldrich, St. Louis, MO) density gradient centrifugation, as described (Biller et al., 2007). $CD8^+$ and $CD4^+$ T cell subsets were always identified by a combination of canine specific anti-CD3 together with anti-CD4 or anti-CD8 mAbs. A typical phenotype analysis strategy is shown in Fig. 1.

To analyze the production of IFN-γ and IL-4, purified PBMC were cultured overnight (ON) in presence of Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin, all purchased from Sigma–Aldrich. This approach has been widely described as useful in the study of established cytokine profile in human and animal models (Terrazzano et al., 2005; Papadogiannakis et al., 2009; Olsen and Sollid, 2013). Intracellular staining with the mAbs recognising dog IFN-γ, IL-4 or isotype-matched controls (Serotec) was performed by a fixing/permeabilization kit (Caltag, Burlingame, CA), following the manufacturer's instructions. To avoid extracellular cytokine export, the cultures were incubated in the presence of 5 μg/ml of Brefeldin-A (Sigma–Aldrich), as described (Terrazzano et al., 2005; Papadogiannakis et al., 2009).

All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using Forward Scatter (FSC) and Side Scatter (SSC) parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by using a two laser equipped *FACScalibur* apparatus and the *Cell Quest analysis software* (Becton Dickinson, Mountain View, CA).

2.7. Statistical analysis

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

3. Results

3.1. Leishmania infected dogs show a significant increase in peripheral $CD8^+$ T cells

First of all, we focused our investigation on the distribution of helper ($CD4^+CD3^+$) and cytotoxic ($CD8^+CD3^+$) T lymphocytes in

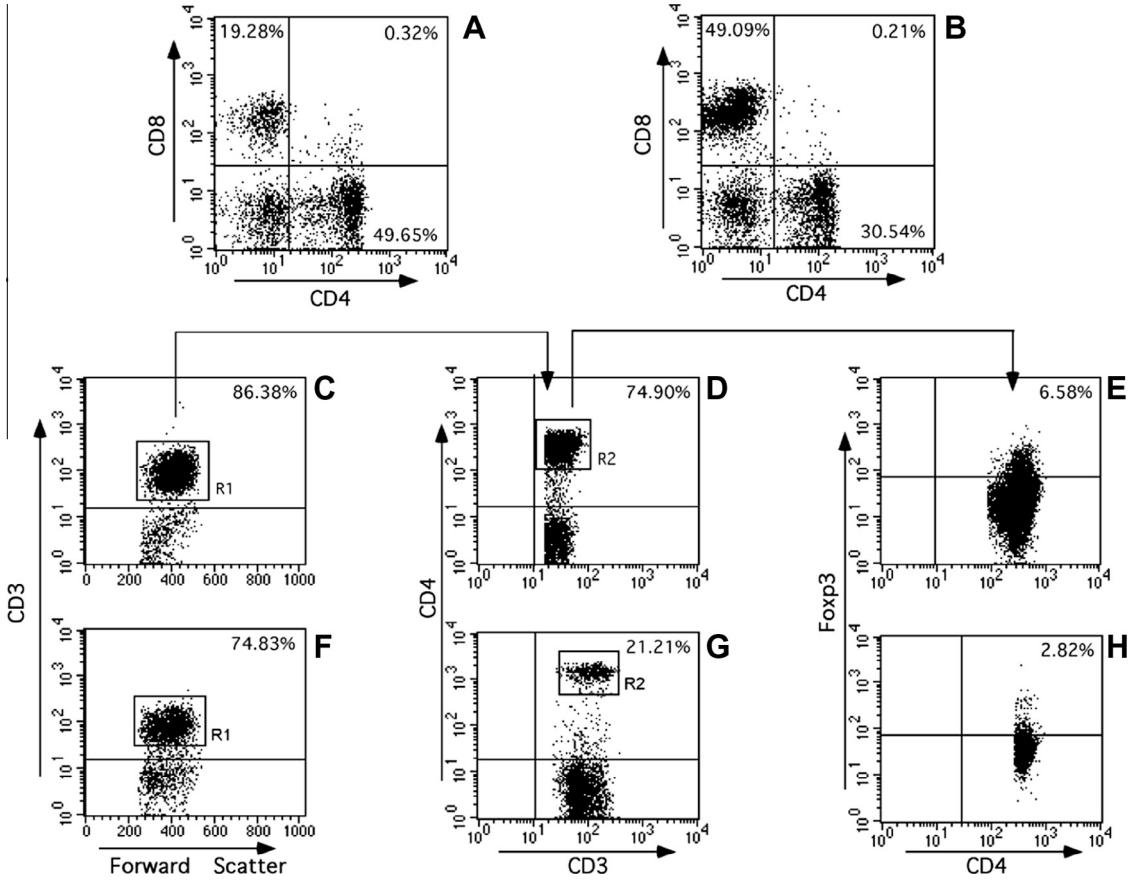

Fig. 1. Flow cytometry analysis strategy for phenotype studies. Panel A and B refer typical staining profiles for $CD4^+$ T and $CD8^+$ T cells obtained in a healthy control and a *Leishmania* infected animal, respectively; C–E Panels show the analysis strategy employed to identify $CD4^+Foxp3^+$ Treg subset in PBMC sample obtained from a control dog. As shown, T cell region (defined by CD3 staining as R1) has been employed to identify the $CD4^+$ T cell subset (R2); in this region the presence of Foxp3 transcription factor allowed the identification of the $CD4^+Foxp3^+$ Treg subset. D–H Panels show a typical analysis for $CD4^+Foxp3^+$ Treg subset in a *Leishmania* infected dog.

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peripheral blood of LD animals as a whole or categorised according to the presence (LD-S) or absence (LD-A) of clinical signs of disease and/or clinicopathological abnormalities, as well as to the IFAT titer (1:80-1:160; LD-L or >1:160; LD-H). In order to maintain the biological complexity of immune subsets, we used immunofluorescence and multi-parametric flow cytometry detection. Fig. 1 shows a typical phenotype analysis strategy. The study has been performed on whole blood or on the bulk of PBMC and the distinct cell subsets have always been defined by co-expression of CD3 molecule, strictly dependent on the presence of a functional T cell Receptor (TCR). As shown in Fig. 2-A, *Leishmania* infected dogs (LD) are characterised by significant increase ($p < 0.0001$) of the percentage of cytotoxic T cell effectors ($CD8^+CD3^+$) compared with healthy controls (67.47 ± 1.85 in LD vs 44.2 ± 2.5 in healthy controls). This observation has been confirmed by the significant decrease of the percentage (31.94 ± 2.05 in LD vs 55.76 ± 2.36 in controls; $p < 0.0001$) of $CD4^+CD3^+$ T cell subset (Fig. 2-C). No significant differences have been observed considering the occurrence of a High versus Low IFAT titer (LD-H vs LD-L) as well as the presence of a symptomatic versus an asymptomatic disease (LD-S vs LD-A). Panels B and D of the Fig. 2 show the comparison of the cell count obtained in the different animal groups. As shown, the significant increase in $CD8^+CD3^+$ T cells (1224 ± 135 in LD vs 366.9 ± 17 in controls; $p < 0.0001$) is confirmed in all the disease subgroups in comparison with healthy control animals (Fig. 2-B). The data on $CD4^+CD3^+$ T cells showed a significant decrease (564.5 ± 55.6 in LD vs 731 ± 26.19 in controls; $p < 0.05$) of this cell subset in comparison with controls in all the LD animals, except the LD-A sub-group (Fig. 2-D) in which no significant differences have been observed with controls (701 ± 59 in LD-A vs 731 ± 26.19 in healthy animals). Notably, a slight no significant increase in the number of $CD3^+$ lymphocytes have been observed in the *Leishmania* infected animal in comparison with controls (data not shown).

Fig. 3 shows that a significant difference (0.53 ± 0.04 in LD vs 1.83 ± 0.15 in controls; $p < 0.0001$) in the $CD4^+/CD8^+$ ratio characterises our cohort of *Leishmania* infected animals. This observation has been confirmed in all the sub-groups of *Leishmania* infected animals, regardless of the IFAT titer or the occurrence of a symptomatic versus asymptomatic disease.

3.2. A reduced level of regulatory T cells and an increase in interferon-γ producing T lymphocytes characterises Leishmania infected dogs

Fig. 4 refers to the comparative analysis of Treg level in the peripheral blood of the pathological cohort and control group. As shown, significant reduction of the percentage of Treg (Fig. 4-A) in the peripheral blood of the *Leishmania* infected cohort has been observed (5.39 ± 0.72 in LD vs 7.17 ± 0.63 in controls; $p < 0.05$). As shown, the reduced Treg percentage significantly correlates with the occurrence of a high IFAT titer (4.35 ± 0.95 in LD-H vs 7.17 ± 0.63 in controls; $p < 0.01$). Notably, when the Treg count was analysed in the pathological cohort versus controls (Fig. 4-B),

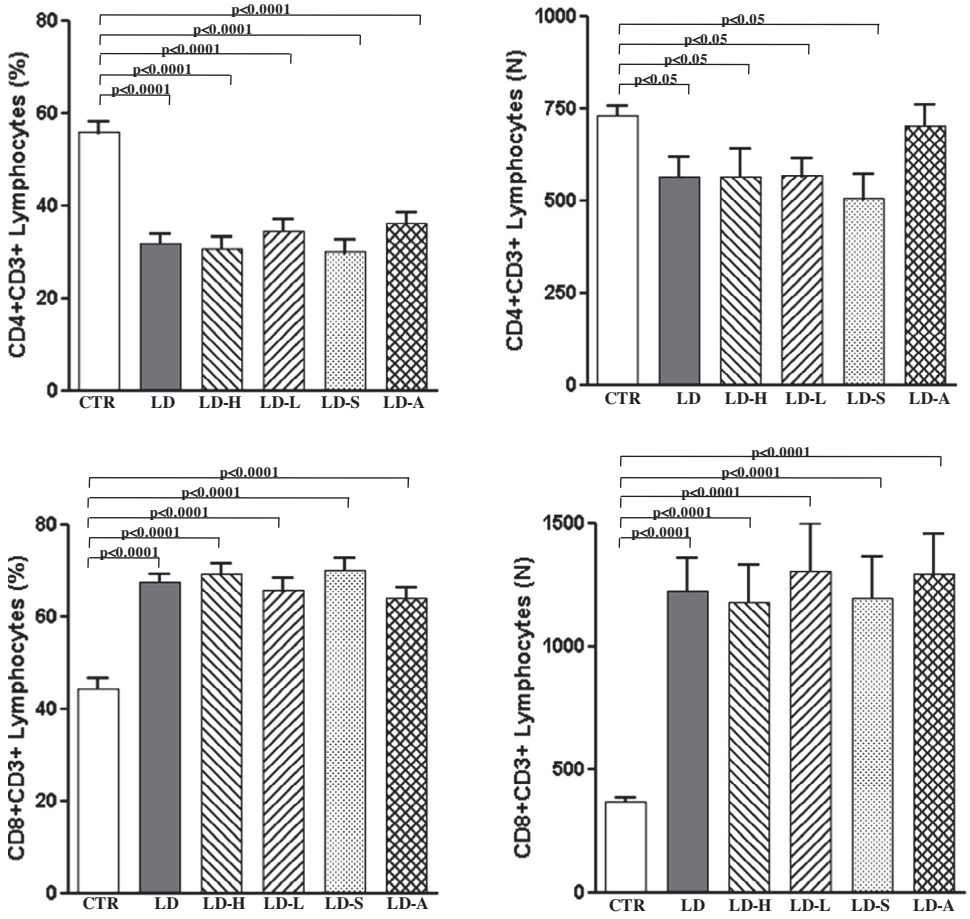

Fig. 2. Significant increase of $CD8^+$ T lymphocyte subset characterises *Leishmania* infected dogs. Panel A and B refer percentage and count ($\times 10^{-6}$/L) of $CD8^+CD3^+$ lymphocytes in peripheral blood of healthy animals (CTR; white column); *Leishmania* infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Panel C and D refer percentage and count ($\times 10^{-6}$/L) of $CD4^+CD3^+$ lymphocytes in peripheral blood of the dogs categorised as previously indicated. Statistical analysis has been performed by using Mann–Whitney test. *P* values are indicated only for significant comparisons.

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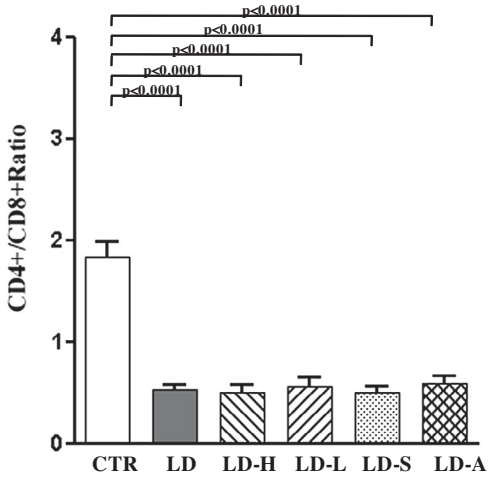

Fig. 3. $CD4^+/CD8^+$ ratio is significantly reduced in *Leishmania* infected dogs. Referred values have been observed in peripheral blood of healthy animals (CTR; white column); *Leishmania* infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Statistical analysis has been performed by using Mann–Whitney test. *P* values are indicated only for significant comparisons.

the difference appeared more consistent, regardless of the pathological subgroup considered (12.74 ± 1.59 in LD vs 27.79 ± 1.94 in controls; $p < 0.0001$; 13.28 ± 2.3 in LD-H; $p < 0.001$; 11.80 ± 1.2 in LD-L; $p < 0.0001$; 12.78 ± 2.16 in LD-S; $p < 0.0005$; 12.77 ± 1.18 in LD-A; $p < 0.0001$). Unlike CD4 + Treg that are largely defined by the expression of Foxp3, Foxp3 expression in CD8 + clones was not correlated with their suppressive activity (Hu et al., 2012, 2013). In this study, we checked for Foxp3 expression $CD8^+$ effectors, but very faint expression was observed in both controls and *Leishmania* infected dogs (data not shown).

The cytokine profile has been largely described as relevant for *Leishmania* spreading control (Chamizo et al., 2005; Carillo and Moreno, 2009; Boggiatto et al., 2010). In order to assess whether the immunological profile by us described (high $CD8^+CD3^+$ T lymphocytes and reduced Treg cells), might be related with the induction of a specific cytokine profile in T lymphocytes, we analysed the IFN-γ and IL-4 production in T lymphocytes of LD animals as compared with healthy controls by immunofluorescence and flow cytometry detection. The analysis has been performed after an ON culture of PBMC, isolated from the peripheral blood of the animals, with Medium or PMA plus Ionomycin. This experimental approach is expected to allow the analysis ex vivo of the cytokine profile acquired by the T cells in vivo (Terrazzano et al., 2005; Olsen and Sollid, 2013). As shown in Fig. 5, the percentage of T_H1 T cells (specifically producing IFN-γ and negative for IL-4) in LD animals is significantly increased as compared with controls ($p < 0.05$) regardless of the grouping criteria used. No significant difference between the controls and the infected cohort was observed in the basal production of IFN-γ, detected in Medium cultures.

4. Discussion

In our report we describe a significant increase of $CD8^+CD3^+$ T lymphocytes and T_H1 T cells ($IFN\text{-}\gamma^+IL\text{-}4^-CD3^+$ lymphocytes) accompanied by a significant reduction of Tregs lymphocytes in a cohort of *Leishmania* infected dogs regardless of the IFAT titer or the occurrence of clinical symptomatic disease.

Reduced percentage of $CD4^+$ T cells has been described in CL, suggesting that CD8-dependent IFN-γ production and lysis of infected-macrophages can mediate protective immunity during the disease (Guarga et al., 2000). Increased number of $CD8^+$ T effectors has been referred to preferentially characterise asymptomatic animals (Reis et al., 2006, 2009). In addition, higher counts of T lymphocytes, mainly due to increased $CD8^+$ T lymphocytes and accompanied by a decreased $CD4^+/CD8^+$ ratio, were described to characterise asymptomatic animals with positive serology and positive molecular results (Coura-Vital et al., 2011). Furthermore, the relevance of variations in the percentage of $CD3^+$ and $CD4^+$ T cells in mildly and severely infected dogs has been highlighted (Miranda et al., 2007) while prolonged allopurinol monotherapy was observed to improve the number of circulating $CD4^+$ T cells, but not to restore their number to within the normal range (Papadogiannakis et al., 2010).

Our results indicate that increase of $CD8^+$ T cells, in presence of normal levels of T lymphocytes can be observed in CL, regardless of the IFAT titer or the presence of clinical signs of disease. Such

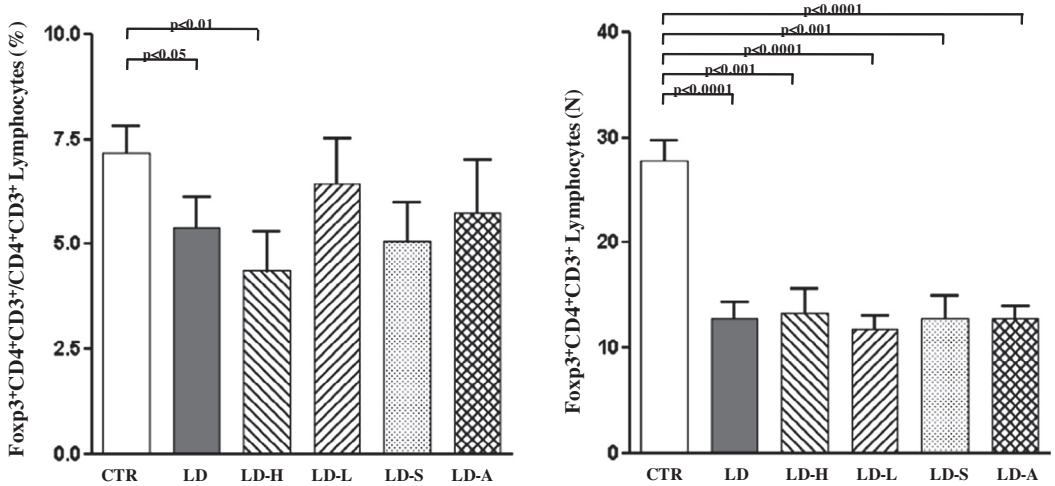

Fig. 4. Significant reduction of percentage and Treg count have been observed in *Leishmania* infected dogs. Panel A and B refer percentage and count ($\times 10^{-6}$/L) of $CD4^+CD3^+Foxp3^+$ (Treg) cells in healthy animals (CTR; white column); *Leishmania* infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Statistical analysis has been performed by using Mann–Whitney test. *P* values are indicated only for significant comparisons.

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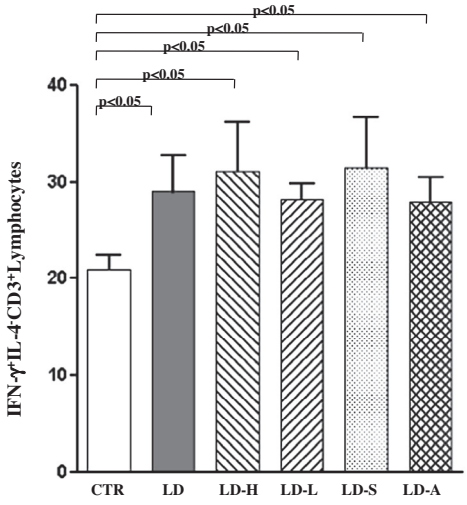

Fig. 5. Significant increase of IFN-γ^+IL-4$^-$CD3$^+$ T lymphocytes characterises *Leishmania* infected dogs. Referred values have been observed in peripheral blood of healthy animals (CTR; white column); *Leishmania* infected dogs (LD; grey column); infected animals with an IFAT titer > 1:160 (LD-H; right striped column); infected animals with an IFAT titer 1:80–1:160 (LD-L; left striped column); infected animals showing symptomatic disease (LD-S; pointed column); infected animals showing asymptomatic disease (LD-A cross-lined column); Statistical analysis has been performed by using Mann–Whitney test. *P* values are indicated only for significant comparisons.

observations confirm the relevance for CD8$^+$ T lymphocytes in the control of intracellular-parasites (Guarga et al., 2000; Reis et al., 2006, 2009; Miranda et al., 2007; Coura-Vital et al., 2011).

A complex network of peripheral mechanisms, co-evolved to prevent or dampen immune-mediated diseases, usually accounts for the regulation of the activation, expansion and recruitment of T cell effectors in the infected microenvironment. Regulatory systems include mechanisms intrinsic to the antigen activation and T cell differentiation as well as those mediated by regulatory suppressor immune-populations, as represented by the Treg subset. The involvement of such regulatory network is of critical relevance in the presence of infectious agents usually associated with chronic diseases, as represented by *Leishmania.* To investigate on such issue, we focused the complex network of regulatory/effector T cell populations in a cohort of 45 *Leishmania* infected dogs, categorised according to their IFAT titer and the occurrence of a symptomatic versus asymptomatic disease. In order to analyse T cell behaviour, maintaining the biological complexity of the immune-regulatory networks, we chose to analyse T cells in the whole blood samples or in the bulk PBMC preparations, by using immune fluorescence and multi parametric flow cytometry detection.

Our data consistently indicate CD8$^+$ T effector cells as a key effector population in CL. Thus the critical role for adaptive immunity in clearing intracellular parasites (Reis et al., 2006, 2009) has been confirmed in our model. Moreover, our results indicate that in active CL an inflammatory response, characterised by increased levels of CD8$^+$ T cells and T_H1 T cells, might be sustained by a decrease of Treg. Thus, parasite persistence could maintain adaptive cytotoxic and pro-inflammatory response by reducing Treg-dependent mechanisms. Several observations, particularly on the murine cutaneous leishmaniasis model, demonstrated that T_H1 and T_H2 responses via their signatory cytokines, IFN-γ, and IL-4 respectively, were counter-regulatory. However, later studies using gene-deficient mice have questioned the precise role of the T_H2 response and IL-4 in regulating the T_H1 response. Depending on the parasite strain/species/host model studied the T_H2 response could either promote infection or at best be irrelevant to disease progression (Alexander and Brombacher, 2012). The presence of reduced Tregs represents a key mechanism to optimise T cell activation and effector functions in the presence of persistent infection. Tregs have been described to modulate recruitment and activation of immune effectors (Sakaguchi, 2005). Thus, in active CL, Treg could hamper inflammatory responses required for infection clearance, in such way exacerbating the risk that the unbridled parasite growth could lead to severe disease. On the contrary, Treg recruitment in infected tissues might prevent the onset of severe immune-mediated pathology. Thus, fine tuning of immune regulatory networks is of critical relevance to avoid immune pathology and protect host against pathogens. Such intriguing scenario could underlie immune-mediated pathology, likely accounting for the presence of immune-mediated auto-immune processes, very common in this parasitic disease (Kharazmi et al., 1982; Ferrer, 1992; Terrazzano et al., 2006; Solano-Gallego et al., 2009; Cortese et al., 2009, 2011). These data may provide new insights into the pathogenesis of immune-mediated alterations associated with CL. Further studies are in progress in order to more specifically address such issue.

Conflict of interest

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

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T cell activation induces CuZn superoxide dismutase (SOD)-1 intracellular re-localization, production and secretion

Giuseppe Terrazzano [a,b,1], Valentina Rubino [b,1], Simona Damiano [c], Anna Sasso [c], Tiziana Petrozziello [c], Valentina Ucci [c], Anna Teresa Palatucci [b], Angela Giovazzino [b], Mariarosaria Santillo [c], Bruna De Felice [d], Corrado Garbi [e], Paolo Mondola [c,*], Giuseppina Ruggiero [b,**]

[a] Dipartimento di Scienze, Università della Basilicata, 85100 Potenza, Italy
[b] Dipartimento di Scienze Mediche Traslazionali, Università di Napoli "Federico II", 80131 Napoli, Italy
[c] Medicina Clinica e Chirurgia, Università di Napoli "Federico II", 80131 Napoli, Italy
[d] Dipartimento di Scienze e Tecnologie Ambientali Biologiche e Farmaceutiche, II Università di Napoli, 81100 Caserta, Italy
[e] Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli "Federico II", 80131 Napoli, Italy

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ABSTRACT

Reactive oxygen species (ROS) behave as second messengers in signal transduction for a series of receptor/ligand interactions. A major regulatory role is played by hydrogen peroxide (H_2O_2), more stable and able to freely diffuse through cell membranes. Copper–zinc superoxide dismutase (CuZn-SOD)-1 is a cytosolic enzyme involved in scavenging oxygen radicals to H_2O_2 and molecular oxygen, thus representing a major cytosolic source of peroxides. Previous studies suggested that superoxide anion and H_2O_2 generation are involved in T cell receptor (TCR)-dependent signaling. Here, we describe that antigen-dependent activation of human T lymphocytes significantly increased extracellular SOD-1 levels in lymphocyte cultures. This effect was accompanied by the synthesis of SOD-1-specific mRNA and by the induction of microvesicle SOD-1 secretion. It is of note that SOD-1 increased its concentration specifically in T cell population, while no significant changes were observed in the "non-T" cell counterpart. Moreover, confocal microscopy showed that antigen-dependent activation was able to modify SOD-1 intracellular localization in T cells. Indeed, was observed a clear SOD-1 recruitment by TCR clusters. The ROS scavenger N-acetylcysteine (NAC) inhibited this phenomenon. Further studies are needed to define whether SOD-1-dependent superoxide/peroxide balance is relevant for regulation of T cell activation, as well as in the functional cross talk between immune effectors.

1. Introduction

T cell activation is a complex phenomenon in which intracellular signals, mediated by the engagement of TCR, are integrated by a variety of ligand/receptor interactions whose outcome is to finely tune antigen-dependent T cell response [1]. T lymphocytes play a pivotal role in the orchestration of the immune response and TCR-mediated signaling is a critical event to properly channeling the immune response and to obtain pathogen control and self-tolerance [2].

Several studies have been suggesting that TCR-dependent T cell activation induces ROS production [3–5]. Different enzymatic sources, such as the mitochondrial respiratory chain [6], lipooxygenases, NADPH oxidases NOX2 and DUOX1 [7,8], have been described to contribute to ROS generation upon TCR triggering. In the light of these observations, the involvement of multiple anti-oxidant enzymes in fine tuning of antigen-dependent T cell response can be hypothesized.

TCR stimulation generates both H_2O_2 and superoxide anion [8,10] and antioxidant enzymes specific for H_2O_2 enhance and/or prolong TCR-dependent ERK activation, while those specific for superoxide anion have no effect [11].

ROS include oxygen superoxide, hydrogen peroxides, hydroxyl radicals and peroxides. They represent a normal product of cellular metabolism and play relevant roles in innate defense against pathogens [12]. Several receptor/ligand interactions, as represented by TGF-beta [13], insulin [14], angiotensin II [15] and EGF [16] have been correlated to the presence of ROS. In this context, ROS appear to act as key second messenger regulating several crucial cellular responses, as protein kinase activation, gene expression and cell proliferation/apoptosis [17].

* Correspondence to: P. Mondola, Dipartimento di Medicina Clinica e Chirurgia, Università di Napoli "Federico II", Via Pansini, 5, 80131 Napoli, Italy. Tel.: +39 081 7463225; fax: +39 081 7463494.
** Correspondence to: G. Ruggiero, Dipartimento di Scienze Mediche Traslazionali, Università di Napoli "Federico II", Via Pansini, 5, 80131 Napoli, Italy. Tel.: +39 081 7463311; fax: +39 081 7463252.
E-mail addresses: mondola@unina.it (P. Mondola), giruggie@unina.it (G. Ruggiero).
[1] GT and VR equally contributed to this work.

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H_2O_2 is more stable than other short-lived ROS molecules (1 minute half-life). It is electrically neutral and it can diffuse inside the cell and freely through cell membranes. In addition, H_2O_2 can be rapidly generated and easily scavenged by numerous mechanisms, thus sharing several features with well-known second messengers [18–20].

SOD molecules mediate scavenging of ROS, to H_2O_2 and molecular oxygen. They belong to a large family of isoenzymes that mediate cellular response to oxidative stress and represent the main enzymatic source of peroxides [21]. All mammalian cells express both the intra-mitochondrial Mn-SOD and the cytosolic dimeric CuZn-SOD (SOD-1), while the tetrameric extracellular CuZn-SOD isoenzyme seems to be selectively expressed by specific cell populations [22,23]. Hyperoxia and copper availability accelerate both the synthesis and activity of Cu,Zn SOD [24]. Significant control of ROS signaling depends on its spatially restricted production at intracellular sites, where redox-regulated signal occurs [25]. In this context, SOD-1 recruitment has been described in redox-dependent TNF-alpha and IL-1 receptor-induced endosomes [26,27]. In addition, SOD-1 associates with Rac-1-regulated NADPH oxidase complexes in different mouse tissues and cell lines [28].

SOD-1 may be released *in vitro* by fibroblasts, hepatocytes [29], human neuroblastoma cells [30] and Sertoli cells [31]. The extracellular release of such enzyme is related to specific stress conditions [32]. ER/Golgi involvement in SOD-1 secretion has been described [33–36], while it is unclear how this cytosolic protein can be targeted into the ER/Golgi network.

SOD-1 is constitutively secreted by microvesicles in some cell lines through an ATP dependent mechanism [37]. The intracellular increase of the enzyme can be observed in neuroblastoma SK-N-BE cells when they are exposed to oxidative stress [37]. Recently, it has been shown that SOD-1 secretion is induced by high level of extracellular K^+ in GH3 rat pituitary cells [38] and that the enzyme interacts with membrane of neuroblastoma SK-N-BE cells activating a phospholipase/protein kinase C pathway, able to increase intracellular calcium [39,40].

Receptor–ligand interactions, involving members of hematopoietin receptor super family and EGF, have been described to mediate extracellular H_2O_2 generation [41,42]. Moreover, exogenously added H_2O_2 is able to induce signals in the absence of ligands, whereas catalase is able to inhibit such effect [43,44]. A role for SOD-1 in modulating ROS-dependent intra-cellular and inter-cellular signaling might be hypothesized.

Communication between immune cells involves the secretion of several proteins, like the cytokines, and the presence of their receptors on neighboring cells. This type of intercellular "dialog" may involve the release of membrane vesicles, like exosomes. These vesicles can affect cell physiology inducing intracellular signaling and conferring them new biological properties [45,46]. Peripheral blood human T cells, T cell clones and Jurkat T cells are able to release microvesicles in the culture medium. The microvesicle production is finely regulated and, notably, it increases upon TCR triggering [47].

In previous papers, we showed that cytosolic SOD-1 is secreted by several cell types [29,30,37] and it is also released in primary lymphoid organs, as represented by human thymus [48]. These observations suggest a paracrine role for SOD-1.

Multiple cytokines have been observed to regulate the expression of the tetrameric form of extra-cellular SOD-1 [49], while no data are available on the role of dimeric, cytosolic SOD-1 in functional adaptive immune effectors. Therefore, the role for SOD-1 in ROS-dependent signaling as well as in the communication between immune effectors needs to be addressed.

This study is aimed to investigate whether cytosolic SOD-1 might be part of the molecular network involved in TCR triggering. With this purpose SOD-1 intracellular level and localization, as well as SOD-1 microvesicle secretion have been investigated in TCR-triggered human T lymphocytes.

2. Material and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 healthy donors, after informed consent, by centrifugation of peripheral blood on Ficoll-Paque cushion (GE Healthcare, Uppsala, Sweden) gradient. T cells have been isolated from PBMC by using a negative isolation kit (Invitrogen Corporation, Carlsbad, CA, USA) and following the manufacturer's instructions. PBMC or T cells (1×10^6/ml) were cultured in 96 well flat-bottomed plates (Falcon) in RPMI 1640 medium with 2% FCS (Invitrogen, Carlsbad, CA, USA). TCR triggering was performed by anti-CD3 mAb (Becton Dickinson, Mountain View, CA, USA) at 5 ng/ml or by using anti-CD3/anti-CD28 beads (Invitrogen), at 0.3 bead/cell. This activation strategy has been largely demonstrated to mimic antigen-dependent T cell triggering. To analyze TCR-dependent SOD-1T cell export, distinct experiments were performed in the presence of Brefeldin-A, (BFA) at 5 μg/ml or of 1 mM methylamine, all purchased from Sigma-Aldrich (Milan, Italy), as described [37]. Cell viability was evaluated by using Propidium Iodide (PI) (Sigma-Aldrich) labeling and flow cytometry detection [37] as well as by analyzing lactate dehydrogenase (LDH) activity in culture supernatants by using the Roche Molecular Biochemical LDH kit (Mannheim, Germany). Written informed consent (model n. 5526 of Azienda Ospedaliera Universitaria "FEDERICO II") was obtained from each donor at the time of venous peripheral blood donation. All the experiments done by using blood donations were performed and analyzed anonymously, without any biographical reference to donors.

2.2. ELISA

The quantitative detection of human SOD-1 in medium of cultured PBMC was carried out using the Bender Med System kit (Bender Med System Diagnostic, Vienna, Austria), as described [37]. Results were always normalized for total protein content of the tested sample. SOD-1 ELISA detection has been always performed on culture supernatants immediately frozen at −80 °C. Protein concentrations were determined according to the method of Lowry et al. [50] using BSA, as standard.

2.3. RNA preparation, semi-quantitative RT-PCR and DNA sequencing

Analysis of SOD-1 specific RNA has been performed, as described [51]. Briefly, total RNA was extracted with High Pure RNA isolation kit (Roche Italia, Milano, Italia), according to the manufacturer's instructions. Traces of contaminated DNA were removed with DNAse I treatment. Quantification was achieved in a single reaction by using the housekeeping β-actin gene as internal standard. To rule out genomic DNA contamination we performed a negative control that contained RNA instead of cDNA. The signal intensities of PCR products were separated on a 1.2% agarose gel and were visualized by ethidium bromide staining. The products' signal intensities were determined by computerized densitometric analysis using Fotoplot software. The expression of SOD-1 was normalized to β-actin mRNA levels. To check the specificity of the amplified products, DNA bands were eluted from the gel and purified; sequence analysis was determined by the Big Dye Terminator Cycle Sequencing method (ABI-PRISM Sequencer 310 Perkin-Elmer).

2.4. Microvesicle isolation and western blotting for SOD-1 detection

To purify the membrane microvesicle-containing fraction, supernatants were collected immediately after culture and treated, as described [52]. Briefly, they were sequentially centrifuged at 500 *g* for 15 min to remove cellular debris and again at 10,000 *g* for 20 min. The obtained supernatant was collected and further centrifuged at 100,000 *g* for

2 h. The resulting pellet was then collected and considered to represent the enriched membrane vesicle fraction. Western blotting analysis of the purified material was performed as previously described [37]. Comparative analysis of SOD-1 was performed by using 40 μg of total proteins.

2.5. Immunofluorescence and flow cytometry analysis

Intracellular SOD-1 content was evaluated anti-SOD-1 mAb and FITC labeled anti-mouse IgG secondary antiserum (Sigma-Aldrich) staining of permeabilized cells and immunofluorescence technique. A commercial fixing/permeabilization kit, purchased from Becton Dickinson was always employed, following the manufacturer's instructions. For the analysis of SOD-1 content in distinct cell subsets and to evaluate T cell activation after TCR triggering, co-staining with FITC, PerCP or APC labeled anti-CD3, anti-CD45 and anti-CD69 mAb was performed. Labeled antibodies and isotype-matched controls were purchased from Becton Dickinson. T cell staining and activation were performed by anti-CD3 mAb recognizing different CD3 epitopes. Cell death was always less than 5% as evaluated by using PI (Sigma-Aldrich) staining. Immunofluorescence, flow cytometry and data analysis were performed by using a two laser equipped Becton–Dickinson FACSCalibur flow cytometer and the Cell Quest analysis software.

2.6. Fluorescence microscopy

PBMC or purified T cells (0.5×10^6) were adhered to polylysine-coated glass slides for 16–18 h at 37 °C. When indicated, the above populations were stimulated with anti-CD3 mAb CLB-CD3/4E at 1:100 ascites dilution or anti-CD3/anti-CD28 beads (at 0.3 bead/cell) and 1 mM NAC [53]. Cells were incubated at 37 °C and immediately fixed with 3% paraformaldehyde solution. Fixed cells were incubated with FITC labeled anti-CD3 and anti-human Cu,Zn SOD-1 rabbit antibody (Santa Cruz Biotechnology, CA, USA) for 45 min in a humidified chamber, washed three times with PBS and incubated with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Life Technologies) for additional 45 min at 37 °C in the same conditions. After 3 washes with PBS the glass slides were mounted using a 50% solution of glycerol in PBS and examined with a Zeiss LSM 510 confocal microscope with a 63× oil immersion objective (N.A. 1,4) at room temperature. Pictures were taken from selected fields of control and treated samples.

2.7. Cell to cell aggregate evaluation

To evaluate cell aggregation, PBMCs were cultured in 96 wells flat bottomed microtiter plates (Falcon) in the presence of Medium, anti-CD3 and 1 mM NAC (Sigma-Aldrich), as indicated. This NAC concentration was demonstrated in preliminary experiments to completely block ROS formation, as described [54]. Contrast phase microscopy analysis was performed with a Leitz DIAVERT microscope with a 10× objective at room temperature. Pictures were taken from selected fields by using a digital Nikon Coolpix Camera. NAC treatment was unable to significantly affect T cell viability and proliferation, as evaluated by PI staining after 1 to 5 h of culture and ^{3}H thymidine incorporation after 72 h of culture. Quantification has been performed by counting the number of cell aggregates (identified by the presence of at least 8 clustered cells) in the cell culture of 1×10^5 PBMC plated on the flat-bottomed microtiter wells.

2.8. Statistical analysis

Statistical evaluation of data, by InStat 3.0 software (GraphPad Software Inc., San Diego, California, USA), has been performed by means of the Mann–Whitney test or Paired *t* test, as indicated. Two-sided p values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. TCR triggering induces both intracellular increase and BFA-dependent secretion of SOD-1 in human T lymphocytes

To investigate whether antigen-dependent T cell activation induces SOD-1 production and extracellular secretion, we measured the release of this enzyme in supernatants of PBMC cultured from 15 min to 18 h in presence of Medium alone or with anti-CD3 mAb, that induces the TCR triggering mimicking antigen-dependent activation of T lymphocytes. As shown in Fig. 1A, human PBMC cultures secreted small amount of SOD-1 that remained substantially stable from 15 min till to 18 h of culture. Anti-CD3 treatment slightly, but significantly ($p < 0.05$), increased such basal secretion. The increment was detectable after 4 h of culture and reached the highest level after 18 h of activation. This effect was independent on cell damage, always evaluated by PI labeling (Fig. 1B) and significantly correlated with TCR-dependent activation, as revealed by the up-regulation of the activation molecule CD69 (Fig. 1C). Therefore, TCR-triggering was associated with the induction of SOD-1 secretion in human lymphocytes.

To ascertain whether such secretion was sustained by up-regulation of SOD-1 gene transcription, we analyzed SOD-1 specific mRNA. As shown in Fig. 2, an increase of more than 70% of SOD-1 specific mRNA has been observed in the cultures treated with anti-CD3 mAb as compared with resting cells. Therefore, antigen-dependent T cell activation induced SOD-1 secretion that was sustained by the increase of SOD-1 transcription in the whole PBMC population.

To evaluate whether SOD-1 export might be part of micro-vesicle production upon TCR triggering, we purified the micro-vesicle fraction from the supernatants of 18-hour cultures of PBMC and purified T cells. TCR triggering of PBMC was performed with anti-CD3 mAb. In order to mimic costimulatory signals, usually mediated by accessory cells, a combination of anti-CD3/anti CD28 beads was used to fully activate purified T cells, as described [1]. Fig. 3 shows western blotting analysis of SOD-1 in the enriched membrane vesicle fractions. Fig. 3A–B reports one representative experiment, while Fig. 3C refers the analysis of data obtained in all the six experiments performed in PBMC cultures. The comparison revealed the occurrence of a mean 25% increase of SOD-1 content in the enriched membrane vesicle fraction obtained from anti-CD3 treated lymphocytes ($p < 0.01$). As shown, in the enriched membrane vesicle fraction isolated from anti-CD3/anti-CD28 treated T cells, a more consistent increase of SOD-1 content was observed (Fig. 3D–F). Indeed, a mean increase >120% of SOD-1 content was evidenced in three experiments performed with purified T lymphocytes ($p < 0.05$).

To investigate whether other cell populations, present in PBMC, contribute to SOD-1 production and secretion in response to TCR-triggering, we analyzed intra-cellular SOD-1 levels in the T cell subset and in the "non-T" counterpart in a mixed context. This evaluation was performed by the combination of immune fluorescence and flow cytometry detection, to preserve the biological complexity of antigen-dependent T cell response and allow specific detection of SOD-1 in the T cell subset and in "non-T" population (Fig. 4A). As shown (Fig. 4B), very low amount of intracellular SOD-1 was observed in all the "resting" lymphocytes (T and non-T cells).

After TCR-triggering, the up-regulation of SOD-1 intracellular level was observed only in T lymphocytes if compared to non-T cells (Region 1 *versus* Region 2 in Fig. 4C).

To investigate the pathway involved in SOD-1 secretion, we analyzed intracellular SOD-1 retention in T cells in the presence of BFA (Fig. 4D) or methylamine (Fig. 4E) described to block ER/Golgi intracellular network and cell endocytosis, respectively [51,52]. In this regard, BFA but not methylamine treatment induced significant increase of

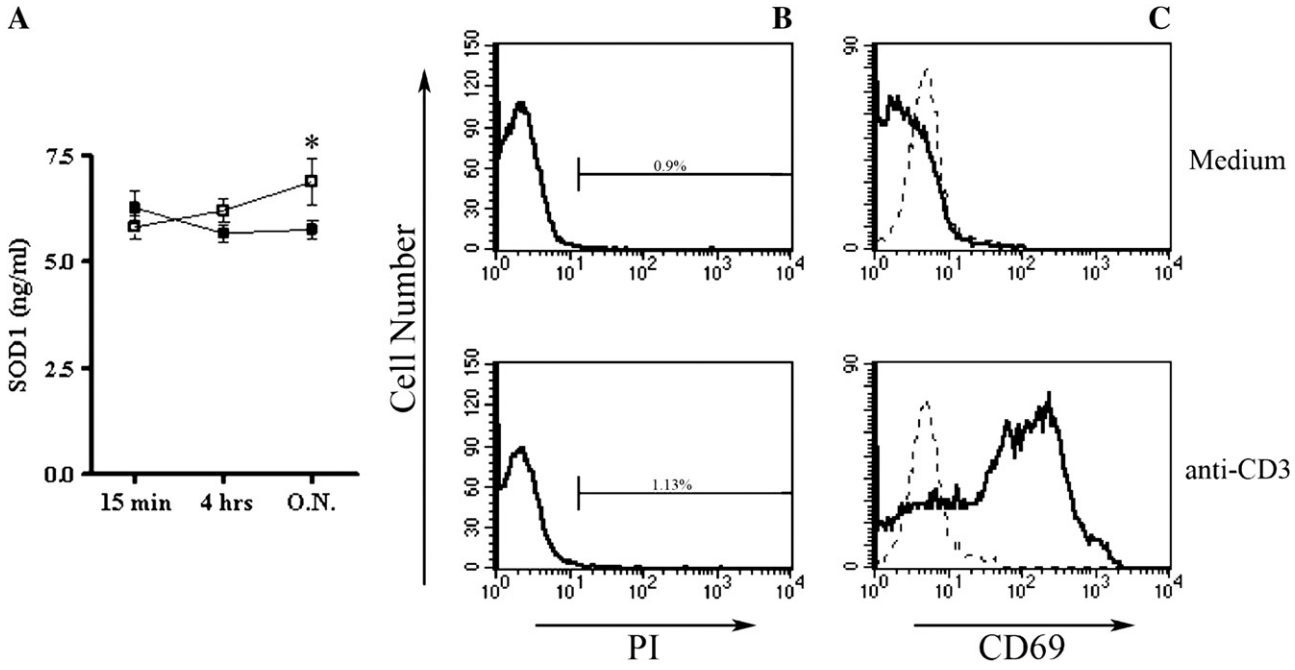

Fig. 1. SOD-1 concentration in anti-CD3 triggered cultures of human lymphocytes. (*A*) SOD-1 amount in supernatants of PBMC cultured in the presence of Medium (black squares) or anti-CD3 (white squares). SOD-1 concentrations were analyzed in undiluted samples by using ELISA assay; results were normalized for total protein content of the tested sample. Each point refers mean value obtained in five independent experiments; error bars indicate SEM. * indicates the occurrence of statistically significant ($p < 0.05$) higher SOD-1 concentration in anti-CD3 treated cultures. (B and C) PI and CD69 labeling of PBMC cultured O.N. with Medium or anti-CD3, as indicated. Results refer to one of five independent experiments. As shown (B), no significant differences have been observed in PI staining profiles of PBMC cultured with Medium and anti-CD3; (C) CD69 staining profile (bold line) of PBMC cultured with Medium or anti-CD3, as indicated; broken line indicates isotype control; as shown, activated PBMCs were characterized by significant increase of the activation molecule CD69.

the enzyme content in T cells. No significant changes in intracellular levels of SOD-1 were observed in "non-T" cell population, in the same experimental conditions (Fig. 4F). Notably, none extracellular SOD-1 increase was detected by ELISA in anti-CD3 cultures incubated with BFA (data not shown).

Fig. 4G–I reports the statistical comparisons of SOD-1 intracellular amount in PBMC (Fig. 4G), in T cells (Fig. 4H) and "non-T" cells (Fig. 4I), as evaluated by considering the mean fluorescence intensity (MFI) values obtained in all the 4 experiments performed. As shown, no significant changes in SOD-1 levels were observed in the absence of TCR triggering. Thus, SOD-1 amount was strictly dependent on antigen-mediated T cell activation. Indeed, anti-CD3 treatment significantly increased SOD-1 intracellular level ($p < 0.005$) in the whole lymphocyte population (Fig. 4G). This effect specifically characterized the T cell subset ($p < 0.005$; Fig. 4H), while no differences were observed in the "non-T" population (Fig. 4I). Moreover, the block of ER/Golgi network, mediated by BFA treatment, was observed to mediate significant ($p < 0.05$) intra-cellular SOD-1 retention only in T lymphocytes (Fig. 4H). Similar results have been obtained by anti-CD3/anti-CD28 triggering of purified T cells (data not shown). As control, intracellular accumulation of Interferon-gamma was specifically detected in TCR triggered cultures treated with BFA (not shown).

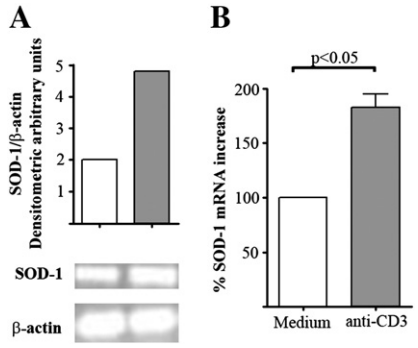

Fig. 2. Anti-CD3 treatment induces significant increase of SOD-1 mRNA in human lymphocytes. (A) Densitometric arbitrary units ratio between SOD-1 and beta actin in Medium and anti-CD3 treated cultures. Results refer one of five independent experiments. mRNA was measured by RT-PCR, as detailed in Material and methods section. (B) Comparative analysis of SOD-1 mRNA percent increase in all the five experiments performed. For each experiment mRNA amount in Medium cultured PBMC was considered the reference value (100) for calculation of percent increase in the anti-CD3 treated culture. As shown, a mean increase of more than 70% of SOD-1 specific mRNA has been observed in anti-CD3 treated PBMC. Error bars indicate SEM. Statistical analysis has been performed by using Paired *t* test.

3.2. TCR and SOD-1 co-localize and cluster after TCR-triggering in human T cells

We analyzed SOD-1 and TCR cellular localization by confocal microscopy after 2 min of culture in the presence of Medium alone or with anti-CD3. Fig. 5 shows TCR and SOD-1 co-staining after 2 min of culture with Medium alone (Fig. 5A) or anti-CD3 (Fig. 5B and C). As expected, the homogeneous surface TCR distribution observed in resting T cells (Fig. 5A) was completely changed by anti-CD3 triggering (Fig. 5B and C). Indeed, significant TCR clustering (Fig. 5B and C) characterized activated T cells. SOD-1 staining revealed a quite homogeneous intracellular distribution of the enzyme in resting T cells; staining profiles also confirmed the presence of SOD-1 at very low levels in human T lymphocytes (see Fig. 4).

Notably, confocal microscopy revealed that TCR triggering was able to induce a clustered distribution of SOD-1 enzyme (Fig. 5B and C). Merged images clearly showed that TCR clusters have been recruiting intracellular SOD-1, whose localization strictly reflected TCR distribution (Fig. 5B and C). TCR/SOD-1 co-localization disappeared 20 min after anti-CD3 treatment (not shown). To preserve the physiological complexity, we chose to perform the analysis in PBMC population as a whole and we identified T cells by labeling with specific antibodies. In this model, TCR triggering is allowed by the physiological cross talk between T cells and autologous antigen

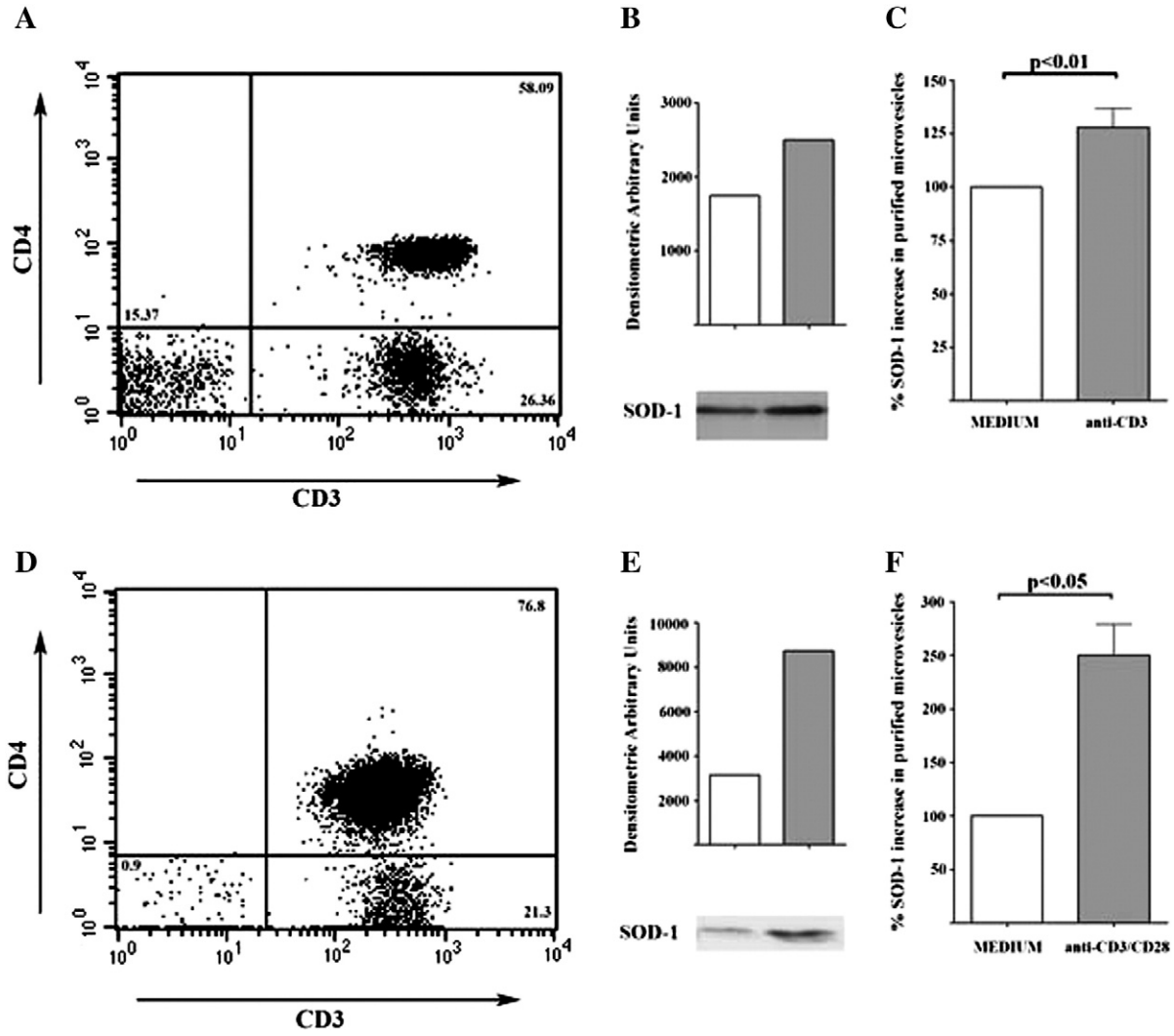

Fig. 3. TCR-dependent T cell activation increases SOD-1 containing microvesicle secretion by human T lymphocytes. (A) Flow cytometry analysis of PBMC population. As shown, T lymphocytes ($CD3^+$ cells) represent less than 85% of the total population; (B) western blot of enriched membrane vesicle fractions, isolated from culture supernatants of Medium (white column) and anti-CD3 treated PBMC (gray column), as detailed in Material and methods section. Densitometric analysis shows increased presence of SOD-1 in TCR triggered PBMC. Results refer one representative experiment of the six performed; (C) Analysis of percent increase of SOD-1 containing microvesicle in supernatants derived from anti-CD3 treated PBMC in six experiments. For each experiment, SOD-1 amount in Medium cultured PBMC was considered the reference value (100) for calculation of percent increase in the anti-CD3 treated culture. As shown, a mean increase of 25% was observed in the microvesicle-enriched fraction obtained from the supernatants of anti-CD3 treated PBMC. (D) Flow cytometry analysis of a typical purified T cell population isolated by using negative selection strategy, as indicated in the Material and methods section. As shown, T lymphocytes ($CD3^+$ cells) represent more than 98% of the total population; (E) western blot of enriched membrane vesicle fractions, isolated from culture supernatants of Medium (white column) and anti-CD3/antiCD28 treated T cell cultures (gray column), as detailed in Material and methods section. Densitometric analysis shows increased presence of SOD-1 in the sample obtained from TCR triggered T cells. Results refer one representative experiment of the three performed; (F) analysis of percent increase of SOD-1 containing microvesicle in supernatants derived from anti-CD3/anti-CD28 treated T lymphocytes in three experiments. For each experiment, SOD-1 amount in Medium cultured T cells was considered the reference value (100) for calculation of percent increase in the anti-CD3/anti-CD28 treated cultures. As shown, an increase of more than 120% was observed in the microvesicle enriched fraction obtained from the supernatants of anti-CD3/anti-CD28 treated purified T cells. Error bars indicate SEM. Western blot comparative analysis of SOD-1 has been always performed on aliquots of enriched microvesicle fractions containing 40 μg of total proteins. Statistical analysis has been performed by using Paired *t* test.

presenting cells (APC). Notably, we never observed a TCR clustering decoupled from SOD-1 co-localization. No significant changes in SOD-1 intracellular localization were observed in "non-T" population after anti-CD3 triggering (not shown). Notably, SOD-1/TCR intracellular co-clustering was observed also in anti-CD3/anti-CD28 triggered purified T cells (not shown).

To investigate whether SOD-1/TCR co-localization in anti-CD3 activated T cells is dependent on ROS bioavailability, we performed experiments in the presence of the ROS scavenger NAC at 1 mM concentration. In this condition, TCR clustering was significantly reduced (from 70 to 95% in NAC/anti-CD3 co-cultures). As shown, NAC significantly inhibited both TCR and SOD-1 clustered localization (Fig. 5D). Indeed, TCR was homogeneously distributed on cell membrane, similarly to what was observed in resting condition (Fig. 5A). SOD-1 co-staining in anti-CD3/NAC treated lymphocytes also resembled basal images with the presence of small areas of faint cytosolic accumulation (Fig. 5D). Merged images revealed a clear-cut distinct distribution of TCR and SOD-1 in anti-CD3/NAC treated T cells. Thus, ROS availability significantly affected activation-dependent TCR/SOD-1 re-localization in human T cells.

To ascertain whether ROS availability might also affect the cell-to-cell aggregation dependent on TCR triggering, we analyzed the effect of NAC incubation on early cell clustering (usually detectable after 45 min of incubation with anti-CD3). As shown in Fig. 6, anti-CD3-sitimulation was able to induce cell aggregation after 1 h of treatment (Fig. 6C). Such effect became more evident after 3 h of incubation (Fig. 6G). Anti-CD3/NAC co-treatment severely impaired anti-CD3 induced cell clustering after 1 h of incubation (Fig. 6D).

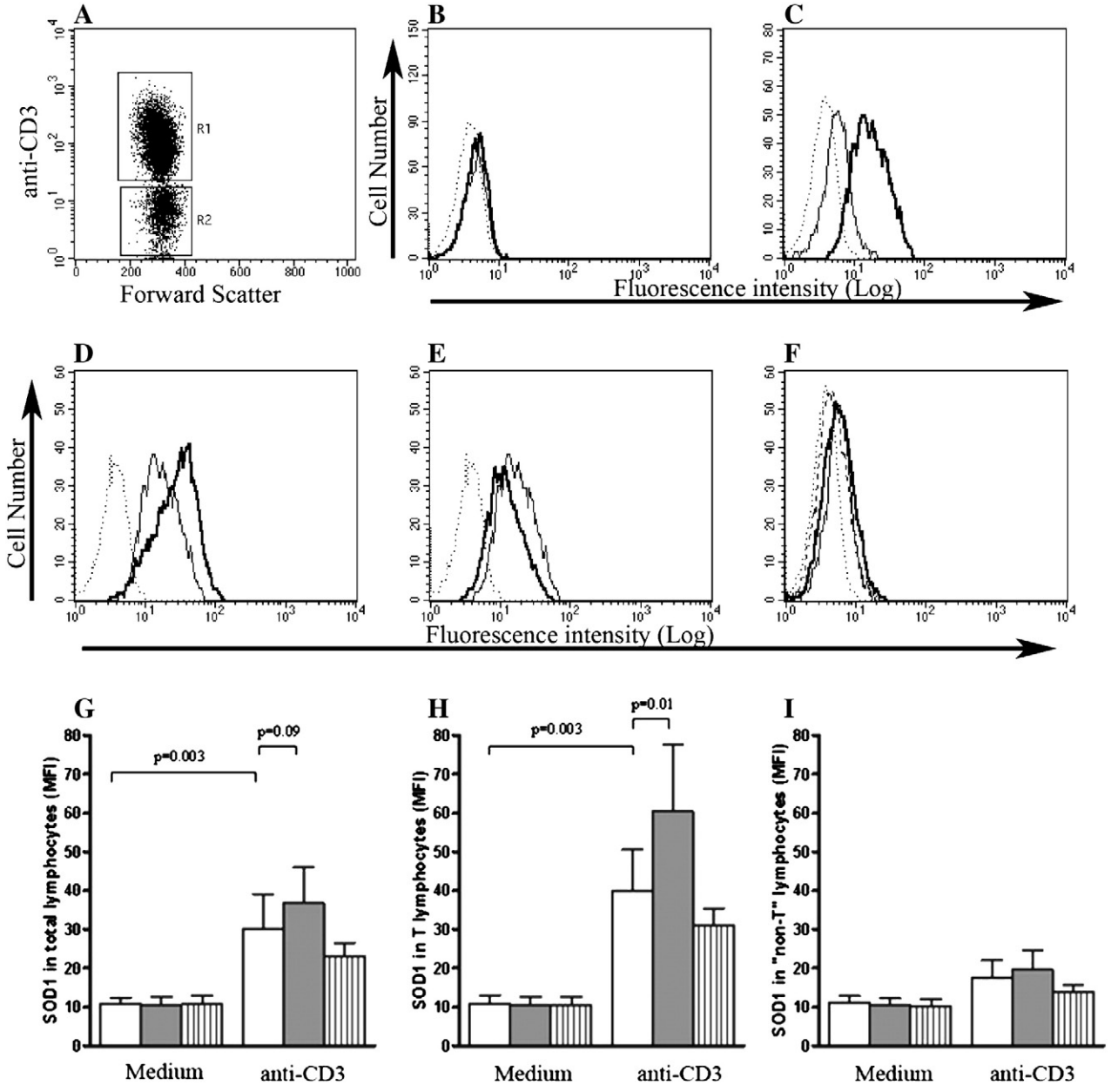

Fig. 4. Significant increase of intracellular SOD-1 and BFA-dependent SOD-1 export can be specifically demonstrated in anti-CD3 triggered human T cells. (A) Gating criteria for the identification of the "T cell subset" (R1) and of the "non-T population" (R2) in a PBMC culture; (B) SOD-1 staining profile of T cells (bold line) and "non-T population" (plain line) in O.N. Medium cultured PBMC. Dotted lines show the isotype control. As shown, similar, very low amount of intracellular SOD-1 characterizes both population. (C) SOD-1 staining profile of T cells (bold line) and "non-T population" (plain line) in anti-CD3 cultured PBMC. Dotted lines show the isotype control. As shown, specific increase of SOD-1 intracellular content can be observed in the T cell subset (bold line) as compared with the "non-T" counterpart (plain line); (D) SOD-1 staining in T cell population (R1) cultured with anti-CD3 alone (plain line) or in the presence of BFA (bold line). Dotted lines show the isotype control. As shown, anti-CD3/BFA co-culture increases intracellular SOD-1 content in T cell population. (E) SOD-1 staining in T cell population (R1) cultured with anti-CD3 alone (plain line) or in the presence of methylamine (bold line). Dotted lines show the isotype control. As shown, no significant changes in SOD-1 intracellular levels can be observed in anti-CD3/methylamine co-cultures. (F) SOD-1 staining profiles of "non-T population" (R2) in anti-CD3 cultures (plain line), in BFA/anti-CD3 co-cultures (bold line) or in methylamine/anti-CD3 cultures (broken line). Dotted lines show the isotype control. As shown, no significant changes in SOD-1 intracellular content have been observed. Results refer one of 4 independent experiments. (G–I) Column graphic reports the means of SOD-1 fluorescence intensities observed in 4 independent experiments in the whole PBMC population (G), in the T cells (H) and in the "non-T" population (I). White columns indicate SOD-1 levels in cells, cultured as indicated; gray columns indicate SOD-1 in BFA co-treated cultures; striped columns refer SOD-1 in methylamine co-treated cultures.

Quantification has been performed by direct counting of cell aggregates, identified by the presence of at least 8 clustered cells, in the microtiter wells. Comparative analysis showed a cell aggregate inhibition of 83.64 ± 1.62 in anti-CD3/NAC co-treated cultures in 6 independent experiments; ($p < 0.05$). This inhibition was transient and progressively decreased, likely mirroring the ROS scavenging activity of NAC. Indeed, after 3 h of NAC/anti-CD3 incubation a percentage of clustering inhibition of 55.35 ± 2.23 was observed in 6 independent experiments (Supplementary Table S1). The inhibiting effect of NAC co-incubation completely disappeared after 6 h of anti-CD3/NAC co-treatment. NAC co-incubation was unable to mediate significant effects on cell viability and proliferation (not shown).

4. Discussion

This study revealed that SOD-1 is part of the network of molecules involved in antigen-dependent T cell response. SOD-1 was recruited by antigen triggered TCR and its intracellular content was specifically upregulated in human T cells after 16–18 h of anti-CD3 incubation. Moreover, SOD-1 was secreted by a BFA-dependent

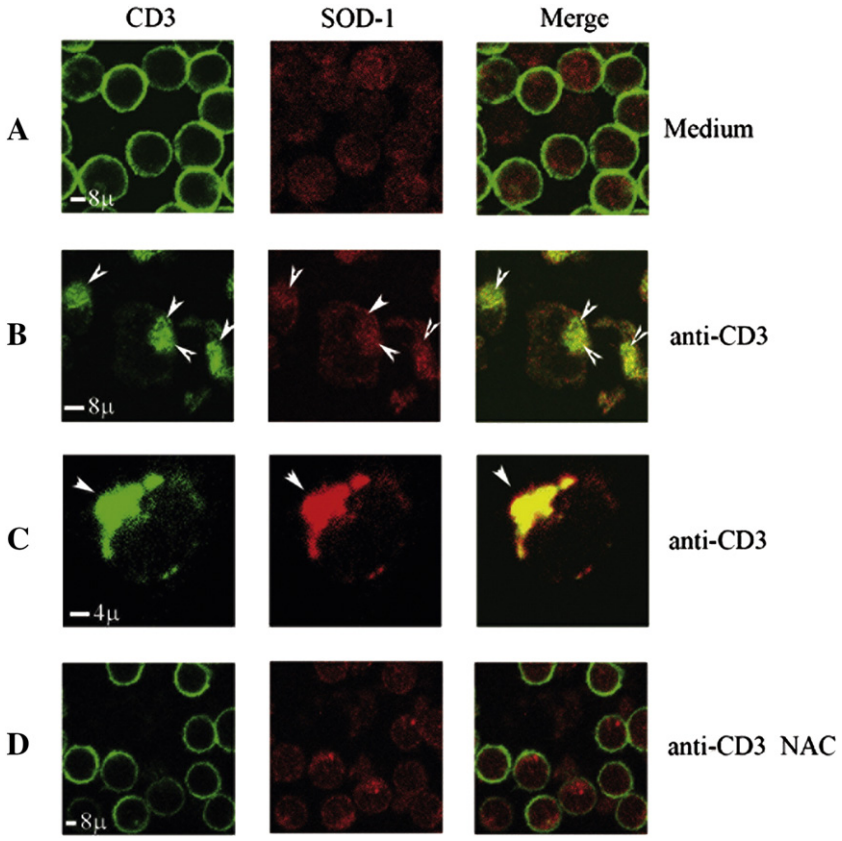

Fig. 5. Anti-CD3 triggering induces ROS-dependent TCR and SOD-1 co-clustering in activated lymphocytes. (A) Confocal microscopy image of CD3 (green) and SOD-1 (red) in resting T cells. A homogeneous, distinct, membrane and intracellular distribution of the TCR and SOD-1 can be appreciated. (B and C) After 2 min of anti-CD3 treatment a clustered distribution of TCR can be observed (white arrows). SOD-1 distribution becomes strongly clustered and resembles that of TCR (white arrows). This cell re-localization is better showed in panel C where a single cell has been focused. Merged images show that TCR clusters recruit intracellular SOD-1, whose localization strictly reflects TCR distribution (white arrows). (D) anti-CD3/NAC co-incubation induces a homogeneous TCR surface distribution. SOD-1 localization also resembles basal images, with the presence of small areas of faint cytosolic accumulation. Merged images reveal a clear-cut distinct distribution of TCR and SOD-1.

microvesicle pathway by TCR triggered T cells. These effects have been observed maintaining the biological complexity of antigen-dependent T cell response and confirmed in purified T cells activated by anti-CD3/anti-CD28 beads.

We showed that extra-cellular SOD-1 is increased in PBMC cultures after anti-CD3 treatment. This effect was accompanied by both the induction of SOD-1 mRNA and increase of SOD-1 containing micro-vesicles in culture supernatants. Moreover, we identified the T cell population as the specific target for SOD-1 induction and extra-cellular export. Therefore, TCR-dependent activation behaves as a triggering element for SOD-1 production and secretion by human T cells.

SOD-1 production is induced in neuroblastoma SK-N-BE cells after oxidative stress [30,37]. Moreover, other and our data showed that cytosolic SOD-1 is secreted by many cell lines carrying out a paracrine modulatory role [30,31,37] and SOD-1 extracellular export was by us described in primary lymphoid organs [48].

Induction of extra-cellular export of SOD-1 after TCR-triggering proposes a more complex physiological involvement of such enzyme in T cell activation. In this study, we described that a BFA-dependent secretion mechanism [54] characterized SOD-1 micro-vesicle inter-cellular trafficking upon antigen-dependent immune response. No effect has been observed in the presence of methylamine that impairs cell endocytosis [55]. Thus, a major involvement of endocytic recycling pathways might be excluded. Such mechanism, previously described in neuronal model [30–34,37], represents an intriguing issue for further investigations.

SOD-1 is a cytosolic protein lacking signal peptide and consequently considered to be excluded from ER translocation. The small amount of wild type SOD-1 detected in ER–Golgi apparatus [36], does not support the direct involvement of this organelles in SOD-1 secretion. Moreover, the possible interference of BFA, a classical inhibitor of ER–Golgi dependent protein secretion, in vesicular pathways not directly involving ER–Golgi apparatus cannot be excluded. In this context, our data propose that SOD-1 could be part of the micro-vesicle-dependent pathways functioning as secondary messenger between immune cells [45–47].

A number of data indicate that exogenously added H_2O_2 induced signals in the absence of ligands, whereas catalase is able to inhibit such effect [42,43,56]. Moreover, the observations that production of catalase characterizes many pathogens [57] and that viral infection modulates H_2O_2 production [58] confirm the multiple roles played by extracellular H_2O_2 in the activation processes of lymphocytes [59].

We consistently found that antigen-dependent T cell triggering mediated changes in the intra-cellular localization of SOD-1 that was observed to co-localize with clustered TCR. This event was dependent on ROS availability since it was impaired by NAC co-treatment. ROS production is an essential component in signaling cascades that mediate actin cytoskeleton rearrangements. Small G protein Rac, a key element in the network assembly of actin in lamellipodia [60–62] participates in activation-dependent ROS production by different cell types [63,64]. Moreover, Rac-mediated ROS production results in the downmodulation of Rho activity thus regulating cellular morphology and migratory behavior [65]. SOD-1 associates with Rac-1 regulated NADPH oxidase complexes in different mouse tissues and cell lines [28]. In this context, massive ROS scavenging is expected to disrupt ROS-dependent regulation of cell contractility and motility. This event could account for the TCR/SOD-1 intracellular redistribution (Fig. 5). The impairment of early cell aggregation in

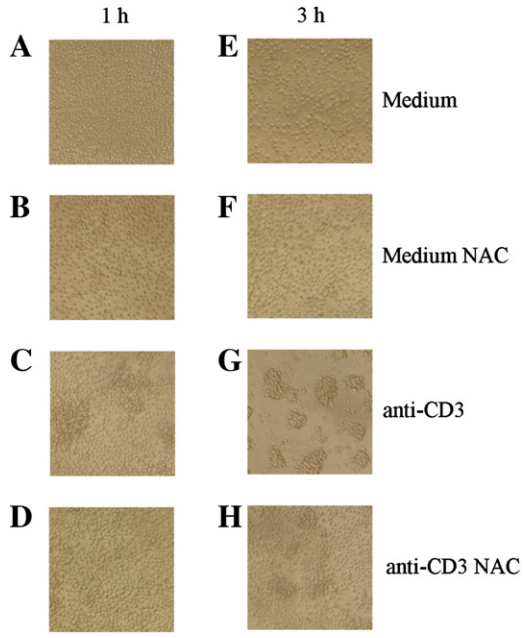

Fig. 6. NAC treatment inhibits early activation-induced aggregation of TCR triggered lymphocytes. Contrast phase microscopy images showing 1 and 3 hour cultures of PBMC incubated with Medium (A and E), NAC (B and F), anti-CD3 (C and G) or anti-CD3 and NAC (D and H). As shown, significant cell aggregation is observed in anti-CD3 triggered cells. Lymphocyte clustering is significantly inhibited in the anti-CD3/NAC co-treated cultures; (D) after 1 h of incubation. Quantification is performed by counting cell aggregates, identified by the presence of at least 8 clustered cells. Comparative analysis (see Supplementary Table S1) shows a 83.64 ± 1.62 inhibition of cell aggregates in anti-CD3/NAC co-treated cultures in 6 independent experiments; ($p < 0.05$). (H) After a 3 hour period the inhibiting effect of NAC treatment is observed to be lowered (55.35 ± 2.23 inhibition of cell aggregates in 6 independent experiments). Quite normal clustering of anti-CD3 treated PBMC is observed at longer culture time in anti-CD3/NAC co-treated cells (not shown). Results show one representative experiment of the six performed.

presence of anti-CD3/NAC co-treatment (Fig. 6) strongly supports such hypothesis.

Compelling evidences indicate that ROS, together with their essential role in innate antimicrobial defense [12], are critically involved in the regulation of antigen-dependent response of adaptive immune effectors [10,11,66–69]. Exposure of T cells to oxidant agents, such as pervanadate or H_2O_2, induces and/or enhances TCR signaling during T cell activation [70–72]. TCR-dependent signaling generates both superoxide anion and H_2O_2 that selectively regulate antigen-dependent proliferation and Fas ligand expression by T effectors [10]. An oxidative signal implies its tight regulation and transient character. Thus, in the presence of multiple intracellular ROS sources [6–9], the involvement of multiple anti-oxidant mechanisms in fine tuning of antigen-dependent T cell response can be hypothesized. Indeed, Mn-SOD/SOD-2 a major mitochondrial antioxidative enzyme has been consistently associated with T cell activation [73] and a role of catalase, glutathione and thioredoxin has been also proposed [74].

In the models of ROS generation upon stimulation of receptors it has been shown that H_2O_2 is the relevant oxidant species that regulate signaling [18–20]. Notably, H_2O_2 has a short half-life in the reducing environment of the cytosol, and it acts close to its site of production. Thus, an important aspect of ligand-dependent TCR activation might be the rapid translocation of receptors to a source of H_2O_2 or, *vice versa*, the clustering of such a source to the receptor. To this regard, our data strongly support the hypothesis that SOD-1 intracellular localization in antigen-triggered T cells could provide H_2O_2 generation in the cell compartment specifically involved in tuning antigen-dependent signals.

A number of data suggested the role for H_2O_2 as key modulator of protein phosphorylation on either serine–threonine and tyrosine residues [75]. Indeed, all protein tyrosine phosphatases (PTPs) contain an essential cysteine residue in the signature active enzyme site motif that has been demonstrated to be target of specific H_2O_2 oxidation. The H_2O_2-mediated inhibition of PTP activity is expected to result in a shift of protein tyrosine kinases toward protein phosphorylation. The involvement of SOD-1 in such regulatory pathways has been suggested [76].

Our data on SOD-1 intracellular re-localization upon TCR-triggering suggests that SOD-1 could directly modulate kinase/phosphatase activity related to proximal TCR signaling. The evidence [10] that anti-CD3 induced ERK phosphorylation requires H_2O_2 but is independent on superoxide anion, strongly supports such hypothesis. Thus, subcellular compartmentalization of H_2O_2 generating enzymes (like SOD-1) could represent a relevant element in achieving the superoxide/peroxide balance required to optimize antigen-dependent T cell response.

Taken in all, our data suggest that SOD-1 is part of the molecular network involved in antigen-dependent T cell response. At the best of our knowledge, this is the first observation revealing a relationship between SOD-1 secretion/intracellular re-localization and the antigen dependent T cell activation. Further studies are needed to investigate on the involvement of SOD-1 in the regulation of TCR signaling cascades as well as in the functional cross talk between immune effectors.

5. Conclusion

This study reports for the first time that SOD-1, a major physiological regulator of cytosolic superoxide/peroxide balance, is part of the molecular network involved in antigen-dependent T cell activation. Indeed, we observed: i. mRNA induction and increased levels of extra-cellular SOD-1 containing micro-vesicles in anti-CD3 triggered cultures; ii. increase of intra-cellular SOD-1 and BFA-dependent SOD-1 microvesicle secretion in TCR-triggered T cells; iii. TCR/SOD-1 co-localization was observed in anti-CD3 treated T cells.

Further studies are needed to establish whether SOD-1 is involved in modulating ROS-dependent intra-cellular and inter-cellular signaling in antigen triggered human T cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.10.020.

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