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New Therapeutic Targets in Pediatric ITP

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1. INTRODUCTION

1.1 What is Immune Thrombocytopenia

Immune thrombocytopenia (ITP) is the most common childhood piastrinopenia, characterised by isolated thrombocytopenia and defined by a peripheral blood platelet count of $<100\times10^9$/L [1, 2]. It is an autoimmune disorder caused by platelets destruction mediated by autoantibodies [2-5].

There are several clinic manifestations of the disease: patients could be asymptomatic, or they could manifest mild mucocutaneous to life-threatening bleeding [1]. ITP could be characterised by severe bleeding in only 5% of patients, but bleeding could induce hospitalization within 5 years after diagnosis in 15% of patients [1, 6, 7]. Furthermore, ITP patients are also characterized by fatigue and by a compromission of health-related quality of life [8]. They are more predisposed to the development of venous thromboembolism then general population and this condition could compromise patients health even more considering their high risk of bleeding [9].

ITP could be a primary condition or secondary to other causes or diseases [1], such as the use of certain drugs, lymphoproliferative disorders, infections and other immune disorders [1]. Its incidence is of about 2-4 cases per 100000 persons, with a first peak between 20-30 years of age with a female predominance and another peak after 60 years of age without differences in sex distribution [1, 10]. Some patients experience one episode of ITP and successfully immediate remission, nevertheless chronic ITP occurs in 70% of adult patients [1]. Certainly, ITP remission determined by treatment or spontaneous requests many years after diagnosis [1]. ITP in children is usually benign and its remission occurs approximately 6-18 months after diagnosis, even though 20-30% of patients develop chronic ITP [2, 11].

ITP pathophysiology is complex and is not yet well-known. The main concern is that antibody-coated platelets are prematurely destroyed in the spleen and in the liver through interaction with Fcγ receptors (FcγRs) [12].

In patients in which other causes of thrombocytopenia have been excluded, ITP is defined as a platelet count below 100000 per cubic millimeter [1, 13]. It is important to investigate the clinical history of patients, for example the use of drugs, in order to exclude any other causes of thrombocytopenia and to also understand secondary causes of ITP [1]. In ITP patients, the peripheral-blood smear shows a decreased number of platelets without other abnormalities, even though several patients have large platelets [1]. A specific diagnostic test for ITP has not been developed; indeed, autoantibodies against platelets are found only in about 50-60% of ITP patients and their levels evaluation is not recommended as diagnostic method [14, 15]. Moreover, the examination of also bone marrow is not recommended as diagnostic method in ITP patients and it is performed only in patients with other hematologic alterations or when they do not respond adequately to the treatment [1].

1.2 Immune Thrombocytopenia Definition and Classification

ITP was originally known as idiopathic thrombocytopenic purpura [13]. Successfully, the term “idiopathic” was replaced by “immune” in order to highlight the immune-mediated process of ITP, and the term “primary” was used to replace “idiopathic” in order to assess the absence of any other causes [13]. Since bleeding symptoms are absent or minimal in most patients, the term "purpura" was determined to be inappropriate [13, 16, 17]. However, the acronym ITP was preserved and it is currently used as an abbreviation for immune thrombocytopenia [13].

It was necessary to establish a uniform predefined cutoff level of platelet for ITP diagnosis [13]. The actually threshold for ITP diagnosis is

characterised by a platelet count less than 100 X 10^9/L [13]. This novel threshold was preferred compared to the commonly used levels of less than 150 X 10^9/L; indeed, several healthy patients exhibited a platelet count between 100 and 150 X 10^9/L, with a 10-year probability to develop severe thrombocytopenia of about 6,9 % [13, 18]. Also in non-Western population a platelets value between 100 and 150 X 10^9/L was observed in healthy subjects [19-21]; moreover, the novel cutoff level also allows the exclusion of pregnant women who show physiological pregnancy-related thrombocytopenia, which does not request other clinical investigations [13, 22].

The definition of "secondary ITP" is referred to all forms of immune-mediated thrombocytopenia which are caused by other diseases or drugs exposure [13] and also includes rare ITP, such as fetal and neonatal alloimmune thrombocytopenic purpura and post-transfusion purpura [13]. It is clinically important the distinction between primary and secondary ITP, because they request different treatments and show distinct natural histories [13]. For example, in case of ITP secondary to another disorder, the treatment is aimed to this disorder [13, 23, 24]; however, ITP induced by drugs exposure undergo in remission when drug administration is stopped, and in case of most severe ITP it requested only platelets transfusion ad initial treatment [13, 25].

Since there are not clinical or laboratory parameters of disease duration, it was proposed a new classification of ITP [13]. The term "newly diagnosed ITP" indicates all ITP cases at diagnosis, while "persistent ITP" is referred to all patients which do not achieve spontaneous remission or do not show response after stopping treatment between 3 and 12 months from diagnosis [13]. However, during this period there is a significant possibility of spontaneous remission of the disease, therefore it is possible to avoid aggressive and invasive therapeutic interventions [13, 26, 27]. Finally, the

term “Chronic ITP” is referred to patients with ITP for more than 12 months [13]. Considering the severity of the disease, ITP is distinguished in mild, moderate and severe [13], on the basis of thrombocytopenia degree, closely related to the risk of bleeding [13].

Recently, the International Working Group (IWG) on ITP has introduced new terminology, standard definitions and outcome criteria for diagnosis and management of ITP [13, 28].

The term “Severe ITP” is referred to patients with clinically significant bleeding. This condition is characterised by bleeding symptoms at presentation for which the treatments are mandatory, or by new bleeding symptoms which request additional treatments with a different platelet-enhancing agent or an increased dose of drugs [13]. The terms “mild” and “moderate” are less indicate to use because of their vagueness [28]. IWG recommend to not evaluate only the platelet count to establish treatment effectiveness because there are not tools of standardized bleeding and quality of life assessment for ITP in literature [1]. Considering these evidences, Rodeghiero *et al.* have described an ITP-specific Bleeding Assessment Tool (ITP-BAT), a standardized data collection which provides a precise and specific definition of bleeding manifestations and of their severity degree, making data collection and communication between investigators easier [28].

ITP-BAT provides specific terms for haemorrhagic manifestations in ITP in order to develop an ITP-specific bleeding assessment [28]. Bleeding symptoms could affect: skin (S), visible mucosae (M), and organ (and internal mucosae) (O). The bleeding grade should be established by a physician or trained nurse at presentation and at each follow-up visit. The range of bleeding grade is between 0 and 4 for epistaxis and for bleeding in the organ domain, except ocular and intracranial bleeding (grade 0 and 2 to 4). Instead, for the other bleeding sites (skin and mucosal) four grades (0 to

3) were used. Grade 5 is used only for fatal bleeding [28]. Therefore, after attributed the highest grade for each domain, the SMOG index is obtained [28].

1.3 Cells and biological mechanisms involved in Immune Thrombocytopenia

The pathophysiology of ITP is complex and not yet fully understood [1]. In particular, both autoantibodies-mediated platelet destruction and bone marrow impaired platelet production are involved in the pathophysiology of ITP [29]. Indeed, in 1951 Harrington and collaborators demonstrated for the first time that platelet destruction in ITP is induced by a "plasma-derived factors", which are then identified as antiplatelet antibodies [30].

Platelets targeted by autoantibodies are prematurely degraded in the spleen and in liver or through interaction with FcγRs [1, 12] (Figure 1).

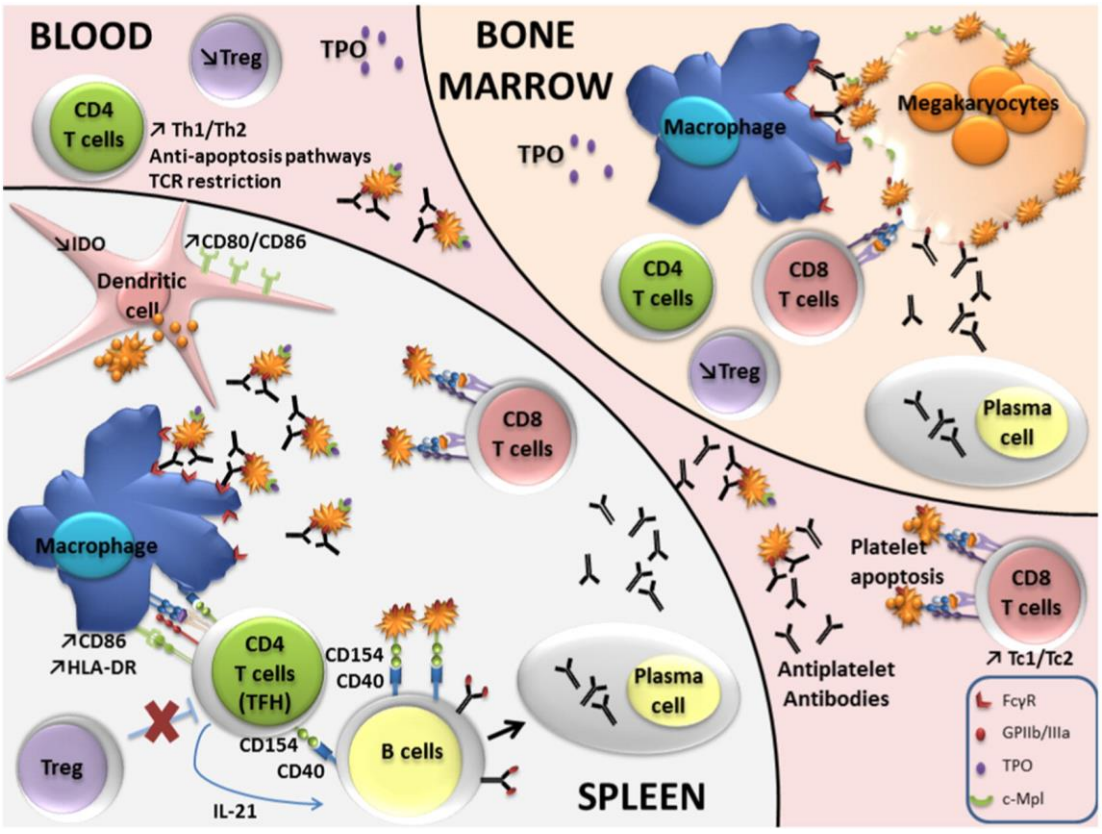

Figure 1 - Platelet peripheral destruction in Immune Thrombocytopenia. Opsonized platelets were degraded by splenic macrophages, which present a platelet-derived antigen responsible for activation of autoreactive CD4+ T cells. These cells contribute to B cells activation,which recognize native platelet

antigens expressed on the surface of follicular dendritic cells (Audia S, Mahévas M, Samson M, Godeau B, Bonnotte B. Pathogenesis of immune thrombocytopenia. Autoimmun Rev. 2017 Jun;16(6):620-632. doi: 10.1016/j.autrev.2017.04.012. Epub 2017 Apr 17. PMID: 28428120.).

FcγRs are cell-surface glycoproteins which are involved in the relation between humoral and cellular immunity [31]. There are three classes of FcγRs on human leukocytes: FcγRI, II (with two isoforms IIa and IIb) and III (with two isoforms IIIa and IIIb). They have different functions and, in particular, it is possible to distinguish them in activating receptors (FcγRI, IIa, and III) and in inhibitory receptor (FcγRIIb). Therefore, the stimulation of FcγRI, IIa, and III induces the activation of inflammatory cells, such as monocytes/macrophages or neutrophils, while FcγRIIb stimulation induces an inhibition of the activating FcγRs [31]. The imbalance of the activating and inhibitory FcγRs is associated with the pathophysiology of several autoimmune diseases, such as ITP [31].

Most of the autoantibodies produced in ITP are of the IgG class and principally recognize platelet glycoproteins (GP) IIb/IIIa and Ib/IX, but there are several ITP patients having antibodies directed to multiple platelet antigens [30, 32-35]. Furthermore, autoantibodies not only can determine platelet destruction through complement activation and desialylation [1, 36, 37], but they also inhibit megakaryocyte function [1, 38].

Since anti-platelet antibodies are not revealed in up to 50% of ITP patients, other mechanisms of platelet destruction may be involved. For example, in ITP patients changes in lymphocyte populations are reported, observing not only B-cell hyperfunction, which determines an increased autoantibodies production [2, 39-41], but also an increase of T helper (Th) 1 and Th17 phenotypes [42] and a decrease of regulatory T cells number and activities, with consequent increased T-cell-mediated cytotoxicity [37, 43]. Effectively, B cells require the intervention of platelet specific and autoreactive CD4 + T cells in order to trigger a deleterious immune response [33]. Accordingly, it has been demonstrated that *in vitro* GPIIIa stimulation

induced a strong CD4+ T cell proliferation and an increased IL-2 production in peripheral blood mononuclear cell (PBMC) cultures isolated from ITP patients compared to healthy subjects [33, 44, 45]. Increased levels of T cells were also observed in peripheral blood of ITP patients [33, 46]. Therefore, in recent years it has been studied the role of CD4+ T cells in the pathogenesis of ITP; furthermore, recent studies demonstrated that also CD8 + T cells play a role in ITP, by contributing to platelets disruption and by counteracting normal thrombocytopoiesis in bone marrow [33].

ITP pathogenesis not only involved T cells and B cells, but also other cell populations [47]. Impairment of dendritic cells, NK cells, mesenchymal stromal cells (MSCs) and macrophages are involved in ITP development [47-50].

Definitely, ITP pathogenesis is very complex, so more studies are needed to better understand it and to find new therapeutic strategies against ITP.

1.3.1 T Cells involvement in ITP

The balance of Th1/Th2 cells ratio is the main characteristic which defines the cellular immune response; its imbalance is observed in several immune-mediated diseases, such as ITP [33]. Th1 cells are activated by IL-12 and IFN-γ and they are responsible for IFN-γ and IL-2 release. Instead, Th2 cells are stimulated by IL-4 and produce several cytokines, such as IL-4, IL-5, IL-9, IL-10 and IL-13 [33, 51].

In ITP patients an imbalance of Th1/Th2 ratio is observed, with a major presence of Th1 cells [33, 48]. Initially, Th1 cells involvement in ITP pathogenesis was studied by revealing the levels of Th1-released cytokines in patients peripheral blood; in particular, it has been demonstrated Th1 prevalence in ITP patients by detecting serum high levels of IFN-γ, IL-2 and TNF-α [12, 48, 52, 53]. Moreover, high levels of IFN-γ, IL-10 and IL-2 were detected in serum of both paediatric and adult ITP patients, thus

suggesting Th0/Th1 polarization [33, 54, 55]. These results were confirmed also by measuring RNA levels and IFN-γ intracellular levels in T cells [48, 56, 57].

In contrast, a decrease of Th2 is observed with a consequent increase of Th1/Th2 ratio both in the circulation and in the spleen [48, 56, 58, 59]. Indeed, several studies have demonstrated not only a reduction of Th2-released cytokines in serum of both paediatric and adult ITP patients, but also a reduced percentage of Th2 cells in peripheral blood of ITP patients [33, 56, 59, 60].

As mentioned above, it has been clarified that CD8 + T cells are also involved in the pathogenesis of ITP; these cells are responsible for platelets degradation in periphery and also for inhibiting their production in bone marrow [48]. It has been observed that CD8 + T cells express high levels of proteins involved in their cytotoxic capacity, such as perforin, granzyme A and granzyme B, leading to platelet apoptosis [48, 61-63]. Moreover, Li *et al*. demonstrated that CD8 + T cells co-cultured *in vitro* with megakaryocytes induced and impairment of platelet production [48, 64]. It has also been reported that T cells recruitment in bone marrow is very high in ITP patients [65] and that increased levels of granzyme A and B, resulted by CD8+ T cells activation, were present in several ITP patients [66].

1.3.2 Mesenchymal Stromal Cells involvement in ITP

Mesenchymal stromal cells (MSCs) are non-hematopoietic multipotent cells which are able to differentiate in adipocytes, chondroblasts, and osteoblasts [48, 67-70]. They can be isolated from bone marrow and from other human tissue, such as adipose tissue, umbilical cord blood, dental pulp, muscle, tendon, skin, and, in recent years, it has been demonstrated that also human breast milk could be a source of MSCs [71-74].

MSCs release chemokines, cytokines, and extracellular matrix protein, which are all responsible for improving hematopoietic stem cells survival, for modulating immune effector cells activity and function, and for inhibiting T-cells [69, 75-77]. They have immunomodulatory and anti-inflammatory properties [69, 78] and this aspect is of crucial importance for the management of several immune-mediated disorders [69]. MSCs exert immune-suppressive properties by releasing soluble factors, such as IL-6, IL-10, transforming-growth factor β (TGF-β), interferon-γ (INF-γ), prostaglandin E2 (PGE2), and nitric oxide (NO) [69, 70, 79, 80].

MSCs can regulate immune response by influencing activity of several immune cells, such as T and B cells, dendritic cells, regulatory T cells (T regs), and natural killer (NK) cells [69]. In particular, MSCs from healthy subjects are able to modulate both Th1 and Th2 cells activity, determining a reduction of interferon (IFN)-γ release by Th1 and an increase of IL-4 release by Th2 [70, 81]. Moreover, these cells are also responsible for increasing Tregs rate and for reducing IFN-γ release by NK cells [70].

MSCs obtained from ITP patients bone marrow are characterized by an anormal morphology and they show a reduced proliferation rate and an increased apoptosis [48, 70, 82, 83]. Their activities are severely impaired, so that they are unable to inhibit the proliferation and functions of activated T cells [70, 83].

Considering these evidences about MSCs important role in modulating immune and inflammatory responses and their intense impairment in ITP, they could be considered an important possible target for novel therapeutic strategies to counteract ITP.

1.3.3 Macrophages involvement in ITP

Macrophages are immune phagocytic mononuclear cells with a key role in immune and inflammatory responses. There are two different

macrophage activation phenotypes: the classically activated macrophages (M1) and the alternatively activated macrophages (M2) [49, 84, 85]. M1 macrophages are characterised by pro-inflammatory, anti-microbial, and anti-tumor activities and are responsible for increased pro-inflammatory cytokines release, such as TNF-α, IL-6, IL-1β, and Nitric Oxide Synthase (iNOS). They are activated by TNF-α, IFN-γ, and bacterial lipopolysaccharide (LPS) [49, 84-86]. Instead, M2 macrophages are stimulated by IL-4, IL-13, IL-10, and by Phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway. They have anti-inflammatory and immunosuppressive properties, mediated by anti-inflammatory cytokines release, such as IL-10 and Transforming Growth Factor-β (TGF-β), and exert also pro-angiogenic and pro-fibrotic properties, contributing to tumor progression [49, 84-86]. Furthermore, both M1 and M2 macrophages are involved in iron metabolism. Indeed, M1 phenotype is responsible for an increase of iron internalization and, then, of iron intracellular concentration [87, 88]. Intracellular iron high levels cause pro-inflammatory cytokines expression and reactive oxygen species (ROS) production, thus contributing to the inflammatory state [88]. Conversely, M2 macrophages are responsible for iron release and, consequently, are characterized by low intracellular iron concentration, which inhibits pro-inflammatory cytokines expression and induces a reduction iNOS expression [88].

In ITP pathogenesis macrophages are considered both effector cells, contributing to platelet depletion, and antigen presenting cells, stimulating the adaptative immune response [48]. In particular, in ITP patients, high levels of M1 macrophages and, consequently, an increased M1/M2 ratio are reported. This M1/M2 ratio alteration is responsible for an increased pro-inflammatory cytokines release, with consequent impairment of inflammatory status and immune response [49, 89-91].

Considering the impairment of M1/M2 ratio in ITP, the research of new therapeutic strategies could be aimed to find new drugs able to modulate macrophage phenotype polarization.

1.4 Current Treatments of ITP

ITP patients require treatment when bleeding manifestations appear and/or when their platelet count is lower than 20-30 $X10^9$/L [48, 92]. Platelet count is the most important index of activity in ITP patients [93].

ITP treatments are requested in order to rapidly but transiently increase platelet count, to maintain a stable platelet count and, finally, to obtain ITP remission [93, 94].

Interestingly, most recently the American Society of Hematology has proposed a different management of ITP treatment between adults and children [95]. In particular, while it suggests to administer treatment in newly diagnosed adults with a platelet count $< 30 \times 10^9$/L, at the same time, it recommends that *"children with no bleeding or mild bleeding be managed with observation alone regardless of platelet count"* [14]. This decision to use two different approaches between adults and children is due to the low probability to develop bleeding in ITP children with mild or no bleeding symptoms have a low, although the platelet count is low [95]. Therefore, the main difference between pediatric and adult ITP consists in the different initial management, which involves the observation in children [95].

1.4.1. First-Line Therapy in ITP

First-line therapy includes corticosteroids and, in case of severe bleeding or in emergency situations, high dose intravenous immunoglobulins (IVIG), which are needed to induce a rapid increase of platelet count [92].

Steroids are used in several inflammatory diseases and in ITP they are able to induce a transient response in almost 80% of cases [48, 96]. In ITP

steroids are able to restore Th1/Treg balance [48, 97] and to increase both circulating Treg number [48, 98], and myeloid-derived suppressor cells in a murine model of ITP [48, 99]. The main corticosteroid regimen used in ITP consists in standard-dose prednisone [100]. Therapy also includes High Dose Dexamethasone (HD-Dexa). Prednisone is administered 1 to 2 mg/kg orally per day for 1 to 2 weeks, with a gradual taper and discontinuation by 6 to 8 weeks [93], while HD-Dexa is administered as one or more cycles of 40 mg orally once daily for 4 days [100, 101]. An initial response to corticosteroid is observed in up to 80% of ITP patients [93]; instead, long-term responses are observed in only 20 to 40% of newly diagnosed patients disease [93, 101]. In patients which non respond to therapy, corticosteroid administration should be interrupted [100]. HD-Dexa shows anti-inflammatory effects which are more potent than prednisone ones. It is reported that HD-Dexa is responsible for the increase of M2-like macrophages which are involved in suppressing autoimmunity, and is also able to elevate the Th1/Th2 ratio [89, 102].

Although short courses of HD-Dexa avoid the onset of toxicities associated with prolonged corticosteroid exposure, such as osteoporosis and weight gain, HD-Dexa administration induces acute toxicities, such as hypertension, cognitive impairments, and hyperglycaemia [100]. Indeed, for this reason, ITP patients with diabetes, hypertension or cardiovascular disease are excluded for studies of HD-Dexa [103, 104].

The American Society of Hematology recommend that IVIG should be initially administered as a single dose of 1 g/kg and repeated in non-responding patients [100, 105]. IVIG are administered as emergency therapy when an increase of bleeding score is observed and they induce a response in about 80% of patients [48, 106]. IVIG are generally administered in combination with steroids in order to increase their efficacy [48]. Their activities are complex and are promoted by several mechanisms:

activating FcγR blockade, inhibitory FcγRIIb upregulation, cytokine production regulation, dendritic cells maturation and autoantibodies neutralization [48, 107]. Most of these mechanisms induced by IVIG were obtained from studies conducted in animal models [48]. In studies conducted in human ITP, it has been demonstrated that IVIG are able to induce an increase of platelet lifespan by causing a reduction of splenic platelet clearance [48, 108]. IVIG causes several side effects, among them headache, aseptic meningitis and acute kidney injury [109-112]. Moreover, another uncommon complication of IVIG administration is the haemolysis from passive transfer of anti-A and anti-B haemagglutinins which could be observed in non-blood group O patients, when IVIG preparations consist in high-titre anti-A or anti-B antibodies and large cumulative doses of IVIG (>100 g over 2–4 days) [100, 113]. IVIG could also be associated to a high thrombosis risk, which is about 1% per year for arterial or venous thrombosis for patients who are regularly treated with IVIG [100, 114, 115]. Therefore, it has been observed that IVIG are able to induce an increase in platelet count more rapidly than corticosteroids [100, 116].

1.4.2 First-Line Therapy in Children

ITP children are principally asymptomatic at diagnosis or show bleeding confined to the skin or oral mucosa [100, 117]. The decision about treatment initiation depends on thrombocytopenia degree, bleeding severity and the presence of other risk factors for bleeding [100, 105, 118]. The America Society of Hematology establishes that in children with no bleeding or mild bleeding (petechiae) have to be only observed, without considering the platelet count [100, 105]. This decision was made for several reasons: firstly, remission occurs spontaneously within 3 to 6 months in newly diagnosed ITP children [100, 117, 119]; secondly, first-line therapy with corticosteroids or IVIG seems to not modify long term disease remission

rates [100, 120]; finally, in children severe bleeding is rare (only in 3% of children at diagnosis) [100, 119, 121].

Several dosing regimens of corticosteroid have been used in children, for example prednisone at a dose of 1 to 2mg/kg/day or short courses of high-dose dexamethasone (24 mg/m2/day, maximum 40 mg per day, for 4 days) [100]. Insteas, IVIG is typically administered as a single dose of 0.8 to 1 g/kg [100].

1.4.3 Second-Line Therapy in ITP

The American Society of Hematology recommends splenectomy or treatment with Thrombopoyetin Receptor Agonists (TPO-RAs) in adult patients in which ITP persists for more than 3 months, also after treatments with corticosteroids [122]. Splenectomy induces a prolonged response in 60–70% cases [48, 123]. Furthermore, patients responsive to splenectomy show a decreased clonal expansion of T cells [48, 124]. Considering that in the spleen there are also long-lived plasma cells which are responsible for specific antiplatelet antibodies release, splenectomy induces also their removal [125].

Since a key role in ITP pathogenesis is played by B cells, the use of drugs responsible for their degradation has been recommended [48]. Among these types of drugs, rituximab has been used for years [126]. It is a chimeric mono clonal antibody directed against CD20, a transmembrane glycoprotein expressed on B cells surface, which could induce complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity or induction of B cells apoptosis [48, 127]. Rituximab induces a response in 40% and 30% of patients at 1 and 2 years of follow-up and 20% of patients still respond after 5 years [128, 129]. It is known that rituximab is responsible for depletion of B cells, but not of plasma cells, which indeed do not express CD20. Rituximab is clinical efficient because its B cell depletion inhibits the

generation of short-lived plasma cells, responsible for antiplatelet antibodies production. Nevertheless, antiplatelet antibodies produced by long-lived plasma cells are not influenced by B cells depletion, indeed in the spleen or in the bone marrow of rituximab-refractory ITP patients it is possible to observe these types of cells [130, 131]. Rituximab, in addition to inducing B cell depletion, has other important effects: it could induce a reversion of T cell polarization abnormalities, an increase of Treg functions in responder patients, and a reduction of splenic and circulating TFH [125, 132].

Veltuzumab represents another B cell depleting therapy which is efficient in ITP [133]. It is a humanized anti-CD20 antibody which differs from rituximab both in structure and in function [133]. Indeed, it shows framework regions and amino acid differences, which are responsible for slower off-rate and increased complement-dependent cytotoxicity [134-136]. It induces B cells depletion and a rapid patients response [137].

In adults in which ITP has lasted for more than 3 months and that are corticosteroid dependent or have no response to corticosteroids, the American Society of Hematology recommends the use of a TPO-RAs rather than rituximab [122]. TPO-RAs induce a response rate of about 70-80% and they act by inducing platelet production by megakaryocytes [138]. It has been reported a prolonged response after their discontinuation in up to 15% of ITP patients, an effect problably due to their potential immunomodulating properties [139, 140]. The currently used TPO-Ras are romiplostim and eltrombopag, but several studies are underway on other TPO-RAs, such as lusutrombopag, avatrombopag, or hetrombopag [141-143].

1.4.4 Second-line Therapy in Children

In children who have not responded to first-line therapies, second-line therapies are proposed [122]. Second-line therapies include splenectomy, TPO-RA, and rituximab. In particular, the American Society of Hematology

recommends the use of TPO-RAs rather than rituximab and splenectomy in ITP children who, firstly, do not respond to first-line therapy and, also, have non-life-threatening mucosal bleeding and/or diminished Health-Related Quality of Life (HRQoL) [122]. Moreover, it is recommended the use of rituximab rather than splenectomy in these patients [122].

Eltrombopag is currently the only TPO-RA approved by US Food and Drug Administration for the treatment of chronic ITP in children [144]. There are two multicenter, double-blind, placebo controlled clinical trials which demonstrated efficacy of ELT in increasing platelet counts, decreasing bleeding and the need for concomitant ITP therapies with relatively few adverse effects: PETIT (Efficacy and Safety Study of Eltrombopag in Pediatric Patients With Thrombocytopenia From Chronic ITP) and PETIT2 (Study of a New Medication for Childhood Chronic ITP, a Blood Disorder of Low Platelet Counts That Can Lead to Bruising Easily, Bleeding Gums, and/or Bleeding Inside the Body) [144]. Headache, nausea, and hepatobiliary laboratory abnormalities are the main reported ELT-related adverse effects. Unfortunately, long-term safety data in children are limited, but studies in adults are promising as they have not revealed a clinically significant increase in the incidence of thrombosis, cataract formation, or medullary fibrosis [144].

1.5 Eltrombopag as immune modulator drug

ELT is an orally available TPO-RA approved for chronic ITP treatment in both pediatric and adult patients, when the first-line therapy fails [144-147]. Its main property consists in stimulating hematopoietic stem cells to promote platelets production [147], but it also exerts immunomodulating properties [31, 43, 148, 149]. Indeed, Bao *et al.* demonstrated for the first time that TPO-RA improved *in vitro* Treg suppressive functions in chronic ITP patients and consequently a reduction of effector T helper functions,

suggesting these agents as immunomodulating drugs [43]. Moreover, Li *et al.* demonstrated that in chronic ITP patients TPO-RA administration restored Breg cells functions, which are instead strongly impaired in ITP [148].

It has been also demonstrated that TPO-RA are responsible for inhibiting T-cells response to platelet auto-antigens. In a mouse ITP model generated by Foxp3 Treg depletion, TPO-RA treatment not only induced an increase in platelets production, but also inhibited both T cells responses to platelet autoantigens and anti-platelet autoantibodies production [149].

Liu and collaborators observed that TPO-RA are able to modulate FcγR in ITP [150]. They demonstrated that ELT induced an increased expression of FcγRIIb and a reduction of FcγRIIa and FcγRI expression on monocytes from patients with ITP, thus counteracting the activation of monocytes and, consequently, reducing the inflammatory state [150].

Novel therapeutic approaches could be based on ELT immunomodulating properties in order to ameliorate the inflammatory profile and the immune system function.

1.6 Eltrombopag as iron chelator

Recently, it has been reported that ELT has also iron chelating properties, binding the main intracellular form: iron (III) [151, 152]. It has been demonstrated that in leukemia cells ELT induces a reduction of labile iron [153]. On this basis, Vlachodimitropoulou *et al.* demonstrated that ELT could mobilize cellular iron, suggesting a possible role for ELT in treating iron overload conditions, either alone or in combination with clinically licensed chelators. Successfully, they clarified the iron-binding properties of ELT [152]. They observed that ELT promoted cellular iron mobilization more than additive (synergistic) with deferasirox. In particular, they demonstrated a particular shuttling mechanism, through which ELT rapidly

donated the chelated iron to deferasirox [152]. This mechanism has also been observed when ELT was combined with the otherwise ineffective extracellular hydroxypyridinone chelator, CP40 [152]. Definitely, ELT was suggested as a powerful iron chelator, able to reduce intracellular iron concentration and to promote iron mobilization when combined with clinically available chelators [152].

Punzo *et al.* demonstrated that ELT exerted iron chelating properties also in bone tissue and that its effects were more effective when administered in combination with deferasirox, the most common used iron chelator, suggesting a synergic effect [151]. Moreover, in 2020 Argenziano and collaborators confirmed the iron chelating properties of ELT also in Osteosarcoma cell lines [154].

Recently, it has been demonstrated that ELT administered in combination with deferasirox reduced intracellular iron concentration also in acute myeloid leukemia cell line; this reduction was related not only to iron chelating properties of these drugs, but also to ELT capibility to modulate iron metabolism by inhibiting its uptake and by promoting its release [155].

Therefore, the emerging ability of ELT to chelate iron and modulate its metabolism could be a new strength for the use of ELTs for the treatment of ITP.

1.7 Iron Metabolism and its involvement in inflammation

Iron is an important element in mammal cells [156]. It is involved in several biological processes, such as cellular metabolism and signaling [157, 158], cell growth and differentiation, DNA synthesis and repair, immunity, synthesis of hemoglobin, neurotransmission, and others [159, 160]. Its metabolism is finely regulated and its alteration could damage cells, tissues and organ functions [159, 161]. There are several key proteins

responsible for regulating iron metabolism: hepcidin, ferroportin (FPN-1), ferritin, transferrin (TF) and transferrin receptor (TFR-1) [162]. Hepcidin is a peptide hormone responsible for degradation and inhibition of FPN-1 [163]. FPN-1is the only known iron exporter, responsible for iron release by cells [164]. TF binds serum iron and transports it to target cells expressing Transferrin Receptor 1 (TFR-1), which is an iron transporter responsible for its internalization [165]. Among these proteins, hepcidin is considered the key protein in modulation of iron metabolism and it could be considered also as inflammatory marker [166]. Indeed, during inflammation the high levels of pro-inflammatory cytokines and, in particular, high levels of interleukin (IL)-6 are responsible for increase of hepcidin levels [221]. So, hepcidin, by binding FPN-1, induces its degradation and, consequently, inhibits iron release by cells [167]. Iron accumulation causes cell damage, inducing reactive oxygen species (ROS) production, which determines DNA damage, protein and lipid modifications, tumor microenvironment alteration and contributes to tumor onset and progression [168-170] (Figure 2).

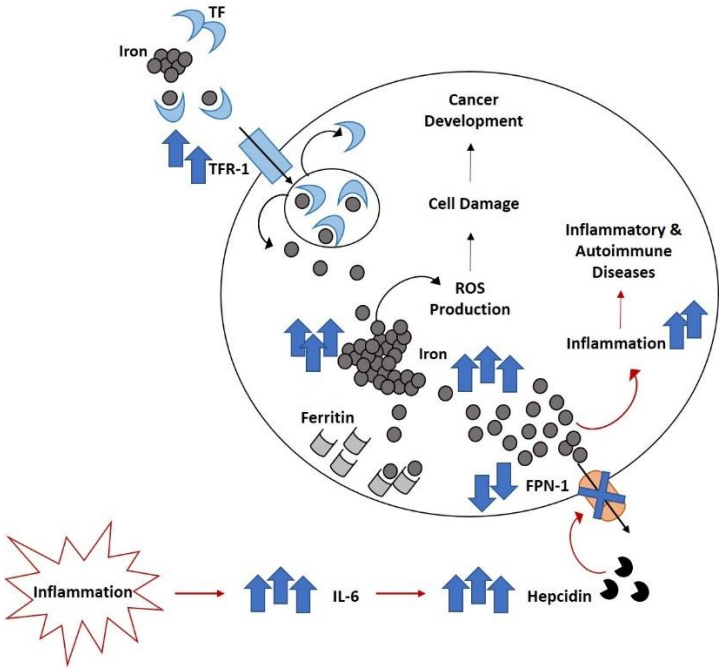

Figure 2 – Iron metabolism alteration. Inflammation induces an increase of IL-6 levels, which is responsible for FPN-1 degradation and, consequently, for intracellular iron concentration increase. TFR-

1 over expression and increased levels of ferritin also contribute to iron accumulation, which induced cell damage, by inducing ROS production and contributing to inflammatory state. TF: Transferrin; TFR-1: Transferrin Receptor 1; ROS: Reactive Oxygen Species; FPN-1: Ferroportin 1; IL-6: Interleukin-6 (Di Paola A, Tortora C, Argenziano M, Di Leva C and Rossi F. Iron Metabolism: From Inflammation to Cancer. Ann Hematol Oncol. 2021; 8(6): 1351. http://doi.org/10.26420/annhematoloncol.2021.1351).

Furthermore, iron has a key role also in immune response by modulating macrophages function [221]. It is known that in chronic inflammation and autoimmune diseases iron retention by macrophages has an important pathogenetic role [171-173]. Indeed, macrophages intracellular iron could influence their inflammatory response. M1 and M2 macrophages present a different iron turnover. In particular, M1 phenotype is characterised by a reduced expression of FPN-1 and increased levels of ferritin, so they are more predisposed to iron accumulation. Contrarily, M2 macrophage are responsible for metabolizing and exporting iron, presenting lower intracellular iron concentrations [174]. Iron levels could influence macrophage polarization [175]. The presence of low intracellular iron levels in macrophages is responsible for inhibiting pro-inflammatory cytokines expression, while when these levels are high it is possible to observe an increase of pro-inflammatory cytokines production [176-178]. In case of chronic inflammation, macrophages are more predisposed to the internalization of iron through erythrophagocytosis and the the iron transporter DMT1. Moreover, inflammation also causes a reduction of FPN-1 expression both directly, and indirectly by increasing levels of hepcidin, thus determining a reduction of iron release by cells and, consequently, an increase of intracellular iron concentration [179]. In autoimmune diseases and in case of chronic inflammation, iron accumulation in macrophages seems to be involved in pathogenesis. It has been reported that increased intracellular iron induces a phenotype switch of macrophages toward a pro-inflammatory phenotype in the injured spinal cord [180]. Also in ITP, it is reported a prevalence of M1pro-inflammatory macrophages, which are involved in an increased release of pro-inflammatory cytokines [49, 91].

Conversely, iron deprivation in human macrophages induces anti-inflammatory responses; indeed, it has been demonstrated that iron deprivation in human macrophages, stimulated by Lipopolysaccharide (LPS), reduced both levels of the proinflammatory cytokines IL-1β and tumor necrosis factor (TNF-α), suggesting that iron chelation could prevent LPS polarization toward M1 pro-inflammatory phenotype [181].

Considering the important involvement of iron and of all proteins responsible for its metabolism regulation in inflammation and considering the altering inflammatory state in ITP, it could be useful to use it as innovative therapeutic target to counteract inflammation and alteration of immune system in ITP patients.

1.8The Endocannabinoid System

The endocannabinoid (EC) system is an endogenous signalling system composed by [182-184]:

- endogenous cannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are synthetized "on demand" and, once they have fulfilled their function, they are hydrolysed;
- their selective receptors, cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2);
- the enzymes involved in their synthesis and degradation: N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and Fatty acid amide hydrolase (FAAH) for AEA; Diacylglycerol lipase (DAGL) and Monoacylglycerol lipase (MAGL) for 2-AG.

CB1 and CB2 receptors are two 7-transmembrane-domain and G protein-coupled receptors (GPCR), which are differently localised (202) (Figure 3).

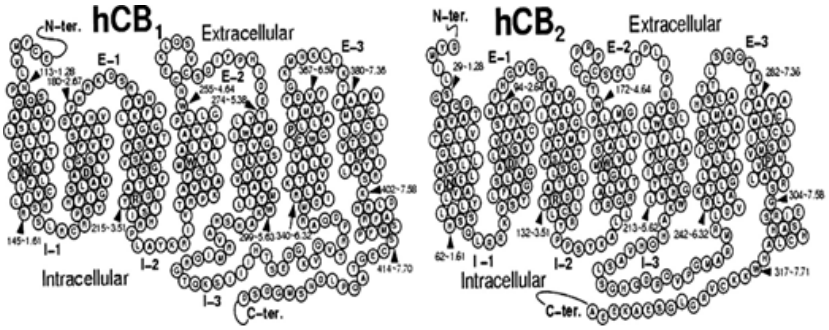

Figure 3 – Structure of CB1 and CB2 receptors. CB1 and CB2 receptors are two 7-transmembrane-domain and G protein-coupled receptors (GPCR), which are differently localised (Reggio PH. Endocannabinoid binding to the cannabinoid receptors: what is known and what remains unknown. Curr Med Chem. 2010;17(14):1468-86. doi: 10.2174/092986710790980005. PMID: 20166921; PMCID: PMC4120766).

The CB1 receptor is principally expressed in the central nervous system (CNS), and, more in particular, on spinal cord and dorsal root ganglia, and on brain presynaptic neurons [185, 186]. The CB2 receptor is, instead, mainly localized in periphery, in particular on immune cells, such as macrophages, B cells, NK, MSCs, and on lymphatic organs, thymus, spleen, and thymus [182, 187].

The EC system takes part in several biological processes, such as organism development, pain management, and regulation of appetite is involved in many biological functions: regulation of appetite, pain management, and also organism development since the earliest stage of gestation [188].

The EC system play an important role in modulating both inflammatory and immune response [182]; this is an important property of EC system because through its modulation it is possible to manage different diseases [182] (Figure 1). Obviously, considering the different localization of CB1 and CB2 receptors, their modulation led to different effects. Indeed, while CB1 exerts its function in CNS, by acting as anti-inflammatory mediator and by modulating the release of neurotransmitter at axonic terminals [189], CB2 act in the peripheral regions, by regulating immune response [182].

It has been demonstrated that a variant of CB2 encoding gene (*rs35761398*), which determines the production of a less functional receptor variant, CB2 Q63R, is closely associated to autoimmune disorders [190]. Indeed, RR homozygote subjects are more prone to the onset of autoimmune diseases compared with QQ homozygote subjects [190].

The EC system is able to inhibit immune cell proliferation and pro-inflammatory mediator release, such as chemokines, cytokines, ROS [191]. In particular, cytokines are compounds responsible for positively or negatively influence inflammatory state and they are released by macrophages in the earliest stage of innate immune response and by T-cells during the adaptive immune response [192]. In autoimmune diseases the immune tolerance is compromised, and there is an alteration of inflammatory response, with a prevalence of pro-inflammatory state which is responsible for tissue damage [193]. Several authors highlighted the important role of cannabinoids in regulate cytokines release; in particular, AEA reduces the inflammation by decreasing the release of IL-6 and nitric oxide by lipopolysaccharide (LPS)-activated macrophages in vitro [194]. Moreover, also the partial agonist of CB1 and CB2, tetra-hydro-cannabinol (THC), determines the inhibition of IL-12 and interferon gamma (IFN-γ) release by Th1 cells, thus inducing anti-inflammatory and immunosuppressive effects [195]. Definitely, all the drugs which act on EC system exert immune-suppressive effects, by inducing a reduction of T and B cells proliferation, an inhibition of antibodies production by B cells, a decrease of cytokines and chemokines release by NK cells, a reduction of macrophages migration, phagocytosis and pro-inflammatory cytokines release, and finally an improvement of MSCs immune-suppressive and anti-inflammatory properties [79, 80, 196-200].

1.9 The Endocannabinoid System in ITP

Considering the well-known immunomodulating properties of CB2 and the correlation between its non-functional variant Q63R and several autoimmune diseases, Rossi and collaborators in 2011 for the first time decided to investigate the presence of this variant also in ITP [201]. In particular, they genotyped 190 ITP Italian children for the CNR2 rs35761398 variant [201]. Successfully, the same study was conducted also in an Egyptian ITP child population [202]. These studies revealed a strong correlation between Q63R polymorphism and the susceptibility to childhood ITP. This disease is characterised by an alteration of T cells function and by an increased Th1/Th2 ratio which results in an increase release of pro-inflammatory cytokines [182, 201, 202]. CB1 and CB2 receptors are involved in modulation of IL-10 production; in particular, it has been demonstrated that their selective stimulation promotes an increase in the levels of this cytokine release [203, 204], thus confirming the EC system anti-inflammatory property [182].

In ITP the immunosuppressive and anti-inflammatory properties of MSCs are strongly improved. These cells are involved in inhibition of T and B cells proliferation [205], but in ITP patients MSCs lose this property together with their proliferation capability [82, 206]. Interestingly, CB2 receptor is more frequently expressed in healthy MSCs than in MSCs obtained from ITP pediatric patients [69]. Moreover, it has been demonstrated that AEA and 2-AG are able to regulate platelets function and survival. In particular, AEA is responsible for inhibiting platelets apoptosis [202], and 2-AG acts as megakaryopoietic agent [207]. Another important effect of 2-AG is its capability to stimulate the maturation and promote proliferation of a human megakaryocytic cell line [208].

Considering the involvement of EC system in immune and inflammatory responses and, in particular, its involvement also in ITP, it could be considered a possible noteworthy therapeutic target to manage ITP.

2. AIM OF THE STUDY

In these three years of my PhD program, I had the opportunity to carry on the project on the identification of novel and innovative therapeutic targets for ITP with the research group directed by Professor Francesca Rossi. The research activity was conducted in the Laboratory of Molecular and Cellular Biology of the Experimental Medicine Department of the University of Campania "*Luigi Vanvitelli*".

ITP is an autoimmune disease caused by autoantibodies-mediated platelet destruction and it represents the more frequent piastrinopenia in childhood, characterised by isolated thrombocytopenia. It is characterized by an alteration of immune and inflammatory responses; in particular, the increase of Th1/Th2 and of M1/M2 ratios induces an impairment of immune and inflammatory responses, with a consequent alteration of cytokines release. Interestingly, macrophages in ITP act as effector cells, phagocytizing platelets, and as antigen presenting cells, stimulating auto-antibodies against platelets production by B cells.

Recently, it has been demonstrated that also MSCs are involved in ITP pathogenesis through their immune modulatory functions. Indeed, MSCs are able to inhibit B and T cells proliferation and activation. The immune modulating ability of these cells are strongly impaired in ITP patients, indeed ITP MSCs are characterized by a high rate of apoptosis and a reduced ability to inhibit T-cell proliferation.

Corticosteroids represent the main first-line therapy for ITP management, among them HD-Dexa is the most commonly used. It is able to induce an increase of anti-inflammatory M2 macrophages which suppress autoimmunity, but also increased the Th1/Th2 ratio. Unfortunately, HD-Dexa has long term side effects. Therefore, the research of innovative strategies to manage ITP could be useful.

It has been reported that glucocorticoids are able to modulate the immunosuppressive properties of MSCs. Moreover, also CB2 receptor has a key role in regulating MSCs properties. CB2 receptor has an important role in immune modulation; indeed, it inhibits the activation of immune cells and reduces the cytokines production. Furthermore, the presence of CB2 functional variant has been observed in several inflammatory/immune diseases, and also in childhood ITP.

Considering these evidences, during the first year of my PhD program, we evaluated whether JWH-133, a CB2 receptor selective agonist, and Dexa are able to restore the immunomodulatory and anti-inflammatory properties of MSCs in ITP patients in order to suggest new therapeutic approaches and to reduce Dexa concentration and, consequently, its side effects.

ELT is known to be a drug approved as second-line therapy in childhood chronic ITP in order to stimulate platelet production. It is also an immune modulating drug, able to improve Treg and Breg functions, determining a reduction of Th1 and B cells activity, and, consequently, a modulation of inflammatory and immune responses. Recently, it has been demonstrated that ELT also exerts iron chelating properties, by binding intracellular iron (III). Iron is an important element for mammal cells, involved in several physiologic biological processes. The alteration of its metabolism and, in particular, its intracellular accumulation could be responsible for cellular damage. It seems that high iron intracellular levels are closely related to the inflammatory state; in particular, inflammation induces an increased release of IL-6, which is responsible for the production of high levels of hepcidin. Hepcidin is involved in FPN-1 degradation and, consequently, it determines iron accumulation. Therefore, iron could be used as a novel therapeutic target in several iron-overload and inflammatory diseases.

Since in ITP there is a prevalence of pro-inflammatory M1 macrophage phenotype, and considering that ELT exerts immune modulating and iron

chelating properties, during second year we investigate a new possible mechanism of action of ELT in regulating macrophage phenotype polarization. In particular, we evaluated ELT capability to induce macrophage phenotype switch from the M1 pro-inflammatory phenotype to the M2 anti-inflammatory type in ITP pediatric patients, in order to reduce inflammatory state and restore the immune system function.

Finally, during the third year, we decided to investigate ELT effects also on MSCs. In particular, since in ITP the impairment of inflammatory state could led to an increase of intracellular iron concentration also in MSCs with a consequent compromission of their survival and activities, we evaluated ELT capability to restore MSCs viability, survival and immune-modulating properties in ITP patients.

3. MATERIALS AND METHODS

3.1 Source of MSCs

During the first year, the study was performed using MSCs isolated from the bone marrow (BM) of two newly diagnosed ITP children (median age 7 ± 2 years) and two healthy donors (median age 7 ± 2 years). ITP patients were enrolled in the Department of Women, Child and General and Specialized Surgery of University of Campania "Luigi Vanvitelli" and were free of any glucocorticosteroids therapy for at least 3 months. Healthy donors were enrolled in Hematology Department of Bambino Gesù Hospital.

During the last year, MSCs were obtained from the BM of ten newly diagnosed ITP children (median age 6 ± 2 years) and ten healthy donors (median age 6 ± 2 years). ITP patients and healthy donors were enrolled in the Department of Women, Child and General and Specialized Surgery of University of Campania "Luigi Vanvitelli".

All procedures performed were in accordance with the Helsinki Declaration of Principles, the Italian National Legislation, and the Ethics Committee of the University of Campania "Luigi Vanvitelli", which formally approved the study. Written informed consent was obtained from parents, and assent was acquired from children before any procedures.

3.2 Mesenchymal Stromal Cells Cultures

MSCs were obtained from mononuclear cells, isolated from bone marrow by density gradient centrifugation (Ficoll 1.077 g/mL). Mononuclear cells were diluted in complete culture medium consisted of a low-glucose Dulbecco's Modified Eagle Medium (LG-DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mM L-glutamine, and plated in non-coated T25

polystyrene culture flasks. Non-adherent cells were discarded after 48 h, while adherent cells were cultured at 37 °C in a humidified atmosphere with 5% CO2. The culture medium was replaced twice a week until 80% confluence was reached; then MSCs were split, re-plated for expansion and harvested until the sixth passage (P0–P6). MSCs were characterized by flow cytometry using monoclonal antibodies, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), specific for these antigens: HLA DR, HLA A-B-C, CD45, CD34, CD13, CD14, CD31, CD80, CD90, CD73, and CD105. Labeled cells were acquired using a FACSCanto flow cytometer, and data were elaborated using FACSDiva software.

For the first step of the study, MSCs mRNA and proteins were isolated from each passage to analyse CB2 expression and density.

While, for the study conducted during the third year of PhD, MSCs supernatants and proteins were isolated from each passage. In particular, supernatants derived from CTR MSCs and ITP MSCs at P6 were collected to analyze IL-4, IL-10, IFN-γ and IL-6 release with an enzyme-linked immunosorbent assay (ELISA) and to perform Iron Assay. Furthermore, ITP MSCs were treated with ELT [6 μM] at P6. After 48h of ELT incubation, cells were harvested for protein extraction, and cell cultures supernatants were collected to analyze IL-4, IL-10, IFN-γ and IL-6 release with several ELISA and to perform Iron Assay.

3.3 MSCs and T Cells Co-Culture

Peripheral blood mononucleated cells (PBMCs) were isolated from peripheral blood obtained from two healthy subjects (median age 7 ± 2 years) using Ficoll density gradient centrifugation, and were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin with or without mitogen-induced stimulation by phytohemagglutinin (PHA).

Activated lymphocytes (1.25 ± 10^5 cells/well) were then co-cultured with MSCs (2.5 ± 10^4 cells/well) on a 24-well plate at a ratio of 5:1 in the RPMI medium, and treated with LPS [500 ng/mL] to mimic inflammatory condition. After 3 days, JWH-133 [2.5 μM] and Dexa [100 nM], alone or in combination, were added to the plates for 24 h. The supernatants of co-cultures were collected to analyse TNF-α release with an ELISA, while T-cells were used to perform the cell viability assay.

3.4 Source of Macrophages

Macrophages were obtained from the peripheral blood of ten ITP children (median age 6 ± 2 years) and ten healthy donors (median age 6 ± 2 years). ITP patients were enrolled in the Department of Women, Child and General and Specialized Surgery of University of Campania "Luigi Vanvitelli" and in Hematology Department of Bambino Gesù Hospital. They were free from any therapy for at least 15 days. Healthy donors were enrolled in Hematology Department of Bambino Gesù Hospital.

3.5 Macrophages Cultures

Macrophages were obtained from PBMCs, isolated by density gradient centrifugation (Ficoll 1.077 g/mL), diluted at $1X10^6$ cells/mL in α-Minimal Essential Medium (α-MEM) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 g/mL streptomycin and L-glutamine, and plated in 24-well Cell Culture Multiwell. In order to obtain fully differentiated human macrophages, the PBMCs were cultured for 15 days in presence of 25 ng/mL recombinant human macrophage colony-stimulating factor (rh-MCSF). Culture medium was replaced twice a week. Cells were cultured at 37°C in a humidified atmosphere with 5% CO2. Macrophages derived from ITP patients were treated with ELT [6 μM] from second medium change until the last one. After 15 days of differentiation, cells were harvested for

protein extraction, and cell cultures supernatants were collected to perform Iron Assay and to analyze IL-4, IL-10, IL-6, TNF-α, and IFN-γ release with ELISA.

3.6 Drugs and Treatments

Lypopolysaccharide (LPS), JWH-133, Dexamethasone (Dexa), and AM630 were dissolved in PBS containing dimethyl sulfoxide (DMSO). DMSO final concentration on cultures was 0.01%. Non-treated cultured cells were maintained in incubation media during the relative treatment time with or without vehicle.

CTR-MSCs, ITP-MSCs, and ITP-MSC–T-cell co-cultures were treated with LPS [500 ng/mL], JWH-133 [2.5 μM], Dexa [100 nM], and AM630 [1 μM] alone or in combination for 24 h. AM630 was applied for 15 min before JWH-133 treatment.

Eltrombopag (ELT) was dissolved in sterile water at a concentration of [10 mM]. Cells were treated with ELT at the final concentration of [6 μM]. The concentrations of ELT were determined following a pilot Dose-Response experiment. Non-treated cultured cells were maintained in incubation media during the relative treatment time with and without vehicle (sterile water). Macrophages obtained from healthy donor were treated with LPS at the final concentration of [500 ng/mL)]. DMSO final concentration on cultures was 0.01%. Non-treated cultured cells were maintained in incubation media during the relative treatment time with or without vehicle (DMSO 0.01%).

3.7 Total RNA Extraction and Reverse Transcription Quantitative Polymerase Chain Reaction (RTqPCR)

Total RNA from MSC cultures was extracted using Qiazol® (Qiagen, Hilden, Germany). mRNA extraction consists in four steps:

- separation;

- precipitation;

- washing;

- solubilization.

Successfully, the first-strand cDNA was obtained from approximately 1000 ng of mRNA, after a reaction of reverse transcription. The transcript levels of CB2 were detected by RT-qPCR using a CFX96 Real-Time PCR system and using I-Taq Universal SYBR® Green Master Mix (Bio-Rad). The cycling conditions were 10 min at 95 °C (for the initial denaturation), followed by 40 cycles of 15 s at 94 °C (for the denaturation), and 1 min at 68 °C (for the annealing/extension/data collection). The β-actin gene was used as the reference gene for the normalization of the real-time PCR products. The PCR primers used to detect each gene were designed using the Primer 3 program and synthesized by Sigma Aldrich, and are reported in table 1:

Gene	Forward primer	Reverse primer
CB2	5'-AAGGCTGTCTTCCTGCTGAA-3'	5'- CACAGAGGCTGTGAAGGTCA-3'
β-ACT	5'-GCGAGAAGATGACCCAGATC-3'	5'-GGA TAGCACAGCCTGGATAG-3'

Table 1- Human oligonucleotides (forward and reverse) used for RTqPCR, designed using Primer 3 program.

We performed the assays in technical duplicate for each subject and tested the linearity and efficiency of the experiments over dilutions of cDNA including five orders of greatness. To confirm the specificity of the reactions, we performed the dissociation curve analysis of amplification products. To analyse the data and achieve the relative gene expression levels we used the 2^{-DDCt} method.

3.8 Protein Isolation; Western Blot

Proteins were extracted using a radio-immunoprecipitation assay (RIPA) lysis buffer. During the first phase of this project, CB2 and Bcl2 proteins were characterized in total lysates from MSCs cell cultures by Western blotting. Membranes were incubated overnight at 4 °C with a rabbit polyclonal anti-CB2 antibody (1:500 dilution, abcam) and mouse monoclonal anti-Bcl-2 (1:200 dilution, Santa Cruz). A mouse polyclonal anti-β-Tubulin antibody (1:5000 dilution, Elascience) was used to check for comparable protein loading and as a housekeeping protein. We performed single experiments on each one of the two ITP patients' samples (displayed as mean ±SD).

Successively, during second year of PhD experience, iNOS, CD206, IL-6, and DMT1, proteins were detected in total lysates from treated and non-treated macrophages cultures by Western Blotting. Membranes were incubated overnight at 4 °C with these antibodies: rabbit polyclonal anti-iNOS (1:2000 dilution; Invitrogen), mouse monoclonal anti-CD206 (1:200 dilution; Invitrogen), rabbit polyclonal anti-IL-6 antibody (1:500 dilution; abcam), and mouse monoclonal anti-DMT1 antibody (1:100 dilution; Santa Cruz). A mouse monoclonal anti-β-Tubulin antibody (1:5000, Elabscience) was used to check for comparable protein loading and as a housekeeping protein.

Finally, during last year, TFR-1, FPN-1, Bcl-2, and pERK proteins were detected in total lysates from MSCs cultures by Western Blotting. Membranes were incubated overnight at 4 °C with these antibodies: rabbit monoclonal anti-TFR-1 (1:1000 dilution; abcam), rabbit polyclonal anti-FPN-1 (1:1000 dilution), mouse monoclonal anti-Bcl-2 antibody (1:50 dilution; Santa Cruz), and rabbit polyclonal anti pERK antibody (1:1000 dilution; Bethyl Laboratories). A mouse monoclonal anti-β-Actin antibody (1:100, Santa Cruz) was used as a housekeeping protein.

Reactive bands were detected by chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate) on a C-DiGit® Blot scanner (LI-COR Biosciences). Images were captured, stored, and analyzed using Image Studio Digits software, version 5.0.

3.9 ELISA

The concentration of several molecules of interest were detected through the enzyme linked immunosorbent assay (ELISA).

Firstly, IL-6, TNF-α, and IL-4 levels were measured in supernatant obtained from MSC culture as well as MSC–T-cell co-cultures, using a commercially available Human Inflammatory Cytokines Multi-Analyte ELISArray Kit.

Then, several ELISA Assays were performed in order to also determine IL-6, IL-4, IL-10, TNF-α, and IFN-γ concentration in macrophages culture supernatants.

Finally, in the last phase of our study, IL-4, IL-10, IFN-γ, IL-6 and Hepcidin levels were measured in supernatants obtained from MSCs cultures pre- and after treatment with ELT.

Briefly, a microplate was coated with monoclonal antibodies that were specific to the compounds of interest. Standards and supernatants were pipetted into the wells of the microplate. A positive control was obtained by pipetting only the standard into the wells. A negative control was obtained by pipetting the standard and cell culture supernatants into non-coated wells. After the plate was washed, enzyme-linked polyclonal antibodies specific for IL-6, TNF-α, IL-4, IL-10, IFN-γ, and hepcidin were added to the wells. The reaction was revealed by the addition of the substrate solution. The optical density was measured at a wavelength of 450 nm by using the Tecan Infinite M200 spectrophotometer. Cytokines concentrations (pg/mL) were

determined against a standard concentration curve. All samples were run in duplicate.

3.10 Annexin V & Dead Cell Assay

Apoptosis in MSCs, treated with JWH-133 and Dexa, was evaluated by a cytofluorometric assay with the Muse cell analyzer machine by using the *"Annexin V & Dead Cell Assay Kit"*. Test was performed after 24 h of compound exposure. Drugs were added alone or in combination at the following concentrations: JWH-133 (2.5 µM) and Dexa (100 nM). The Muse Annexin V & Dead Cell Assay utilizes Annexin V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. A dead cell marker, 7-amino-actinomycin D (7-AAD), is also used as an indicator of cell membrane structural integrity.

Briefly, 100 µL of a cell suspension (1 X 10^5 cells/mL) was mixed with 100 µL of the *Muse Annexin V & Dead Cell Reagent* and incubated for 20 min at room temperature in the dark. The results, automatically displayed, were analyzed with Muse 1.4 analysis software for data acquisition and analysis.

3.11 Count and Viability Assay

To perform the count and viability assay, we isolated the T-cells from the co-culture media. MSCs grow in adhesion, so they remained in the plate. T-cell count and viability were evaluated after 24 h of treatments exposure with the Muse cell analyzer machine with *"Count & Viability Assay Kit"*.

After 48h of ELT [2 µM], [6 µM] and [10 µM] treatment, we perform another count and viability assay on MSCs in order to evaluate drug concentration effects and to establish the best drug concentration. Briefly, 450 µL of the Muse Count & Viability reagent was added to 50 µL of cells suspension (1 X 10^5 cells/mL) and incubated for 5 min at room temperature.

This reagent differentially stains viable and non-viable cells based on their permeability to the two DNA binding dyes present in the reagent. The results were analysed with Muse 1.4 analysis software for data acquisition and analysis.

3.12 Iron Assay

After 15 days of differentiation, macrophages culture supernatants were collected to measure iron (III) concentration, as well as MSCs supernatants, both in CTR and in ITP, pre and after treatment with ELT. The assay was performed by using the Iron Assay Kit (Abcam). Standards and macrophages supernatant was pipetted into the wells and was incubated with an acidic buffer to allow iron release. Then, an iron probe at room temperature for 60 min was added, protected from light. Released iron reacted with the chromogen resulting in a colorimetric (593 nm) product, proportional to the iron concentration. The optical density was measured at a wavelength of 593 nm by using the Tecan Infinite M200 spectrophotometer. Iron (II) and Total Iron (II+III) contents of the test samples (nmol/µL) were determined against a standard concentration curve. Iron (III) content can be calculated as: Iron (III) = Total Iron (II+III) - Iron (II)

3.13 Statistical Analysis

All the experiments were run in duplicate or in triplicate. Statistical analyses on molecular, biochemical, and cellular data were performed using the non-parametric Wilcoxon test, and the Student's t test to evaluate differences between quantitative variables. Data are expressed as mean ± standard deviation (SD). A p value ≤ 0.05 (* or ^) was considered statistically significant.

4. RESULTS

4.1 Expression of CB2 Receptor in MSCs Derived from ITP Patients

We performed a real-time PCR and a Western blot (WB) to evaluate CB2 receptor mRNA and protein expression levels in MSCs isolated from the bone marrow (BM) of ITP patients, at different stages of MSCs growth, from Passage 2 to Passage 8 (P2 to P8) (Figure 4, Table 2A, B).

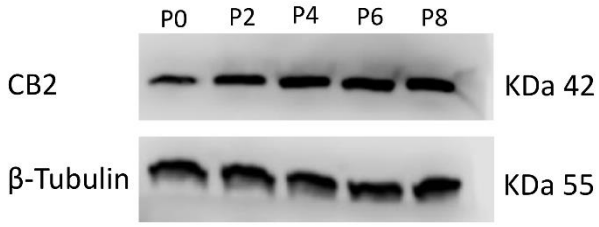

Figure 4 – CB2 protein density from passage 2 to passage 8. CB2 protein density was determined by Western blotting, starting from 15 µg of total lysates. The most representative image is displayed. The proteins were detected using Image Studio Digits software.

(A)	**CB2 Relative Quantification ($2^{-\Delta\Delta Ct}$)**				
	Passages				
Samples	P0	P2	P4	P6	P8
MSC ITP-1	1	2,3*	2,5*	4,8*	6,0*
MSC ITP-2	1	3,0*	1,9*	4,6*	5,4*
(B)	**CB2 Protein signal density**				
	Passages				
Samples	P0	P2	P4	P6	P8
MSC ITP-1	1	1,72*	2,10*	2,26*	2,90*
MSC ITP-2	1	1,80*	2,11*	2,29*	2,44*

Table 2 - CB2 receptor expression levels in MSCs from ITP patients. CB2 mRNA expression (Table 2A) and protein density (Table 2B) in MSCs from two ITP patients at the P0, P2, P4, P6, and P8 passages. mRNA levels were determined by qPCR. Results were normalized for the housekeeping gene β-actin and are shown in Table 2A. The intensity ratios of immunoblots compared to the P0 passage, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-tubulin and are shown in Table 2B. A Wilcoxon test was used to evaluate the statistical differences in mRNA expression and protein density. * $p \leq 0.05$ compared to the P0 passage.

We observed the highest expression of CB2 mRNA and protein density from P6, similarly to what we have previously demonstrated in MSCs isolated from the BM of healthy subjects (MSC-CTR) (75). Therefore, we compared the CB2 expression of ITP-MSCs with CTR-MSCs at P6 (Figure 5, Table 3A, B). Molecular and biochemical analysis showed that, in ITP-

MSCs, CB2 was significantly lower than CTR-MSCs, suggesting that a reduction of CB2 expression could be related to ITP-MSCs impaired function.

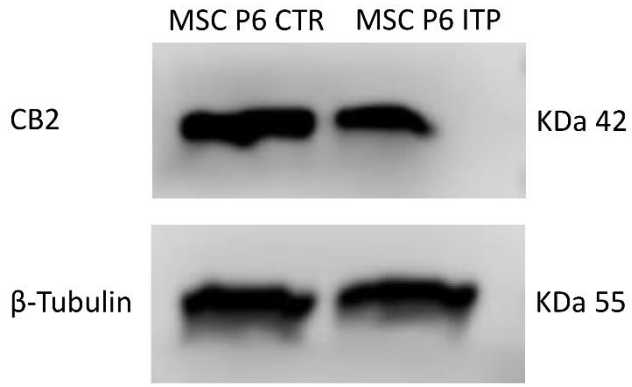

Figure 5 – CB2 Protein density in MSC P6 ITP compared to MSC P6 CTR. CB2 protein density was determined by Western blotting, starting from15 µg of total lysates. The most representative image is displayed. The proteins were detected using Image Studio Digits software.

(A)	
Samples	CB2 Relative Quantification ($2^{-\Delta\Delta Ct}$)
MSC P6 CTR-1	1
MSC P6 CTR-2	1
MSC P6 ITP-1	0,54*
MSC P6 ITP-2	0,62*
(B)	
Samples	CB2 Protein signal density
MSC P6 CTR-1	1
MSC P6 CTR-2	1
MSC P6 ITP-1	0,53*
MSC P6 ITP-2	0,49*

Table 3 - CB2 mRNA expression (Table 3A) and protein density (Table 3B) in MSCs from two ITP patients at the P6 passage compared with levels in MSCs from two healthy donors at the same passage. mRNA levels were determined by qPCR. Results were normalized for the housekeeping gene β-actin and are shown in Table 3A. The intensity ratios of immunoblots compared to MSC P6 CTR, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-tubulin and are shown in Table 3B. A Wilcoxon test was used to evaluate the statistical differences in mRNA expression and protein density. * $p \leq 0.05$ compared to MSC P6 CTR.

4.2 Effects of JWH-133 and Dexa on Cytokine Release

The multi-ELISA assay (Table 4) revealed a significant increase of the pro-inflammatory cytokine interleukin 6 (IL-6) in supernatants of ITP-

MSCs compared to MSCs-CTR. At the same time, ITP-MSCs showed a significant reduction of the anti-inflammatory cytokine, interleukin 4 (IL-4).

(A) IL-6							
Samples	CTR-1	ITP-1 NT	ITP-1 JWH-133	ITP-1 DEXA	ITP-1 D+J	ITP-1 AM630	ITP-1 A+J
pg/mL	7,04	12,87*	9,02^	7,70^	11,01^	17,05^	15,41^
Samples	CTR-2	ITP-2 NT	ITP-2 JWH-133	ITP-2 DEXA	ITP-2 D+J	ITP-2 AM630	ITP-2 A+J
pg/mL	7,18	16,02*	9,83^	8,63^	9,44^	17,38^	17,52^
(B) IL-4							
Samples	CTR-1	ITP-1 NT	ITP-1 JWH-133	ITP-1 DEXA	ITP-1 D+J	ITP-1 AM630	ITP-1 A+J
pg/mL	44,61	31.53*	43,11^	45,26^	39,38^	28,89^	27,48^
Samples	CTR-2	ITP-2 NT	ITP-2 JWH-133	ITP-2 DEXA	ITP-2 D+J	ITP-2 AM630	ITP-2 A+J
pg/mL	45,82	32,68*	42,91^	45,03^	40,67^	29,41^	30,05^

Table 4 – IL-6 and IL-4 cytokines levels. IL-6 (A), and IL-4 (B) from CTR-MSCs and ITP-MSCs were investigated through a multi-ELISA assay after 24 h treatment with JWH-133 (2.5 µM), Dexa (100 nM), and AM630 (1 µM) alone and in combination. The tables show the concentrations (pg/mL) of the two cytokines. A Wilcoxon test was used for statistical analysis. $p \leq 0.05$ was considered statistically significant. * vs. CTR; ^ vs. ITP NT.

JWH-133 [2,5 µM] and Dexa [100 nM], administered alone and in combination, are able to restore IL-6 balance. In particular, their co-administration induces a significant reduction of IL-6 levels, similar to the one induced by Dexa [100 nM] alone. JWH-133 [2.5 µM] and Dexa [100 nM] determines also an increase of IL-4 that is greater when used in combination and comparable to the increases of CTR-MSCs. Moreover, to confirm the CB2 anti-inflammatory properties in ITP-MSCs, we also blocked CB2 with its reverse agonist AM630 [1 µM] that induces an increase of IL-6 and a reduction of IL-4 release, respectively, counteracting the effects induced by JWH-133.

4.3 Effects of JWH-133 and Dexa on ITP-MSCs Apoptosis

We performed a cytofluorimetric assay in order to evaluate JWH-133 [2,5 µM] and Dexa [100 nM] effects on ITP MSCs apoptosis (Table 5A), and also a WB (Table 5B and Figure 6) in order to evaluate Bcl2 protein expression. JWH-133 [2,5 µM] and Dexa [100 nM], administered alone and in combination, induced a significant reduction of total apoptotic cells percentage. Accordingly, the WB revealed an increase of Bcl2 protein density after single treatment and co-treatments.

(A)	**Percentage of Total Apoptotic ITP-MSCs**			
	Treatments			
Samples	NT	JWH-133	DEXA	J+D
MSC ITP-1	41,56	34,24*	30,53*	27,56*
MSC ITP-2	44,94	31,26*	35,27*	31,08*
(B)	**Bcl2 Protein signal density**			
	Treatments			
Samples	NT	JWH-133	DEXA	J+D
MSC ITP-1	1	7,94*	10,25*	7,85*
MSC ITP-2	1	8,48*	8,15*	6,67*

Table 5 - Apoptosis in ITP-MSCs. (Table 5A) Annexin V and PI double-stained apoptosis assay, in MSCs derived from two ITP patients after 24 h treatments with JWH-133 [2.5 µM] and dexamethasone [100 nM], alone or in combination. $p \leq 0.05$ was considered statistically significant compared to the untreated control (NT). (Table 5B) protein density of Bcl-2. The intensity ratios of immunoblots compared to MSC ITP NT, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-tubulin and are shown in Table 5B A Wilcoxon test was used to evaluate the statistical differences. $p \leq 0.05$ was considered statistically significant. * vs ITP NT.

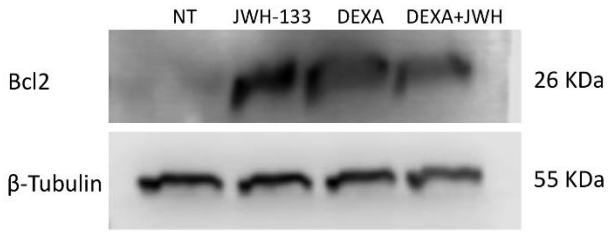

Figure 6 - Bcl2 protein density in MSCs after treatments. Bcl2 protein density in MSCs from two ITP patients was determined by Western blotting, starting from 15 µg of total lysates and after 24 h exposure to JWH-133 [2.5 µM] and Dexa [100 nM] alone and in combination. The most representative image is displayed. The proteins were detected using Image Studio Digits software. The intensity ratios of immunoblots compared to NT, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-tubulin and are shown in Table 5B.

Moreover, to confirm CB2 direct involvement in survival of ITP-MSCs, we performed an apoptotic assay also after CB2 blockage with AM630 [1 µM]. As expected, the inverse agonist induced an increase of the total apoptotic cells percentage compared to the untreated cells and counteracted the positive effects on cellular survival induced by JWH-133 [2.5 µM] in ITP-MSCs (Table 6).

	Percentage of total apoptotic ITP-MSCs			
	Treatments			
Samples	**NT**	**JWH-133**	**DEXA**	**J+D**
MSC ITP-1	45,88	30,59*	52,01*	55,36*
MSC ITP-2	46,22	31,70*	53,60*	55,90*

Table 6 – Percentage of total apoptotic ITP-MSCs. Annexin-V and PI double-stained Apoptosis Assay, in MSCs derived from 2 ITP patients after 24h treatments with JWH-133 [2,5 µM] and AM630 [1 µM], alone and in combination. The table shows the percentage of total apoptotic cells. A Wilcoxon test has been used for statistical analysis. $p \leq 0.05$ has been considered statistically significant. * vs NT (NT).

4.4Effects of JWH-133 and Dexa on ITP-MSCs' Immunosuppression Capacity

To evaluate JWH-133 [2.5 µM] and Dexa [100 nM] effects on immunosuppressive capacity of ITP-MSCs, we co-cultured MSCs from ITP patients with T-cells isolated from healthy subjects. As shown in Table 7, ITP-MSCs were unable to counteract T-cell proliferation. Interestingly, ITP-MSCs reduced T-lymphocyte number in a significantly manner after JWH-133 [2.5 µM] and Dexa [100 nM] administration, alone and in combination.

T Cell Viability					
Sample1	**T cells**	**T cells + MSC**	**T cells + MSC JWH-133**	**T cells + MSC DEXA**	**T cells + MSC J+D**
(Number of cells x 10^6)	7,04	12,87*	9,02^	7,70^	11,01^
Sample 2	**T cells**	**T cells + MSC**	**T cells + MSC JWH-133**	**T cells + MSC DEXA**	**T cells + MSC J+D**
(Number of cells x 10^6)	7,18	16,02*	9,83^	8,63^	9,44^

Table 7 - T-cell viability. The viability of T-cells co-cultured with ITP-MSCswas estimated by a cytofluorimetric assay after 24 h treatment with JWH [2.5 µM] and Dexa [100 nM], alone and in combination. The table shows the results, as cell number x 10^6. A Wilcoxon test was used for statistical analysis. $p \leq 0.05$ was considered statistically significant. * vs. T-cells + MSC.

The CB2 blockage with AM630 [1 µM] significantly counteracted the JWH-133 [2.5 µM] effects, once again confirming the important role of CB2 as a mediator of MSCs immunosuppressive properties (Table 8).

T Cell Viability					
Sample1	**T cells**	**T cells + MSC**	**T cells + MSC JWH-133**	**T cells + MSC AM630**	**T cells + MSC J+A**
(Number of cells x 10^6)	5,86	6,26	4,36*	6,61	6,62
Sample 2	**T cells**	**T cells + MSC**	**T cells + MSC JWH-133**	**T cells + MSC AM630**	**T cells + MSC J+A**
(Number of cells x 10^6)	5,74	6,18	4,43*	6,58	6,38

Table 8 – T-cell viability after AM630 treatment. Viability of T cells co-cultured with ITP-MSCs estimated by a cytofluorimetric assay after 24h treatment with JWH-133 [2,5 µM] and AM630 [1 µM], alone and in combination. The table shows the results, as cell number per 106. A Wilcoxon test has been used for statistical analysis. $p \leq 0.05$ has been considered statistically significant. * vs T cells+MSC.

We also evaluated the levels of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) (Table 9), in supernatants of T-cells, alone and co-cultured with ITP-MSCs, stimulated or not with lipopolysaccharide (LPS) [500 ng/mL]. We did not reveal any differences in TNF-α levels between T-cell ITP-MSCs co-culture and T-cells alone, indicating that the MSCs from ITP patients had lost their capacity to inhibit TNF-α release by T-cells. In T-cells-ITP-MSCs co-culture treated with LPS [500 ng/mL], we observed high levels of TNF-α after the inflammatory stimulus with LPS [500 ng/mL] compared to untreated co-culture. Both JWH-133 [2.5 µM] and Dexa [100 nM] were able to restore the inhibitory properties of ITP-MSCs, reducing TNF-α release and showing a strong synergic effect when used in combination.

TNF-α							
Sample1	**T cells**	**T cells + MSC**	**T cells + LPS**	**T cells + MSC LPS**	**T cells + MSC L + JWH-133**	**T cells + MSC L + DEXA**	**T cells + MSC L + J + D**
pg/mL	44,61	31,53*	43,11^	45,26*	39,38°	28,89°	27,48°
Sample2	**T cells**	**T cells + MSC**	**T cells + LPS**	**T cells + MSC LPS**	**T cells + MSC L + JWH-133**	**T cells + MSC L + DEXA**	**T cells + MSC L + J + D**
pg/mL	45,82	32,68*	42,91^	45,03^	40,67°	29,41°	30,05°

Table 9 - TNF-α release quantification. The release of the pro-inflammatory TNF-α by T-cells alone and co-cultured with ITP-MSCs was investigated by ELISA assay after 24 h treatment with JWH [2.5 μM] and Dexa [100 nM], alone and in combination. LPS [500 ng/mL] was administrated to each sample before treatments. The table shows the concentrations (pg/mL) of TNF-α. A Wilcoxon test was used for statistical analysis. $p \leq 0.05$ was considered statistically significant. * vs. T-cells; ^ vs. T-cells + MSC; ° vs. T-cells + MSC LPS.

Moreover, to understand which of the two cell types released TNF-α, we compared their release in a basal condition and after LPS stimulation, and, as shown in Table 10, MSCs released a negligible amount of TNF-α, with respect to T-cells, after inflammatory stimulus.

TNF-α				
Sample1	**MSC-ITP NT**	**MSC+LPS**	**T cells**	**T cells+LPS**
pg/mL	0,51	0,99*	5,88*	7,62*
Sample 2	**T cells**	**T cells+MSC**	**T cells+LPS**	**T cells+MSC LPS**
pg/mL	0,39	1,08*	5,67*	7,86*

Tabella 10 - TNF-α levels. TNF-α release by ITP-MSCs and T cells investigated by ELISA assay after 24h treatment JWH-133 [2,5 μM] and AM630 [1 μM], alone or in combination. The table shows the concentrations [pg/mL] of TNF-α. A Wilcoxon test has been used for statistical analysis. $p \leq 0.05$ has been considered statistically significant. * vs MSC.

4.5 Effect of Eltrombopag on iNOS and CD206 Proteins Expression in macrophages

We perform a Western Blot in order to evaluate protein expression levels of the M1 macrophage polarization marker, iNOS, and of the M2 macrophage polarization marker, CD206. Firstly, we compared iNOS and CD206 expression levels in macrophages obtained from ITP patients (ITP

NT) with macrophages obtained from healthy donors (CTR NT). We observed higher and lower levels of iNOS and CD206, respectively, in ITP NT than CTR NT, indicating that there was a prevalence of M1 macrophage phenotype in ITP patients. Then, we evaluated the ELT [6 µM] treatment effects on both macrophage polarization markers, and we observed a reduction of iNOS and an increase of CD206 expression levels, both statistically significant, in the treated samples (ITP ELT) compared to the non-treated ones (ITP NT) (Figure 7 A, B).

These results let us suppose that ELT is able to determine a phenotype switch towards the anti-inflammatory M2 macrophage type.

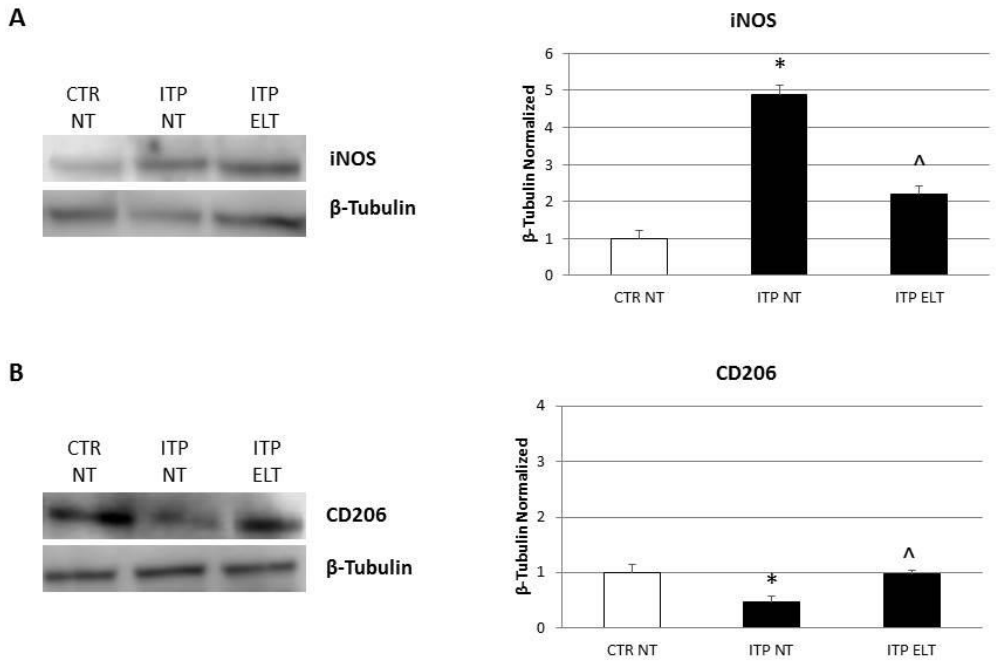

Figure 7 - Effect of Eltrombopag (ELT) on Nitric Oxide Synthase (iNOS) and Cluster of Differentiation 206 (CD206) protein expression. (A) iNOS protein expression levels in Immune Thrombocytopenia (ITP) macrophages compared with CTR (control) macrophages, determined by Western Blot, starting from 20 µg of total lysates after treatment with Eltrombopag [6 µM]. (B) CD206 protein expression levels in ITP macrophages compared with CTR macrophages, determined by Western Blot, starting from 20 µg of total lysates after treatment with ELT [6 µM]. The most representative images are displayed. The proteins were detected using Image Studio Digits software and the intensity of immunoblots compared to the untreated control, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-Tubulin. Histogram shows iNOS (A) and CD206 (B) expression levels as the mean ± S.D. of independent experiments on each individual sample. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to Non-treated Control (CTR NT), ˆ indicates $p \leq 0.05$ compared to ITP NT.

4.6 Effect of Eltrombopag on Pro-Inflammatory Cytokines Release in macrophages

We performed a Western Blot and several enzyme-linked immunosorbent assays (ELISA) in order to understand the effect of ELT [6 μM] on pro-inflammatory cytokines release.

Firstly, we evaluated protein expression levels of the pro-inflammatory cytokine IL-6 by Western Blot, and we observed a very strong increase of its expression levels in ITP NT compared to CTR NT. Accordingly, ELISA also showed a statistically significant increase of IL-6 release in ITP macrophages. ELT [6 μM] treatment induced a significant reduction of IL-6 expression levels and release (Figure 8 A, B). We confirmed these data after also stimulating CTR macrophages with LPS [500 ng/mL], indeed after the inflammatory stimulus with LPS we observed increased IL-6 levels. ELT [6 μM] administration restored the inflammatory state, reducing IL-6 levels (Figure 8 C). Subsequently, we evaluated the release of other two pro-inflammatory cytokines, TNF-α and IFN-γ. In ITP NT, we revealed an increase of their levels and we observed that ELT administration induced a statistically significant reduction (Figure 9 A, B). Moreover, after LPS [500 ng/mL] stimulation, we revealed an increase of TNF-α and IFN-γ levels in CTR NT. ELT [6 μM] treatment induced a remarkable reduction of their release, ameliorating the inflammatory state (Figure 9 C, D).

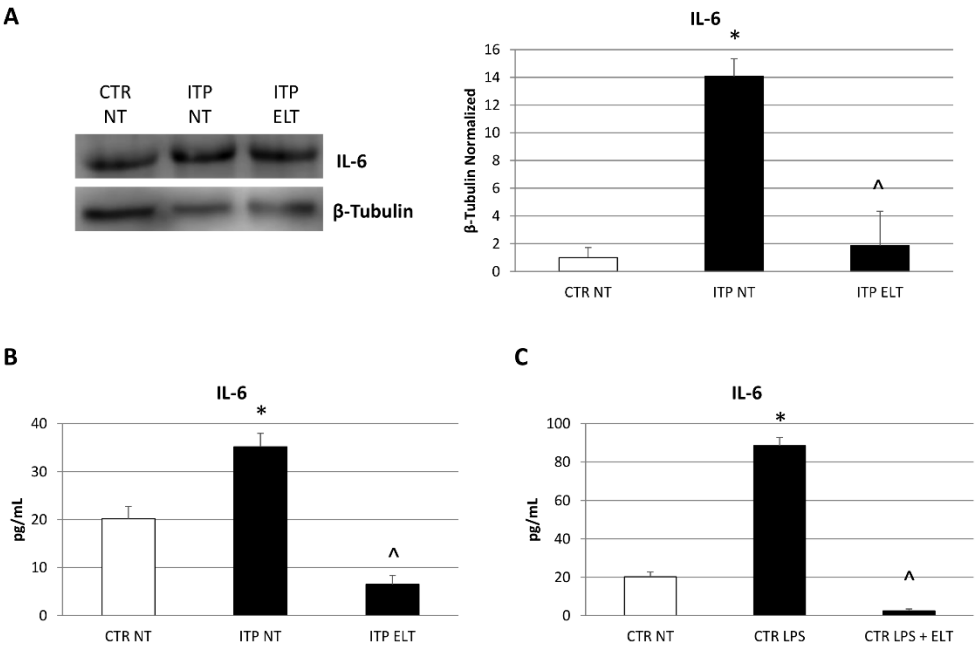

Figure 8 - Effect of ELT on Interleukin-6 (IL-6) protein expression and release. (A) IL-6 protein expression levels in ITP macrophages compared with CTR macrophages, determined by Western Blot, starting from 20 µg of total lysates after treatment with ELT [6 µM]. The most representative images are displayed. The proteins were detected using Image Studio Digits software, and the intensity of immunoblots compared to the untreated control, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein -Tubulin. Histogram shows IL-6 expression levels as the mean ± S.D. of independent experiments on each individual sample. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ^ indicates $p \leq 0.05$ compared ITP NT. (B) IL-6 concentrations (pg/mL) in ITP macrophages compared with CTR macrophages, determined by enzyme-linked immunosorbent assay (ELISA), after treatment with ELT [6 µM]. Histogram shows IL-6 concentration as the mean ± S.D of independent experiments on each individual sample. The cytokines concentration was determined on a standard concentration curve according to the manufacturer's instructions. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ^ indicates $p \leq 0.05$ compared ITP NT. (C) IL-6 concentrations (pg/mL) in ITP macrophages compared with CTR macrophages, determined by ELISA Assay, after treatment with lipopolysaccharide (LPS) [500 nM] and LPS combined with ELT [6 µM]. Histogram shows IL-6 concentration as the mean ± S.D. of independent experiments on each individual sample. The cytokines concentration was determined on a standard concentration curve according to the manufacturer's instructions. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ^ indicates $p \leq 0.05$ compared to CTR LPS.

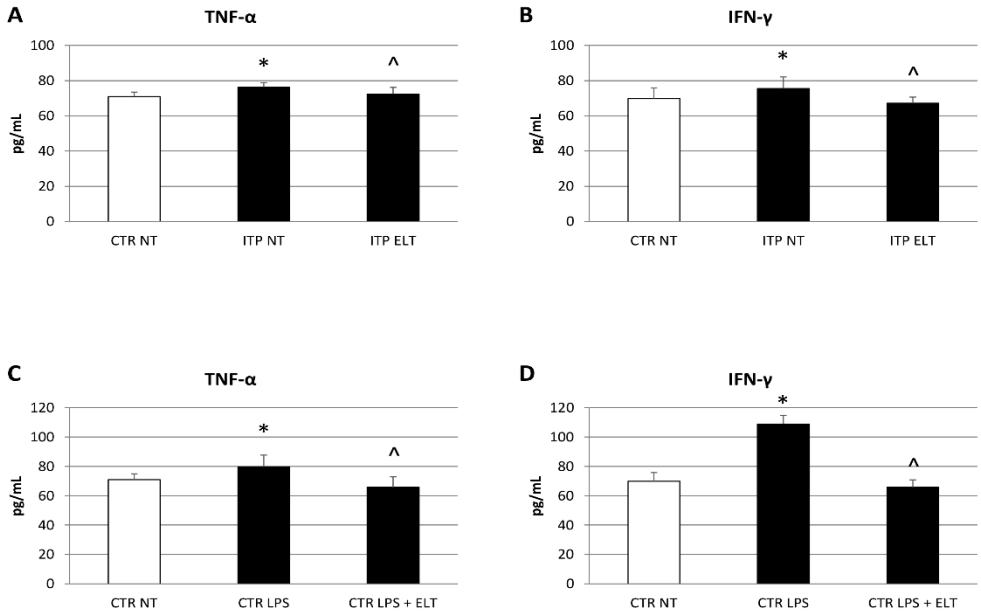

Figure 9 - Effect of ELT on Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ) release. (A, B) TNF-α and IFN-γ concentrations (pg/mL) in ITP macrophages compared with CTR NT macrophages, determined by enzyme-linked immunosorbent assay (ELISA Assay), after treatment with ELT [6 µM]. Histogram shows TNF-α and IFN-γ concentrations as the mean ± S.D. of independent experiments on each individual sample. The cytokines concentration was determined on a standard concentration curve according to the manufacturer's instructions. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ˆ indicates $p \leq 0.05$ compared ITP NT. (C, D) TNF-α and IFN-γ concentrations (pg/mL) in ITP macrophages compared with CTR macrophages, determined by ELISA Assay, after treatment with LPS [500 nM] and LPS combined with ELT [6 µM]. Histogram shows TNF-α and IFN-γ concentrations as the mean ± S.D. of independent experiments on each individual sample. The cytokines concentration was determined on a standard concentration curve according to the manufacturer's instructions. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ˆ indicates $p \leq 0.05$ compared to CTR LPS.

4.7 Effect of Eltrombopag on Anti-Inflammatory Cytokines Release in macrophages

We also analysed the anti-inflammatory cytokines IL-4 and IL-10 levels by ELISA assays. We revealed a decrease of their release in ITP macrophages compared to CTR macrophages. ELT [6 µM] treatment determined a statistically significant increase of both IL-4 and IL-10 in ITP macrophages supernatant, letting us suppose it could ameliorate the inflammatory state (Figure 10 A, B). We confirmed these results in CTR macrophages treated with LPS [500 nM], observing a reduction of IL-4 and IL-10 levels in LPS-treated Macro-CTR. After ELT [6 µM] administration,

we observed an increase of the anti-inflammatory cytokines concentration (Figure 7 C, D).

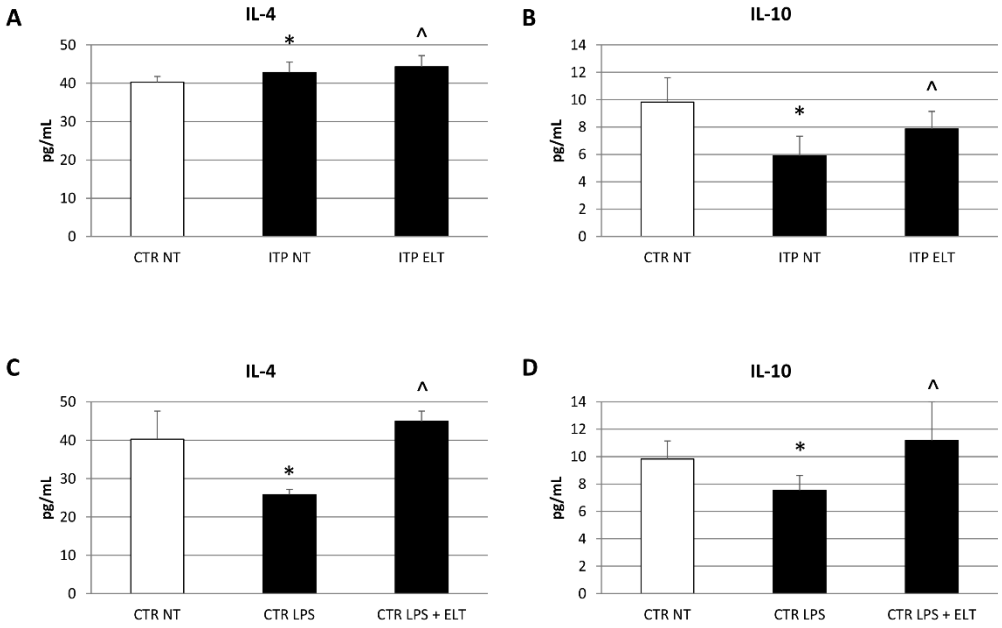

Figure 10 - Effect of ELT on IL-4 and IL-10 release. (A, B) IL-4 and IL-10 concentrations (pg/mL) in ITP macrophages compared with CTR NT macrophages, determined by Enzyme-linked Immunosorbent assay (ELISA Assay), after treatment with ELT [6 µM]. Histogram shows IL-4 (A) and IL-10 (B) concentrations as the mean ± S.D. of independent experiments on each individual sample. The cytokines concentration was determined on a standard concentration curve according to the manufacturer's instructions. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ˆ indicates $p \leq 0.05$ compared ITP NT. (C, D) IL-4 and IL-10 concentrations (pg/mL) in ITP macrophages compared with CTR macrophages, determined by ELISA Assay, after treatment with LPS [500 nM] and LPS combined with ELT [6 µM]. Histogram shows IL-4 (C) and IL-10 (D) concentrations as the mean ± S.D. of independent experiments on each individual sample. The cytokines concentration was determined on a standard concentration curve according to the manufacturer's instructions. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ˆ indicates $p \leq 0.05$ compared to CTR LPS.

4.8 Effect of Eltrombopag on Macrophage Iron Metabolism

By performing a Western Blot, we evaluated the protein expression level of the iron transporter divalent metal transporter 1 (DMT1) in order to understand the effect of ELT on iron metabolism. We observed that DMT1 protein expression level is higher in ITP macrophages than in CTR macrophages. ELT [6 µM] administration determined its expression decrease, thus reducing iron internalization (Figure 11 A). To confirm this result and ELT iron-chelating property, we detected intracellular ferric iron concentration (Fe^{3+}) by performing an Iron Assay. We observed a

significant increase of (Fe3+) in ITP NT compared to CTR NT. ELT [6 µM] administration induced a statistically significant reduction of (Fe3+) (Figure 11 B). We confirmed this result also in CTR macrophages treated with LPS [500 nM], in which we revealed an increase of intracellular iron concentration; after ELT [6 µM] administration, we observed a reduction of (Fe3+) intracellular levels (Figure 11 C).

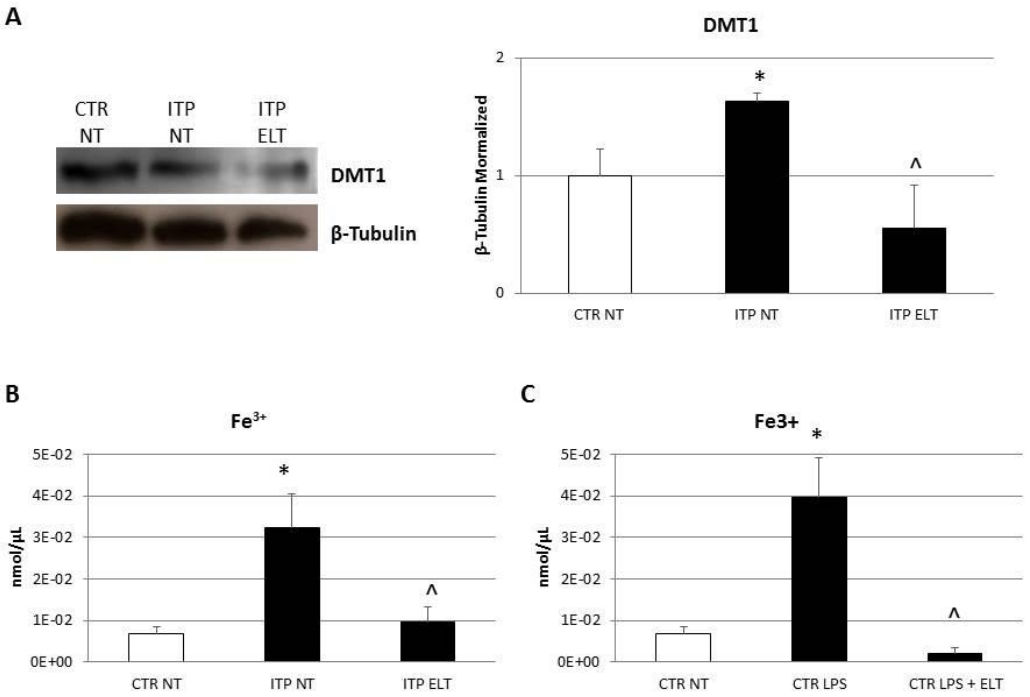

Figure 11 - Effect of ELT on divalent metal transporter 1 (DMT1) protein expression and on Iron release. (A) DMT1 protein expression levels in ITP macrophages compared with CTR macrophages, determined by Western Blot, starting from 20 µg of total lysates after treatment with ELT [6 µM]. The most representative images are displayed. The proteins were detected using Image Studio Digits software, and the intensity of immunoblots compared to the untreated control, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-Tubulin. Histogram shows DMT1 expression levels as the mean ± S.D. of independent experiments on each individual sample. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ^ indicates $p \leq 0.05$ compared ITP NT. (B) Fe3+ intracellular concentrations (nmol/µL) in ITP macrophages compared with CTR macrophages, determined by Iron Assay, after treatment with ELT (6 M). Histogram shows Fe3+ concentration as the mean ± S.D. of independent experiments on each individual sample. A t-test was used for statistical analysis. *indicates $p \leq 0.05$ compared to CTR NT, ^ indicates $p \leq 0.05$ compared to ITP NT. (C) Fe3+ intracellular concentrations (nmol/L) in ITP macrophages compared with CTR macrophages, determined by Iron Assay, after treatment with LPS [500 nM] and LPS combined with ELT [6 µM]. Histogram shows Fe3+ concentration as the mean ± S.D. of independent experiments on each individual sample. A t-test was used for statistical analysis. * indicates $p \leq 0.05$ compared to CTR NT, ^ indicates $p \leq 0.05$ compared to CTR LPS.

4.9 Effect of Eltrombopag on Viability of ITP-MSCs

In order to evaluate the effects of ELT on ITP-MSCs viability, we performed a cytofluorimetric assay to. ELT at concentration of [6 µM] did not alter the percentage of ITP-MSCs viability compared to the untreated cells (NT) (Table 11). This result suggests that ELT [6 µM] is not cytotoxic for ITP-MSCs.

MSC ITP	% VIABLE CELLS
NT	70,66 ± 3,3
ELT [2 µM]	75,4 ± 4,4
ELT [6 µM]	78,07 ± 6,4
ELT [10 µM]	57,82 ± 1,2*

Table 11 – Viability in ITP-MSCs after ELT treatment. Percentage of viable cells in MSCs derived from ITP patients after 48h treatment with ELT at different concentrations [2 µM] [6µM] [10 µM]. The results are presented as the mean percentage ± SD of independent experiments on each individual sample. A t-test has been used for statistical analysis. $p<0.05$ has been considered statistically significant compared to the untreated control (NT).

4.10 Effect of Eltrombopag on Cytokines Release in MSCs

We performed several ELISA (Figure 12 A-D) in order to understand ELT [6 µM] effect on cytokines release. We firstly anlysed the anti-inflammatory cytokines IL-4 and IL-10 levels and we revealed a statistically significant reduction of IL-10 levels in ITP-MSCs with respect to CTR-MSCs. We also found a trend towards a reduction in IL-4 levels, but not in a statistically significant manner. ELT [6 µM] treatment did not influence IL-4 and IL-10 levels in supernatant of ITP-MSCs (Figure 12 A, B). Moreover, we evaluated the pro-inflammatory cytokine Interferon-γ (IFN-γ) release and we demonstrated that in ITP-MSCs a statistically significant increase of this cytokine levels compared to CTR. ELT [6 µM] administration determined

a strong decrease of IFN-γ levels with respect to non-treated ITP-MSCs (ITP NT). We also analysed IL-6 levels in ITP-MSCs supernatants and we confirmed that IL-6 levels are increased in ITP-MSCs compared to CTR. After ELT [6 µM] administration, we revealed a statistically significant reduction of IL-6 release (Figure 12 C, D).

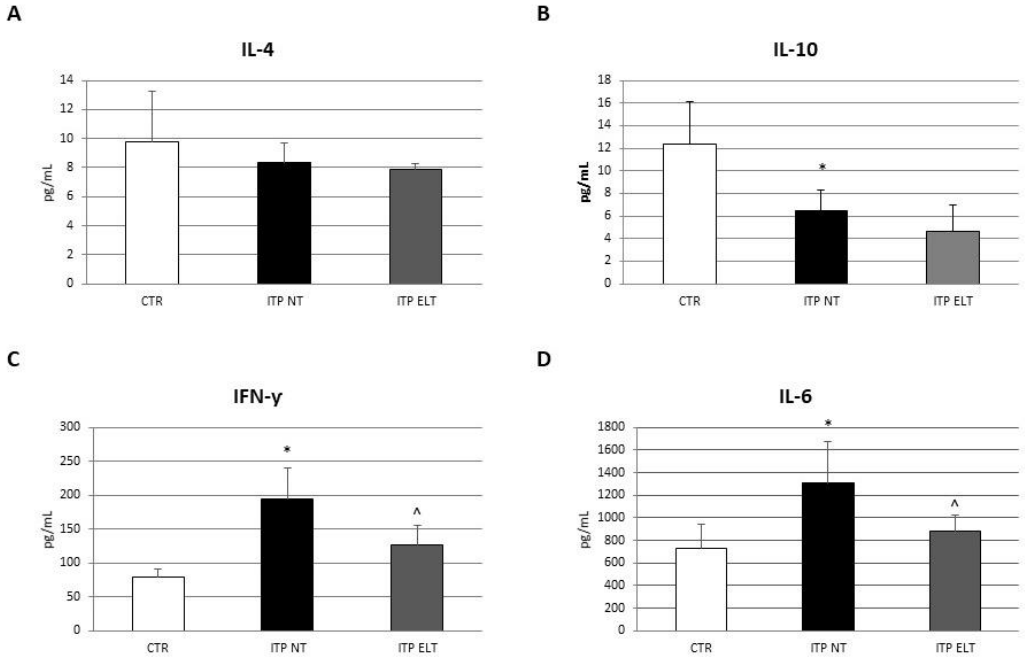

Figure 12 - Pro-inflammatory and anti-inflammatory cytokines levels quantification by ELISA Assay. IL-4 (A), IL-10 (B), INF-g (C) and IL-6 (D) levels in MSCs from 5 CTR and 5 ITP patients before and after 48h treatment with ELT [6 µM] determined through ELISA assay. Histograms show cytokines concentration as the mean ± SD. of independent experiments on each individual sample. A t-test has been used for statistical analysis. *indicates $p \leq 0.05$ compared to CTR. ^indicated $p \leq 0.05$ compared to the untreated control (NT).

4.11 Effect of Eltrombopag on iron metabolism in MSCs

In order to better understand the relation between IL-6 and hepcidin, we perform an ELISA to detect hepcidin levels (Figure 13 A). We revealed an increase of hepcidin levels in ITP-MSCs compared to CTR, confirming the evidence that high levels of IL-6 led to hepcidin up-regulation. Then, we measured intracellular iron concentration [Fe3+] and we evaluated protein expression levels of Transferrin Receptor-1 (TFR-1) and Ferroportin (FPN-1), two important proteins involved in modulation of iron metabolism. TFR-

1 is responsible for iron internalization, while FPN-1 is the only iron transporter responsible for its release by cells. We observed an increased intracellular iron concentration in ITP-MSCs compared to CTR, confirming hepcidin involvement in iron accumulation into cells. We demonstrated that ELT [6 µM] treatment induced not only a strong reduction of iron concentration, confirming its iron chelating properties, but also a reduction of TFR-1 expression levels and an increase of FPN-1 levels, determining a further decrease in intracellular iron levels (Figure 13 B-D).

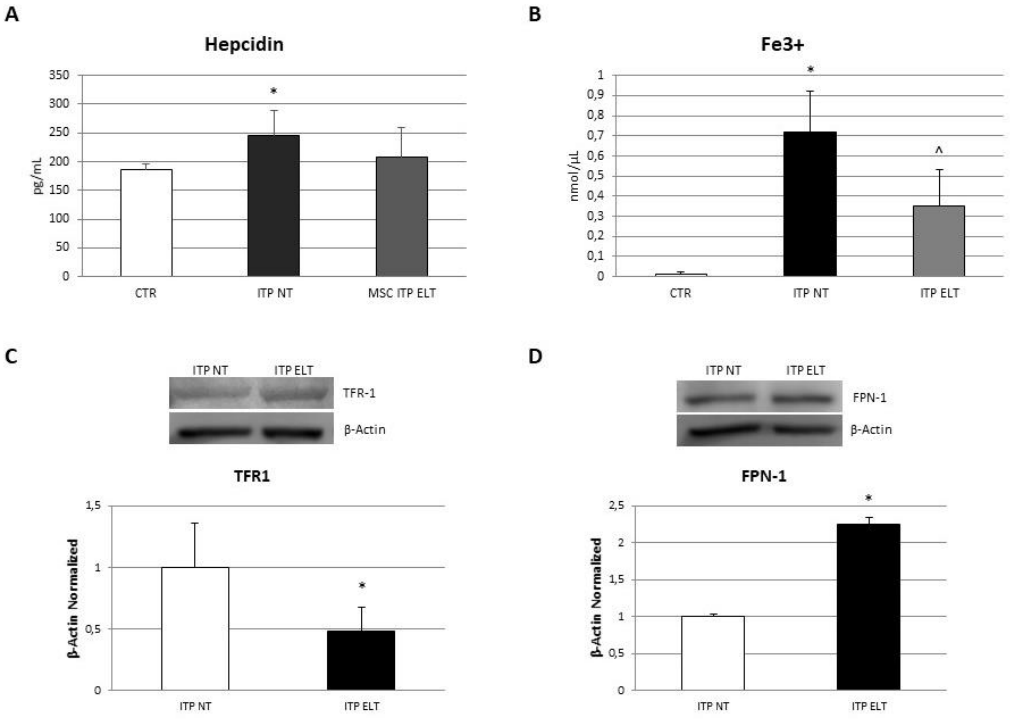

Figure 13 - Evaluation of iron metabolism. Hepcidin (A) from 5 CTR-MSCs and 5 ITP-MSC before and after 48h treatment with ELT [6 µM] investigated through ELISA assay. The experiment was performed for each patient, and the graphs show the concentration of all cytokines [pg/mL] as mean ± SD. A t-test has been used for statistical analysis. $p<0.05$ has been considered statistically significant. *vs CTR; ^ vs ITP-MSC NT. (B) Fe3+ intracellular concentrations (nmol/µL) in 5 CTR-MSC and 5 ITP-MSC before and after 48h treatment with ELT [6 µM] determined by Iron Assay. Histogram shows Fe3+ concentration as the mean ± SD. of independent experiments on each individual sample. A t-test has been used for statistical analysis. *indicates $p \leq 0.05$ compared to CTR. ^indicated $p \leq 0.05$ compared to NT. Transferrin receptor (TFR1) (C) and Ferroportin (FPN1) (D) protein expression levels in MSCs from 5 ITP patients determined by Western Blotting, starting from 15 µg of total lysates, before and after 48h exposure to ELT [6 µM]. The most representative images are displayed. The protein bands were detected through Image Studio Digits software and the intensity ratios of immunoblots compared to CTR, taken as 1 were quantified after normalizing with respective controls. The relative quantification for TFR1 and FPN1 expression, normalized for the housekeeping protein β-Actin, is represented in histogram as mean ± SD of independent experiments on each individual sample. A t-test has been used for statistical analysis. *indicates $p \leq 0.05$ compared to CTR. ^indicated $p \leq 0.05$ compared to NT.

4.12 Effect of Eltrombopag on ITP-MSCs survival and proliferation

To investigate ELT [6 µM] effect on survival and proliferation capability of ITP-MSCs, we performed Western Blotting to evaluate Bcl-2 (Figure 14 A) and pERK (Figure 14 B) protein expression level after treatment. Bcl-2 is an anti-apoptotic protein, while pERK is involved in cell proliferation. ELT [6 µM] administration increased expression levels of both proteins.

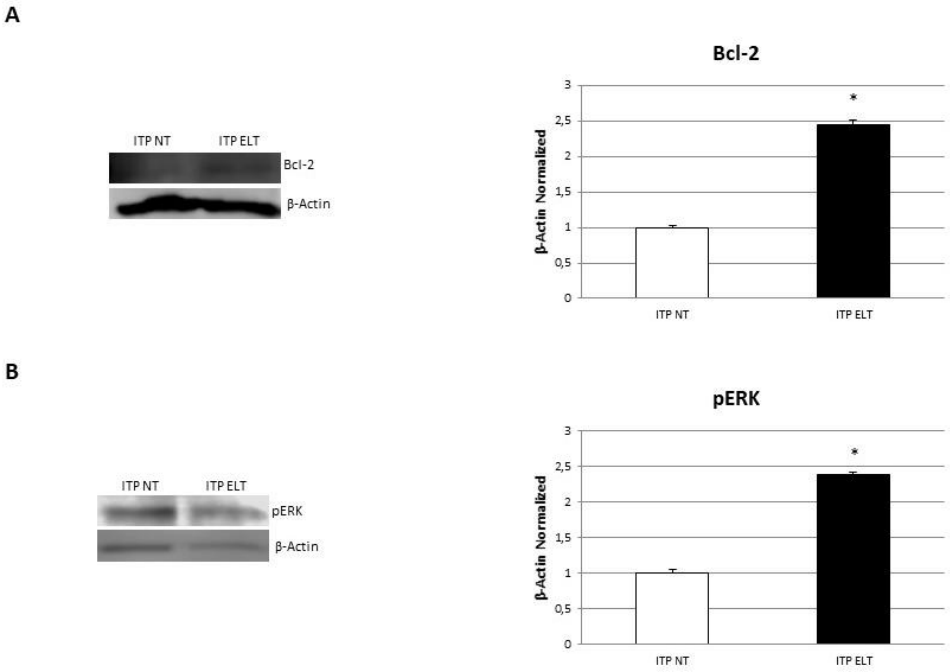

Figure 14 - Evaluation of survival and proliferation. Bcl-2 (A) and pERK (B) protein expression levels in MSCs from 5 ITP patients was determined by Western Blotting, starting from 15 µg of total lysates, before and after 48h exposure to ELT [6 µM]. The most representative image is displayed. The proteins were detected using Image Studio Digits software and the intensity ratios of immunoblots compared to NT, taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-tubulin. The graph represents the relative quantification for Bcl-2 and pERK protein expression as mean ± SD of independent experiments on each individual sample. A t-test has been used to evaluate the statistical differences. *indicates $p \leq 0.05$ compared to NT.

5. DISCUSSION

Immune Thrombocytopenia (ITP) is the most common childhood piastrinopenia, characterized by autoantibodies-mediated platelet destruction [1-5, 209]. The alteration of immune and inflammatory responses plays a key role in ITP pathogenesis [36, 48, 210]. Indeed, in ITP patients the impairment of T cells and macrophages activity and proliferation, together with the alteration of cytokines release, is observed [42, 48, 49, 89-91, 210, 211]. In particular in ITP a prevalence of Th1 and of M1 pro-inflammatory macrophages phenotype are responsible for the alteration of immune and inflammatory processes [33, 42, 48, 49, 89-91].

Interestingly, in recent years it has been demonstrated that also mesenchymal stromal cells (MSCs) are involved in ITP pathogenesis [48, 70, 82, 83]. In physiological conditions MSCs are known to exert immune modulatory properties [69, 78]. Nevertheless, in ITP patients it has been observed a strongly impairment of MSCs proliferation and immunosuppressive capacity [82, 83]. Since their compromission could be involved in ITP development, restoring MSCs function and survival could represent a potential alternative therapeutic approach for ITP.

Glucocorticoid (GC) therapy are the mainstays of an ITP therapeutic regimen [48, 89, 96, 97, 100]. Among them, Dexa is a synthetic GC, which has a strong anti-inflammatory effect and modulates several aspects of ITP pathogenesis [89, 93, 101, 102]. Moreover, it causes several side effects [93]. Therefore, the discovery of new therapeutic strategies to be combined with the classical immunosuppressive therapy would be desirable in order to reduce the side effects associated with these drugs normally used for ITP.

We previously demonstrated that cannabinoid receptor 2 (CB2) is a mediator of anti-inflammatory and immunoregulatory properties of human MSCs [199]. This receptor is responsible for immune regulation by

inhibiting immune cell activation [182, 191]; in particular, it inhibits both T helper cells function and proliferation, and pro-inflammatory cytokine release [79, 80, 196-200]. Moreover, we also observed the presence of a CB2 functional variant in childhood ITP [190].

In these last three years, we focused our attention on the research of new therapeutic target to manage ITP.

During the first year of my PhD experience, we decided to evaluate the effects of CB2 stimulation, with a selective agonist (JWH-133), and also the effects of Dexa administration on ITP-MSCs survival and immunosuppressive capacity. In 2013, we observed that in healthy MSCs, in addition to ITP-MSCs, CB2 shows higher expression at Passage 6 (P6) [69]. Interestingly, CB2 receptor expression was strongly lower in ITP-MSCs than in CTR-MSCs at P6, letting us suppose that its decreased expression could be related to ITP-MSCs impaired function. This result is in agreement with the well-known role of the cannabinoid receptor in immune regulation and provides new insight into the pathogenesis of ITP.

Since cytokines are involved in immune and inflammatory processes, we decided to study their profile in order to better understand pharmacological therapy effects. In particular, we demonstrated that ITP-MSCs were responsible for an impaired cytokines release, contributing to their reduced function. Indeed, we revealed an increased release of pro-inflammatory cytokine interleukin (IL)-6. When we stimulated CB2 with JWH-133, we observed a reduction of this cytokine release, obtaining the same anti-inflammatory effect observed after Dexa administration. Interestingly, this anti-inflammatory effect was observed also when the two drugs were used in combination, suggesting the possible beneficial effect determined by the co-administration of GC with a complementary agent.

ITP-MSCs are characterized both by a slow proliferative capacity and a high rate of apoptosis [48, 70, 82, 83]. Accordingly, we not only observed

that ITP-MSCs in vitro expanded slower, appeared larger and more flattened than CTR-MSCs, but we revealed an increased percentage of cells total apoptosis. To better understand the biological mechanisms responsible for these alterations, we evaluated the expression of an important protein which takes part in intrinsic pathway of apoptosis, Bcl2 [212], in ITP-MSCs after JWH-133 and Dexa administration. We demonstrated that both the drugs, alone or in combination, are able to increase Bcl2 expression in ITP-MSC, thus improving their survival.

T-cells represent the main targets of the immunosuppressive activity of MSCs [77]. Indeed, MSCs determine the inhibition of lymphocyte proliferation and activation [213]. Conversely, in ITP, T cells show a significant proliferation and an increased secretion of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) [214], which is responsible for initiation and regulation of inflammation and immunity [215]. Interestingly, in childhood ITP high levels of TNF-α were observed [52]. Considering all these evidences, and in particular MSCs ability to modulate T cells activity, we decided to co-cultured ITP-MSCs with healthy PHA activated T-cells and then we evaluated lymphocytes viability and TNF-α release after treatments. We observed that Dexa restored the immuno-suppressing properties of ITP-MSCs on T-cells, by determining their viability reduction. These results confirm the main role of GC in suppressing T-cells activity and survival and also of Dexa in promoting MSCs ability to inhibit lymphocyte proliferation [48, 97-99, 216]. Another important and interesting aspect of our study was the evaluation of the effect of CB2 stimulation. In particular we observed a more marked reduction of T-cell viability after JWH-133 administration. Then, we analysed TNF-α levels, in order to understand if treatments could influence T-cells release. We hypothesized that measured TNF-α levels in co-colture corresponded to a T-cells secretion, because it is known that MSCs are responsible for the

release of very lower levels of this cytokine than T-cells. Certain, further investigations are needed to support this hypothesis. Our results demonstrated that ITP-MSCs lost their capability to inhibit TNF-α release by T-cells, and, more interestingly, the co-administration of Dexa and JWH-133 reversed this effect in a strong manner than the single drug administration. This result let us suppose a possible synergism between Dexa and JWH-133. Nevertheless, since these drugs are involved in regulation of T-cell activity [69, 79, 80, 102, 196-198, 200], our result could be explained not properly as a direct effect on T-cells, but rather as an MSC-mediated effect. Undoubtedly, further investigations are needed to better understand the mechanism of these drugs interaction. Therefore, we provided new insight into the pathogenesis of ITP, suggesting the involvement of CB2 receptor in the compromission of ITP-MSC function. For the first time, we demonstrated that CB2 stimulation acts as a mediator of MSCs immunosuppressive properties in ITP compromised immune system. Moreover, once we have confirmed that CB2 receptor could be considered an important anti-inflammatory and immunomodulatory target, we decided to analyse the effect of CB2 inverse agonist, AM630, and we observed that it induced an opposite effect. Finally, we also demonstrated that the co-administration of Dexa and CB2 selective agonist not only restored the immune suppressive and anti-inflammatory properties of ITP-MSCs, but also determined a reduction of their apoptosis by acting on Bcl2 pathway, thus confirming that MSCs are cellular targets of Dexa [217]. All together these results suggest the possibility of using Dexa in combination with CB2 stimulation in ITP, in order to reduce its dose and, consequently, its side effects, by maintaining its therapeutic benefits.

Considering the key role of macrophages in ITP pathophysiology [48], during the second year, we decided to investigate their involvement in immune and inflammatory processes and, consequently, to modulate their

phenotype in order to counteract inflammation and immune system impairment, typical conditions of this disease. In particular, we analysed the possibility to induce a macrophage phenotype switch towards M2 anti-inflammatory type, using Eltrombopag (ELT), an orally available thrombopoietin receptor agonist approved in chronic ITP patients in order to stimulate platelet production [144-147]. It has also immunomodulating properties, determining a reduction of Th1 function and an inhibition of macrophages activation [43, 148-150]. As reported in literature [49, 89-91], we confirmed the prevalence of M1 phenotype, by detecting increased levels of iNOS and reduced levels of CD206 levels in ITP patients macrophages compared to macrophages from heathy donors. iNOS and CD206 are the most used markers to distinguish M1 and M2 phenotypes, respectively [218]. Interestingly, we observed that ELT administration in ITP macrophages induced a strong reduction of iNOS expression and a significant increase of CD206 levels, suggesting that ELT is involved in macrophages phenotype switch toward the anti-inflammatory and immune suppressive M2 type.

We also analysed cytokines profile before and after ELT administration in order to understand its effects on inflammatory state. In accord with other studies, we revealed an alteration of cytokines profile in ITP, detecting high levels of pro-inflammatory cytokines. More in detail, we observed an increase of IL-6 levels, the main pro-inflammatory cytokine released by M1 macrophages. When we treated ITP macrophages with ELT, a strong reduction of IL-6 levels was observed, letting us suppose that this effect was closely related to macrophage polarization from the M1 pro-inflammatory phenotype to the M2 anti-inflammatory one. Furthermore, we confirmed the impairment of cytokine profile in ITP, by detecting also high levels of the pro-inflammatory cytokines TNF-α and Interferon-γ (IFN-γ) in ITP macrophages [48]. IFN-γ is responsible for M1 macrophages activation [49,

84-86]. ELT administration induced a reduction of both pro-inflammatory cytokines levels, most probably by determining a phenotype switch towards the M2 anti-inflammatory one. To support our hypothesis, we decided to analyse ELT effect also on anti-inflammatory cytokine profile and, in particular, on IL-4 and IL-10 levels. The first one is responsible for M2 macrophages polarization, while the second is released by M2 phenotype to counteract inflammation, respectively [49, 84-86]. Firstly, we observed a reduction of these cytokines levels in ITP, thus confirming the impairment of inflammatory state; then, we found that ELT treatment was able to increase their levels, once again letting us hypothesize ELT involvement in macrophage phenotype switch toward M2.

Successfully, we focused our interest on iron metabolism, considering iron a possible novel therapeutic target in ITP. It is known that ELT has also iron chelating properties and that M1 and M2 macrophages are involved in regulation of iron homeostasis with opposite roles [87, 88]. While M1 phenotype is responsible for iron internalization which causes pro-inflammatory cytokines and ROS production, M2 macrophages are responsible for iron release and the consequent low iron intracellular concentration induces a reduction of iNOS and pro-inflammatory cytokines expression [87, 88]. In particular, we detected high levels of DMT1 protein expression and of intracellular iron concentration in ITP macrophages. DMT1 is an important iron transporter responsible for iron intake and involved in macrophage iron recycling [86, 219]. When we treated these cells with ELT, we not only confirmed its iron chelating properties, by reducing intracellular iron concentration, but it also inhibited iron intake in macrophages, by determining a strong reduction of DMT1 expression. Therefore, our results confirm the iron chelating property of ELT and also suggest, once again, that ELT induces M2 macrophage polarization, inducing a reduction of intracellular iron concentration and of DMT1

expression. Therefore, our results provide new insights into the therapy and the management of ITP, confirming ELT also as immune-modulating drug. For the first time, we demonstrated that ELT acts as a mediator of macrophage polarization from M1 pro-inflammatory phenotype to M2 anti-inflammatory one, ameliorating the impaired inflammatory profile and immune response in ITP. We demonstrated that ELT induces a reduction of M1 phenotype marker expression levels, decreases pro-inflammatory cytokines release, and inhibits iron intake by reducing DMT1 expression levels, leading to a macrophage switch towards M2 phenotype.

Finally, during the last year, on the basis of the results obtained after ELT administration on macrophages, we decided to assess its immune modulating effects also in MSCs, whose functions and survival are known to be impaired in ITP [82, 83]. In particular, we evaluated ELT capability to restore MSCs viability, survival and immune-modulating properties in newly diagnosed ITP children. Firstly, we observed an evident reduction of pro-inflammatory cytokines levels, after ELT administration. Inflammation is closely related to iron accumulation, in particular high levels of IL-6 determine an increase of hepcidin levels, which is involved in ferroportin (FPN-1) degradation and, consequently, in inhibition of iron release by cells [167]. Increased levels of intracellular iron are responsible for cells damage, by determining ROS production and consequently cells damage [168-170]. In particular, it has been reported that iron overload modulates cytokines release by MSCs [220]. Since there is a correlation between IL-6 levels and hepcidin activation [221], we hypothesize that the high levels of IL-6 detected in ITP patients could determine hepcidin activation and consequently an accumulation of intracellular iron, thus contributing to MSCs function and viability impairment. Accordingly, we detected increased levels of hepcidin and of Fe3+ in ITP MSCs. Iron accumulation is responsible for MSCs damage, which lost their important functions.

Therefore, using iron as novel possible therapeutic target in ITP could be useful to restore MSCs functionality and signalling mechanisms, impaired by iron-overloaded conditions. Considering the iron chelating properties of ELT [151, 152], which we have amply proved in several cells type [151, 154, 155], and in particular in ITP macrophages, we confirm its capability to chelate iron also in ITP MSC by detecting a reduction of iron intracellular levels. Moreover, we proposed another mechanism of action of ELT, suggesting it not only as an iron chelator, but more precisely as modulator of iron metabolism. In particular, we evaluated its effect on two important key regulator protein of iron metabolism, Transferrin Receptor-1 (TFR-1) and Ferroportin-1 (FPN-1). While TFR-1 is involved in iron internalization by cells, FPN-1 is the only iron transporter responsible for iron release by cells [164, 165]. Interestingly, for the first time, we found that ELT is involved in the modulation of these iron transporter levels. In particular, ELT induced a reduction of TFR-1 protein expression levels together with an increase of FPN-1 levels, inhibiting iron intake and, also, allowing its release, respectively. These effects are both responsible for the reduction of intracellular iron concentration and, consequently, are crucial for restoring the proliferative capacity and the survival of MSCs, damaged by iron excess. Considering that ITP MSCs proliferation and survival are impaired, we decided to evaluate ELT effects also on MSCs proliferation and apoptosis, by analysing the protein expression levels of Bcl-2 and pERK, a protein involved in cell proliferation. Interestingly, we observed an increase of both Bcl-2 and pERK proteins expression, demonstrating ELT beneficial effects on MSCs survival and proliferation. Definitely, our results suggested novel evidence about the immunomodulating properties of ELT, highlighting its capability to counteract the inflammation in ITP-MSCs. For the first time, we demonstrated that ELT is able to act on MSCs survival

and activity directly, restoring their survival and proliferation, and indirectly, by influencing iron intake and release by cells.

6. CONCLUSIONS

In conclusion, although further *in vitro* and *in vivo* studies are needed, our results added important elements to the research of novel therapeutic targets and approaches for ITP. We introduced the possibility to use CB2 agonist together with Dexa in ITP therapy, due to CB2 anti-inflammatory and immune suppressing properties, in order to reduce Dexa dosage and, consequently, its side effects. CB2 stimulation together with Dexa are responsible for the attenuation of inflammatory status in ITP, by determining an improvement of MSCs survival and anti-inflammatory and immunoregulating properties.

We obtained promising results also after treatment with ELT. Indeed, new beneficial effects have been detected in addition to those already known and for which it is currently approved in chronic ITP therapeutic protocols. We decided to use it not for its well-known capability to stimulate platelets production, but for its immunomodulating and iron chelating properties. Interestingly, for the first time, we suggested that ELT induces macrophages switch from M1 pro-inflammatory phenotype to M2 anti-inflammatory type, restraining both inflammation and alteration of immune response. Moreover, we attributed to ELT both a direct effect, by restoring MSCs survival, proliferation and functions, and indirect effect, by influencing iron efflux. Indeed, we proposed a promising and novel therapeutic target for ITP: iron, whose increased levels are closely related to inflammation.

All these data suggest the possibility of using ELT in newly diagnosed ITP not only to stimulate platelets production but also to improve the impaired inflammatory profile and immune response in ITP, by inducing macrophage switch toward M2 phenotype and by restoring the well-known anti-inflammatory and immune-suppressing properties of MSCs.

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