SAPIENZA UNIVERSITÀ DI ROMA

STUDY OF INNATE IMMUNITY AND THE EFFECT OF IVIG INFUSIONS IN PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY AND X-LINKED AGAMMAGLOBULINEMIA

PhD COURSE IN IMMUNOLOGICAL, HAEMATOLOGICAL AND RHEUMATOLOGICAL SCIENCES

CLINICAL IMMUNOLOGY

XXIX CYCLE

PhD STUDENT: DOTT. ALESSANDRO PREZZO

TUTOR: PROF. ISABELLA QUINTI

DIRECTOR: PROF. ANGELA SANTONI

INDEX

INTRODUCTION

1. INTRODUCTION

Primary immunodeficiencies (PIDs) are rare and several genetic defects that influence the development function of immunity [1]. Immunodeficiency disorders include both the hematopoietic and non-hematopoietic arms of host defense. The hematopoietic arms include all the immune system cells derived from the hematopoiesis process and from the subsequent differentiation (such as lymphocytes, monocytes, NK cells, dendritic cells or macrophages) as well as the mediators produced by these cells (such as antibodies, cytokines or enzymes). The non-hematopoietic arms include passive defenses, such as anatomical barriers and pathways, exogenous body secretions, physicochemical environments, ciliary activity or microbiological flora, and active defenses such as physiological responses (elevated body temperatures, tachycardia, vomiting and diarrhea), formation of nitric oxide from arginine and acute-phase reactions. The defects of these host arms defense resulting in a wide spectrum of clinical symptoms, including susceptibility to infections, autoimmunity, inflammation, allergy, and increased incidence of malignancy. PIDs confer predisposition to multiple clinical and immunological phenotypes and an increasing number of PIDs are being shown to confer predisposition to a single type of infection [1]. Despite most humans carry genetic errors of immunity, PIDs are typically rare and classified with familial recessive or dominant tracts. The presence of mutations in patients' genome may be due to the inheritance of mutations with complete or incomplete penetrance from an affected parent, *de novo* dominant lesions in the germ line or somatic mutations.

1.1 Common Variable Immunodeficiency Deficiency (CVID)

In 1971 World Health Organization coined the term "Common Variable Immunodeficiency" (CVID) to describe a number of poorly defined immunodeficiencies characterized by antibody deficiency without a clear Mendelian inheritance [2,3]. Currently, the International Union of Immunological Societies Expert Primary Immunodeficiency Committee refer to CVID as "common variable immunodeficiency disorders", focusing attention to the heterogeneous nature of the disease [4] and the European Society for Immunodeficiencies and the Pan American Group for Immunodeficiency defined CVID as probable in a male or female patient who has marked decrease of IgG (at least 2 SD below the mean age) and a marked decrease in at least one of the isotypes IgM or IgA, and fulfills all of the following criteria (summarized in Table 1) :

1. Onset of immunodeficiency at greater than 2 years of age
2. Absent isohemagglutinins and/or poor response to vaccine

3. Defined causes of hypogammaglobulinemia have been excluded according to a list of differential diagnosis (Table 2)

Clinical criteria for a probable diagnosis (= clinical diagnosis)	Suggestions for alternative diagnosis (i.e. if these criteria are not completely fulfilled)
At least one of the following: - increased susceptibility to infection - autoimmune manifestations - granulomatous disease - unexplained polyclonal lymphoproliferation - affected family member with antibody deficiency AND marked decrease of IgG and marked decrease of IgA with or without low IgM levels (measured at least twice; <2SD of the normal levels for their age); AND at least one of the following: - poor antibody response to vaccines (and/or absent isohaemagglutinins); i.e. absence of protective levels despite vaccination where defined - low switched memory B cells (<70% of age-related normal value) AND secondary causes of hypogammaglobulinaemia have been excluded (**see Table 2**) AND diagnosis is established after the 4th year of life (but symptoms may be present before) AND no evidence of profound T-cell deficiency, defined as 2 out of the following (y=year of life): - CD4 numbers/microliter: 2-6y <300, 6-12y <250, >12y <200 - % naive CD4: 2-6y <25%, 6-16y <20%, >16y <10% - T cell proliferation absent	For patients <4 years old or patients with incomplete criteria please consider "Unclassified antibody deficiency". For patients with evidence of profound T-cell deficiency, please consider Unclassified combined immunodeficiencies.

Table 1. ESID Registry – Working Definitions for Clinical Diagnosis of CVID

Drug induced	Single gene and other defects	Chromosomal anomalies	Infectious diseases	Malignancy	Other systemic disorders
Antimalarial agents	Ataxia telangiectasia	Chromosome 18q- syndrome	HIV	Chronic lymphocytic leukemia	Immunodeficiency by excessive loss of immunoglobulins
Captopril	Autosomal-recessive forms of SCID and other forms of combined	Monosomy 22	Congenital infection with rubella virus	Immunodeficiency with thymoma	
Carbamazepine	Hyper-IgM syndromes	Trisomy 8	Congenital infection with cytomegalovirus	Non-Hodgkin lymphoma	
Glucocorticoids	Transcobalamin II deficiency, hypogammaglobulinemia	Trisomy 21	Congenital infection with Toxoplasma gondii	Monoclonal gammopathy	
Fenclofenac	X-linked agammaglobulinemia	Chromosomal anomalies	EBV		
Gold salts	X-linked lymphoproliferative disorder (EBV-associated)				
Penicillamine	X-linked SCID				
Phenytoin	Some metabolic disorders				
Sulfasalazine					
Anti-CD20 mAbs					

Table 2. Differential diagnosis of CVID

As result of this classification, CVID can be considered as a group of heterogeneous primary antibody failure syndromes characterized by hypogammaglobulinemia [5, 6]. The lack of an efficient antibody production is the result of a B cell dysfunction or a of T cell function impairment with consequently lack of help for antibody production. A lower limit value of IgG at 4.5 g/L for adults has been proposed considering that nearly 95% of CVID patients in a European cohort fulfilled this criterion [7] although some patients present high residual IgG levels (up to 6 g/L) at diagnosis while still showing impaired specific antibody formation [8]. In addition, the level of IgG might vary depending from ethnicity and therefore the definition of hypogammaglobulinemia depends from the region considered [9].

1.2 Definition of CVID

The criteria used [10] for the diagnosis of CVID are:

1. At least one of the characteristic clinical manifestations (infection, autoimmunity, lymphoproliferation).
2. Low level of IgG must be observed at least 2 times, more than 3 weeks apart and the diagnosis of hypogammaglobulinemia should be defined considering the age-adjusted reference range. If the IgG level is very low (<100-300 mg/dL depending on age) and other characteristic features are present, repeated measurement can be omitted.
3. Low level of IgA or IgM
4. If the IgG level is more than 100 mg/dL must also present an impairment of response to T-dependent (TD) and/or T-independent (TI) antigens
5. Other causes of hypogammaglobulinemias must be excluded (Table 2).
6. Genetic studies are generally not required; anyway, the genetic characterization may be useful to set specific therapies (as stem cell therapy).

1.3 Epidemiology of CVID

CVID is the most frequent symptomatic antibody deficiency diagnosed in adulthood [11,12]. Although IgA deficiency occurs with an overall higher frequency, most patients with IgA deficiency are asymptomatic [13]. There are no precise data on the prevalence of CVID, but it was estimated to be between 1: 100.000 and 1: 10.000 of the population, although exist significant difference between countries, probably as result of

different methodologies of study, access to health care, rate at which patients are properly diagnosed or population genetic differences [7].

1.4 Pathogenesis of CVID

In 98% of cases, the genetic defects responsible for CVID patients' condition are unknown. The fundamental characteristics found in all CVID patients is a condition of hypogammaglobulinemia associated with loss of B cell function. The loss of B cell function may be due to an intrinsic defect of B-lymphocytes or T- lymphocytes that do not provide a sufficient support to B-lymphocytes. In the majority of cases, there is a decrease of isotype-switched memory B cells [14,15] and a loss of plasma cells in both bone marrow and mucosal tissues [16,17].

Although B cells *in vitro* are usually able to produce normal amounts of immunoglobulin, in others cases B cells produce only IgM or they are unable to produce immunoglobulin at all [18].

One recent [19] analysis seem distinguished five possible subgroups or defects:

1) B cell production
2) early peripheral B cell maturation or survival
3) B cell activation and proliferation
4) germinal center
5) post-germinal center

Interestingly some patients present an impaired calcium flux after activation of the B cell receptor and an expansion of the CD21-low B cell population. In some patients, it was described also a T cell loss of function, including a reduction of circulating CD4 T cells and impaired proliferation, activation, and secretion of some cytokines (IL-2, IFN-g, and IL-10) with increases of IL-6 and perhaps IL-12 [20-25]. More recently, defects in monocyte/dendritic and impaired innate immune responses have also been demonstrated. [26,27]

1.5 Clinical manifestations

The onset of symptoms appears usually during the first and the third decade of life with a slightly bimodal onset [28,29]. At the time of diagnosis, severe infections are common and complications related to immune dysregulation are present in approximately 20-30% of patients. Up to 80% patients have infection-related structural changes such as bronchiectasis [20,30,31]. CVID patients develop various additional clinical problems, such as autoimmunity, interstitial lung disease, granulomatous disease, liver disease,

gastrointestinal inflammatory disease, lymphoid hyperplasia and/or cancer or lymphoma, each of these with a different prevalence depending on the country considered [10]. Chronic lung disease is the leading cause of mortality in CVID [28,30,32]. Granulomatous disease or atypical sarcoid-like lesions commonly affect lungs, lymph nodes, and spleen (8%-22% of CVID patients). However, skin, liver, bone marrow, kidney, gastrointestinal tract, eyes, and/or brain may also be involved [35-38].

Autoimmunity occurs in 25%-30% of CVID patients [28, 31] and in about 3-5% of CVID patients autoimmunity was the only clinical complication at the time of diagnosis [39]. Thus, CVID patients might display autoimmune manifestations before the appearance of hypogammaglobulinemia, similar to those patients with syndromes of immune dysregulation such as autoimmune lymphoproliferative syndrome [40]. The most common autoimmune diseases include immune thrombocytopenia purpura (ITP) and autoimmune hemolytic anemia (AIHA), while autoimmune neutropenia is much less common [41-43].

Recently, different forms of enteropathy were found in 10-20% of CVID [28] with a high rate of nonmalignant mortality, possibly due to malabsorption [29,44]. Bacterial, protozoal and viral gastrointestinal infections occur in CVID as well as small bowel inflammation. Bowel inflammation is also associated with unexplained persistent chronic diarrhea, weight loss, steatorrhea, and malabsorption with loss of both minerals and fat-soluble vitamins [45–47]. Typically, bowel mucosa shows common histological feature in CVID like villous blunting and crypt distortion with increased lymphocytes (usually CD8 T cells), lymphoid aggregates (lymphoid hyperplasia), and loss of plasma cells [45,48]. Also liver abnormalities, mainly due to nodular regenerative hyperplasia, are common in CVID, in particular hepatomegaly and increased levels of liver enzyme, including alkaline phosphatase [49–51].

At least 20% of CVID present cervical, mediastinal, and abdominal lymphoid hyperplasia and/or splenomegaly [52,53]. Lymph nodes usually show atypical lymphoid hyperplasia, reactive lymphoid hyperplasia, or granulomatous inflammation and these tissues need to be examined for B- and T cell clonality. However, the presence of clonal lymphocytes is not diagnostic of lymphoma because these can be found in CVID lymphoid tissue showing reactive hyperplasia (53). Finally, CVID patients display an increased incidence of cancer compared with the general population, occurring in about 20% of patients and lymphomas are the most common form of malignancy in CVID.

1.6 Laboratory manifestations

One of the main characteristics shared by all CVID patients is a low level of different classes of serum immunoglobulin, in particular a level of IgG less than 4.5 g/L (85%-94% of patients) while IgM levels are variable. Different studies suggest a level between 0.25g/L and 0.4g/L although one study shows that females patients tended to have higher levels. IgA levels are typically low or undetectable in CVID, with 70% of the patients showing values of less than 0.1 g/L [29,44,54,55]. About 20% of the patients with CVID may have very low levels or absence of all immunoglobulin isotypes at presentation [15,28]. Specific antibody production may be variable in some patients with CVID and it might decrease over time [56].

Most patients with CVID will have normal levels of total circulating T cells and natural killer (NK) cells in peripheral blood [29,57]. The number of B cells is normal in the vast majority of CVID, about 10% had an increased level and about 10% of patients had reduced or undetectable levels [55].

CVID patients might also display variable dysfunction within the T cell compartment [58]. One study showed that CD4 T cells were decreased in 29% of CVID patients, while 50% of this cohort demonstrated abnormal proliferative responses to mitogens [44]. Others studies showed restricted T cell receptor repertoires, oligoclonality, increased T cell apoptosis, and reduced expression of CD40L [59–61].

It was shown that also dendritic cells and regulatory T cells may contribute to the pathogenesis of CVID, even if their role are yet to be fully elucidated [23,62,63].

1.7 Genetics

Most CVID cases are sporadic and approximately 5-25% of cases are familial, typically with an autosomal dominant inheritance [64, 65]. Different studies point their attention to the HLA region on chromosome 6p and different HLA-DQ/DR haplotypes could confer either protection or susceptibility to CVID [66]. A genetic linkage study showed mutations on chromosome 4q and 16q associated with CVID, although the mutated genes have not been identified [29,67]. Currently only 2% of CVID cases are explicable by a clear genetic mutation, many patients with these mutations exhibit combined immunodeficiency with clinical characteristics or laboratory abnormalities not commonly seen in CVID, therefore, these disorders are classified as distinct entities on the basis of the defined genetic defects [68]

It was observed that particular polymorphisms of TACI and MSH5 genes are associated with CVID although these same polymorphisms are present in about 1% of healthy individuals [68, 69]. However, the variable nature of CVID suggest a polygenic disease, with multiple potential susceptibility loci for CVID [5].

1.8 IMMUNOGLOBULIN REPLACEMENT THERAPY

Currently the standard therapy for CVID is the IgG replacement that can be given by intravenous (IVIg) or subcutaneous (SCIg) route at varying intervals [70]. Most guidelines suggest to start with a dose of 0.4 to 0.5 g/kg/month for IVIg and 0.4 to 0.6 g/kg/month for SCIg [71]. Higher doses are proposed in case of complications such bronchiectasis, splenomegaly and enteropathy [30,72-74]. IVIg infusions are usually given every 3- or 4-week and typically adverse reactions, such as headaches, nausea and vomiting, flushing, hives, chills, myalgia, arthralgia, or abdominal and/or back pain are reduced slowing the infusion rate. Although complications due to transmission of infectious agents, such as hepatitis B or hepatitis C, are extremely rare [75] it is important to monitor patients on replacement therapy for transmission of unknown or new pathogenic agents. Finally, renal complications can occur patients receiving IVIG and are associated with sucrose-containing products and in patients with preexisting kidney disease [76].

1.9 X-LINKED AGAMMAGLOBULINEMIA

X-linked agammaglobulinemia (XLA) was one of the first immunodeficiencies described with distinctive clinical and laboratory findings. The first symptoms typically appear in the first 24 months of life and include profound hypogammaglobulinemia and strongly reduced numbers of B cells. The genetic defect, that occur with a frequency of about 3-6/million, was found on X chromosome, in the locus that encode a cytoplasmic tyrosine kinase called BTK [77,78].

1.10 CLINICAL FINDINGS IN XLA

XLA patients generally do not present any symptoms during the newborn period due to the maternal immunoglobulin acquired transplacentally. In patients with XLA who have no family history of immunodeficiency the mean age at diagnosis is 2-3 years old [79,80]. Recurrent bacterial infections, particularly otitis, purulent rhinorrhea, conjunctivitis, pneumonitis, diarrhea, and skin infections typically appear in the second half of the first year of life [79,81, 82,83]. *Haemophilus influenzae* and *Streptococcus pneumoniae* infections are common. In addition, XLA patients are susceptible to infection with *Mycoplasmas* and *Ureoplasmas* that may cause persistent pneumonia, arthritis, cystitis, or cellulitis [84,85]. XLA patients are particularly susceptible to chronic enteroviral infections, including vaccine-associated polio while do not appear to have an unusual susceptibility to most viral and fungal infections. Malignancy has been reported. [86-88].

1.11 Laboratory manifestations

XLA patients show markedly decreased numbers of B cells (0.1% of circulating lymphocytes) and a low concentration of all isotypes of serum immunoglobulins [89]. The IgG are usually less than 200 mg/dL, and the IgM and IgA less than 20 mg/dL. Genetic studies showed that the mechanism of rearrangement of immunoglobulins genes was preserved although there may be an over-representation of V, D, or J segments that are typical of a fetal repertoire [90-93]. A definitive diagnosis of XLA can be made if male agammaglobulinemic patients present a family history of hypogammaglobulinemia, <2% B cells, mutation in BTK or the absence of BTK protein or mRNA. Studies performed in bone-morrow show a defect during the development of B cells in the transition between the pro-B cells ($CD34^{+}CD19^{+}sIg^{-}$) stage and pre-B cells ($CD34^{-}CD19^{+}sIg^{-}$) [94].

Generally, 20-25% of patients show neutropenia at the time of diagnosis that can persist for several weeks after IVIg therapy started [79,80], in addition XLA patients might have an increased percentage of monocytes [95].

1.12 The defective gene: BTK

Bruton's tyrosine kinase (BTK) is a member of a family of cytoplasmic tyrosine kinases, called Tec kinases [96,97]. BTK is predominantly expressed in B cell lineage (except in terminally differentiated plasma cell stage), but it is also expressed in myeloid cells [98]. BTK is a member of a family of Src-related cytoplasmic tyrosine kinases that includes Tec, Itk, Rlk, and Bm, that participate in signal-transduction pathways involving growth or differentiation factors [99]. Members of Src-related kinases are characterized by a C-terminal kinase domain preceded by SH2 and SH3 domains, a proline rich tract and an NH2-terminal PH (pleckstrin homology) domain. Typically, SH3 domains bind proline-rich regions and SH2 domains bind phosphorylated tyrosine residues [100] while the PH domain brings BTK to the inner side of the plasma membrane by binding phosphorylated phosphatidyl inositides produced in response to cellular activation [101]. The PH domain is also able to bind the protein kinase C (PKC) and both the α and β/γ subunits of G proteins [102-104]. The proline rich tract that follow the PH region of BTK it was been reported to interact with the SH3 of the Src family members Lyn, Fyn, and Hck [105].

Several studies showed that BTK is activated through a variety of cell surface molecules, in particular the BCR and pre-BCR [106–108] but also others such as IL-5 and IL-6 receptors on B cells [109, 110], the high affinity IgE receptor on mast cells [111]. More recently, a study performed by Ren *et al* [112] showed the importance of BTK in those pathways activated by FcγR pathway in innate immune cells such monocytes/macrophage system.

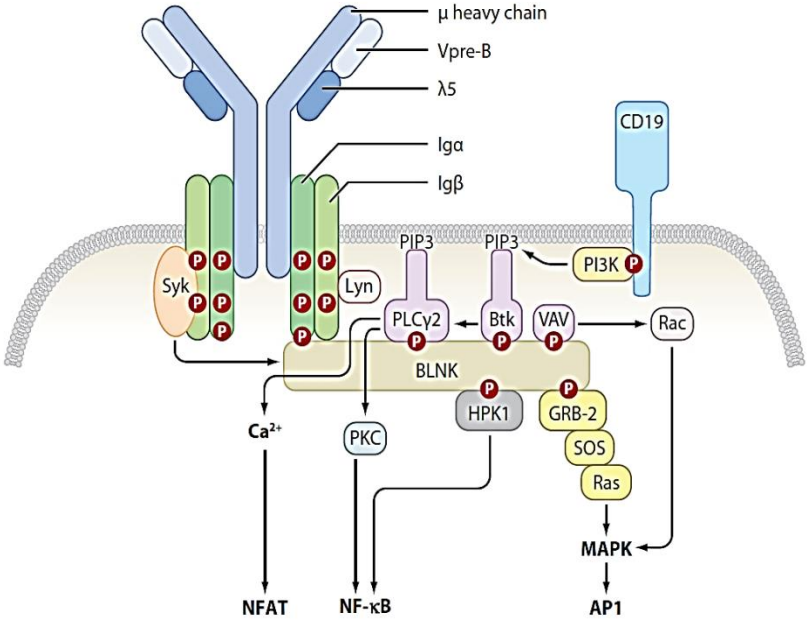


Figure 1. Signal transduction through the pre-B cell receptor [98]. After activation, Syk phosphorylates multiple tyrosine residues in the scaffold protein BLNK. Lyn also phosphorylates BTK and CD19. Phosphatidylinositol 3-kinase (PI3K) bind CD19 and produces PIP3 that work as as a docking site for BTK and PLCγ2. BTK and PLCγ2 bind to phosphorylated tyrosines in BLNK, which allows BTK to phosphorylate PLCγ2.

As shown in Figure 1, upon cell activation BTK moves to the inner side of the plasma membrane, where it is phosphorylated and partially activated by a Src family member [113], then BTK undergoes autophosphorylation [114]. Activated BTK and PLCγ2 bind the scaffold protein BLNK via their SH2 domains, inducing BTK to phosphorylate and activate PLCγ2 [115]. This leads to the production of IP3 that induce the calcium mobilization from the internal storage trough the binding of IP3 with its receptors (IP3R) present on the endoplasmic reticulum. The calcium mobilization results in the activation of the MAP kinases ERK and JNK [116] and others cellular processes such activation of PKC [117]. In addition, BTK phosphorylates several transcription factors and can be found in the nucleus [118,119]

1.13 DIFFERENTIAL DIAGNOSIS

Male and female patients are equally affected by the non-X linked forms of B cells deficiency, [88,120,121] and several defects in B cells lineage are difficult to distinguish from XLA (Table 3). Mutations in mu heavy chain result in a clinical picture similar to that observed in XLA but generally these patients show a more severe phenotype have an earlier onset of symptoms and more severe infectious complications than patients with XLA. Patients with defects in mu heavy chain demonstrate a complete absence of B cells in the peripheral circulation that result in <0.01% of peripheral blood lymphocytes [122] while XLA patients show generally markedly reduced but detectable numbers of B-Cells [89,123]. Moreover, also the concentrations of serum IgG are lower in patients with mutations in mu heavy chain compared to XLA.

Immunologic disorder	Peculiar characteristics
X-linked agammaglobulinemia	Some B-Cells present (mean of 0.1% of peripheral blood lymphocytes); Mutations in Btk
Mu heavy chain deficiency	No B cell present, Undetectable serum IgG; Mutations in mu heavy chain gene
λ5 deficiency	Undetectable λ5 and VpreB in B cell precursors, Mutations in λ5
Myelodysplasia	Chromosomal abnormalities in bone marrow cells
Common Variable Immunodeficiency	Delayed onset of infections, usually have a normal numbers of B-Cells, normal Btk

Table 3. Differential diagnosis in patients with less than 2% of B cells and hypogammaglobulinemia

These observations suggest a beneficial effect of the residual amount of immunoglobulin present in XLA patient, possibly due to Fc receptor-binding function or to antigen binding properties.

In addition, mutations in λ5/14.1, a component of the surrogate light chain, are responsible for the block of B-cell differentiation at the pro-B cell stage [124]. Patients with mutations in λ5/14.1 display profound hypogammaglobulinemia and reduced B cells but unlike in XLA, B cells show a mature phenotype [124].

1.14 IMMUNOGLOBULIN REPLACEMENT THERAPY

IVIg replacement is the standard therapy for patients with XLA. Generally, IVIg are given at a dose of 400 mg/kg every 3/4 weeks [125]. Also SCIg can be a choice for the treatment of XLA, in this case patients can self-administer the Igs, dividing them in weekly doses. There is not a consensus about the use of prophylactic antibiotics in patients with XLA.

1.15 MONOCYTES SUBSETS

Monocytes were recently classified in three different subsets according to their expression of CD14, the receptor of LPS and CD16, a low affinity Fc receptor, in classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}$, $CD16^{+}$) and non-classical monocytes ($CD14^{+}$ $CD16^{++}$) (Figure 2) [126]. Therefore, this new segregation raises the possibility that specialized functions and phenotype can be attributed to these newly defined monocyte subsets. Our knowledge about the three monocytes subsets was recently improved by genome wide analyses [127-129]. The relationship between the different monocytes subsets it is not clear. A study by Cros et al. [127] reported that classical and intermediate monocytes were the most closely related among the subsets, although other studies showed that intermediate and non-classical subsets are more closely related [128, 129], since these two subsets display the highest the number of genes equally expressed between the three subsets [128] suggesting a direct development relationship between these two subsets.

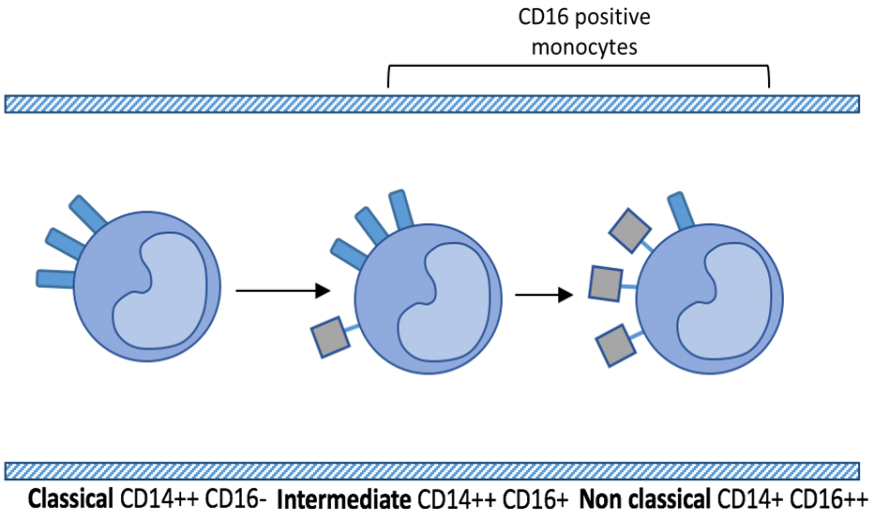


Figure 2 Human monocytes subsets [121]. Classical and intermediate monocytes express by definition, the same level of $CD14^{+}$ while non classical express the higer amount of CD16

There is very poor agreement regarding the cytokines production by the three monocytes subsets. In the study by Cros *et al.* [127], isolated non-classical monocytes were poor producers of several cytokines in response to LPS (including TNF-α, IL-1β, CCL2, IL-10, IL-8, IL-6 and CCL3) but responded strongly to TLR7/8 ligands while the intermediate subset treated with LPS produced the most TNF-α, IL-1β and IL-6. Also Rossol *et al.* [130] showed that the intermediate monocytes stimulated *in vitro* with LPS produced the most TNF-α and IL-1β as well as TNFα when co-cultured with pre-activated T cells, while Wong *et al* [128] showed that non-classical subset are the main producer of TNF-α and IL-1β in response to LPS while equivalent amounts of IL-6 and IL-8 were produced by all three subsets. The data are conflicting also regarding the anti-inflammatory cytokine production: Skrzecynska-Moncznik *et al.* [131] showed that the intermediate monocytes are the main

producers of IL-10 in response to LPS and zymosan but others [127,128,132] showed that classical monocytes were the main producers of IL-10. The different isolation methods used to purify the monocyte subsets might be responsible for the differences in cytokine production reported by the different groups, anyway, the staining in whole blood condition is probably the most ideal in order to minimize nonspecific effects that may occur during isolation and *in vitro* culture [133].

The intermediate monocyte subset expresses the higher levels of MHC class II processing and presentation genes as well surface molecules involved in antigen presenting to T cells, particularly CD40 and CD54 [128, 129]. Indeed, in line with these observations, intermediate monocytes were shown to be the best inducers of SEB-mediated T cell proliferation and the best T cell stimulators with influenza type A antigen [129, 134], even if most of these assays were performed using experimental systems that bypass the antigen phagocytosis. Moreover, also classical monocytes could overexpress MHC class II making them capable to induce T-cell proliferation after activation with SEB [129]. Therefore, it is possible that *in vivo* also classical monocytes can induce T cell proliferation if properly activated as it could happen locally in inflamed tissues where activated monocytes migrate [135, 136]. Conversely the highest expression of MHC I was found in $CD16^+$ monocytes, even if it is not clear if the maximum expression is on intermediate [128] or non-classical [129] subsets. Cros *et al* [127] observed that non-classical monocytes showed a typical patrolling behavior since they continuously roll on endothelium looking for signs of inflammation or damage in order to transmigrate rapidly. In support of this observation, genes associated with cytoskeleton mobility, such as Rho GTPases, RHOC and RHOF, and several upstream Rho activators and downstream effectors were most highly expressed by the non-classical subset [128, 129].

Interestingly, many infection or inflammatory conditions were associated with the expansion of the $CD16^+$ monocytes [137–143]. $CD16^+$ monocytes are often termed ‘‘pro-inflammatory’’ monocytes in line with their ability to produce high amounts of TNF-α and IL-1β [133, 144] and therefore they might play important roles in promoting inflammation. With the new subdivision of $CD16^+$ monocytes in intermediate and non-classical subsets it is clear that it will be important to understand which population is expanded and to deduce their roles in diseases based on phenotypic and functional properties and also from the gene expression profiling studies. As reported by Wang *et al* [145] increasingly evidences appear to show that intermediate monocytes are the monocyte population that most often is expanded in inflammatory and infectious conditions.

It was initially reported an expansion of $CD16^+$ monocytes in sepsis [137] and recently it seems that intermediate is the subset expanded [146]. Concerning the cytokine production, it was already known the

positive association of both pro-inflammatory and anti-inflammatory cytokines with CD16$^+$ monocytes [144, 133,131, 137] but the exact source of these cytokines *in vivo* and the precise contribution of the intermediate and non-classical subsets remain unclear. It was also reported an expansion of intermediate monocytes in tuberculosis (TB) [147],

Castano *et al.* found an increased number of CD16$^+$ monocytes with a more significant expansion of intermediate monocytes than the non-classical subset [138]. The ex-vivo characterization of monocytes subsets form patients with TB showed that the expression of many markers related to maturation, differentiation and function are similar to HD except for higher CD11b and lower HLADR in the non-classical subset of TB patients [138]. Probably CD11b is able to enhance intracellular survival of the *M. tuberculosis* [148], while low HLA-DR expression suggests reduced antigen processing and presentation capability of the expanded subset [149]. On the other hand, during the infection, the CD16$^+$ monocytes produced more TNF-α [138], which has been associated with the control of TB [150], even if it is not known which CD16 $^+$ subset is the main producer of TNF-α.

Also in several viral infections, such as HBV, HCV and HIV, it was reported an expansion of both non classical and intermediate monocytes [151–153]. This expansion is most likely detrimental for HIV patients in that CD16$^+$ monocytes express higher level of CCR5 and CD4 [153-155] and therefore are more permissive to infection by HIV than the classical monocytes. Moreover, the number of intermediate monocytes correlates positively with the increased plasma viral load and decreased CD4$^+$ T cells [153]. As a result of their greater permissiveness to HIV infection, it was strongly suggested that CD16$^+$ monocytes may be acting as Trojan horses, playing an important role in viral dissemination [156, 157]. The increased number of CD16$^+$ monocytes in HIV infection could be explained by their enhanced survival due to the fact that infected monocytes exhibit an anti-apoptotic gene signature [158]. Similarly to HIV, also HCV infect both intermediate and non-classical but not classical monocytes [159] probably due to the higher expression of CD81 in these two subsets compared to the classical monocytes [159]. In fact, CD81 has been reported previously as the primary receptor for HCV [160, 161]. Interestingly, monocytes infected with HCV seem facilitate HIV co-infection and vice versa [159, 162], and the intermediate monocytes were the ones that exhibit coinfection by both HCV and HIV, indicating that they are capable of supporting replication of both viruses [159].

Also in autoimmune disorders, such as rheumatoid arthritis (RA) [130, 141, 163, 164], Crohn's disease (CD) [165–167], it was reported a perturbation in monocyte subsets composition. Several studies showed the expansion of CD16$^+$ monocytes in RA [141,163] even if it remains unclear which is the principal subset

increased. Some studies reported an expansion of non-classical monocytes [164] and others of the intermediate monocytes [130] In addition, which monocyte subset is the main producer of the pro-inflammatory cytokine in RA patients is still an open question. Also in human inflammatory bowel disease (IBD) such as CD or ulcerative colitis (UC) it was observed an expansion of $CD16^+$ monocytes [165, 166]. In particular, Grip *et al* [167] showed in CD an increased number of intermediate monocytes and it is possible that also in UC [166] the subset expanded is the intermediate monocytes. In addition to autoimmune disease, also numerous inflammatory diseases present increased $CD16^+$ monocytes. Typically, several inflammatory diseases show an expansion of intermediate monocytes as in asthma, renal disease, stroke and hemodialysis [168-171], even if also non-classical monocytes can be increased as in the aseptic loosening of implants, coronary artery disease and periodontitis [172-174].

1.16 POLYMORPHONUCLEAR LEUKOCYTES

Polymorphonuclear neutrophils (PMN) are the most numerous cells of the innate immune system and play a key role in the first line of host defense against pathogenic organisms. PMN develop in the bone marrow from the myeloid hematopoietic system and share numerous characteristics with other myeloid cells such as monocytes [175]. PMN are short-lived, terminally differentiated cells and without an activating stimulus, such as inflammatory or infectious stimuli, survive only for a short time in the blood stream (5-90 hours) and then die spontaneously by apoptosis. During microbial infections, circulating microbial products and endogenous pro-inflammatory mediators favor PMN survival, a mechanism critical for their tissue accumulation and effectiveness against pathogens [176, 177]. In response to inflammatory stimuli, PMN migrate quickly from bloodstream to the infection site (24-48h lifespan), where they perform phagocytosis, granule exocytosis and the reactive oxygen species (ROS) production, also called oxidative burst. These processes are crucial for bacterial killing but might also cause tissue injury if excessive or inappropriate [175,178]. Neutrophils express a large number of cell surface receptors for the recognition of microbial invasion, capable to recognize microbial structures (such as TLRs or scavenger receptors). Other receptors (such as Fc-receptors) function as a link between innate immunity and the adaptive immunity, and yet other receptors recognize the inflammatory environment.

A natural consequence of this variety of surface receptors is that in PMN exist a large number of interconnected intracellular pathways activated by surface receptor that can be involved directly in the clearance of pathogens or can inform the cell about the environment or promote additional process

indirectly required for the elimination of pathogens (such as chemotaxis). Taken together, intracellular signal transduction processes provide large amount of complex information to support an efficient antimicrobial immune response. Malfunctions and defects in PMN can play a significant role in various primary immunodeficiencies. Numerous PMN defects have been identified in PIDs such as chronic granulomatous disease (CGD) [179], warts, hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome [180] and leukocyte adhesion deficiency syndromes (LAD) [181]. In particular, CGD patients display recurrent pyogenic infections and granulomatous inflammation caused by the loss of phagocyte superoxide production due to recessive mutations of the four genes encoding subunits of the phagocyte NADPH oxidase [179]. Mutations of CXCR4 are responsible for WHIM that is characterized by deficiency of circulating neutrophils and their accumulation in the bone marrow [180]. LAD syndromes are characterized by defects in adhesion process of PMN and monocytes that results in severe infectious such as omphalitis, pneumonia, gingivitis, and peritonitis and absence of abscess [181]. Neutropenia is also observed in patients with X-linked IgM syndrome (XHIGM), a rare immunodeficiency due to mutations of the gene encoding for CD40L on chromosome X, characterized by normal or elevated serum IgM, reduced levels of IgG, IgA and IgE and defective T cell functions [182]. However, the role of PMN in CVID and XLA has not been fully elucidated. Several abnormalities of innate immune system have been reported in CVID patients, such as defect in differentiation and maturation of dendritic cells (DC) [183, 184], reduced absolute numbers of NK cells [57] and also neutropenia [185]. However, phenotype and functions of PMNs in these patients have not been fully elucidated.

In XLA, although BTK expression is abundant in neutrophils, patients under IVIg therapy are generally healthy [186,187], suggesting that BTK is dispensable outside the B cell compartment. In a recent study, it was demonstrated that TLRs activation in XLA neutrophils is able to induce a normal activation of ERK and JNK kinases and a respiratory burst comparable to that observed in normal controls, probably because other Tec kinase were able to compensate the BTK deficiency [188].

AIM OF THE STUDY

2. AIM OF THE STUDY

Intravenous immunoglobulins (IVIgs), prepared from the collective plasma of several thousand donors are used in treatment of a broad spectrum of diseases. IVIg infusion, administered at replacement dosage, is the standard therapy for primary antibody deficiencies (PAD) such as CVID and XLA, aiming to replace the missing antibodies and thereby to prevent recurrent infections [54,74]. IVIg are also widely used as anti-inflammatory therapy in a variety of acute and chronic autoimmune diseases; indeed, when administered at high dosages they exert an anti-inflammatory effect as demonstrated in different conditions like Kawasaki disease or Guillain-Barré syndrome. *In vitro* studies showed that IVIg has diverse effects on the immune system [189–191]. Poor information is available on the *in vivo* effects of IVIg administered at replacement dosages on human innate immune cells, including the monocyte/macrophage system [192]. Only recently, the mechanism by which IVIg mediated the effects on polymorphonuclear leukocytes (PMN) has been studied [193]. A recent study in patients with Common Variable Immune Deficiency (CVID) showed that IVIg infusion decreased the number of non-classical pro-inflammatory monocytes *in vivo* and suppressed the production of pro-inflammatory cytokines such as TNF-α in response to lipopolysaccharide (LPS) *in vitro* [194]. Moreover, IVIg untoward reaction has been also ascribed to neutrophils involvement in that IVIg might trigger PMN activation and degranulation [195–197]. On the other hand, other studies showed that IVIg might dampen the overall activity of PMN by inducing apoptosis [198–200], by decreasing the pro-inflammatory activity [201] and by inhibiting PMN degranulation [202]. The different effect of IVIg on PMN activity depend by the dose administered. IVIg were able to activate PMN functions and prolong their survival *in vitro,* when tested at low concentration, while at higher concentration IVIg inhibit PMN activation in response to LPS and reduce the LPS-induced prolongation of lifespan [193]. However, caution should be used in that the different procedures used for PMN isolation might differently modulate cell responses, providing divergent results [203–205].

In light of these previous observations, mainly conducted *in vitro*, in this study we analyzed, in a cohort of CVID patients, the phenotype and functions of PMN and monocytes by the study of the expression of a panel of surface receptors, involved in cellular functionality, and by the study of oxidative burst and the phagocytic ability with the aim to understand if CVID patients display an altered innate immune compartment and if the IVIg infusion might affect monocytes and PMN functions. The effect of IVIg was evaluated by studying the PMN and monocytes before and after administration of IVIg *in vivo*. In detail, it was analyzed the expression of:

- CD11b: a component of the phagocytic receptor αMβ2 or CD11b/ CD18. This receptor is expressed on human polymorphonuclear (PMN) leukocytes, NK cells and mononuclear phagocytes and it is involved in adhesion and phagocytosis process [206].

- CD11c: integrin molecule member of αXβ2 receptor that overlaps the properties of αMβ2 integrin in the adherence of neutrophils and monocytes to stimulated endothelium and in the phagocytosis of complement coated particles, overexpressed in inflammatory diseases such as rheumatoid arthritis (RA) [207, 208].

- Siglec 9: member of transmembrane sialic acid-binding proteins CD33-related, involved in adhesion process, broadly expressed on human blood phagocytes before IVIg [209,210], with a postulated inhibitory activity on the immune response through host and bacterial sialoglycans recognition [16,17]

- CD181: a high affinity receptor for interleukin 8. Its activation induces (primarily in neutrophils) chemotaxis and also phagocytosis through the increase of intracellular Ca^{2+}, exocytosis and the respiratory burst. [211]

- CD66b: granulocyte activation marker involved in neutrophils cell adhesion, cell migration and pathogen binding [212,213]. In addition, monoclonal antibodies against the CD66 family members trigger an activation signal that regulate the adhesive activity of CD11b/CD18 [214]. CD66b is able to stimulate neutrophils and its crosslinking is able to induce the secretion of preformed interleukin-8 (IL-8) [215].

- CD16: low affinity Fc receptor (also called FcγRIIIA). Since IgG FcγRs contribute to the anti-inflammatory action of IVIg [216] and that CD16 receptor is involved in the mechanism of CD11b expression [217], both CD16 and CD11b may play *in vivo* critical roles in inflammatory responses [218]

In order to understand if the ability of innate immune cells, from CVID patients, to respond to bacterial stimulation is fully preserved, we also studied the expression of all these receptors after providing opsonized *E. coli* as stimulus before and after the IVIg infusion. In monocytes, the expression of these receptors was analyzed on all the three subsets. Moreover, considering the relationship between the expression of adhesion molecules and oxidative burst [219], we studied on PMN and monocytes the oxidative burst (also known as respiratory burst), a crucial reaction which consist in the rapid release of reactive oxygen species (ROS), necessary to degrade internalized particles. Given the dependence of the oxidative burst by phagocytosis, we also analyzed the phagocytic ability of monocyte and PMN with the purpose to understand whether phagocytosis is altered in CVID patients. The study of these processes provides insights on the overall killing ability of PMN and monocytes and hence on their ability to respond effectively to infections. In order to better understand the *in vivo* effect on PMN and monocytes of the immunoglobulins administration, we evaluated these processes also after IVIg infusion. In addition, we replicated the study on XLA which patients who receive an IVIg treatment similar to that of CVID patients. These experiments might be useful to understand whether the results obtained in CVID are also replicable in other PIDs. Moreover, considering that calcium mobilization is involved in ROS production and that FcγR clustering induce Ca^{2+} mobilization through the axis BTK-PLCγ2, it is possible that the lack of BTK in XLA patients could affect the calcium flux from internal storage and so compromise the ROS production [112]. Because numerous receptors utilize BTK as intracellular mediator, XLA patients can present defective responses of immune cells to activating stimuli; indeed, it was shown that BTK inhibitor could block degranulation and FcγR-mediated cytokine production in monocytes/macrophages [112]. Considering that calcium mobilization is the main event downstream the BTK activation as a result of FcγR clustering, we investigated if in XLA patients the calcium chelation may influence the ROS production and phagocytosis in monocytes and PMN.

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 CVID PATIENTS

Twenty-three CVID patients (12 males and 11 females; age range of 15–74 years; mean age: 50 ± 16.8 years) diagnosed according to the criteria of the European Society for Immune Deficiencies (http://www.esid.org) were enrolled in the study. All CVID patients were on replacement treatment with a cumulative monthly dosage of 300–400 mg/kg of IVIg administered every two-three weeks (Supplementary Table 1). Seventeen healthy donors (HD) (8 males and 9 females; age range 24–65 years; mean age 42.6 ± 13.6 years) were included as controls. The infusion time ranged from 1.5 h to 3 h. The infusion speed was established according to the individual tolerability. The mean IVIg dose administered on the day of blood sampling was 28.9 ± 3.6 g. None of the patients was on steroids or immunosuppressive drugs at the time of the study. All subjects provided informed consent for blood sampling and processing, in accordance with the Institutional Review Board of the Sapienza University of Rome and with the Declaration of Helsinki.

3.2 XLA PATIENTS

Six male patients with XLA (age range of 20–60 years; mean age: 36.7 ± 15.4 years) and ten healthy donors (HD) male (age range of 27–58 years; mean age: 39.6 ± 9.8 years) were enrolled for the study. Patients were in a clinically stable situation without fever and not hospitalized. XLA was diagnosed according to the criteria of the World Health Organization scientific group for PIDs: mutation in BTK, absent BTK mRNA, absent BTK protein in monocytes or platelets, low levels of circulating B cells (measured by levels of $CD19^+$ positive cells in blood samples), decreased or absent immunoglobulins in serum and a typical clinical history with recurrent bacterial infection or a positive family history. XLA patients were on replacement treatment, with monthly dosage of 400-600 mg/kg of IVIg administered every three-four weeks (Supplementary Table 2). The infusion time ranged from 1.5 hours to 3 hours. The infusion speed was established according to the individual tolerability. All subjects provided informed consent for blood sampling and processing, in accordance with the Institutional Review Board of the Sapienza University of Rome and with the Declaration of Helsinki.

3.3 BLOOD SAMPLES PREPARATION

Heparinized whole blood samples were collected immediately before IVIg administration and within one hour after the infusion. These time points were chosen based considering that the highest increase of several cytokines plasma concentration occurs within one hour after IVIg infusion [220]. Total peripheral blood neutrophils and monocytes count was determined from blood cell counts and white blood cell differentials. For evaluating circulating cell without the harm of cell loss related to the density gradient centrifugation procedure, peripheral blood was lysed using lysing buffer (Becton Dickinson, BD) for 15 min at room temperature. Samples were washed twice for the following staining.

3.4 ISOLATION OF PMN

In a subgroup of CVID patients (n = 12) and HD (n = 8) isolation of PMN was performed using a double gradient formed by layering an equal volume of Histopaque-1077 over Histopaque-1119 (Sigma-Aldrich). Anticoagulated whole blood was layered onto the upper Histopaque-1077 and centrifuged at room temperature. The supernatant, plasma and remaining fluid were discarded and the PMN suspension (lower layer), transferred to a new tube and washed with isotonic phosphate buffered saline (PBS). The cells were centrifuged, washed by addition of PBS solution and suspended in PBS solution.

3.5 PHENOTYPIC ANALYSIS OF MONOCYTE SUBPOPULATIONS

Total peripheral blood monocyte count was determined from blood cell counts and white blood cell differentials. Peripheral blood were lysed with a fixed volume of Becton Dickinson lysing buffer lysed for 15 min at room temperature, washed twice and stained (at 4°C for 30 min) with combinations of fluorochrome-labeled antibodies. Monocytes subpopulations were phenotypically identified using anti-CD45, HLA-DR, CD14, and CD16 mAbs (BD, Becton- Dickinson Biosciences, Franklin Lakes, NJ), gating on $CD14^{+}$ $HLA\text{-}DR^{+}$ monocytes and then classified according to their expression of CD14 and CD16 into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non classical monocytes ($CD14^{+}CD16^{++}$) using a 4-color flow cytometry single platform assay. An isotype control (IgG1, BD) was run in parallel in order to set a threshold for distinguish intermediate monocytes ($CD16^{+}$) from classical monocytes ($CD16^{-}$). Monocyte subpopulations were then analyzed in parallel for the expression of surface receptors CD181, CD11b, CD11c and Siglec 9 using various combinations of fluorochrome-labeled antibodies. The corresponding isotype controls (IgG1, BD) for each receptor was run in parallel. 30.000 events were counted per sample. Flow cytometric analysis

was done with a FACSCalibur instrument (BD) using CellQuest (BD) and FlowJo (TreeStar, Ashland, Ore) software. Results were expressed as geometric mean Fluorescence Intensity (MFI) of any given marker within the defined population. All antibodies were obtained from BD Biosciences.

3.6 ANALYSIS OF RECEPTORS EXPRESSION ON PMN

The expression of CD181, CD66b, CD11b, CD11c, CD16 and Siglec 9 was evaluated on PMN from whole blood samples lysed for 15 min at room temperature and performing a staining at 4°C for 30 min with combinations of fluorochrome-labeled antibodies. Samples were washed, suspended in ice cold PBS and analyzed by flow cytometry. Whereas the conditions in which the PMN are analyzed seem to influence their responses [203-205] we verify the differences between whole blood condition and isolation PMN in terms of receptors expression, therefore in a subgroup of patients we performed the analysis of CD11b, Siglec 9 and CD16 expression both in whole blood condition and on isolated PMN (1-2 x 10^5cells). In CVID patients PMN were identified by forward scatter (FSC) and side scatter (SSC) characteristics and gating on CD14 negative events in order to exclude monocytes (Supplementary Fig. 1A–D). In parallel, we performed the same procedure using CD15 antibody to further identify neutrophils (Supplementary Fig. 1E–H). In XLA patients PMN were identified by forward scatter (FSC) and side scatter (SSC) characteristics and gating on CD15 positive events (Supplementary Fig. 3). 30.000 events were counted per sample. Flow cytometric analysis was done with a FACSCalibur instrument (BD) using CellQuest (BD) and FlowJo (TreeStar, Ashland, Ore) software. Results were expressed as geometric mean Fluorescence Intensity (MFI) of any given marker within the defined population. All antibodies were obtained from BD Biosciences

3.7 MONOCYTES AND PMN STIMULATION BY ESCHERICHIA COLI

100 µL of whole blood, collected before and after IgG administration, were added to 20 µl of pre-cooled opsonized not labeled whole Escherichia coli (*E. coli*) at a concentration of 1-2x10^9/ml (Glycotope, Biotechnology). Samples were incubated in water bath for 20 min at 37°C and lysed for 15 min at room temperature. Cells stimulated were stained at 4°C for 30 min with fluorochrome-conjugated antibodies in various combinations to evaluate the expression of the receptors described above. 30.000 events were counted per sample. Flow cytometry analysis were done with a FACSCalibur instrument using CellQuest and FlowJo software.

3.8 MONOCYTES AND PMN OXIDATIVE BURST ACTIVITY

The evaluation of leukocyte oxidative burst was determined with PHAGOBURST assay (Glycotope, Biotechnology), according to the manufacturers' instructions. Whole blood samples (100 µl) were incubated in a water bath for 20 min at 37°C with opsonized *E. coli* (1-2x10^9/ml)). For PMN we provided also PMA (1.62 mM) as strong stimulus. The intracellular production of superoxide anions and hydrogen peroxide in monocytes and neutrophils in response to phagocytosis of bacteria was tested by using of the fluorescence probes DHR 123. The amount of ROS produced was reported as MFI. In addition, we performed in XLA patients the study of oxidative burst also using BAPTA-AM (Unimed Scientifica) as calcium chelator, in order to verify the Ca^{2+}-dependence production of ROS triggered by FcγR stimulation. Whole blood samples were pre-treated with 100 µM of the calcium chelator BAPTA-AM for 30 min and then they were incubated in a water bath for 20 min at 37°C with opsonized *E. coli* (1-2x10^9/ml) according to the manufacturers' instructions of PHAGOBURST assay.

FSC and SSC characteristics were used to identify the PMN population and to gate out other cells or debris. 30.000 events were counted per sample. The oxidation lead to fluorescence detected by flow cytometry, using the blue-green excitation light (488 nm argon-ion laser).

3.9 MONOCYTES AND PMN PHAGOCYTOSIS

The quantitative determination of leukocyte phagocytosis was determined with PHAGOTEST assay (Glycotope, Biotechnology), according to the manufacturers' instructions. Whole blood samples (100 µl) were incubated in a water bath for 10 min at 37°C with FITC-labeled *E. coli* (2x10^9/ml). The percentage of cells having performed phagocytosis (granulocytes and monocytes) was analyzed as well as their MFI (number of ingested bacteria). In addition, we tested in XLA patients, the effect of calcium chelator on phagocytosis in order to verify the Ca^{2+}-independence of the process. Whole blood samples were pre-treated with 100 µM of the calcium chelator BAPTA-AM for 30 min and then they were incubated in a water bath for 10 min at 37°C with FITC-labeled *E. coli* (2x10^9/ml) according to the manufacturers' instructions of PHAGOTEST assay. FSC and SSC characteristics were used to identify the monocytes and PMN populations and to gate out other cells or debris. 30.000 events were counted per sample. Cells were analyzed by flow cytometry using the blue-green excitation light (488 nm argon-ion laser).

3.10 STATISTICAL ANALYSIS

Data were analyzed using the Mann-Whitney U test for unpaired two groups or by paired Wilcoxon test. Correlations were calculated by means of linear regression analysis. Data were analyzed with StatView 5.0.1 software (SAS Institute, Cary, NC). A p value equal or less than 0.05 was considered as statistically significant.

RESULTS

4. RESULTS

4.1 IDENTIFICATION OF MONOCYTE SUBSETS

The expression of CD14 and CD16 receptors allowed identifying three monocyte subsets [11]: classical monocytes showing high CD14 expression and the absence of CD16 expression ($CD14^{++}CD16^{-}$), intermediate monocytes showing a high level of CD14 with low CD16 expression ($CD14^{++}CD16^{+}$), and non-classical monocytes expressing a low level of CD14 with high CD16 expression ($CD14^{+}CD16^{++}$). Initially, a threshold was set on CD45 staining in order to exclude non-leucocyte events. In the $CD45^{+}$ side/scatter plot, a gate was set around monocyte population (Fig. 3A). Events in this gate were shown in a CD14/HLA-DR plot and here a gate was defined including $CD14^{+}$ and HLA-DR^{+} events (Fig. 3B). To determine the boundary between intermediate and classical monocyte subsets an isotype IgG control was used (Fig. 3C). Events in this gate were shown in a CD14/CD16 plot and here three different subsets of monocyte were identified (Fig. 3D-F).

4.2 MONOCYTE SUBSETS IN CVID PATIENTS AND HD. EFFECT OF IVIG INFUSION.

Absolute counts of total monocytes, of classical and non-classical monocytes did not show any significant difference between CVID, and HD. Data from a representative HD and from a CVID patient before and after IVIg infusion were shown in Fig. 3D–F. Classical monocytes were the prevalent monocyte subset in patients and HD (78,6 % ± 10,2 vs 77,9 ± 8,2 respectively) while intermediate monocyte frequencies were higher in CVID patients than in HD (11,4 ± 6,7 vs 6,1 % ± 1,1 respectively*, $p= 0.008$*) and non classical were lower in CVID patients than in HD (10,0 ± 5,3 vs 16,1 % ± 8,5 respectively $p= 0.04$) (Fig. 4). Within 1 h after IVIg infusion, while the absolute leukocytes count did not change, we observed an average reduction of 23% of the total monocyte population (table 4). However, this decrease was not uniform within monocytes subsets since the frequency of non-classical monocyte did not change (from 10,0 ± 5,3 to 7,7 ± 5,5 p= ns) (Fig. 4A), the percentage of intermediate monocytes decreased (from 11,4 ± 6,7 to 7,7 ± 5,4 $p= 0.002$) (Fig. 4B) reaching a value similar to that found in HD and the percentage of classical monocytes increased (from 78,6 ± 10,2 to 84,6 ± 8,1 p= 0,009) (Fig. 4C) (table 4).

	Total leukocytes (cells/mm³)	Total monocytes (cells/mm³)	Classical monocytes (cells/mm³)	Intermediate monocytes (cells/mm³)	Non classical monocytes (cells/mm³)
HD	7000±2375	400±12	311±33	24.3±4.4	64.3±34
CVID before IVIg	6190±2290	383.3±124.5	307±122.1	40.1±20.3▲*	36±18
CVID after VIg	5994±2258	294±95.5•***	250±88.1•***	21.1±13.5•***	22.9±19.4•**

Table 4. Peripheral blood monocytes, and monocyte subsets in healthy donors, patients with CVID before IVIg and 1 h after IVIg administration in vivo. Results are expressed as absolute counts. p values are shown for absolute numbers: p▲: HD vs CVID before IVIg; p●: CVID before IVIg vs CVID after IVIg. *p <0.05; **p<0.01; ***p<0.001.

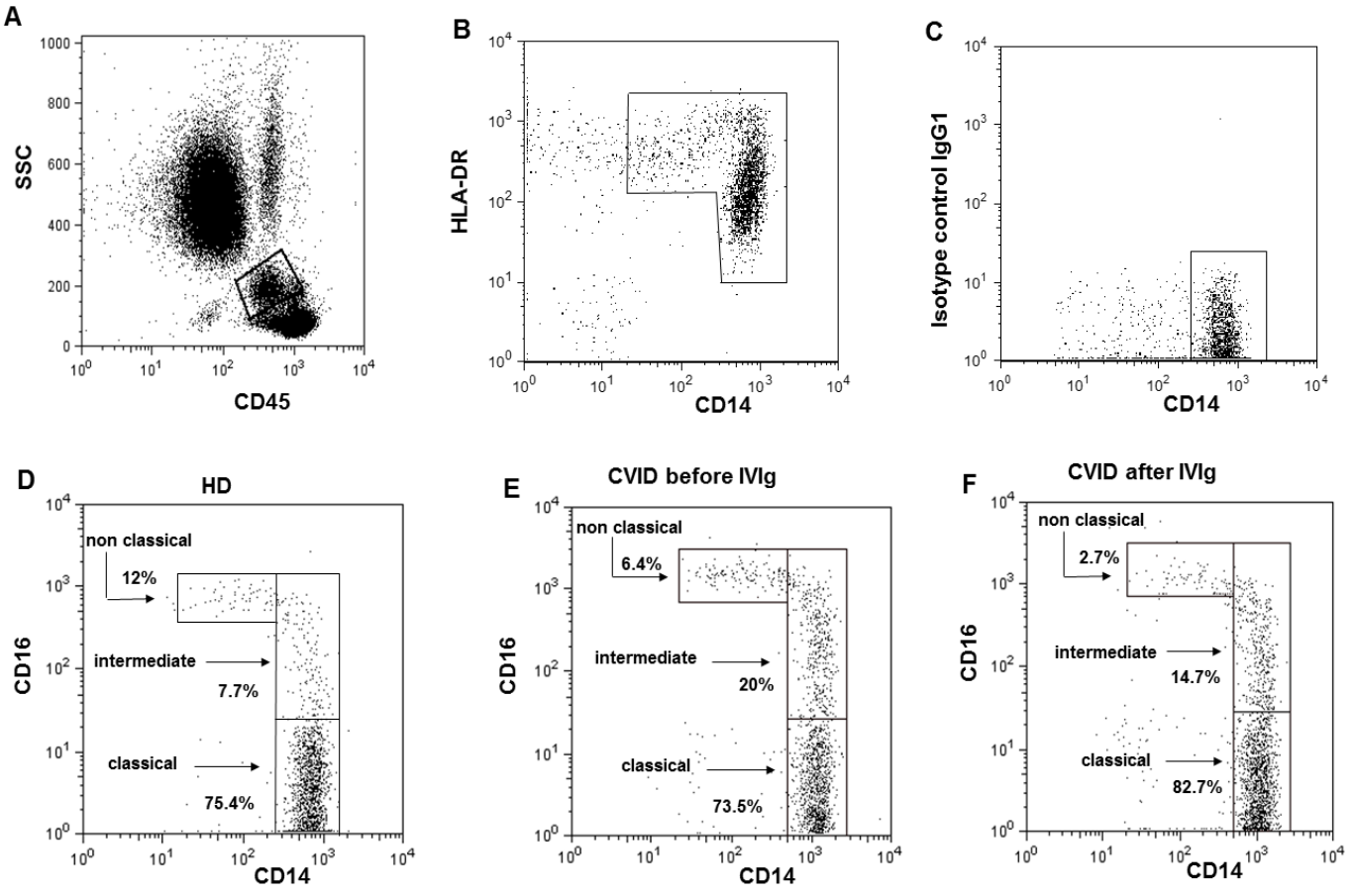


Figure 3 Identification of monocyte subpopulations. Plot A: CD45+monocytes and adjacent lymphocytes, including NK cells. Cells were gated to exclude CD14-/HLA-DR (plot B, cells outside the gate). To determine the boundary between intermediate and classical monocyte subsets an isotype control was used (plot C). Monocyte subsets were analyzed for CD14 and CD16 expression in a representative healthy donor (plot D) and in a CVID patient before (plot E) and after IVIg infusion (plot F). Percentages denote mean values.

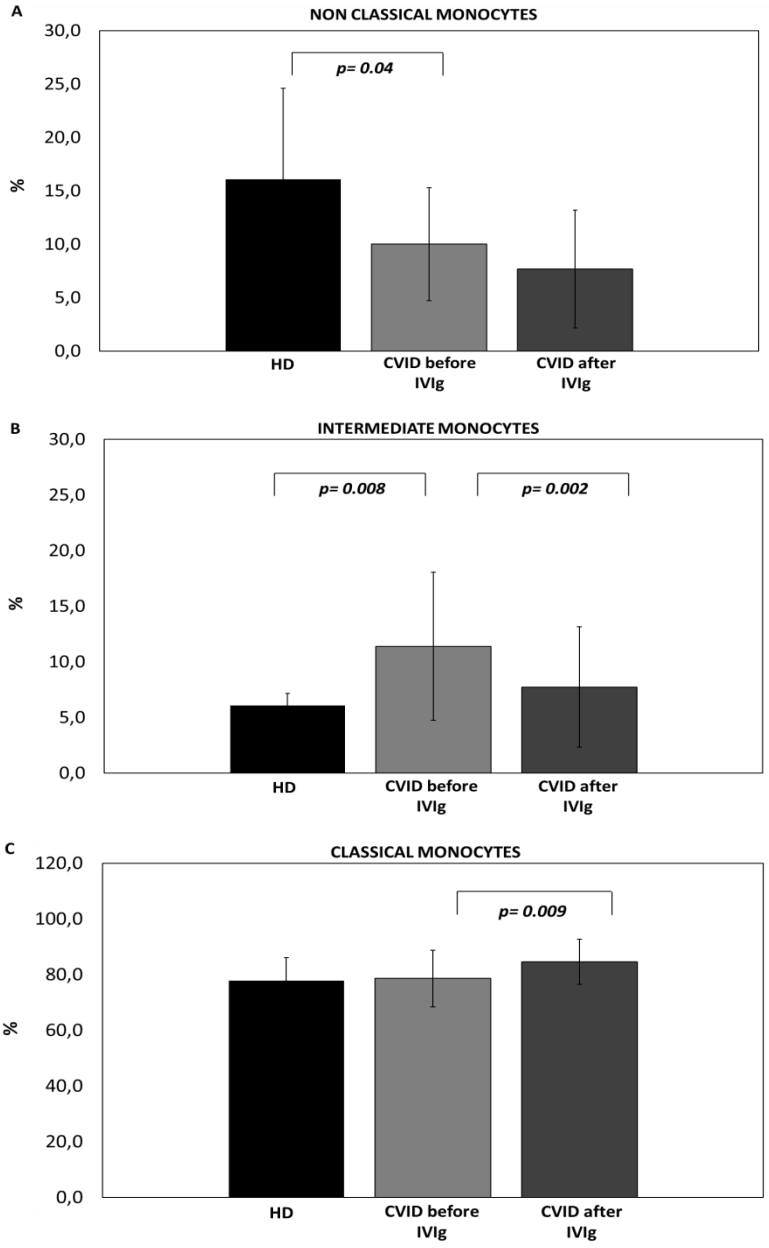


Figure 4. Frequencies of non-classical (A), intermediate (B) and classical (C) monocytes. Data show monocyte frequencies (expressed as % of total monocytes) from blood samples collected immediately before and within 1 h after IVIg infusion of HD and CVID patients. Results are expressed as percentages. Bars denote standard deviation.

4.3 RECEPTORS EXPRESSION ON MONOCYTES IN CVID PATIENTS AND HD. EFFECT OF IVIG INFUSION

In CVID and HD, the expression of CD11b was higher in classical than in intermediate monocytes, while it was almost undetectable in non-classical monocytes (Fig. 5 A). A significant difference in CD11b expression between HD and CVID was evident in the classical monocyte subset (58 ± 12.7 MFI vs 93.8 ± 25.9 MFI respectively *p= 0.006*), while no difference was observed in intermediate monocytes (38.3 ± 20.3 MFI vs 45.5 ± 22.6 MFI respectively) and non-classical monocytes (2.9 ± 1.4 MFI vs 2 ± 0.7 MFI respectively). After IVIg, CD11b expression on classical monocytes decreased (from 93.8 ± 25.9 MFI to 69.2 ± 30.7 MFI *p= 0.006*) and returned to values similar to those observed in HD. The CD11b expression on intermediate and non-classical monocytes did not change after IVIg (from 45.5±22.6MFI to 46.3±26.8 MFI, from 2±0.8 MFI to 2.4±0.9MFI respectively) (Fig.5A).

The postulated inhibitory activity of Siglec 9 on the immune response through host and bacterial sialoglycans recognition [221,222] prompted us to verify its expression on monocyte subsets at baseline and after IVIg. In HD and in CVID, Siglec 9 expression was evident on all monocyte subsets, and it was higher on classical and on intermediate than on non-classical monocytes. A significant difference in Siglec 9 expression between HD and CVID was evident on the classical monocyte subset (93.4 ± 26.5 MFI vs 157.3 ± 89.8 MFI respectively *p= 0.04*). No difference was observed on intermediate monocytes (112.2±22.3MFI vs 146.6±82.4MFI respectively) and on non-classical monocytes (58.3 ± 10 MFI vs 64.1 ± 22.4 MFI respectively) (Fig. 5B). After IVIg, Siglec 9 expression on classical monocytes slightly decreased (from 157.3 ± 89.8 MFI to 132.2 ± 61.1 MFI, *p= 0.003*) but did not change on intermediate (from 146.6±82.4MFI to 141.8±62.2 MFI) and on non-classical subsets (from 64.1 ± 22.4 MFI to 59.8 ± 12.8 MFI) (Fig. 5B). All significant p values are shown in figures.

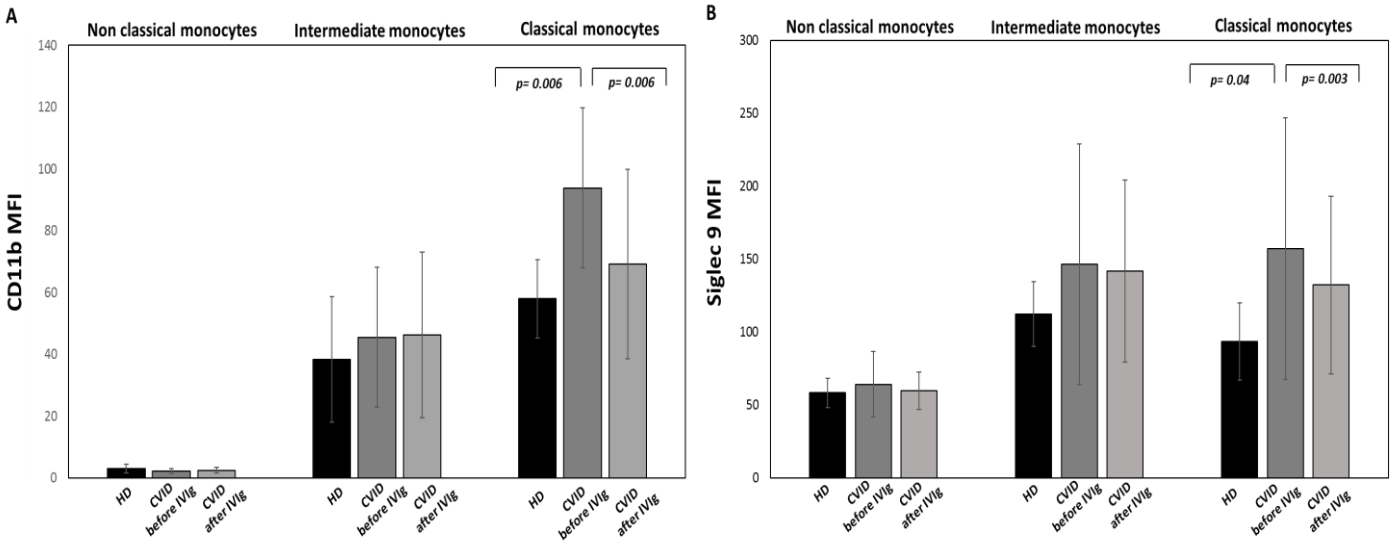


Figure 5. Effect of IVIg on CD11b (A) and Siglec 9 (B) expression in classical, intermediate and non classical monocytes of CVID patients. CD11b and Siglec 9 expression decreased after IVIg infusion. Bars denote the standard deviations.

The role of CD11c in adherence and phagocytosis process and its overexpression in inflammatory disease such as RA [207,208] led us to investigate its expression in CVID patients. Both in CVID and HD the expression of CD11c showed no significant differences among the three monocytes subsets (Fig. 6). Despite to what observed for CD11b, we did not find any difference in CD11c expression level between HD and CVID as shown in Figure 6 (non classical 48,8± 14,8 MFI vs 57,1± 35.2 MFI; intermediate 59,9 ± 24.3 MFI vs 65,4 ± 17 MFI; classical 40,2 ± 19.3 MFI vs 43.3 ± 7.9 MFI respectively). After IVIg, CD11c expression did not change in any monocytes subsets (non classical: from 57,1± 35.2 MFI to 59.2 ± 39.2 MFI; intermediate: from 65,4 ± 17 MFI to 64.0 ± 25.7 MFI; classical: from 43.3 ± 7.9 MFI to 40.2± 10.8 MFI).

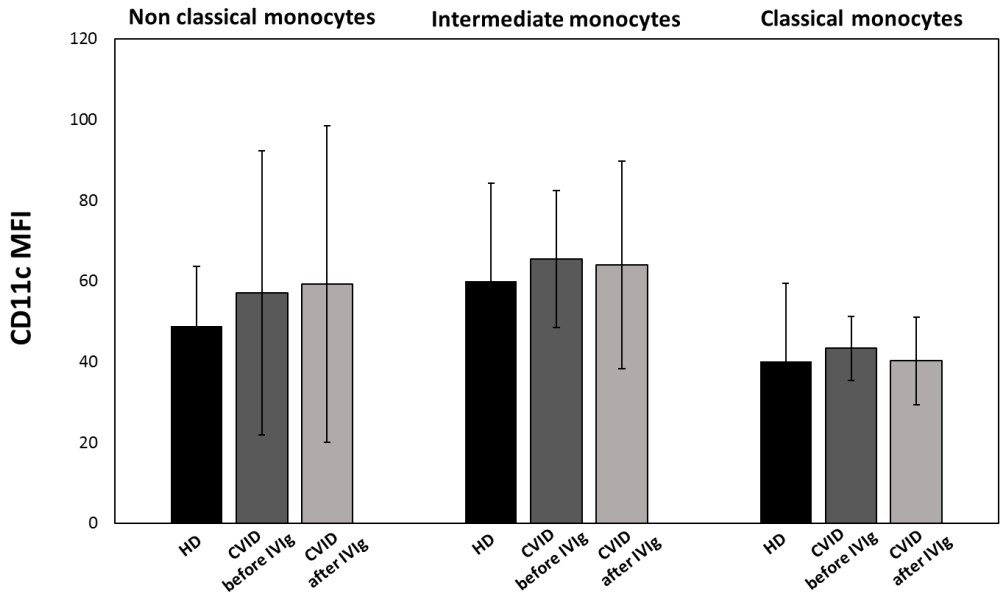


Figure 6. CVID patients show a similar CD11c expression on monocytes subsets compared to HD. After IVIg infusion the expression of CD11c on monocytes subsets remained unaltered. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation.

Since the role played by CD181 in monocytes activity, we evaluated its surface expression with the aim to obtain insights regards the activation status of monocytes and their responsiveness to IL-8. As shown in Figure 7 we found that, in all monocytes subpopulations, CD181 was expressed at similar level in HD and CVID (non classical 7 ± 1 MFI vs 7.5± 1.7 MFI; intermediate 9,8 ± 2.8 MFI vs 11 ± 3.5 MFI; classical 9,6 ± 1.7 MFI vs 9.9 ± 2.6 MFI respectively) and that IVIg administration did not induce any variation of its expression (non classical: from 7.5± 1.7 MFI to 7.4 ± 1.5 MFI; intermediate: 11 ± 3.5 MFI to 10.3 ± 4.1 MFI; classical: from 9.9 ± 2.6 MFI to 9.7± 2.8 MFI).

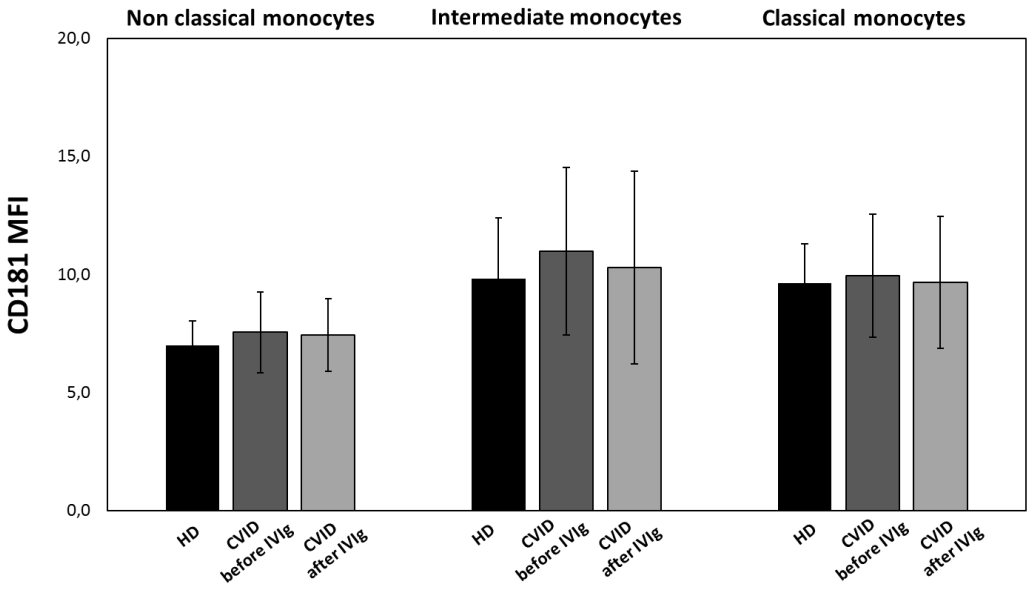


Figure 7. CVID patients show a similar CD181 expression on monocytes subsets compared to HD. After IVIg infusion the expression of CD181 on monocytes subsets remained unaltered. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation.

4.4 ESCHERICHIA COLI STIMULATION OF MONOCYTES SUBSETS

In order to assess if IVIg might affect the monocytes' ability to respond to opsonized *E. coli* in terms of receptors expression, we evaluated the expression of CD11b, CD11c, Siglec 9 and CD181 on the overall monocytes population. We found that monocytes from CVID patients were able to overexpress all the receptors analyzed after stimulation with *E. coli* and that these overexpression were similar to those observed in healthy donors. Moreover, we found that the overexpression of all receptors was preserved also after IVIg infusion. In detail: CD11b, HD from 57.1 ± 13.2 MFI to 129.5 ± 38.9 MFI, CVID before IVIg: from 80.4 ± 23.9 MFI to 125.7 ± 28.5 MFI, CVID after IVIg: from 67.2 ± 31.8 MFI to 126 ± 36.9 MFI, (Fig. 8); Siglec 9, HD: from 123.5 ± 86.6 MFI to 168.7 ± 62.9 MFI, CVID before IVIg: from 129.6 ± 23.1 MFI to 174.1 ± 31.4 MFI, CVID after IVIg from 116.2 ± 24 MFI to 172.6 ± 23.4 MFI, (Fig. 9); CD181, HD: from 9.7 ± 1.2 MFI to 26.4 ± 8.7 MFI, CVID before IVIg: from 9.0 ± 1.6 MFI to 24.5 ± 5.0 MFI, CVID after IVIg from 9.6 ± 2.9 MFI to 26.0 ± 3.7 MFI, (Fig. 10); CD11c, HD: from 37.0 ± 18.5 MFI to 97.6 ± 37.1 MFI, CVID before IVIg: from 41.3 ± 12.6 MFI to 104.0 ± 23.2 MFI, CVID after IVIg from 41.6 ± 10.7 MFI to 109.3 ± 23.1 MFI, (Fig. 11). All p values are shown in figures.

Finally, we also studied how the overexpression of these receptor, induced by opsonized *E. coli*, was distributed among the three monocytes subsets finding that it occurred in all subsets except the expression of Siglec 9 on non classical monocytes that do not seem to be induced by the stimulus, suggesting that non classical monocytes could not be mainly involved in phagocytosis (considering the damping effect of Siglec 9 when it recognize sialic acid residues), although for all other receptors we observed the overexpression even in non classical subset (Tab. 5). The receptors' overexpression seem was preserved also after IVIg infusion showing that IVIg replacement did not affect the PMN ability to respond to opsonized bacteria (Tab.5)

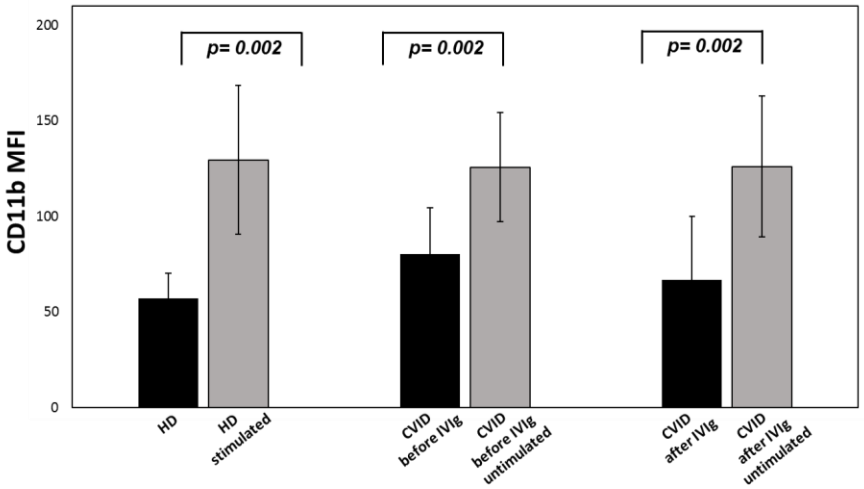


Figure 8. Effect of E. coli stimulation on CD11b expression in HD and CVID patients before and after IVIg infusion. CD11b expression increased after E. coli stimulation before IVIg infusion as well as 1 h after IVIg. Bars denote the standard deviations.

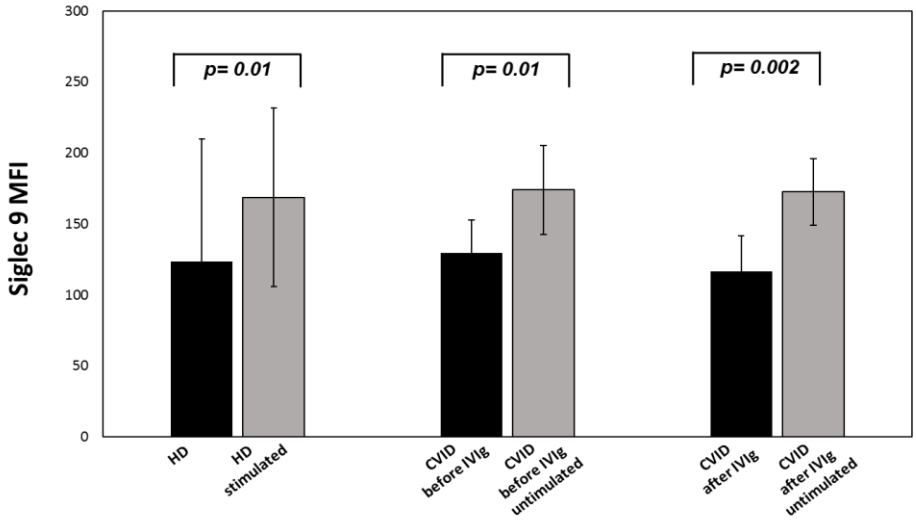


Figure 9. Effect of E. coli stimulation on Siglec 9 expression in HD and CVID patients before and after IVIg infusion. Siglec 9 expression increased after E. coli stimulation before IVIg infusion. Siglec 9 overexpression was preserved after the infusion. Bars denote standard deviations.

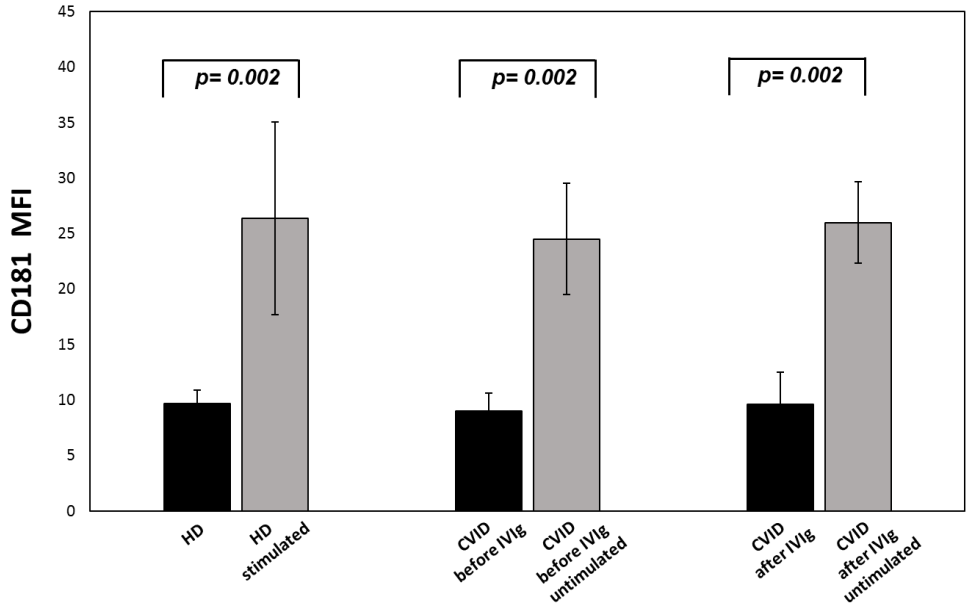


Figure 10. Effect of E. coli stimulation on CD181 expression in HD and CVID patients before and after IVIg infusion. CD181 expression increased after E. coli stimulation before IVIg infusion. CD181 overexpression was preserved after the infusion. Bars denote standard deviations.

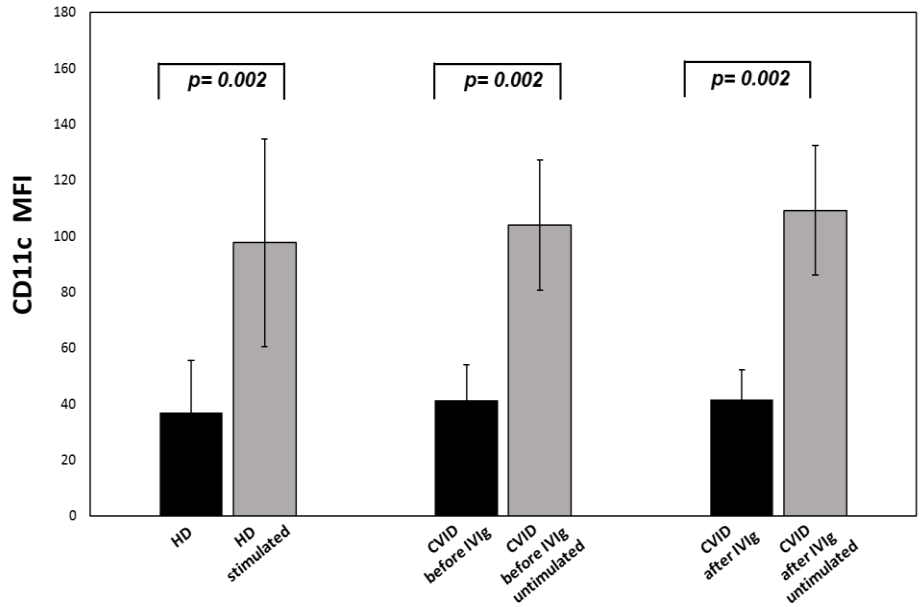


Figure 11. Effect of E. coli stimulation on CD11c expression in HD and CVID patients before and after IVIg infusion. CD11c expression increased after E. coli stimulation before IVIg infusion. CD11c overexpression was preserved after the infusion. Bars denote standard deviations.

4.5 RECEPTORS EXPRESSION ON PMN IN CVID PATIENTS AND HD AND *E. COLI* STIMULATION. EFFECT OF IVIG INFUSION

The activation status of PMNs is strongly influenced by their ability to perform phagocytosis, respiratory burst and adhesion processes, therefore we evaluated the expression level of CD181, CD66b, CD11b, CD11c, CD16 and Siglec 9 receptors. As shown in Fig 12 PMN from CVID and HD showed an overlapping expression of all receptors suggesting that unlike monocytes, PMNs of CVID patients exhibit a normal phenotype. After IVIg infusion the expression of all receptors remained unaltered except the expression of CD181 that decrease after IVIg infusion (*p=0.0005*) (Fig 12, Table 6). Moreover, we found that PMN stimulation by opsonized *E. coli* induced an overexpression of CD66b, CD11b, CD11c, CD16 and Siglec 9 receptors both in CVID and in HD and that also after IVIg infusion the overexpression was preserved (Table 6) suggesting that the pathway by which these receptors were overexpressed, through the clustering of FcγRs, was intact in CVID and that IVIg do not alter in any way the process. On the contrary and in line with previous studies [223] we found a decreased expression of CD181 after stimulation with *E. coli* both in HD and CVID and the reduction of CD181 expression was confirmed also after IVIg infusion (table 6).

Finally, we found that the overexpression of CD11b induced by *E.coli*, although occur both in patients and in HD, it is higher in CVID (5.2 ± 6.5 vs 20.2 ± 21.1 respectively). The reason for the greater overexpression of CD11b in CVID is attributable to only 8 patients who responded excessively to the stimulation, however, we did not find an association between clinical data and receptors expression.

		HD			CVID before IVIg			CVID after IVIg		
Receptor		Non classical	Intermediate	Classical	Non classical	Intermediate	Classical	Non classical	Intermediate	Classical
CD11b (MFI)	UN	2.9±1.4	38.3±20.30	58±12.7	2±0.7	44.2±23.1	90.9±27.0	2.3±0.7	45.95±27.9	65.8±31.3
	ST	10±5.8 †	103.3±31 †	129.4±39.4 †	5.6±3.2 •	93.4±26.5 ‡	126±28.9 *	5.8±3.3 ▪	99.4±46.2 ‡	121.7±44.5 ‡
CD181 (MFI)	UN	7,0±1	9,8±2.6	9,6±1,7	7,5±1,7	11,0±3,5	9,9±2,6	7,4±1,5	10,3±4,1	9,7±2,8
	ST	31,8±2,1 ‡	34,0±2,1‡	26,1±2,1‡	23,6±5,2‡	28,4±4,3‡	25,4±5,6‡	26,4±3,8‡	28,4±3,1‡	24,3±3,4‡
CD11c (MFI)	UN	48,8±14,8	59,9±24,3	40,2±19,3	57,1±35,2	65,4±17,0	43,3±7,9	59,2±39,2	64,0±25,7	40,2±10,8
	ST	104,7±43,4‡	115,3±57,9‡	105,8±47,7‡	114,4±40,2‡	140,4±19,4‡	111,7±23,8‡	112,6±32,5‡	116,7±20,9‡	103,6±28,1‡
Siglec9 (MFI)	UN	57.5±9.9	103.8±25.9	93.4±26.5	64.1±22.5	146.6±82.4	159.6±93.4	59.8±12.8	141.8±62.2	131.8±66.1
	ST	47±15.3	138.7±35.1†	150.7±37.8†	75.3±32.3	168.2±89.8 †	202.2±67.8◦	65.0±27.6	173.6±57.0‡	188.1±54.5‡

Table 5 Receptors expression on monocytes' subsets from HD and CVID patients. Values are expressed as Mean Fluorescence Intensity (MFI). UN: Unstimulated; ST: Stimulated with E. coli. ‡ *p= 0.002* • *p= 0.003* * *p= 0.01* ▪ *p= 0.005* † *p= 0.02* ◦ *p= 0.007*

Receptor		HD	CVID before IVIg	CVID after IVIg
CD11b (MFI)	UN	1.3±0.3	1.7±1	2±1.4
	ST	5.2 ± 6.5 *	20.2 ± 21.1‡	22.8 ± 24.5‡
CD181 (MFI)	UN	133.2±22.7	139.5±26.0	117.5±30.3
	ST	111.5±33***	101.6±28.9***	92.2±21.9***
CD11c (MFI)	UN	21.8±12.1	19.4±6.2	18.3±9.0
	ST	47.7±17.8***	53.4±15.7●	46.8±20.5◊
Siglec 9 (MFI)	UN	49.7 ± 16.4	53.4 ± 8.6	52.2 ± 9.3
	ST	77.1 ± 25.6 *	83.1 ± 15.6‡	79.2 ± 14.8‡
CD66b (MFI)	UN	360.2±99.2	369.5±104.1	385.5±127.2
	ST	991.2±305.2***	1159±506.4◊	1073.7±499.0 ◊
CD16	UN	2826.4 ± 922.6	2428.3 ± 738.7	2274.2 ± 730.3
	ST	3246.1 ± 959.3 ◦	3092.3 ± 1103.9 ▪	2861.3 ± 874.7▪

Table 6. Receptors expression on PMNs from HD and CVID patients. Values are expressed as Mean Fluorescence Intensity (MFI) Statistical significance, determined by the nonparametric Wilcoxon Signed Rank, is indicated as p value * p=0.012 ‡ p= 0.0007 ◦ p=0.04 ▪p=0,003 ***p=0.001 ●p=0.0002 ◊p=0.0001

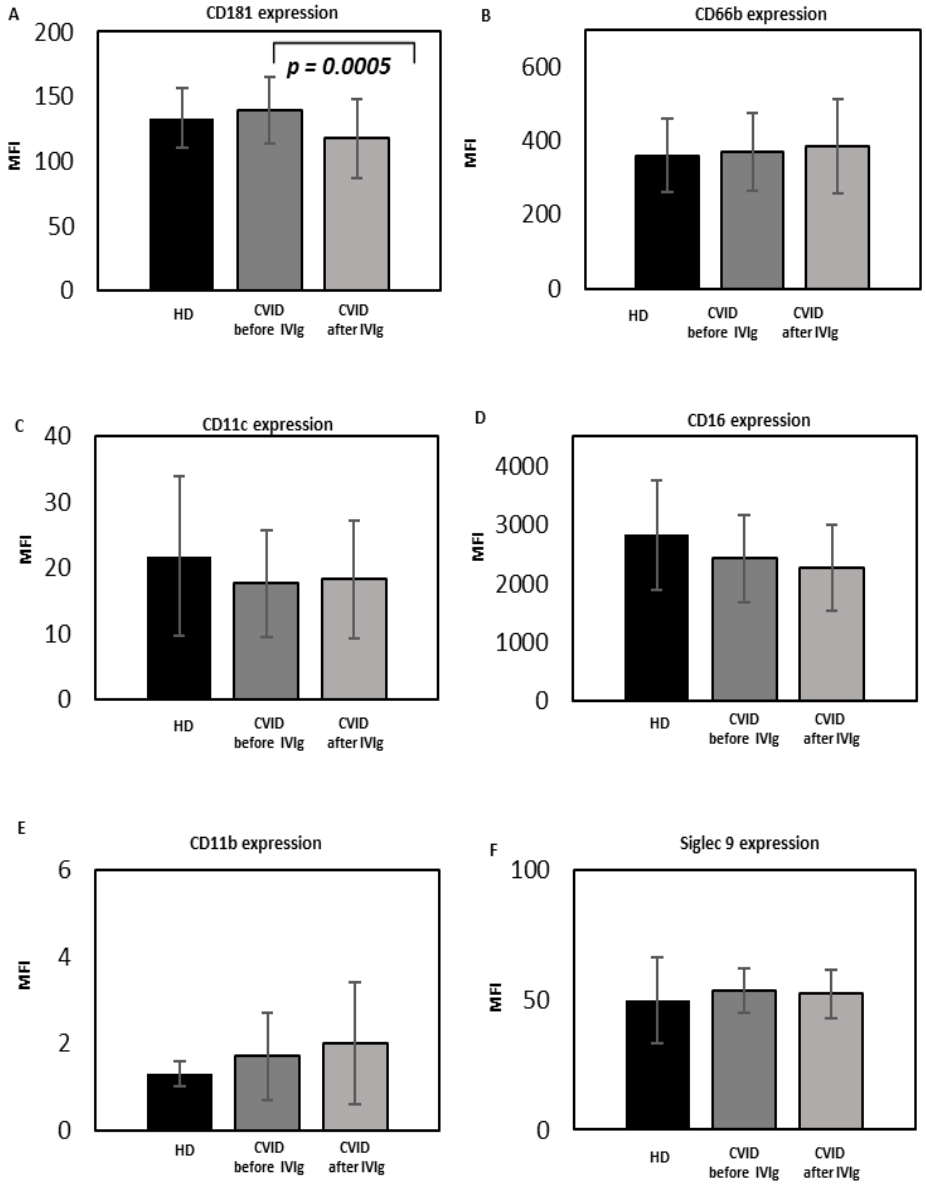


Figure 12. CD181, CD11b, CD11c and Siglec 9 expression on PMN from HD and CVID patients before and after IVIg infusion. Whole blood samples were analyzed for the expression of CD181, CD66b, CD11b, CD11c, CD16 and Siglec 9 before and after IVIg infusion. The expression of all surface receptors was evaluated by performing a staining at 4°C for 30 min with specific fluorochrome-labeled antibody. Samples were washed, suspended in ice-cold PBS and analyzed by flow cytometry. CVID patients and HD show a similar receptors expression. Soon after IVIg infusion only the expression of CD181 decrease (Plot A). Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, determined by the nonparametric Wilcoxon Signed Rank, is indicated as p value.

4.6 ISOLATED PMN SURFACE RECEPTORS ANALYSIS AND *IN VIVO* EFFECT OF IVIG INFUSION

In a subgroup of CVID patients we analyzed the expression of CD16, CD11b and Siglec 9 both in isolated PMN and whole blood condition, in order to clarify if isolation might affect the surface receptors expression. PMN isolated from CVID and HD showed a similar expression of CD16 (CVID: 1715 ± 261.6 MFI vs HD: 1929 ± 100.4 MFI); CD11b (CVID: 18.7 ± 11.8 MF vs HD: 20.5 ± 7.2 MFI) Siglec 9 (CVID: 49.6 ± 2.3 MFI vs HD: 54.8 ± 4.5 MFI). IVIg administration did not alter the receptors expression (CD16: 1631.5 ± 355.6 MFI; CD11b: 19.8 ± 7.7 MFI; Siglec 9: 47.8 ± 2.7 MFI) (Fig. 13A–C). Our results show that, while the CD16 and Siglec 9 expression was similar in both experimental conditions, the expression of CD11b is increased on isolated PMN (*p = 0.01*) (Fig. 13B) demonstrating that the isolation procedure might alter surface receptors expression.

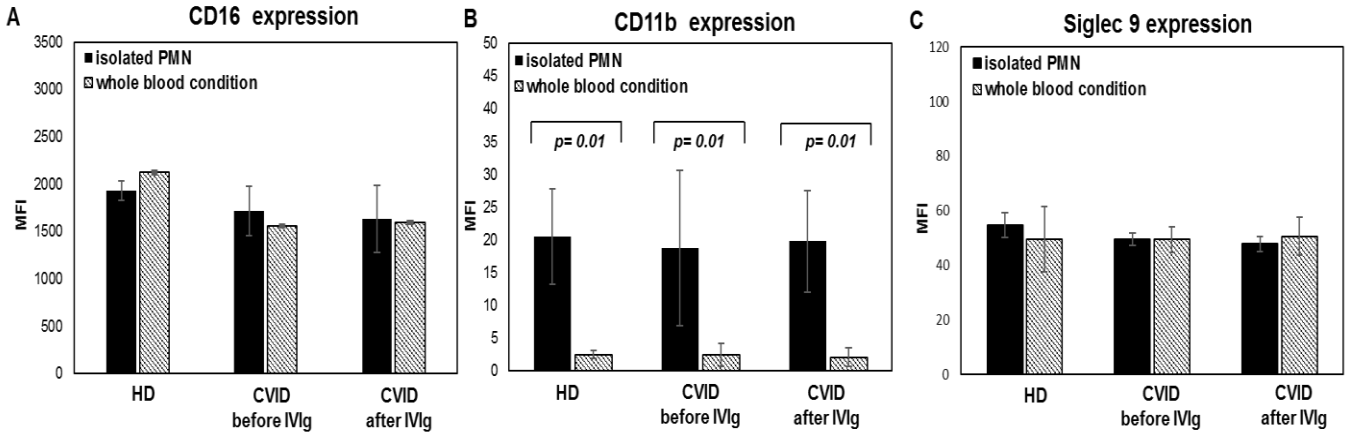


Figure 13.Comparison between the expression of CD16 (A), CD11b (B) and Siglec 9 (C) on isolated PMN and whole blood in CVID and HD patients.. The expression of these receptors on isolated PMN from HD is similar to that observed on PMN from CVID patients and remain unaltered after IVIg infusion. CD16 and Siglec 9 expression was similar on PMN analyzed in whole blood condition or on isolated PMN. CD11b on isolated PMN showed an enhanced expression.

4.7 MONOCYTES OXIDATIVE BURST

The intracellular production of superoxide anions and hydrogen peroxide by monocytes in response to phagocytosis of bacteria was tested by the use of the fluorescence probes DHR 123. The oxidation leads to fluorescence detected by flow cytometry, using the blue-green excitation light (488 nm argon-ion laser). We analyzed the MFI of cells producing reactive oxygen species after *E. coli* stimulation (Fig. 14A). *E. coli* stimulation induced a comparable monocyte oxidative burst in HD and in CVID patients (150.8 ± 52.4 MFI vs 166.7±97.9 MFI, respectively) (Fig. 14B), even if the data obtained in CVID were more dispersed than those obtained in HD. In particular, a very high level of oxidative burst was evident in three CVID patients, anyway the amount of ROS production did not correlate with any clinical complications. After IVIg infusion, monocyte

oxidative burst slightly decreased in all CVID patients (from 166.7 ± 97.9 MFI to 141.1 ± 92.1 MFI, *p=0.004*), as shown in Fig. 14B, suggesting a slight damping effect of IVIg infusion.

4.8 PMN OXIDATIVE BURST

The PMN oxidative burst was evaluated by using opsonized *E. coli* and PMA in whole blood conditions. *E. coli* induced a similar oxidative burst in CVID and HD (CVID: 519.8 ± 203.1 MFI; HD: 508.8 ± 137.7 MFI), once again a greater data dispersion was observed in CVID (Fig. 15A–B). One hour after IVIg, the *E. coli*-induced oxidative burst was comparable to that observed prior the infusion (before IVIg: 519.8 ± 203.1 MFI; after IVIg: 539.5 ± 301.4 MFI) (Fig. 15C–D). On the contrary, PMA induced a more intensive oxidative burst was in CVID than in HD (940.2 ± 317.1 MFI and 470.4 ± 280.3 MFI, respectively, *p= 0.004*) (Fig. 16A–B). In CVID patients the PMA-triggered oxidative burst decreased from 940.2 ± 317.1 MFI to 771.2 ± 348.8 MFI (*p= 0.02*) after IVIg infusion (Fig. 16C–D). *E. coli*–induced oxidative burst on PMN and on monocytes of individual CVID patients were strongly related in samples collected before IVIg (R = 0.81, *p < 0.0001*) and after IVIg (R = 0.8, *p < 0.001*). Unfortunately, we could not found any clinical associations with PMN oxidative burst and PMN surface receptors expression.

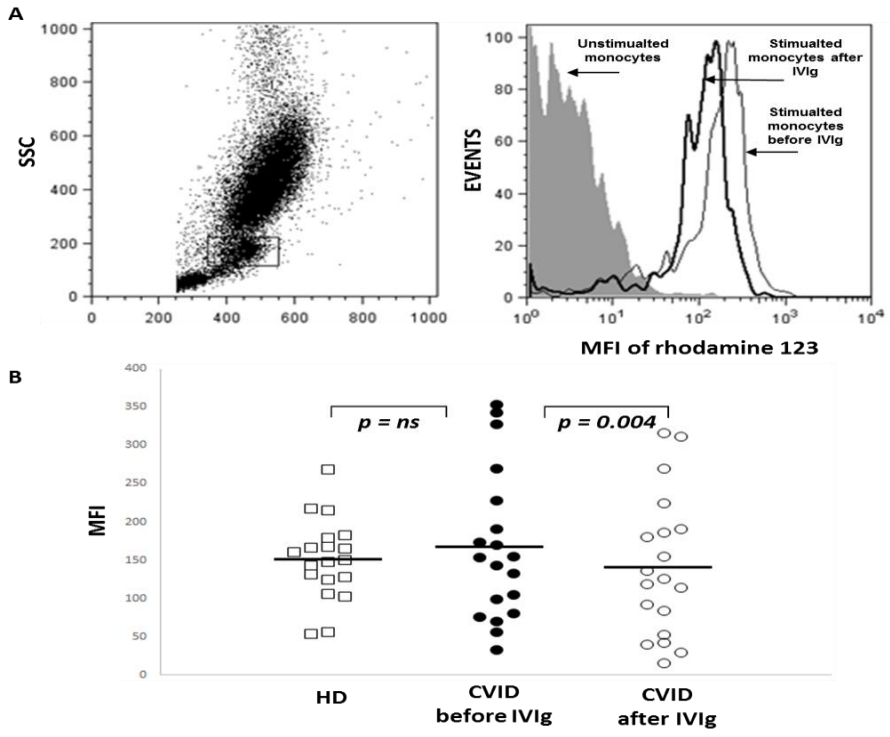


Figure 14. Monocytes oxidative burst after *E. coli* stimulation in a representative CVID patient before and after IVIg infusion. Panel A: dot plots showing the electronic gate of monocytes population. Histograms denote fluorescence intensities in unstimulated monocytes (solid histograms) and in *E. coli* stimulated monocytes before IVIg infusion (thin lines) and after IVIg infusion (thick lines). The x-axis denotes the fluorescence of rhodamine 123 (R 123) produced by the oxidation of dihydrorhodamine 123 (DHR 123). Panel B: monocytes oxidative burst after *E. coli* stimulation in HD, in CVID pre-IVIg and 1 h post-IVIg administration. Bars denote mean values.

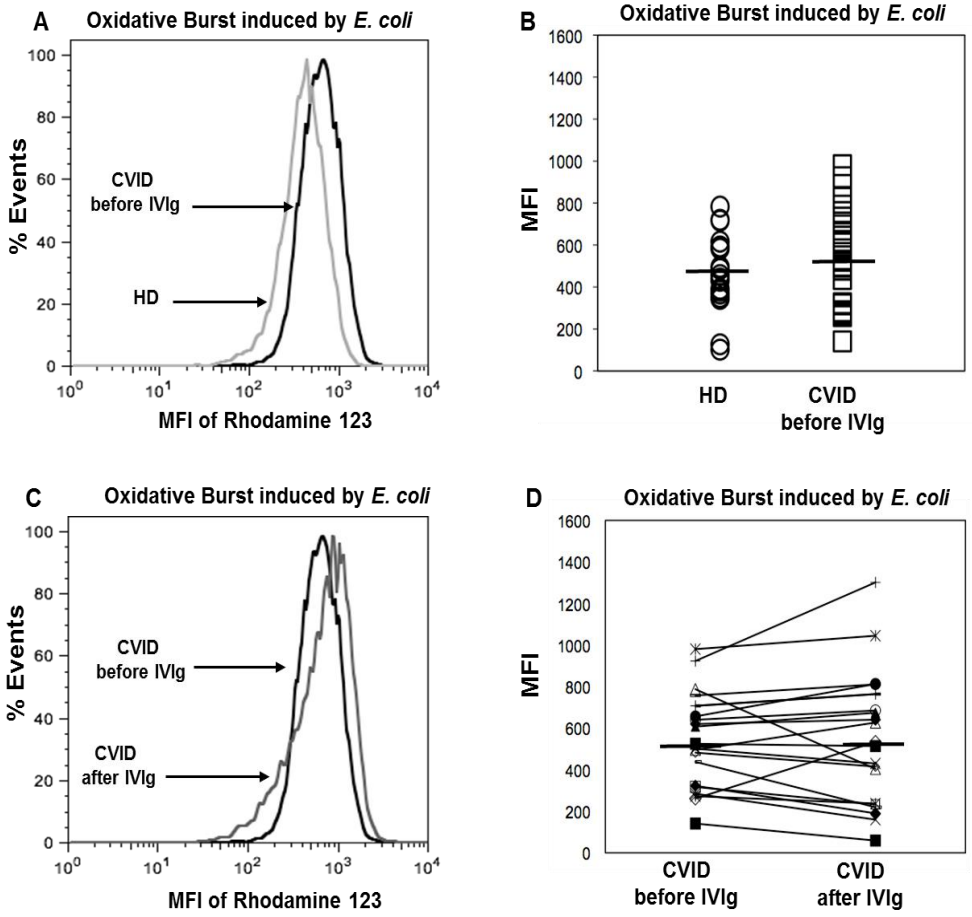


Figure 15. PMN oxidative burst induced by E. coli in HD and CVID patients. The quantitative leukocyte oxidative burst evaluated by incubating whole blood samples in a water bath at 37 _C for 20 min with opsonized E. coli (1–2 _ 10^9/ml). The conversion of DHR 123 to R 123 were used to evaluated the intracellular ROS production. CVID patients showed a normal PMN oxidative burst when stimulated by opsonized E. coli (A, B). IVIg administration didn't affect PMN ROS production (C, D). PMN population was identified by flow cytometry using FSC and SSC characteristics. Results are expressed as Mean Fluorescence Intensity. A horizontal bar denotes the mean values. Statistical *significance indicated as p value were determined by the nonparametric Mann-Whitney test (B) and Wilcoxon Signed Rank test (D).*

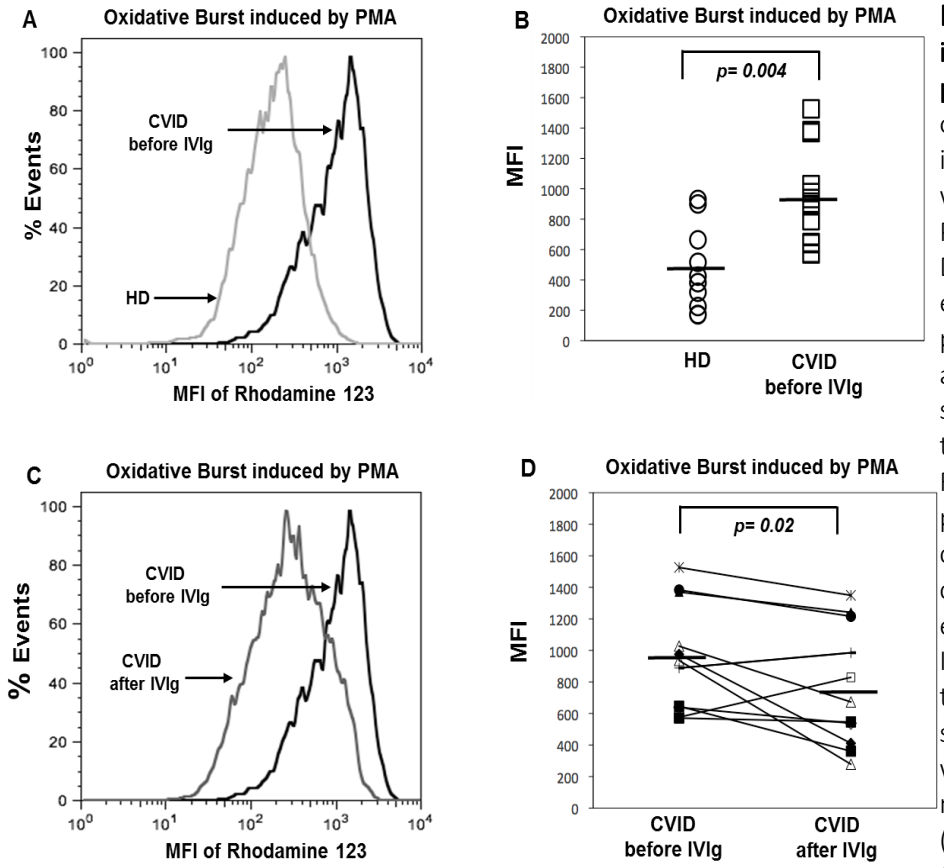


Figure 16 PMN oxidative burst induced by PMA in HD and CVID patients. The quantitative leukocyte oxidative burst was evaluated by incubating whole blood samples in a water bath at 37°C for 20 min with PMA (1.62 mM). The conversion of DHR 123 to R 123 were used to evaluated the intracellular ROS production. CVID patients showed an increased oxidative burst when stimulated with PMA in comparison to HD (A, B). IVIg infusion reduced ROS production (C, D). PMN population was identified by flow cytometry using FSC and SSC characteristics. Results are expressed as Mean Fluorescence Intensity. A horizontal bar denotes the mean values. Statistical significance indicated as p value were determined by the nonparametric Mann-Whitney test (B) and Wilcoxon Signed Rank test (D).

4.9 MONOCYTES AND PMN PHAGOCYTOSIS

Since phagocytosis is a fundamental step for the resolution of infections, we evaluated in CVID patients the process using FITC-labeled opsonized *E. coli* with the aim to test the ability of monocytes to internalize bacteria through FcγR. Our results show that monocytes from CVID patients perform a similar phagocytosis compared to HD (CVID: 428.1 ± 137.0 MFI vs HD: 386.8 ± 154.4 MFI) (Fig. 17A) but one hour after IVIg, phagocytosis ability decreased (from 428.1 ± 137.0 MFI to 373.1 ± 120.4 MFI, *p=0.02*) (Fig. 17A), similarly as observed for the oxidative burst. We observed also a normal phagocytosis performed by PMN from CVID (CVID: 612.0 ± 214.5 MFI vs HD: 663.8 ± 160.8 MFI) (Fig. 17B). After IVIg infusion, phagocytosis remains unaltered (612.0 ± 214.5 MFI to 582.5 ± 179.1 MFI) (Fig. 17B) demonstrating that IVIg treatment did not affect the ability of PMNs of CVID patients to perform a bacterial phagocytosis as the subsequent oxidative burst.

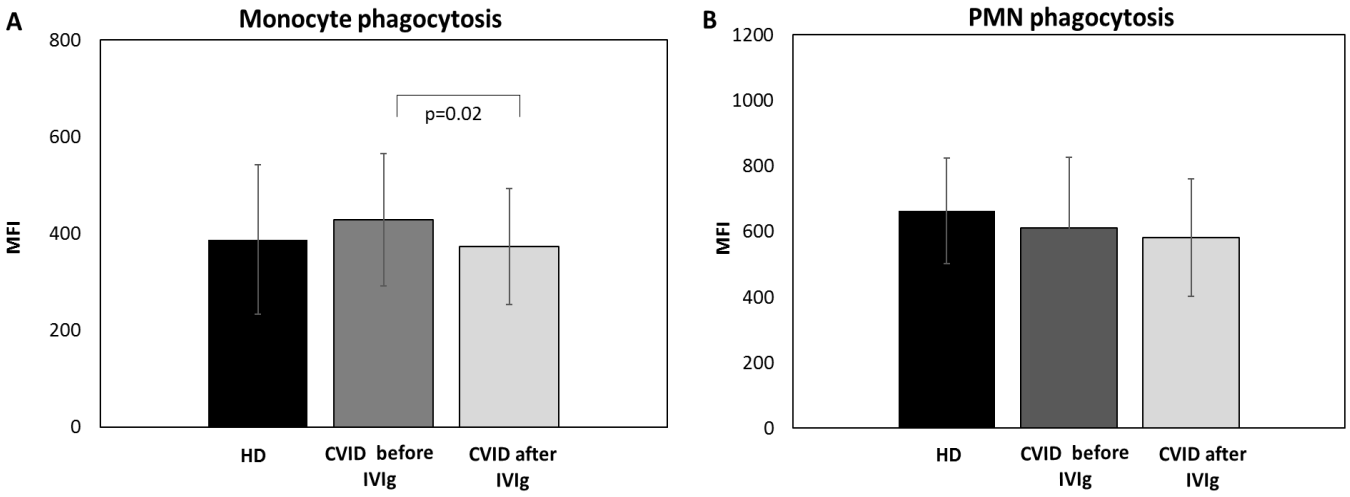


Figure 17. Monocytes and PMN phagocytosis in CVID patients and HD. The quantitative determination of leukocyte phagocytosis was tested by incubating whole blood samples in a water bath for 10 min at 37°C with FITC-labeled E. coli ($2x10^9$/ml). The percentage of cells having performed phagocytosis (granulocytes and monocytes) was analyzed as number of ingested bacteria and results are expressed as Mean Fluorescence Intensity. CVID patients show an increased monocytes phagocytosis (plot A) than HD while PMN (plot B) performed a similar phagocytosis compared to HD. After IVIg administration monocytes phagocytosis decreased, while it remains unchanged in PMN. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, determined by the nonparametric Mann Whitney and Wilcoxon Signed Rank test is indicated as p value

4.10 MONOCYTE SUBSETS IN XLA AND THE EFFECT OF IVIG INFUSION

Monocytes subsets were identified according to their expression CD14 and the CD16 in classical, intermediate and in non classical (Supplementary Fig. 2). We observed that the classical monocytes were the most frequent subpopulation in XLA patients and HD (XLA: 76.8 ± 3.8% and HD: 84.4 ± 3.3%). Furthermore, we observed a similar percentage of non-classical monocytes in XLA and HD (XLA before IVIg: 5.9 ± 2.7% and HD: 7.0 ± 3.0%), while the intermediate subset was increased in patients compared to HD (XLA before IVIg: 17.2 ± 2.9% and HD: 8.4 ± 3.2%, *p= 0.01*) (Fig. 18). After the IVIg infusion, we observed a decrease of intermediate monocytes percentage (from 17.1 ± 2.9% to 14.2 ± 3.2%, p 0.04) even if they remain more represented respect to HD (14.2 ± 3.2% vs 8.4 ± 3.2%, respectively p =0.01). The frequency of non-classical and classical monocytes did not change after the infusion (from 5.9 ± 2.7% to 5.5 ± 2.4%; from 76.8 ± 3.8% to 80.2 ± 3.2% respectively) (Fig. 18).

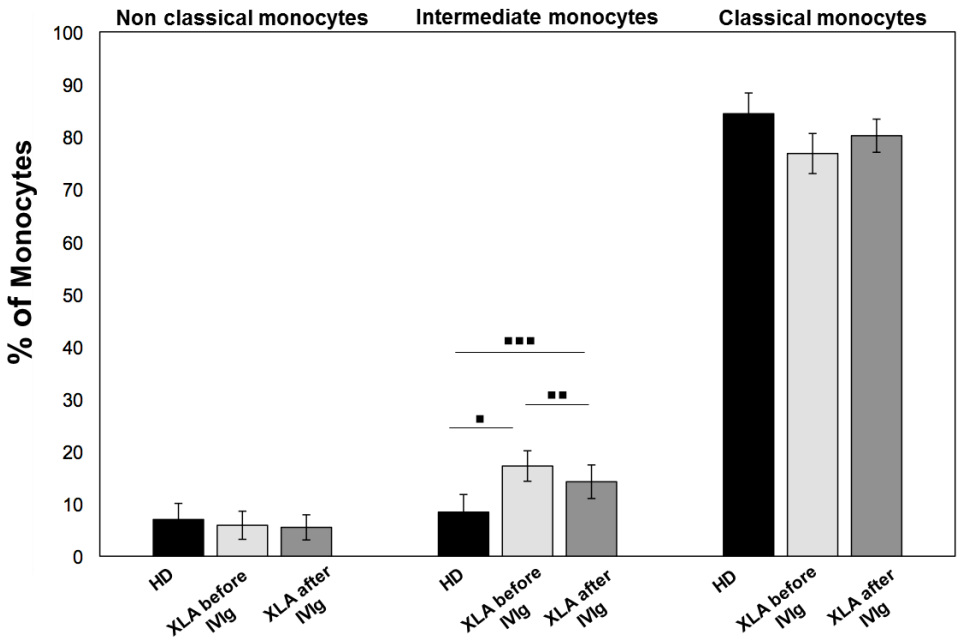


Figure 18. Frequencies of non classical, intermediate and classical monocytes from HD and XLA patients before and after IVIg infusion. Whole blood samples were analyzed for the expression of CD16 and CD14 before and after IVIg infusion. Histograms show that non classical and classical monocyte frequencies from XLA patients are similar to that observed on HD and that IVIg infusion did not change their frequency. Intermediate monocytes percentage is increased in XLA patients (▪p 0.01), IVIg infusion induce their reduction (▪▪p 0.04) even if remained at higher level respect to HD (▪▪▪p 0.01). Results are expressed as percentages. Histograms denote mean values and bars standard deviation. Statistical significance as determined by the nonparametric Mann Whitney and Wilcoxon Signed Rank test is indicated as ▪ p value.

4.11 MONOCYTES SURFACE RECEPTORS IN XLA

With the aim to obtain insights regards the activation status of monocytes we evaluated the surface expression of CD181, CD11b, CD11c and Siglec 9 as receptors involved in cellular responses. As shown in Table 6 we found that, in the three monocytes subpopulations, all these receptors were expressed at a similar level in XLA patients and in HD and that IVIg administration did not induce any variation of their expression showing that monocytes from XLA had a normal phenotype (Fig. 19A-D).

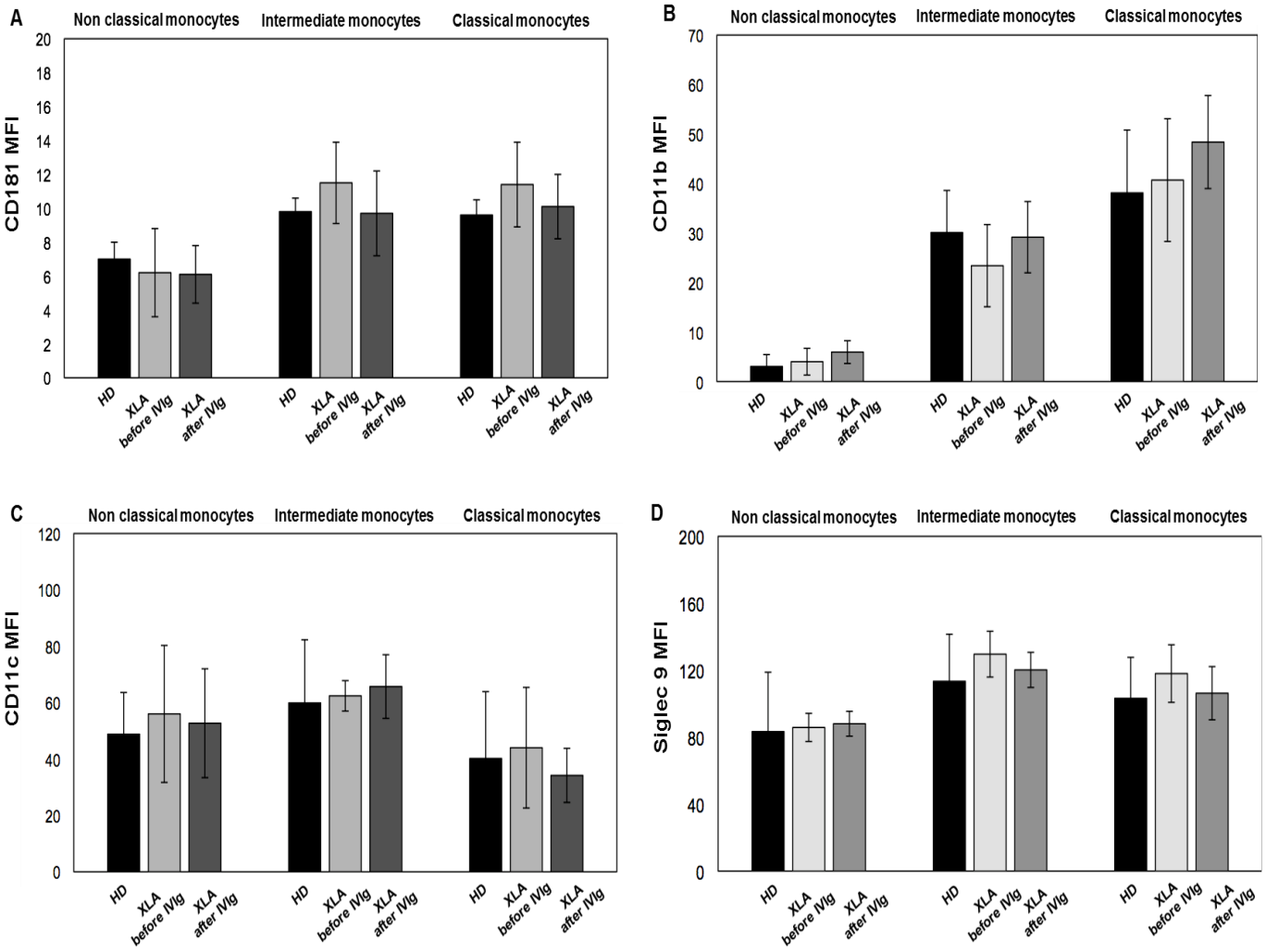


Figure 19 CD181 (A), CD11b (B), CD11c (C) and Siglec 9 (D) expression on monocytes subsets from HD and XLA patients before and after IVIg infusion. Whole blood samples were analyzed for the expression of CD181, CD11b, CD11c and Siglec 9 before and after IVIg infusion. The expression of all surface receptors was evaluated by performing a staining at 4°C for 30 min with specific fluorochrome-labeled antibody. Samples were washed, suspended in ice-cold PBS and analyzed by flow cytometry. XLA patients show a similar CD181, CD11b, CD11c and Siglec 9 expression on monocytes subsets compared to HD. After IVIg infusion the expression of all receptors on monocytes subsets remained unaltered. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation.

		HD			XLA before IVIg			XLA after IVIg		
Receptor		Non classical	Intermediate	Classical	Non classical	Intermediate	Classical	Non classical	Intermediate	Classical
CD181 (MFI)	UN	7.1 ± 1	9.8 ± 2.6	9.6 ± 1.7	6.2 ± 0.8	11.5 ± 2.4	11.4 ± 2.5	6.1 ± 0.9	9.7 ± 2.5	10.1 ± 1.9
	ST	22.3 ± 1.5	15.2 ± 3.6	15.6 ± 1.4	18.6 ± 1.4	17.2 ± 3.5	17.8 ± 2.7	18.4 ± 1.9	13.9 ± 4.7	15.1 ± 2.2
CD11b (MFI)	UN	3 ± 2.4	30.1 ± 8.5	38.1 ± 12.7	4 ± 2.7	23.4 ± 8.3	40.7 ± 12.4	5.9 ± 2.3	29.2 ± 7.2	48.4 ± 9.4
	ST	8.6 ± 4	72.8 ± 9.8	96.7 ± 12.1	9 ± 6.6	62 ± 25.2	75.5 ± 18.8	14.1 ± 8.6	70.3 ± 27	75.4 ± 26.1
CD11c (MFI)	UN	40.2 ± 19.3	59.9 ± 24.3	48.8 ± 14.8	56 ± 22.4	62.4 ± 5.4	50.2 ± 10	52.7 ± 23.7	65.7 ± 21.4	38.4 ± 7.7
	ST	128.5 ± 10.6	117.3 ± 21.8	101.4 ± 9.5	140.5 ± 0.7	120.5 ± 2.1	111.5 ± 0.7	141 ± 9.9	112 ± 15.6	107 ± 14.1
Siglec 9 (MFI)	UN	83.3 ± 35.5	113.3 ± 28.1	103.2 ± 24.5	85.8 ± 8.4	129.6 ± 13.7	118 ± 17.2	88 ± 7.4	120.2 ± 10.5	106.3 ± 15.
	ST	102.2 ± 42.6	151.6 ± 38.4	146.5 ± 38.4	138.9 ± 56.5	151.5 ± 22.6	177.8 ± 55.2	96.8 ± 24.2	143.6 ± 27	143.5 ± 34.9

Table 7 Receptors expression on monocytes' subsets from HD and XLA patients. Values are expressed as Mean Fluorescence Intensity (MFI) ± standard deviation. All the receptors showed a significant increase of their expression after stimulation (*p=0.03*). UN: Unstimulated; ST: Stimulated with E. coli

4.12 STIMULATION OF MONOCYTES IN XLA PATIENTS

With the aim to verify if IVIg influence the monocytes' ability to express CD181, CD11b, CD11c and Siglec 9 following stimulation through FcγR, we provided opsonized *E. coli* as stimulus and we analyzed the expression of surface receptors on monocytes subsets. We found that opsonized *E. coli* induced, in whole blood conditions, a similar increase of all receptors in XLA patients and HD (Table 7). The overexpression of these receptors induced by *E. coli* was preserved also after IVIg infusion, demonstrating that *in vivo* IVIg replacement did not influence the monocytes ability to overexpress surface receptors in response to bacterial stimulus (Table 7).

4.13 PMNS SURFACE RECEPTORS

The activation status of PMNs is strongly influenced by their ability to perform phagocytosis, respiratory burst and adhesion processes; therefore, we evaluated the expression level of CD181, CD66b, CD11b, CD11c, CD16 and Siglec 9 receptors. As shown in Table 8, PMN from XLA and HD showed an overlapping expression of all receptors with the exception of CD66b that was overexpressed in XLA patients (XLA before IVIg 484.7 ± 66 MFI vs HD 333.5 ± 29 MFI, *p=0.001*). After IVIg infusion the expression of all receptors remained unaltered (Fig. 20A-F) demonstrating, like previously shown in CVID patients, that IVIg did not influence the PMNs' phenotype.

	HD		XLA before IVIg		XLA after IVIg	
Receptor	**Unstimulated MFI**	**Stimulated MFI**	**Unstimulated MFI**	**Stimulated MFI**	**Unstimulated MFI**	**Stimulated MFI**
CD181	337.3 ±16.8	205.8 ±102.9▪	361.5 ± 57.3	198 ± 76.5‡	317.5 ±98.3	169±69.3‡
CD66b	333.5 ± 29	1310 ± 162.6▪	484.7 ± 66	1365.7 ±287.7‡	486.7 ±116.4	1240 ± 654‡
CD11b	1.4 ± 0.3	4.2 ± 3.1▪	1.9 ± 1.3	21.4 ± 5.4‡	2.1 ± 1.3	24.3 ±4.8‡
CD11c	20 ± 7.7	48.7 ± 11▪	24.8 ± 10.5	50.4 ± 14.4‡	24.4 ±10.6	47.9 ±13.6‡
CD16	3555.6 ± 1144.5	4336.6 ±1539.5▪	3012 ± 952.8	3758.8 ± 1035.2 ‡	2753.8 ±794.4	3594.6 ±1033.6‡
Siglec 9	56.1 ± 15.5	99 ± 38.7▪	61.7 ± 11.9	97.2 ± 27.4‡	61.2 ±16.1	81.7 ± 28.1‡

Table 8. Receptors expression on PMNs from HD and XLA patients. Values are expressed as Mean Fluorescence Intensity (MFI), Statistical significance, determined by the nonparametric Wilcoxon Signed Rank test, is indicated as p value. ▪p=0.005; ‡p=0.03

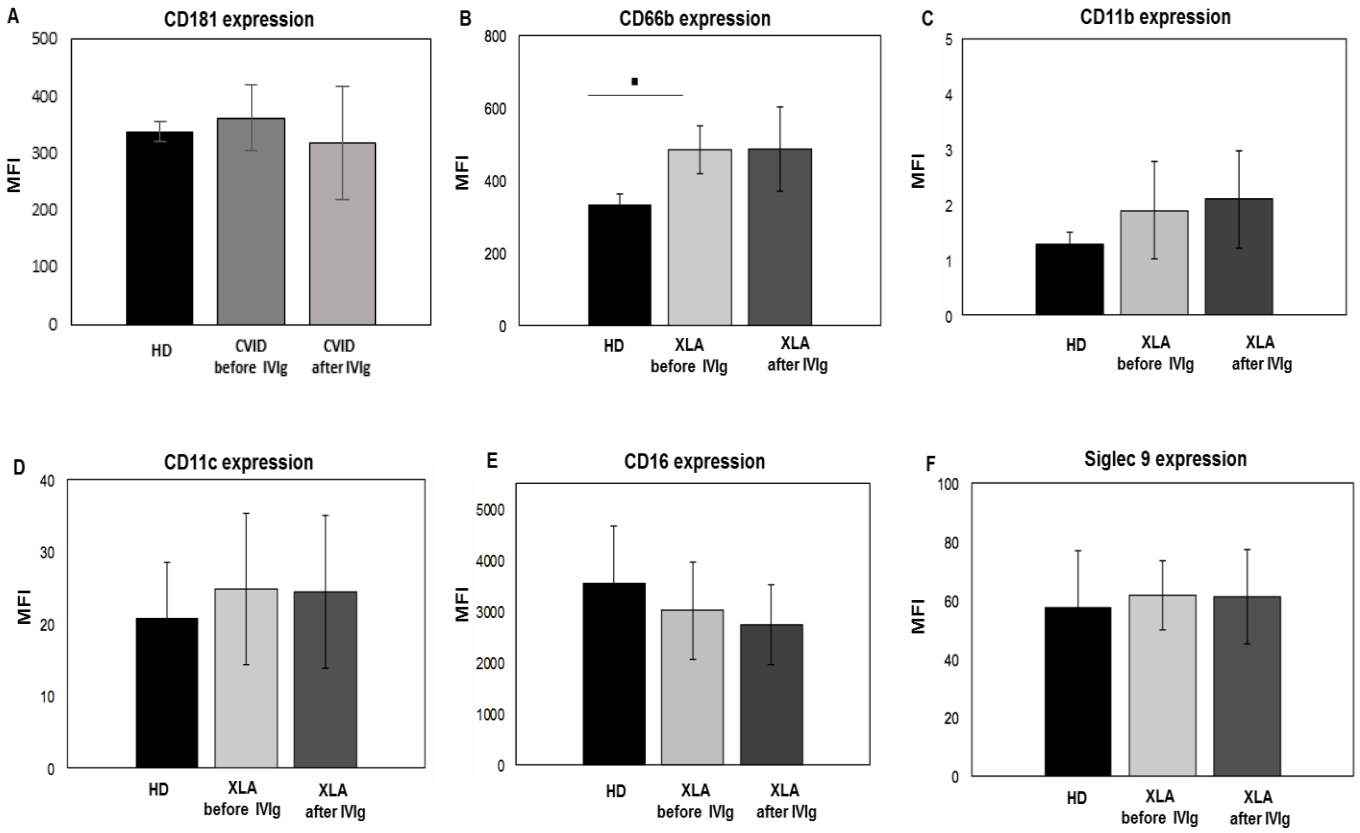


Figure 20 CD181 (A), CD66b (B) CD11b (C), CD11c(D), CD16 (E) and Siglec 9 (F) expression on PMN from HD and XLA patients before and after IVIg infusion. Whole blood samples were analyzed for the receptors' expression. The expression of all surface receptors was evaluated by performing a staining at 4°C for 30 min with specific fluorochrome-labeled antibody. Samples were washed, suspended in ice-cold PBS and analyzed by flow cytometry. XLA patients and HD show a similar CD181, CD11b, CD11c, CD16 and Siglec 9 expression, while CD66b expression was overexpressed on PMN from XLA patients compared to HD (▪p 0.01). After IVIg infusion, the expression of all receptors remained unaltered. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, determined by the nonparametric Mann Whitney test, is indicated as ▪p value.

4.14 STIMULATION OF PMNS IN XLA PATIENTS

PMN stimulation by opsonized *E. coli* induced an overexpression of all the analyzed receptors on PMN from XLA patients and HD except CD181, which was reduced after stimulation, in line with that, previously observed in CVID patients (Table 7). As indicated in table 7, PMNs from XLA patients showed a greater increase of CD11b compared to HD as previously seen in CVID patients. The receptors' overexpression induced by *E. coli* was preserved also after IVIg infusion showing that IVIg replacement did not affect the PMN ability to respond to opsonized bacteria.

4.15 MONOCYTES OXIDATIVE BURST IN XLA PATIENTS

The oxidative burst reflects the killing ability of innate immune cells and involve many cellular component and pathways. The lack of BTK activity might influence the respiratory burst considering that is a process that require Ca^{2+} mobilization from internal storage. Therefore, we evaluated the monocytes ROS production in XLA patients after stimulation *ex vivo* with opsonized *E. coli* and PMA (Fig. 21A-B). We found that both *E. coli* and PMA induced a comparable oxidative burst in monocytes from XLA patients and HD (*E. coli*: 176.5 ± 33.6 MFI vs 185.2 ± 40 MFI, respectively; PMA: 375 ± 30.6 MFI vs 350.3 ± 50.2 MFI, respectively). After IVIg infusion, monocyte oxidative burst induced by *E. coli* slightly decreased (from 176.5 ± 33.6 MFI to 152.8 ± 33.3 MFI, p 0.004) as previously observed on CVID monocytes, but remain unaltered when PMA was provided (from 375 ± 30.6 MFI to 356 ± 28.7 MFI) as shown in Fig. 21A-B.

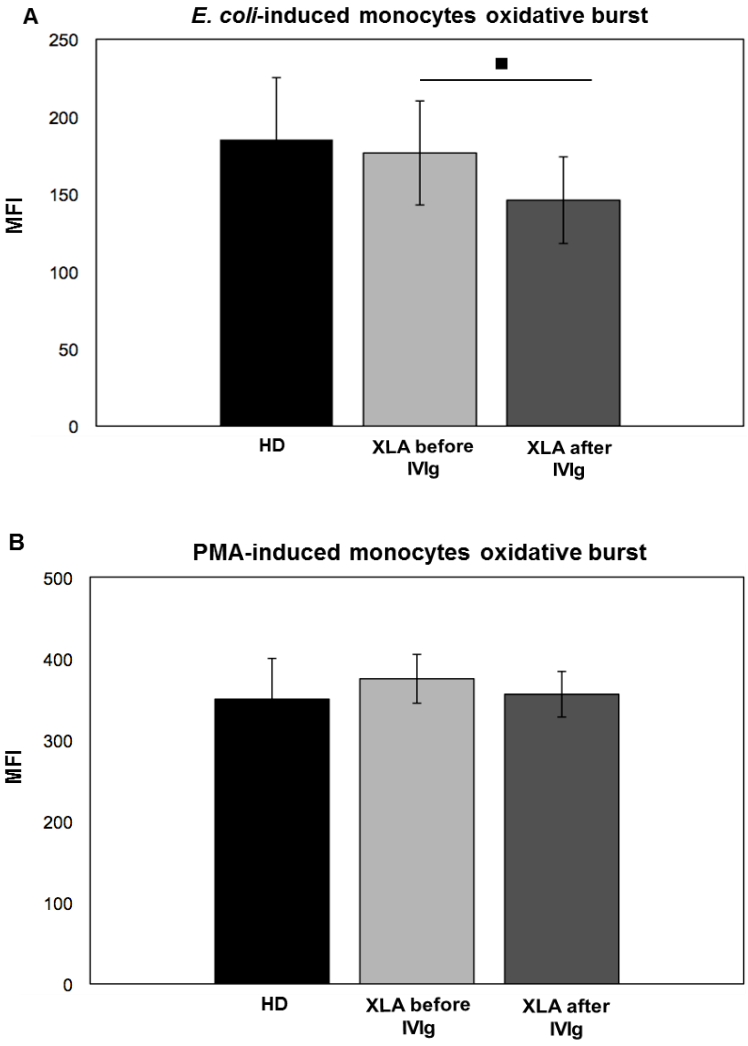


Figure 21 Monocytes oxidative burst in XLA patients and HD. The quantitative evaluation of leukocyte oxidative burst was observed by incubating whole blood samples in a water bath at 37°C with opsonized E. coli (1-2x10^9/ml) and PMA (1.62mM). The conversion of DHR 123 to R 123 were used to evaluated the intracellular ROS production. XLA patients show a similar monocytes E. coli-induced (**plot A**) and PMA-induced (**plot B**) oxidative burst compared to HD. After IVIg, monocyte oxidative burst slightly decreased (■p 0.004), but remain unaltered when PMA was provided. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, determined by the nonparametric Wilcoxon Signed Rank test, is indicated as ■p value

4.16 PMN OXIDATIVE BURST IN XLA PATIENTS

The PMNs intracellular production of superoxide anions and hydrogen peroxide in response to phagocytosis was tested by using the fluorescence probes DHR 123. We analyzed the MFI of cells producing reactive oxygen metabolites after *E. coli* and PMA stimulation. Both stimuli induced a comparable PMN oxidative burst in XLA patients and HD (*E. coli*: 187.8 ± 27.5 MFI vs 201.8 ± 13.7 MFI, respectively; PMA: 519 ± 21.2 MFI vs 515 ± 140 MFI, respectively) (Fig. 22A-B). One hour after IVIg, the *E. coli*-induced ROS production remains unchanged (from 187.8 ± 27.5 MFI to 181.6 ± 41.3 MFI, p ns) as well as that PMA-induced (from 519 ± 21.2 MFI to 490 ± 41.3 MFI, p ns) (Fig. 22A-B).

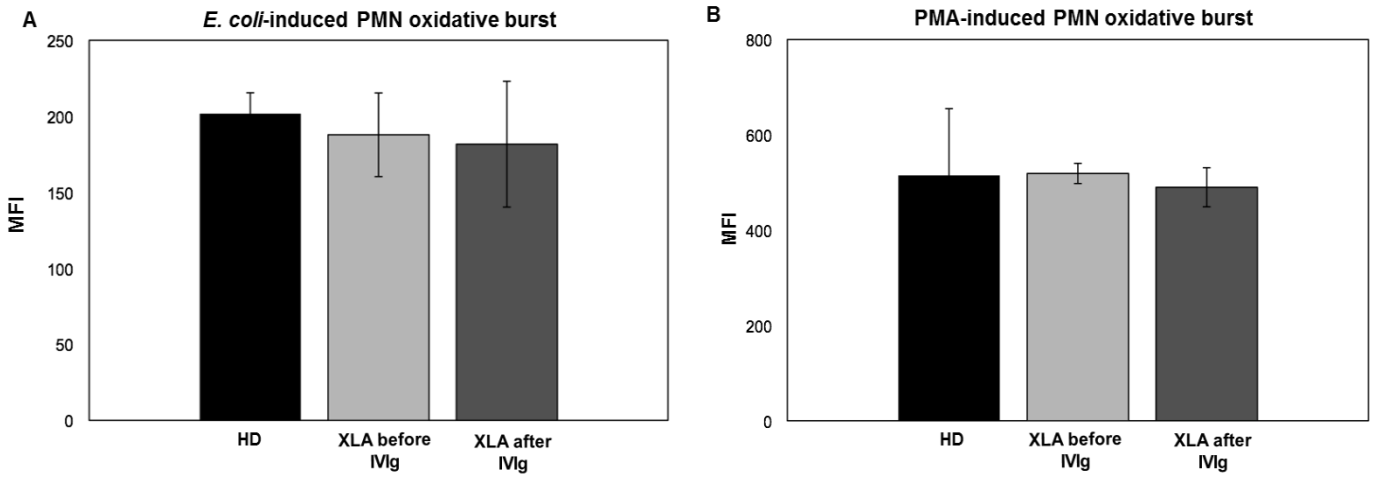


Figure 22. PMN oxidative burst in XLA patients and HD. The PMNs intracellular production of superoxide anions and hydrogen peroxide was tested by using the fluorescence probes DHR 123 incubating whole blood samples in a water bath at 37°C with opsonized E. coli (1-2x10^9/ml) and PMA (1.62mM). XLA patients show a comparable PMN E. coli-induced (plot A) and PMA-induced (plot B) oxidative burst to HD. After IVIg, PMNs ROS production remain unaltered. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation.

4.17 MONOCYTES AND PMN PHAGOCYTOSIS IN XLA PATIENTS

We evaluated in XLA patients the phagocytosis using FITC-labeled opsonized *E. coli* with the aim to test the ability of monocytes and PMN to internalize bacteria through FcγRs. Our results show that monocytes from XLA patients perform a normal phagocytosis compared to HD (XLA: 418.7 ± 13.9 MFI vs HD: 430.8 ± 116.6 MFI) (Fig. 23A). One hour after IVIg, phagocytosis ability decreased (from 418.7 ± 13.9 MFI to 318.2 ± 13.3 MFI, p 0.043) (Fig. 23A) as previously seen in CVID. Supporting the strong dependence of oxidative burst from phagocytosis process, we found a positive correlation between oxidative burst and phagocytosis

performed by monocytes both before and after IVIg infusion (p=0.027, R 0.862; p=0.043, R 0.824, respectively) (Fig 24 A-B).

Regards the phagocytic activity of PMN we observed that also PMN from XLA perform a normal phagocytosis via FcγR compared to HD (XLA: 490.7 ± 80.5 MFI vs HD: 587.3 ± 155 MFI) (Fig. 23B) and that after IVIg infusion, phagocytosis remain unaltered (from 490.7 ± 80.5 MFI to 469.5 ± 70.4 MFI) (Fig. 23B) demonstrating that, as previously shown in CVID , also in XLA patients IVIg infused a replacement dosage did not affect the ability of PMNs to perform a bacterial phagocytosis.

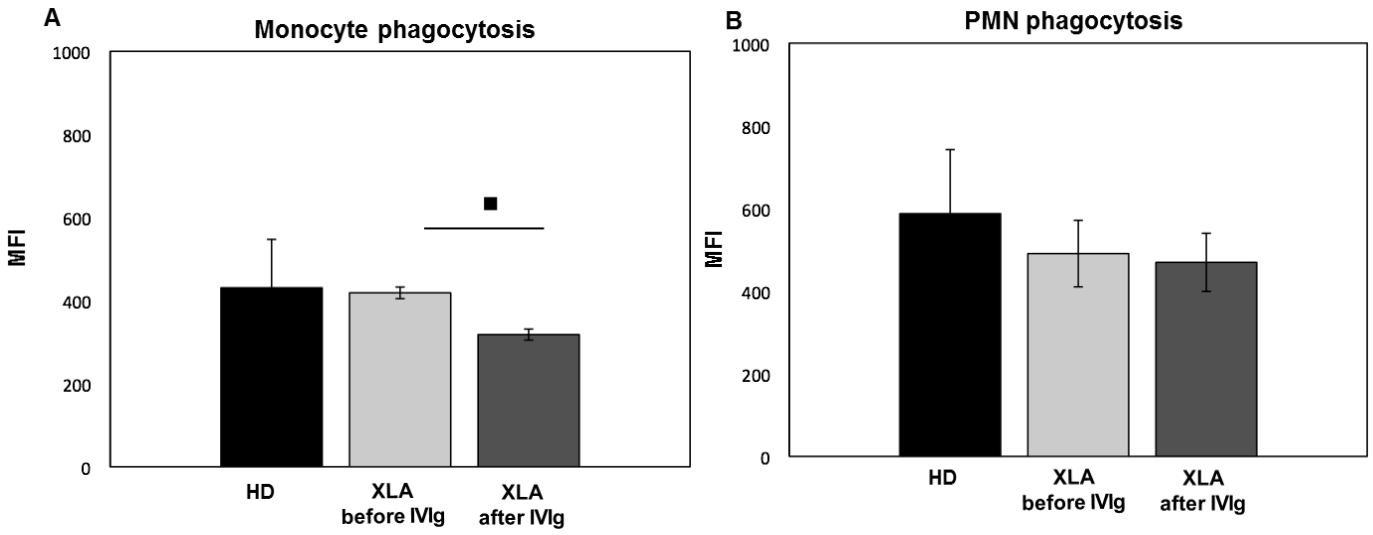


Figure 23. Monocytes and PMN phagocytosis in XLA patients and HD. The quantitative determination of leukocyte phagocytosis was tested by incubating whole blood samples in a water bath for 10 min at 37°C with FITC-labeled *E. coli* ($2x10^9$/ml). The percentage of cells having performed phagocytosis (granulocytes and monocytes) was analyzed as number of ingested bacteria and results are expressed as Mean Fluorescence Intensity. XLA patients show a similar monocyte (plot A) and PMN (plot B) phagocytosis compared to HD. After IVIg administration monocytes phagocytosis decreased (▪p 0.043), while it remains unchanged in PMN. Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, determined by the nonparametric Wilcoxon Signed Rank test, is indicated as ▪ p value

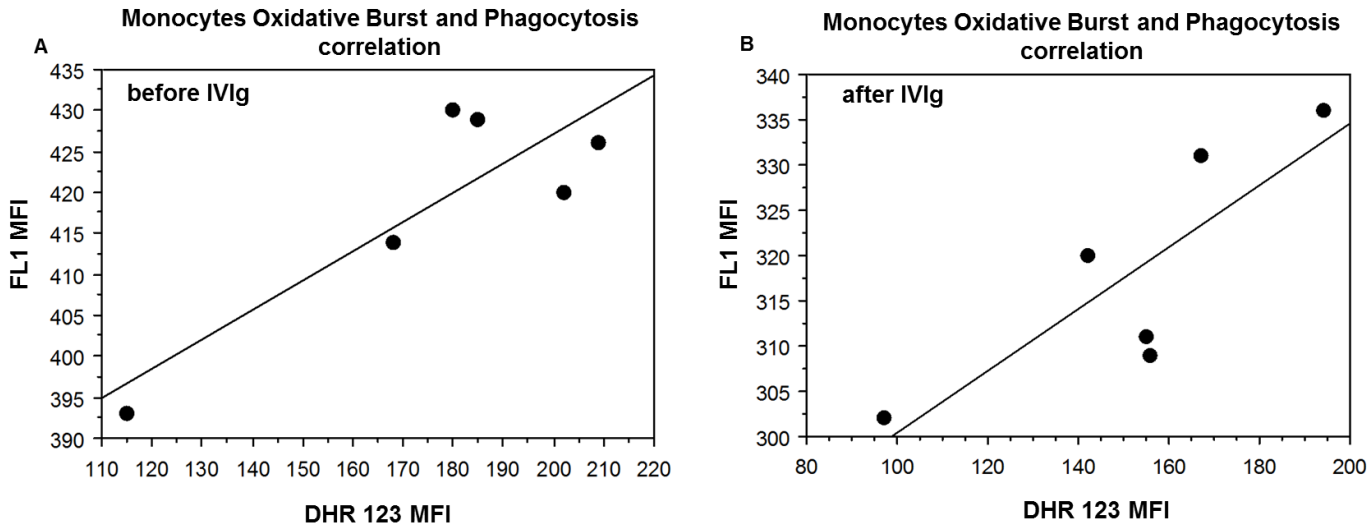


Figure 24. Correlation between monocytes oxidative burst and phagocytosis before IVIg infusion. Plot A and plot B denote a positive correlation between monocytes oxidative burst and phagocytosis before (p 0.027, R 0.862) and after IVIg infusion (p 0.043, R 0.824) respectively. Correlations are calculated by means of linear regression analysis.

4.18 CALCIUM CHELATION ASSAY ON MONOCYTES

In order to verify the importance of Ca^{2+} mobilization in oxidative burst and phagocytosis process in XLA patients, we pre-treated whole blood samples with calcium chelator BAPTA-AM. We observed a lower reduction of *E. coli*-induced oxidative burst on monocytes from XLA compared to HD (*p=0.003*), indeed the average reduction in monocytes from XLA it is about 50% (from 176.5 ± 33.6 MFI to 79.8 ± 19 MFI, *p= 0.02*) and about 75% in monocytes from HD (from 185.2 ± 40 MFI to 46.2 ± 4 MFI, *p= 0.01*) (Fig. 24A-C) suggesting that in XLA patients the role of calcium mobilization for the ROS production is less essential respect to HD. On the contrary, the calcium chelation did not affect the oxidative burst induced by PMA (HD: from 350.1 ± 50 MFI to 342.5 ± 35.3 MFI, p ns; XLA: from 375.8 ± 30.2 MFI to 345 ± 25.3 MFI, p ns) (Fig. 24 D-F), since it is able to directly bind PKC and so inducing the oxidative burst bypassing the FcγR engagement, BTK activation and Ca^{2+} mobilization.

In parallel, to verify the independence of phagocytosis from Ca^{2+} mobilization, we tested the phagocytic ability of monocytes pre-treating whole blood with BAPTA-AM. We observed a regular phagocytosis both in XLA patients and HD (XLA: from 418.7 ± 13.9 MFI to 400 ± 15.2 MFI; HD: from 430.8 ± 116.6 MFI to 415 ±

109.9 MFI,) confirming that, unlike the ROS production, calcium mobilization is not required for the phagocytic process (Fig. 25A-B)

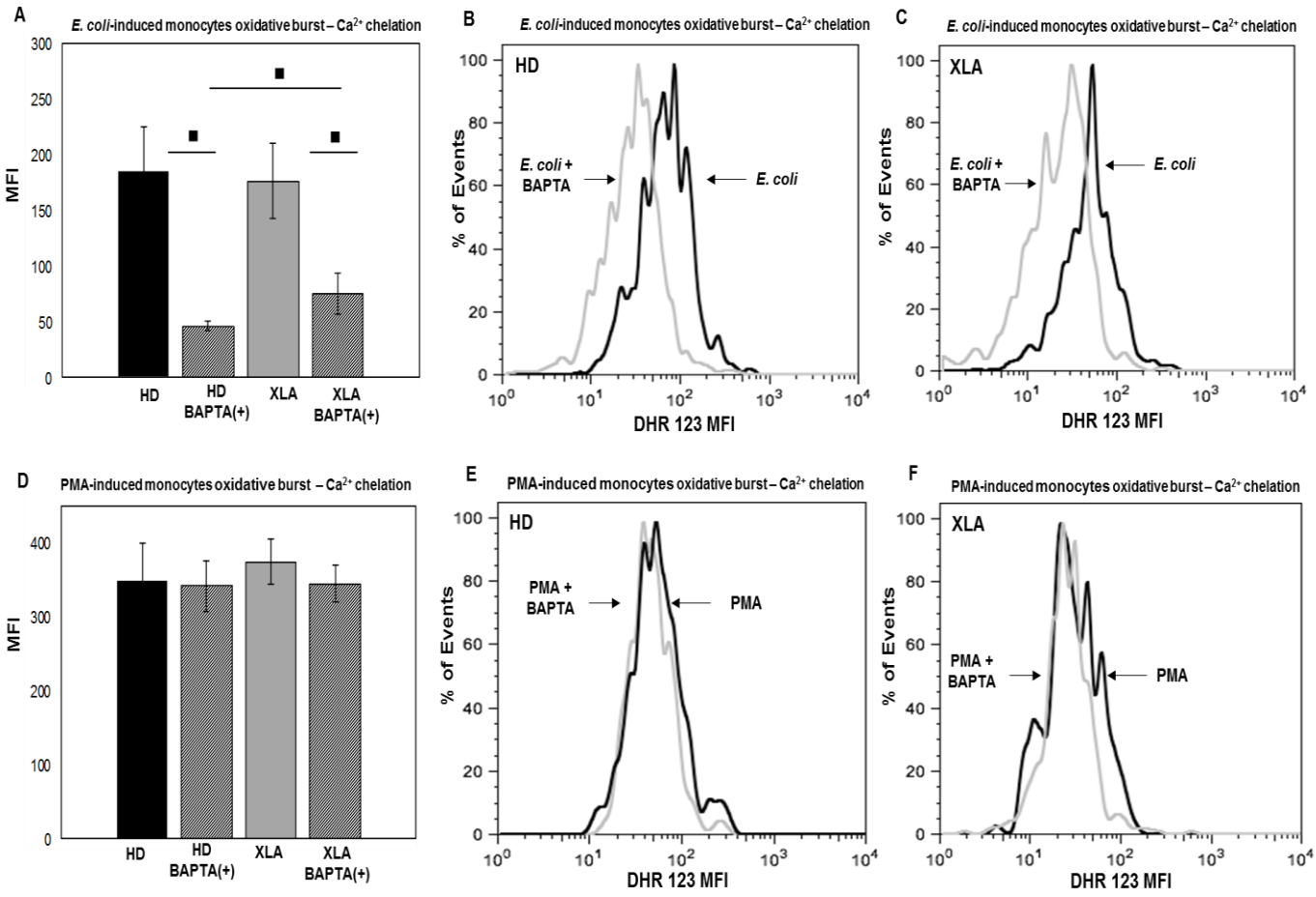


Figure 25 Monocytes oxidative burst – Ca^{2+} chelation. Whole blood samples were pre-treated with 100 µM of the calcium chelator BAPTA-AM for 30 min and incubated in a water bath for 20 min at 37°C with opsonized E. coli (1-2x10^9/ml) and PMA (1.62 mM). The conversion of DHR 123 to R 123 was used to evaluate the intracellular ROS production. FSC and SSC characteristics were used to identify the monocytes population. The reduction of E. coli-induced oxidative burst was lower in XLA than HD (plot A) (▪*p=0.003*) An average reduction of 75% E. coli-induced oxidative burst from HD (▪*p=0.01*) and of 50% from XLA patients (▪*p=0.02*) was observed (plot A), while BAPTA-AM did not affect the oxidative burst induced by PMA both HD and XLA patients (plot D). Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, indicated as p value, was determined by the nonparametric Mann-Whitney test and Wilcoxon Signed Rank test. Plot B and C show monocytes E. coli-induced oxidative burst in representative HD and XLA patient, black peak BAPTA-AM(-) and gray peak BAPTA-AM(+). Plot E and F show monocytes PMA-induced oxidative burst in representative HD and XLA patient, black peak BAPTA-AM(-) and gray peak BAPTA-AM(+).

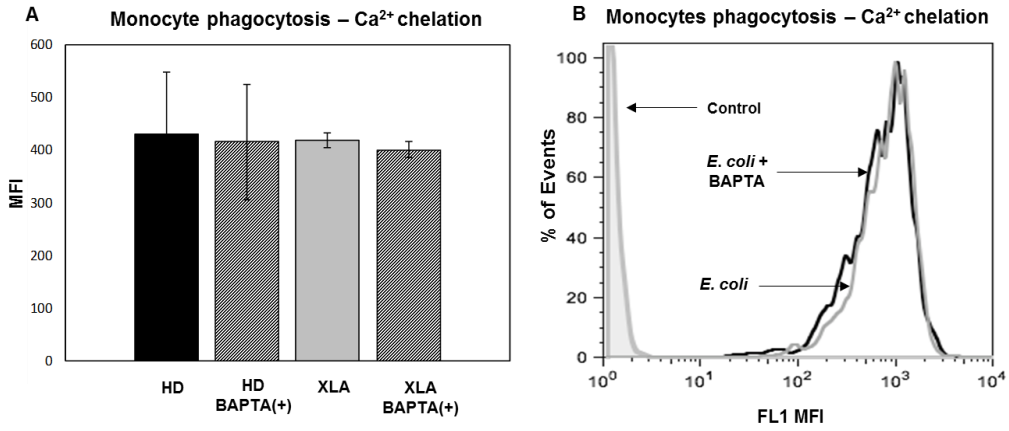


Figure 26 BAPTA pre-treated monocytes phagocytosis in XLA patients and HD . A regular phagocytosis was observed on monocytes (plot A) pre-treating whole blood samples with 100 µM of calcium chelator BAPTA-AM. In plot B is show monocytes phagocytosis of representative XLA patient, black peak BAPTA-AM(-) and gray peak BAPTA-AM(+). Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation.

4.19 CALCIUM CHELATION ASSAY ON PMN

The role of Ca^{2+} mobilization in ROS production and phagocytosis was also evaluated on PMN from XLA patients by pre-treating whole blood samples with BAPTA-AM. Similarly to monocytes, we observed a reduction of *E. coli*-induced oxidative burst on PMN from HD and XLA patients (HD: from 201.8 ± 13.7 MFI to 50.4 ± 3.7 MFI, *p= 0.01*; XLA: from 187.8 ± 27.5 MFI to 90.2 ± 15.4 MFI*, p= 0.02*) (Fig. 26A-C). Also on PMN we observed a greater reduction in HD respect the XLA patients (*p= 0.003*). On the contrary, providing the PMA as a stimulus, the oxidative burst is not affected (HD: from 515.1 ± 140 MFI to 500 ± 80.3 MFI; XLA: from 519 ± 21.2 MFI to 499 ± 22.3 MFI) (Fig. 26D-F), since PMA induces the process after direct binding to PKC. Confirming the independence from Ca^{2+} mobilization of phagocytic process, we found similar values of phagocytosis before and after incubation with BAPTA-AM both in XLA patients than in HD (XLA: from 490.7 ± 80.5 MFI to 485.4 ± 75.2 MFI, p ns; HD: from 587.3 ± 155 MFI to 578 ± 135.4 MFI) (Fig. 27A-B).

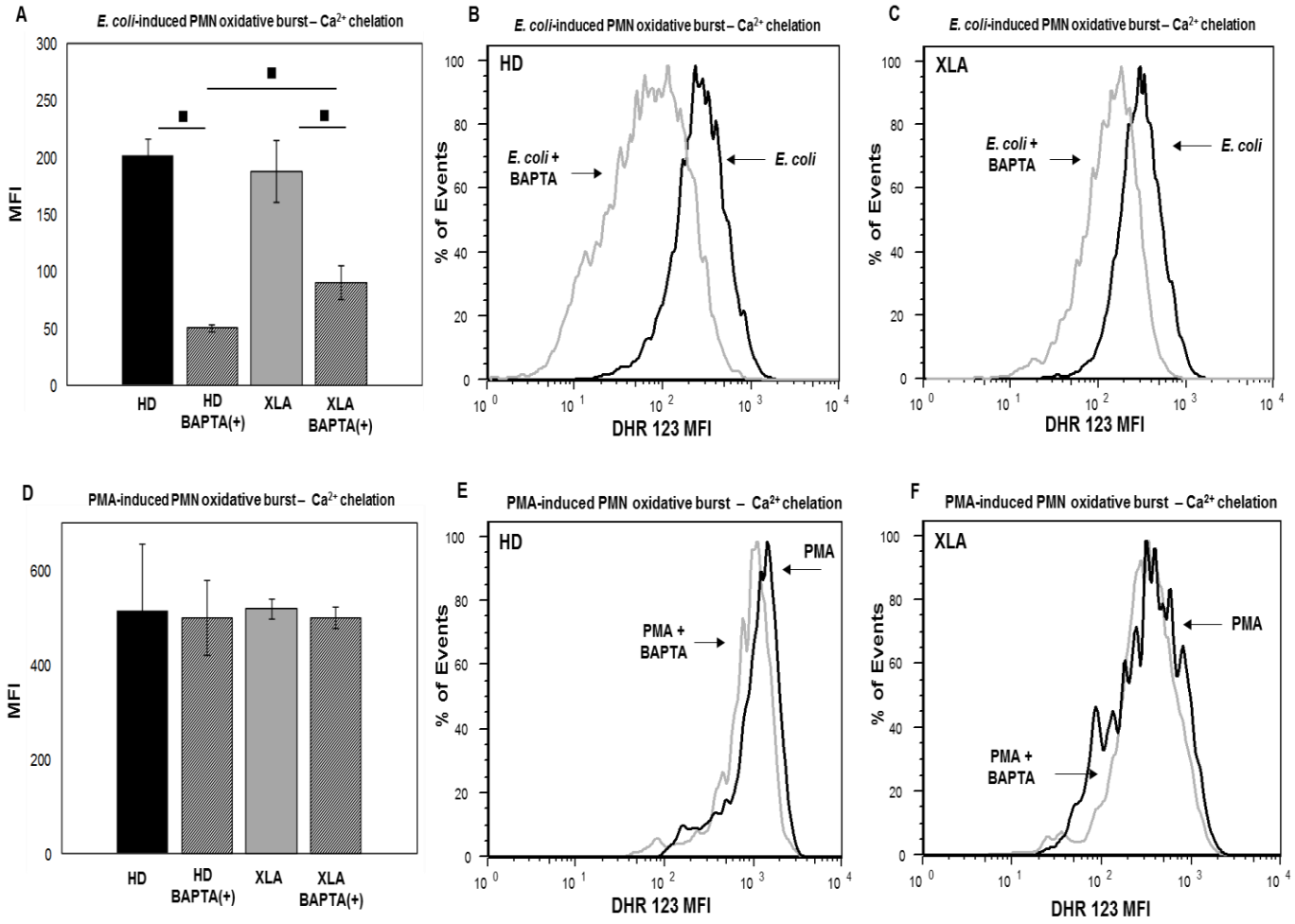


Figure 27. PMN oxidative burst – Ca2+ chelation. Whole blood samples were pre-treated with 100 µM of the calcium chelator BAPTA-AM for 30 min and incubated in a water bath for 20 min at 37°C with opsonized *E. coli* (1-2x10^9/ml) and PMA (1.62 mM). The conversion of DHR 123 to R 123 was used to evaluate the intracellular ROS production. FSC and SSC characteristics were used to identify the PMN population. Also in PMN the reduction of E. coli-induced oxidative burst was lower in XLA than HD (plot A) (▪*p=0.003*). An average reduction of 75% E. coli-induced oxidative burst from HD (▪*p=0.01*) and of 50% from XLA patients (▪*p=0.02*) was observed (plot A), while BAPTA-AM did not affect the oxidative burst induced by PMA both HD and XLA patients (plot D). Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation. Statistical significance, indicated as p value, was determined by the nonparametric Mann-Whitney test and Wilcoxon Signed Rank test. Plot B and C show PMN E. coli-induced oxidative burst in representative HD and XLA patient, black peak BAPTA-AM(-) and gray peak BAPTA-AM(+). Plot E and F show PMN PMA-induced oxidative burst in representative HD and XLA patient, black peak BAPTA-AM(-) and gray peak BAPTA-AM(+).

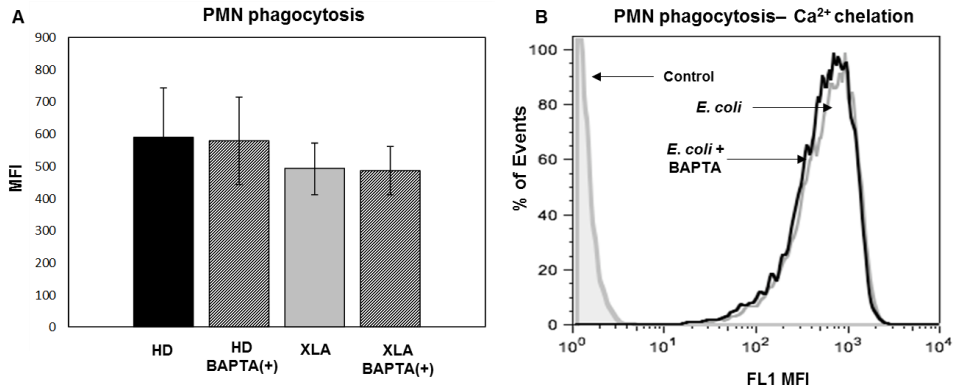


Figure 28 BAPTA pre-treated PMN phagocytosis in XLA patients and HD. A regular phagocytosis was observed on PMN (plot A) pre-treating whole blood samples with 100 µM of calcium chelator BAPTA-AM. In plot B is show PMN phagocytosis of representative XLA patient, black peak BAPTA-AM(-) and gray peak BAPTA-AM(+). Results are expressed as Mean Fluorescence Intensity. Histograms denote mean values and bars standard deviation.

DISCUSSION

5. DISCUSSION

In 2010, a study from Ziegler-Heitbrock L. *et al.* pointed the attention on a new classification and nomenclature of human monocyte subsets [126]. It distinguished classical and non-classical monocytes and it introduced a new subset called intermediate monocytes. Before this classification, intermediate monocytes were not considered as an individual subset because of its low number and transitional nature. Recent studies through gene expression profiling confirmed the unique nature of intermediate human monocytes [128]. Recently, clinical studies demonstrated an expansion of these cells in several inflammatory and autoimmune diseases like asthma, rheumatoid arthritis and HIV infection [145]. Several studies showed intermediate monocyte subset as the main producer of TNF-α and other pro-inflammatory cytokines [128,133,144]. Moreover, in CVID patients the expansion of CD14bright $CD16^{+}$ monocytes were associated with T cell activation [224]. For these reasons, intermediate monocytes are now often called pro-inflammatory monocytes. CVID is characterized by multiple abnormalities in the immune system beyond humoral immunity involving dendritic cells (DCs), CD8 T cells, CD4 T cells, invariant natural killer T (iNKT) cells and regulatory T cells (Tregs) [225–227]. We demonstrated that CVID patients displayed an increased frequency of pro-inflammatory intermediate monocytes ($CD14^{++}CD16^{+}$). Interestingly, in line with our observation on CVID, also XLA patients show an increased percentage of intermediate monocyte, in agreement with their underlying inflammatory condition. This observation suggests that a greater frequency of intermediate monocytes could be a feature shared between different PAD. On the contrary we found an increased expression of CD11b and Siglec 9 only on classical monocytes from CVID patients while monocytes from XLA patients showed a normal phenotype, suggesting that the overexpression of these receptors can be a peculiar characteristic of CVID and possibly not verifiable in others PADs. CD11b is a component of the phagocytic receptor αMβ2 (CD11b/CD18) [228] that plays an important role in phagocytosis process [206]. Its overexpression on classical monocytes is probably caused by a continuous chronic stimulation considering the inflammatory status of CVID patients, even if further investigations are necessary, while Siglec 9, a member of transmembrane sialic acid-binding proteins CD33-related [209,210] has a postulated inhibitory activity on the immune response through host and bacterial sialoglycans recognition [221,222]; therefore although CD11b and Siglec 9 have different biological activities, our hypothesis is that the simultaneous overexpression of both receptors is a system to respond to infections and to avoid an excess of immune response. However, differently from a previous study [229] our results show that the functionality of monocytes, expressed as oxidative burst and phagocytosis, was normal in CVID patients and these

observation were also confirmed in XLA patients. This discrepancy from the previous study might be mainly due to the different stimuli used for the induction of oxidative burst, in that Aukrust *et al* [229] used non-opsonized zymosan acting via TLR2 [230] and PMA, while we used a physiological stimulus, the opsonized *E. coli* that acting via FcγRs [231].

Our observations clearly indicate that the monocyte compartment is not affected by the immune abnormalities of CVID patients and that in XLA patients the lack of a functional BTK did not affect the ROS production as well the phagocytic process. Conversely, it remains possible that the expansion of the intermediate subset may contribute to the inflammatory status of patients.

The effect of IVIg on innate immunity cells, it has been mainly analyzed by *in vitro* models providing conflicting results [197,202,232]. A recent study [194] has evaluated the *in vivo* effect of IVIg on classical and non-classical monocytes of patients affected by CVID showing a reduction in the number of non-classical $CD14^{+}CD16^{++}$ monocytes and a concomitant decrease of TNF-α, after 4h from IVIg administration. Therefore, we studied the *in vivo* effect of IVIg replacement, taking into consideration the most recent classification of monocytes. We observed in CVID patients an average reduction of about 23% of the total monocyte population immediately after IVIg infusion but, more importantly, this reduction was not equally distributed among the three monocytes subsets but it involved mainly the intermediate monocytes. in line with the observations made on CVID patients, we reported in XLA patients a prompt reduction of intermediate subset after IVIg infusion. This seems to confirm the normalizing effect of IVIg infusion on monocytes distribution among the different subpopulations. The reduction of the pro-inflammatory monocytes might be a mechanism through which IVIg infusion exert an anti-inflammatory effect, even when infused at replacement dosages. The mechanism by which IVIg are able to reduce the intermediate subset remains unclear although it is possible that the apoptotic process is involved. It was hypothesized that anti-Fas antibody-mediated Fas (CD95/APO-1) ligation and activation of caspases may be involved in that IVIg preparations contain agonistic and antagonistic anti-CD95 antibodies. These antibodies might interact with CD16+ monocytes that constitutively up-regulated pro-apoptotic genes making these cells particularly susceptible for apoptotic death [194, 233].

In addition, we found that IVIg induced a reduction of the expression of CD11b and Siglec 9 in CVID patients but exclusively on the monocytes subsets that overexpress them, suggesting that IVIg could have a "normalizing" effect only when the surface receptor are overexpressed while IVIGs do not affect the receptor expression when monocytes have a normal phenotype, as observed in XLA patients. The reduction of Siglec

9 was possibly due to the binding of antibodies contained in IVIg preparations and Siglec 9 by a specific interaction [198,234]. These naturally occurring autoantibodies might contribute to the anti-inflammatory effects of IVIg via cell death regulation. All these effects could be an explanation for the anti-inflammatory effect that IVIg exert and for which is not known a univocal explanation. It is therefore possible that IVIg exert an anti-inflammatory effect by modulating the function of monocytes, an hypothesis corroborated by the slight reduction of the phagocytic capacity and oxidative burst observed in CVID and XLA patients after the infusion. A possible explanation for the reduction of ROS production by monocytes after the infusion is probably related to the reduced phagocytosis ability. It is possible that IVIg may transiently bind the Fc receptors on monocytes' surface and so limiting their availability to the bind with opsonized *E. coli*. In this way, IVIg administrated at replacement dosage might exert a dampening effect on monocytes compartment by reducing their fundamental functions. The anti-inflammatory role of IVIg exerted on monocytes was also showed by Siedlar et al [194] while other studies showed that the positive role of IVIg in CVID was due to a modulation of cellular functions of B cells, T cells, and dendritic cells [184,235-238]. Nevertheless, we observed that intravenous IgG administration did not affect the monocytes' functional ability to respond to a bacterial stimulation through FcγR, in terms of receptors expression, also confirming that in these processes the BTK kinase activity is not involved or more probably, it is bypassed by other redundant pathways. Although the oxidative burst and phagocytosis were reduced after IVIg infusion, they remain within the normal range, suggesting that IVIg do not severely affect the ability to appropriately respond to pathogens.

With the aim to bring to light the possible variation of PMN phenotypes in CVID and XLA patients, we analyzed a pool of receptors involved in their activation and cellular responses. Recognition and adhesion of serum-opsonized bacteria to human neutrophils were reported to be mediated by the binding of C3b and iC3b to specific CRs, including the integrins CD11b/CD18 (CR3) and CD11c/CD18 (CR4) [251-254]. Differently from what previously seen on monocytes, we found that the expression of all receptors analyzed was similar in CVID and XLA patients compared to HD, with the exception of an overexpression in XLA patients of CD66b, known as a granulocyte activation marker. This might indicate that in XLA patients PMNs have an altered activation status. Further investigations are necessary to clarify if the absence of BTK could induce an altered PMNs' responses to pathogens. Differently from that observed at resting state [239], *E. coli* stimulation induced a similar up-regulation of CD11c, CD66b, CD16 Siglec 9 expression in CVID, XLA and HD, demonstrating that the PMNs' ability to respond to opsonized pathogen through receptors' overexpression

is unaltered and that the BTK kinase activity is not involved in this process or it is bypassed by other redundant pathways. In line with that observed in previous study [223] we found a similar reduced expression of CD181, the receptor of IL-8, after stimulation with *E. coli*. It is possible that the proteolytic degradation might be responsible for the down-regulation of CD181 after phagocytosis [223]. However, we observed that *E. coli* stimulation induced a stronger CD11b up-regulation in CVID than HD (especially noticeable in eight patients, even if no clinical correlation was found). The wide heterogeneity of this results did not surprise considering that CVID encompasses a group of heterogeneous disorders. In fact, the number of potential distinct entities within CVID is high [10]. The clinical and immunological heterogeneity might also explain the different results obtained in our study and the results published by Casulli *et al* who showed a low CD11b expression on PMN at resting state in CVID [239]. In addition, a considerable variability in the CD11b expression in leukocytes exists also among healthy donors [240]. Therefore, it might be important to analyze the expression of this receptor in larger populations. Moreover, the procedure used for PMN isolation might also induce a more variable expression of certain receptors [203-205]. With the aim to understand how the isolation procedure can affect the expression of surface receptors of PMNs, in a subgroup of CVID patients we repeated the analysis of the expression of CD16, CD11b and Siglec 9 on isolated PMN. While Siglec 9 and CD16 expression was similar on PMN from whole blood and on isolated PMN, the expression of CD11b was greatly increased on isolated PMN, showing the importance of the technique used. However, we confirmed that there were no significant differences between healthy donors and CVID.

Similarly to what observed by Kutukculer *et al.* in CVID pediatric population [241], we showed a normal *E. coli*-induced oxidative burst performed by PMN from CVID patients and this result was also confirmed in XLA patients. On the contrary, in CVID the oxidative burst was increased when induced by PMA. This difference might depend by the mechanism of action of these stimuli. While opsonized *E. coli* engage the phagocytosis through Fc receptors, in a multi-step process that comprises the formation of phagosome [242,243], PMA is a chemical activator of oxidative burst used with the aim to minimize the effect of potential differences on the surface receptor status among individuals [240]. Indeed, PMA is able to permeate through the plasma membrane and to interact directly with protein kinase C (PKC) by inducing the ROS production [244,245] and thus by reproducing the overall activity of respiratory burst [240]. Our results suggest that PMN from CVID patients are capable to perform a normal oxidative burst when it is triggered by phagocytosis but when the phagocytic step is avoided the oxidative burst is enhanced. As also shown by Casulli *et al* [239] if PMN were pretreated with TLR1/2 and TLR4 agonists or TNF-α and then stimulated with N-formyl-Met-Leu-Phe (fMLP),

the ROS production was reduced. Therefore, it appears that different stimuli activate different PMN pathways resulting in different ROS production.

Currently, the effects of IVIg on PMN from CVID patients has not been fully elucidated [193,226] and the few published studies were mainly conducted *in vitro*. Different mechanisms, such as inhibition of the complement cascade, modulation of cytokine production, neutralization of autoantibodies and the modulation of inhibitory FcγR expression have been suggested as mechanism responsible for the anti-inflammatory activity of IVIg administered at high dosages [246]. Several theories have been postulated on the mechanisms through which IVIg preparations exert their immunoregulatory properties at replacement dosages and different studies proposed an involvement of different type of cells. Some studies demonstrated that natural anti-Siglec 9 autoantibodies present in IVIg might lead to a neutrophil apoptosis that is enhanced by pro-inflammatory cytokines [234,247,248]. Others studies indicated that IVIg are capable to inhibit PMN degranulation [202] and to decrease their pro-inflammatory activity [201]. Higurashi *et al* [196] showed that IVIg trigger *in vitro* ROS production by neutrophils primed with TNF-α and Teeling *et al* [197] showed that IVIg could induce *in vitro* neutrophils degranulation even if with a wide variability between different donors. Moreover, Casulli *et al* [193] showed an increased CD11b/CD18 expression and an enhanced ROS production when PMN from healthy donors were incubated *in vitro* with IVIg at low dosages (1–5 mg/ml), a concentration similar to that reached after IVIg administration in CVID patients *in vivo*. Some evidence brought to light that the PMN isolation step might induce the expression of surface molecules undetectable in whole blood that it might interfere with PMN function [249] and it might alter cellular interactions necessary to preserve PMN viability [250].

Our results suggest that IVIg might exert an anti-inflammatory effect at replacement dosages by acting on the monocyte compartment but probably not on PMN. Indeed, in this study we found that the results on the effects of IVIg effects on monocytes are not replicated on PMN. We showed that IVIg did not affect the receptors expression on PMN in all experimental condition used with the exception of CD181 that decrease after IVIg infusion in CVID patients. Actually, a lower expression of CD181 could result in a lower responsiveness of PMNs to IL-8. Considering that IL-8 is a chemoattractant for neutrophils, and that it can to induce phagocytosis and oxidative burst, it is possible that the reduction of its expression induced by IVIg may contribute to an anti-inflammatory effect that infusions of immunoglobulins exert a replacement dosage. Further investigations are necessary and it might be possible that *in vitro* effect of IVIg does not

recapitulate the *in vivo* phenomenon since many cellular and mediator interactions are lacking in the in vitro assays.

Moreover we found that the PMNs' intensity of oxidative burst after IVIg infusion overlapped that observed before infusion in CVID and XLA patients. This ability to perform regular oxidative burst after IVIg when *E.coli* is provided as a stimulus is also reflected by the fact that we found a regular phagocytosis even after the infusion. The finding that only on PMNs from CVID patients the IVIg infusion reduce the oxidative burst when triggered by PMA is in line with the previous observations that suggest a normalizing effect of immunoglobulins infusion only when a cell phenotype or function are altered. Although our results show that PMN from CVID and XLA patients under treatment with immunoglobulin for several years have a normal phenotype and function, further investigations are necessary to verify the PMN receptor expression and functionality in naïve untreated patients and to better understand the mechanisms underlying the different ROS production according to the stimuli provided.

The study of the oxidative burst and phagocytosis performed in XLA patients provided us important information regard the killing ability of innate immune cells in these patients and prompted us to further investigate the role of BTK in these functions. The oxidative burst is a crucial reaction performed by innate immune cells needed to degrade internalized particles and bacteria; it is a process dependent from Ca^{2+} mobilization that is the main event downstream BTK activation, BTK is able to activate PLCγ2 which generates DAG and IP3 that trigger the release of Ca^{2+} from endoplasmic reticulum [255] (Fig. 28). Interestingly, our results show that the respiratory burst induced through FcγRs in XLA patients is preserved both on monocytes and on PMN, suggesting that BTK is not essential for calcium mobilization and consequently for the ROS production. Our observations are supported by others studies that reported different pathways for Ca^{2+} mobilization [262-264]. Surprisingly, we found that BAPTA-AM exerted a greater inhibition of oxidative burst in HD than XLA, suggesting that oxidative burst in XLA patients appear less dependent from Ca^{2+} mobilization compared to HD. Our hypothesis is that the residual ability to produce ROS, following stimulation through FcγR, observed in patients, is probably due to a greater efficacy of Ca^{2+} independent stimulation of PKC, indeed PKC is normally maintained in an inactive conformation [256-258] and it can be activated both by DAG, produced by PLCγ2, and by Ca^{2+} [259] (Fig. 28). A support to our idea comes from the observation that BAPTA-AM did not affect the ROS production when PMA was provided as stimulus. In fact, phorbol esters are able to bind the same site of DAG in PKC and are able to induce its activation [260, 261] (Fig 28). Moreover, our observations are also in line with the conclusions made by Ren *et al.* [112] who

hypothesized that the lack of BTK kinase activity within a whole organism could have limited effects on the FcγR-mediated processes.

In agreement with Ren *et al* [112], we found that the phagocytosis process performed by monocytes and PMN was fully functional, suggesting that BTK kinase activity (and the downstream Ca^{2+} mobilization) is not required for phagocytosis. Indeed, consistent with previous studies [265-268] we observed that the phagocytic process remains unaltered in PMN and monocytes pre-treated with the Ca^{2+} chelator BAPTA-AM, supporting the independence of phagocytosis from Ca^{2+} mobilization. Our results observed in XLA patients

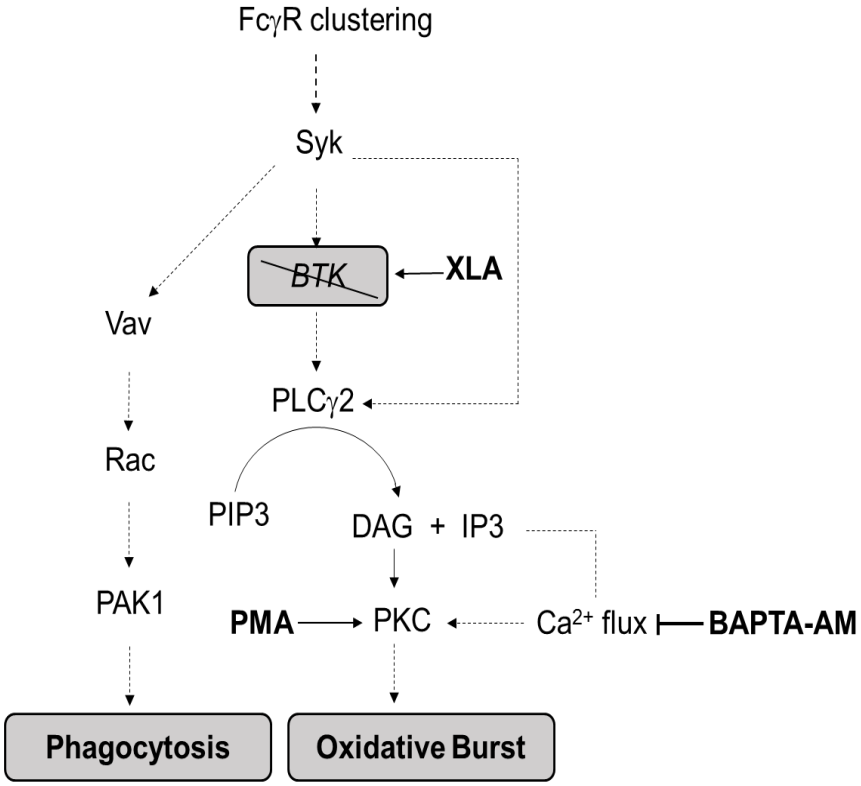


Figure 28 Schematic representation of signaling pathways for phagocytosis and oxidative burst. As a result of FcγR clustering, two separate pathways lead to phagocytosis, through a calcium independent way, or to the oxidative burst in a calcium dependent manner. Syk induce the activation of Vav and the downstream signaling that lead to the phagocytosis process. The main pathway for the activation of the oxidative burst consist in the activation of BTK by Syk and the consequent phosphorylation of PLCγ2. PLCγ2 activated produce DAG and IP3 from PIP3. DAG directly activate PKC while IP3 bind IP3R on endoplasmic reticulum inducing Ca^{2+} mobilization that lead to PKC activation and finally to the oxidative burst. The lack of BTK in XLA patients is effectively bypassed by the direct stimulation of PLCγ2 by Syk. The inhibition of Ca^{2+} mobilization by BAPTA-AM strongly reduce the oxidative burst. PMA is able to bind PKC to the same binding site of DAG and bypass both the production of DAG and the calcium flux from endoplasmic reticulum.

confirm also those obtained by Mangla *et al.* [269] on Xid mice and by Ren *et al* using *in vitro* a BTK inhibitor [112].

In conclusion, taken together our results show that XLA and CVID patients had an increased frequency of pro inflammatory monocytes and that IVIg infusion are able to reduce their percentage, suggesting the hypothesis that the increased percentage of $CD14^{++}$ $CD16^{+}$ monocytes is a common characteristic of these two primary immunodeficiencies with a different pathogenesis but with a common clinical infectious phenotype. In this way it seems that IVIg exert, in these primary immunodeficiencies, a normalizing effect on monocyte compartment by reducing the percentage of intermediate monocytes. This effect may contribute to the anti-inflammatory effect that IVIg exert also at replacement dosages. Actually, our results seem to show that IVIg infusion exert a damping effect on the overall activity of circulating innate immune cells and in particular on monocytes, even if the process analyzed remained fully functional. Finally, the study of calcium chelation suggest that in XLA patients exists redundant pathways that bypass the lack of BTK, but that also exist in XLA patients efficacy Ca^{2+} independent pathways for the oxidative burst induced by FcγR clustering, anyway further investigations are needed to better characterize the pathways involved.

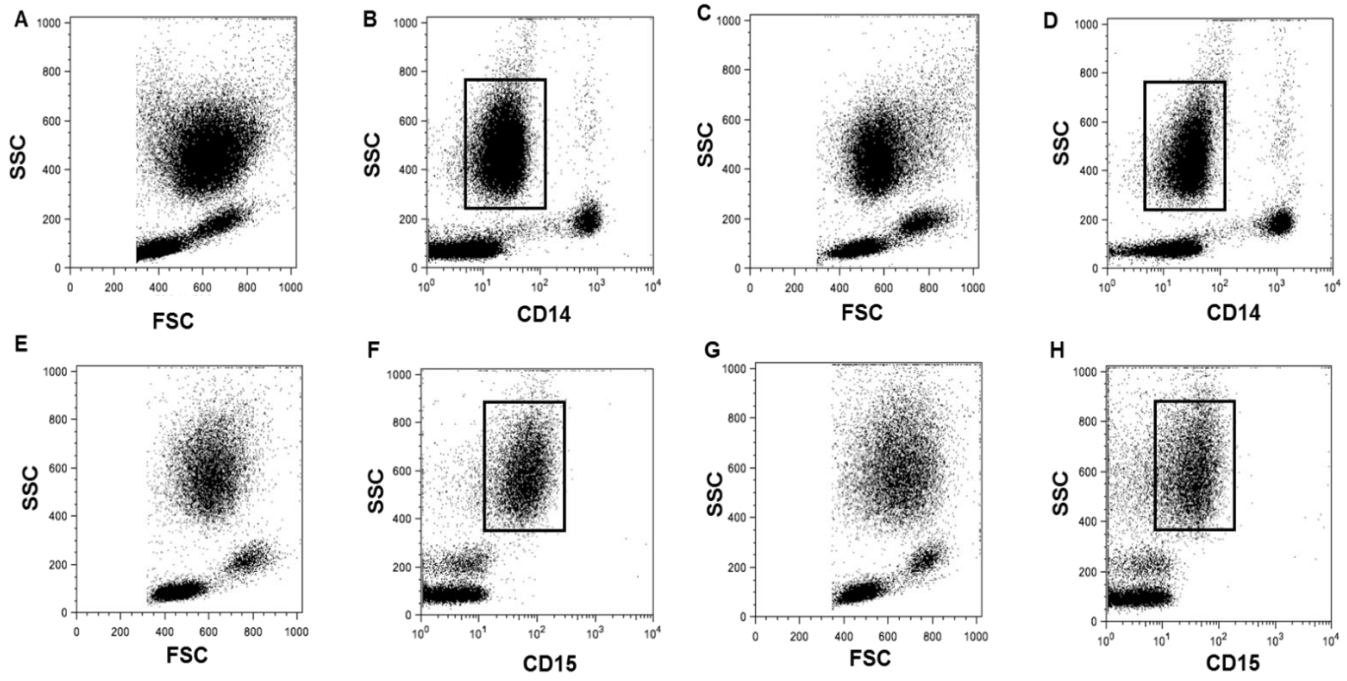
SUPPLEMENTARY

MATERIAL

6. SUPPLEMENTARY MATERIAL

Patient	Age/sex	*Serum IgG (mg/dL)	Monthly cumulative IVIg dose	Neutrophils (10^9/L)	Comorbidities
1	71/F	766	30 g	4.6	Respiratory tract infections
2	74/F	881	30 g	6.4	Celiac disease, chronic gastritis
3	67/M	635	30 g	3.7	Vitiligo, COPD, respiratory tract infections
4	71/M	739	28 g	3.8	Chronic gastritis
5	37/F	697	30 g	6.1	Chronic thyroiditis
6	59/F	553	25 g	5.3	Splenomegaly, chronic gastritis
7	38/F	544	25 g	5.1	Chronic gastritis
8	58/F	634	30 g	4.2	Respiratory tract infections, COPD, chronic gastritis
9	57/F	450	30 g	4.7	Chronic gastritis, respiratory tract infections,
10	52/F	681	30 g	2.5	Respiratory tract infections, COPD
11	31/M	606	40 g	3.4	Thrombocytopenia, splenomegaly
12	40/F	680	30 g	3.8	Pancytopenia
13	55/M	812	30 g	2.9	Chronic diarrhea
14	53/M	633	28 g	6.8	Respiratory tract infections
15	47/M	810	30 g	5.2	Chronic gastritis
16	64/F	770	20 g	1.9	Uveitis, Respiratory tract infections
17	24/M	859	30 g	2.9	Ulcerative recto-colitis
18	15/F	822	25 g	1.5	Autoimmune hemolytic anemia
19	67/M	447	25 g	4.6	Type 2 diabetes mellitus, COPD
20	25/M	720	30 g	4.1	COPD, chronic gastritis
21	34/M	630	30 g	4.9	Chronic diarrhea, COPD
22	64/M	526	30 g	3.1	Respiratory tract infections, COPD
23	47/M	790	28 g	3.7	Respiratory tract infections

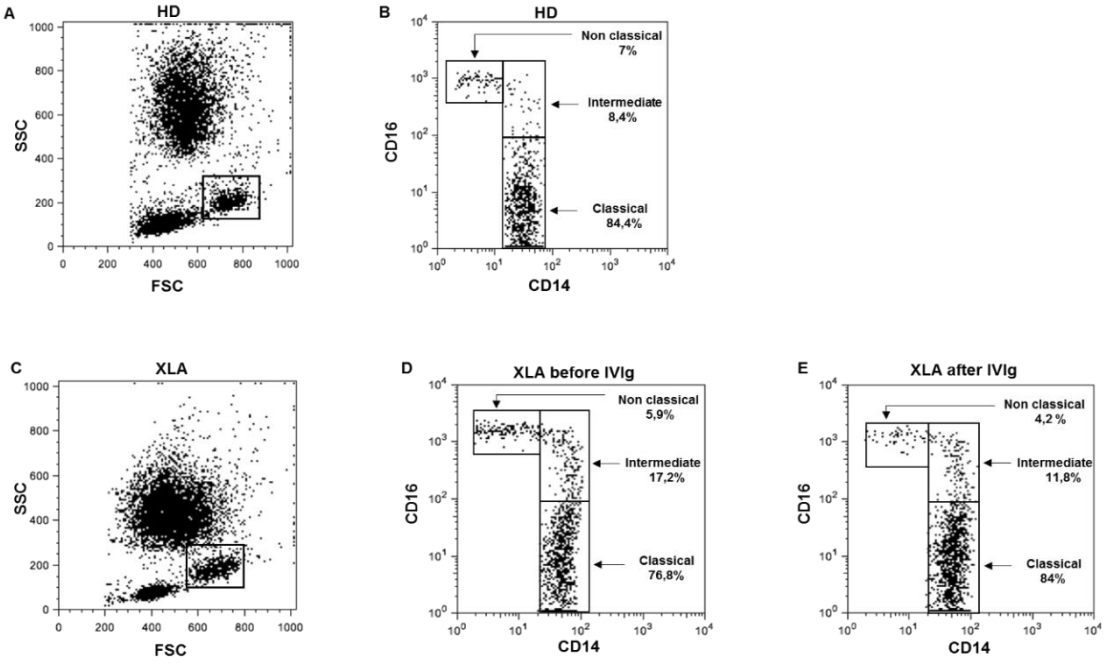
Supplementary table 1 Demographic and clinical data of patients CVID *Serum IgG values refer to pre-infusion levels. F, Female; M, male. COPD: Chronic obstructive pulmonary disease.



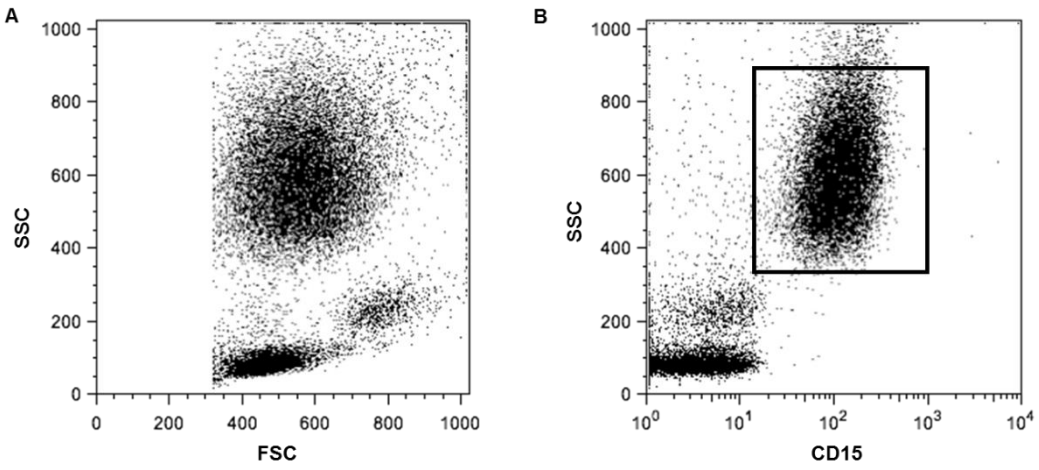
Supplementary Figure 1. **Identification of PMN population.** Peripheral blood samples were lysed for 20 min at room temperature and washed twice. White cells were suspended in ice-cold PBS and stained at 4 °C for 30 min with CD14 antibody. Samples were washed, suspended in ice-cold PBS and analyzed by flow cytometry. PMN were identified by SSC characteristics and CD14 negative in order to exclude monocytes. A representative healthy donor (plot A–B) and a representative CVID patient (plot C–D) are shown. CD15 antibody was used as a specific marker of neutrophils: a representative healthy donor (plot E–F) and a representative CVID (plot G–H) are shown.

Patient	Age/sex	*Serum IgG (mg/dL)	*Serum IgA (mg/dL)	*Serum IgM (mg/dL)	B cells (%)	Monocytes (10^9/L)	Neutrophils (10^9/L)	Comorbidities
1	37/M	910	0.02	0.02	0.13	0.47	4.6	Celiac disease, respiratory tract infections, COPD
2	38/M	660	0.5	0.25	0.04	0.69	6.4	Respiratory tract infections, COPD
3	60/M	750	0.01	0.04	0.15	0.53	3.7	Epilepsy, stroke
4	39/M	680	0.03	0.02	0.2	0.48	3.8	Lymphadenomegaly, neurophaty, conjunctivitis, dermatitis
5	20/M	1090	0.02	0.01	0.2	0.92	6.1	Respiratory tract infections, COPD, dermatitis
6	26/M	240	0.02	0.01	0.1	0.93	5.3	Celiac disease, respiratory tract infections

Supplementary table 2. Demographic and clinical data of patients XLA *Serum IgG, IgA and IgG values refer to pre-infusion levels. M, male. COPD: Chronic obstructive pulmonary disease.



Supplementary figure 2. Identification of monocyte subpopulations. Peripheral blood samples lysed for 20 minutes at room temperature and washed twice. White cells were suspended in ice-cold PBS and stained at 4°C for 30 min with CD14 and CD16 fluorochrome-labeled antibodies. Samples were washed, suspended in ice cold PBS and analyzed by a 4-color flow cytometry single platform. Monocytes subpopulations were phenotypically classified according to their expression of CD14 and CD16 into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$) in a representative HD (plot A-B) and in a representative XLA patient before and after IVIg infusion (plot C-E). Percentages denote mean values.



Supplementary figure 3. Identification of PMN population in XLA patient. Peripheral blood samples were lysed for 20 minutes at room temperature and washed twice. White cells were suspended in ice-cold PBS and stained at 4°C for 30 min with CD15 fluorochrome-labeled antibody. Samples were washed, suspended in ice-cold PBS and analyzed by flow cytometry. PMN were identified by Side Scatter (SSC) and CD15 fluorochrome-labeled antibody. CD15 was used as a specific marker of neutrophils. A representative XLA patient is shown (plot A-B).

REFERENCES

[1] J. L. Casanova, L. Abel, EMBO J. 26, 915 (2007)

[2] Fudenberg H, Good RA, Goodman HC, Hitzig W, Kunkel HG, Roitt IM, et al. Primary immunodeficiencies: report of a World Health Organization Committee. Pediatrics 1971;47:927-46.

[3] Cooper MD, Faulk WP, Fudenberg HH, Good RA, Hitzig W, Kunkel H, et al. Classification of primary immunodeficiencies. N Engl J Med 1973;288:966-7.

[4] Notarangelo LD, Fischer A, Geha RS, Casanova JL, Chapel H, Conley ME, et al. Primary immunodeficiencies: 2009 update. J Allergy Clin Immunol 2009; 124:1161-78.

[5] Orange JS, Glessner JT, Resnick E, Sullivan KE, Lucas M, Ferry B, et al. Genome-wide association identifies diverse causes of common variable immunodeficiency. J Allergy Clin Immunol 2011;127:1360 1367.e6.

[6] Yong PF, Thaventhiran JE, Grimbacher B. "A rose is a rose is a rose," but CVID is not CVID common variable immune deficiency (CVID), what do we know in 2011? Adv Immunol 2011;111:47-107.

[7] Chapel H, Cunningham-Rundles C. Update in understanding common variable immunodeficiency disorders (CVIDs) and the management of patients with these conditions. Br J Haematol 2009; 145:709-27.

[8] Cunningham-Rundles C. How I treat common variable immune deficiency. Blood 2010;116:7-15.

[9]. Kardar G, Oraei M, Shahsavani M, Namdar Z, Kazemisefat G, Haghi Ashtiani M, et al. Reference intervals for serum immunoglobulins IgG, IgA, IgM and complements C3 and C4 in Iranian healthy children. Iran J Public Health 2012;41:59-63.

[10] Francisco A. Bonilla, MD, PhDa, Isil Barlan, MDb, Helen Chapel, MDc, Beatriz T. Costa-Carvalho, MDd et al. , International consensus document (ICON): common variable immunodeficiency disorders, J. Allergy Clin. Immunol. Pract. 4 (2016) 38–5

[11] Leiva LE, Zelazco M, Oleastro M, Carneiro-Sampaio M, Condino-Neto A, Costa-Carvalho BT, et al. Primary immunodeficiency diseases in Latin America: the second report of the LAGID registry. J Clin Immunol 2007;27:101-8.

[12] Boyle JM, Buckley RH. Population prevalence of diagnosed primary immunodeficiency diseases in the United States. J Clin Immunol 2007;27:497-502.

[13] Rachid R, Bonilla FA. The role of anti-IgA antibodies in causing adverse reactions to gamma globulin infusion in immunodeficient patients: a comprehensive review of the literature. J Allergy Clin Immunol 2012;129:628-34.

[14] Warnatz K, Denz A, Drager R, Braun M, Groth C, Wolff-Vorbeck G, et al. Severe deficiency of switched memory B cells (CD27(þ)IgM(_)IgD(_)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 2002;99:1544-51.

[15] Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EURO class trial: defining subgroups in common variable immunodeficiency. Blood 2008; 111:77-85.

[16] Ochtrop ML, Goldacker S, May AM, Rizzi M, Draeger R, Hauschke D, et al. T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency. Blood 2011;118:309-18.

[17] Taubenheim N, von Hornung M, Durandy A, Warnatz K, Corcoran L, Peter HH, et al. Defined blocks in terminal plasma cell differentiation of common variable immunodeficiency patients. J Immunol 2005;175:5498-503.

[18] Bryant A, Calver NC, Toubi E, Webster AD, Farrant J. Classification of patients with common variable immunodeficiency by B cell secretion of IgM and IgG in response to anti-IgM and interleukin-2. Clin Immunol Immunopathol 1990;56:239-48.

[19] Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood 2011;118:6814-23.

[20] Malphettes M, Gerard L, Carmagnat M, Mouillot G, Vince N, Boutboul D, et al. Late-onset combined immune deficiency: a subset of common variable immunodeficiency with severe T cell defect. Clin Infect Dis 2009;49:1329-38.

[21] Thon V, Eggenbauer H, Wolf HM, Fischer MB, Litzman J, Lokaj J, et al. Antigen presentation by common variable immunodeficiency (CVID) B cells and monocytes is unimpaired. Clin Exp Immunol 1997;108:1-8.

[22] Fischer MB, Hauber I, Vogel E, Wolf HM, Mannhalter JW, Eibl MM. Defective interleukin-2 and interferon-gamma gene expression in response to antigen in a subgroup of patients with common variable immunodeficiency. J Allergy Clin Immunol 1993;92:340-52.

[23] Holm AM, Aukrust P, Aandahl EM, Muller F, Tasken K, Froland SS. Impaired secretion of IL-10 by T cells from patients with common variable immunodeficiencyeinvolvement of protein kinase A type I. J Immunol 2003;170:5772-7.

[24] Adelman DC, Matsuda T, Hirano T, Kishimoto T, Saxon A. Elevated serum interleukin-6 associated with a failure in B cell differentiation in common variable immunodeficiency. J Allergy Clin Immunol 1990;86:512-21.

[25] Martinez-Pomar N, Raga S, Ferrer J, Pons J, Munoz-Saa I, Julia MR, et al. Elevated serum interleukin (IL)-12p40 levels in common variable immunodeficiency disease and decreased peripheral blood dendritic cells: analysis of IL-12p40 and interferon-gamma gene. Clin Exp Immunol 2006;144: 233-8.

[26] Scott-Taylor TH, Green MR, Raeiszadeh M, Workman S, Webster AD. Defective maturation of dendritic cells in common variable immunodeficiency. Clin Exp Immunol 2006;145:420-7.

[27] Yu JE, Knight AK, Radigan L, Marron TU, Zhang L, Sanchez-Ramon S, et al. Toll-like receptor 7 and 9 defects in common variable immunodeficiency. J Allergy Clin Immunol 2009;124:349-56. 356.e1-3.

[28] Gathmann B, Mahlaoui N, Ceredih, Gerard L, Oksenhendler E, Warnatz K, et al. Clinical picture and treatment of 2212 patients with common variable immunodeficiency. J Allergy Clin Immunol 2014;134:116 26.

[29] Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol 1999;92: 34-48.

[30] Chapel H, Lucas M, Patel S, Lee M, Cunningham-Rundles C, Resnick E, et al. Confirmation and improvement of criteria for clinical phenotyping in common variable immunodeficiency disorders in replicate cohorts. J Allergy Clin Immunol 2012;130:1197-1198.e9.

[31] Boileau J, Mouillot G, Gerard L, Carmagnat M, Rabian C, Oksenhendler E, et al. Autoimmunity in common variable immunodeficiency: correlation with lymphocyte phenotype in the French DEFI study. J Autoimmun 2011;36: 25-32.

[32] Kutukculer N, Karaca NE, Demircioglu O, Aksu G. Increases in serum immunoglobulins to age-related normal levels in children with IgA and/or IgG subclass deficiency. Pediatr Allergy Immunol 2007;18:167-73.

[33] Maglione PJ, Overbey JR, Radigan L, Bagiella E, Cunningham-Rundles C. Pulmonary radiologic findings in common variable immunodeficiency: clinical and immunological correlations. Ann Allergy Asthma Immunol 2014;113:452-9.

[34] Maarschalk-Ellerbroek LJ, de Jong PA, van Montfrans JM, Lammers JW, Bloem AC, Hoepelman AI, et al. CT screening for pulmonary pathology in common variable immunodeficiency disorders and the correlation with clinical and immunological parameters. J Clin Immunol 2014;34:642-54.

[35] Fasano MB, Sullivan KE, Sarpong SB, Wood RA, Jones SM, Johns CJ, et al. Sarcoidosis and common variable immunodeficiency: report of 8 cases and review of the literature. Medicine (Baltimore) 1996;75:251-61.

[36] Lin JH, Liebhaber M, Roberts RL, Dyer Z, Stiehm ER. Etanercept treatment of cutaneous granulomas in common variable immunodeficiency. J Allergy Clin Immunol 2006;117:878-82.

[37] Mechanic LJ, Dikman S, Cunningham-Rundles C. Granulomatous disease in common variable immunodeficiency. Ann Intern Med 1997;127:613-7.

[38] Misbah SA, Spickett GP, Esiri MM, Hughes JT, Matthews WB, Thompson RA, et al. Recurrent intra-cranial granulomata presenting as spaceoccupying lesions in a patient with common variable immunodeficiency. Postgrad Med J 1992;68:359-62.

[39] Agarwal S, Cunningham-Rundles C. Autoimmunity in common variable immunodeficiency. Curr Allergy Asthma Rep 2009;9:347-52.

[40] Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. Blood 2010;116:e35-40.

[41] Cunningham-Rundles C. Hematologic complications of primary immune deficiencies. Blood Rev 2002;16:61-4.

[42] Michel M, Chanet V, Galicier L, Ruivard M, Levy Y, Hermine O, et al. Autoimmune thrombocytopenic purpura and common variable immunodeficiency: analysis of 21 cases and review of the literature. Medicine (Baltimore) 2004;83:254-63.

[43] Wang J, Cunningham-Rundles C. Treatment and outcome of autoimmune hematologic disease in common variable immunodeficiency (CVID). J Autoimmun 2005;25:57-62.

[44] Resnick ES, Moshier EL, Godbold JH, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. Blood 2012;119:1650-7.

[45] Agarwal S, Mayer L. Pathogenesis and treatment of gastrointestinal disease in antibody deficiency syndromes. J Allergy Clin Immunol 2009;124:658-64.

[46] Malamut G, Verkarre V, Suarez F, Viallard JF, Lascaux AS, Cosnes J, et al. The enteropathy associated with common variable immunodeficiency: the delineated frontiers with celiac disease. Am J Gastroenterol 2010;105:2262-75.

[47] Aslam A, Misbah SA, Talbot K, Chapel H. Vitamin E deficiency induced neurological disease in common variable immunodeficiency: two cases and a review of the literature of vitamin E deficiency. Clin Immunol 2004;112:24-9.

[48] Daniels JA, Lederman HM, Maitra A, Montgomery EA. Gastrointestinal tract pathology in patients with common variable immunodeficiency (CVID): a clinicopathologic study and review. Am J Surg Pathol 2007;31:1800-12

[49] Malamut G, Ziol M, Suarez F, Beaugrand M, Viallard JF, Lascaux AS, et al. Nodular regenerative hyperplasia: the main liver disease in patients with primary hypogammaglobulinemia and hepatic abnormalities. J Hepatol 2008;48:74-82.

[50] Ward C, Lucas M, Piris J, Collier J, Chapel H. Abnormal liver function in common variable immunodeficiency disorders due to nodular regenerative hyperplasia. Clin Exp Immunol 2008;153:331-7.

[51] Fuss IJ, Friend J, Yang Z, He JP, Hooda L, Boyer J, et al. Nodular regenerative hyperplasia in common variable immunodeficiency. J Clin Immunol 2013;33: 748-58.

[52] Elenitoba-Johnson KS, Jaffe ES. Lymphoproliferative disorders associated with congenital immunodeficiencies. Semin Diagn Pathol 1997;14:35-47.

[53] Gompels MM, Hodges E, Lock RJ, Angus B, White H, Larkin A, et al. Lymphoproliferative disease in antibody deficiency: a multi-centre study. Clin Exp Immunol 2003;134:314-20.

[54] Quinti I, Soresina A, Spadaro G, Martino S, Donnanno S, Agostini C, et al. Long-term follow-up and outcome of a large cohort of patients with common variable immunodeficiency. J Clin Immunol 2007;27:308-16.

[55] Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood 2008;112:277-86.

[56] Robinson M, Smart J, Tang M. Common variable immune deficiency disorders: a paediatric experience. Curr Trends Immunol 2008;9:85-91.

[57] Aspalter RM, Sewell WA, Dolman K, Gerard L, Oksenhendler E, Warnatz K, et al. Deficiency in circulating natural killer (NK) cell subsets in common variable immunodeficiency and X-linked agammaglobulinaemia. Clin Exp Immunol 2000;121:506-14.

[58] Giovannetti A, Pierdominici M, Aiuti F. T-cell homeostasis: the dark(ened) side of common variable immunodeficiency. Blood 2008;112:446. Author reply 446e7.

[59] Farrington M, Grosmaire LS, Nonoyama S, Fischer SH, Hollenbaugh D, Ledbetter JA, et al. CD40 ligand expression is defective in a subset of patients with common variable immunodeficiency. Proc Natl Acad Sci U S A 1994;91: 1099-103.

[60] Giovannetti A, Pierdominici M, Mazzetta F, Marziali M, Renzi C, Mileo AM, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. J Immunol 2007;178:3932-43.

[61] Oliva A, Scala E, Quinti I, Paganelli R, Ansotegui IJ, Giovannetti A, et al. IL- 10 production and CD40L expression in patients with common variable immunodeficiency. Scand J Immunol 1997;46:86-90.

[62] Arandi N, Mirshafiey A, Jeddi-Tehrani M, Abolhassani H, Sadeghi B, Mirminachi B, et al. Evaluation of CD4þCD25þFOXP3þ regulatory T cells function in patients with common variable immunodeficiency. Cell Immunol 2013;281:129-33.

[63] Horn J, Manguiat A, Berglund LJ, Knerr V, Tahami F, Grimbacher B, et al. Decrease in phenotypic regulatory T cells in subsets of patients with common variable immunodeficiency. Clin Exp Immunol 2009;156:446-54.

[64] Finck A, Van der Meer JW, Schaffer AA, Pfannstiel J, Fieschi C, Plebani A,et al. Linkage of autosomal-dominant common variable immunodeficiency tochromosome 4q. Eur J Hum Genet 2006;14:867-75.

[65] Hammarstrom L, Vorechovsky I, Webster D. Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). Clin Exp Immunol 2000;120:225-31.

[66] Kralovicova J, Hammarstrom L, Plebani A, Webster AD, Vorechovsky I. Finescale mapping at IGAD1 and genome-wide genetic linkage analysis implicate HLA-DQ/DR as a major susceptibility locus in selective IgA deficiency and common variable immunodeficiency. J Immunol 2003;170:2765-75.

[67] Schaffer AA, Pfannstiel J, Webster AD, Plebani A, Hammarstrom L, Grimbacher B. Analysis of families with common variable immunodeficiency (CVID) and IgA deficiency suggests linkage of CVID to chromosome 16q. Hum Genet 2006;118:725-9.

[68] Al-Herz W, Bousfiha A, Casanova JL, hatila T, Conley ME, Cunningham- Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency. Front Immunol 2014;5:162.

[69] Liadaki K, Sun J, Hammarstrom L, Pan-Hammarstrom Q. New facets of antibody deficiencies. Curr Opin Immunol 2013;25:629-38.

[70] Stiehm ER, Keller MA, Vyas GN. Preparation and use of therapeutic antibodies primarily of human origin. Biologicals 2008;36:363-74.

[71] Berger M, Jolles S, Orange JS, Sleasman JW. Bioavailability of IgG administered by the subcutaneous route. J Clin Immunol 2013;33:984-90.

[72] Lucas M, Hugh-Jones K, Welby A, Misbah S, Spaeth P, Chapel H. Immunomodulatory therapy to achieve maximum efficacy: doses, monitoring, compliance, and self-infusion at home. J Clin Immunol 2010;30:S84-9.

[73]. Orange JS, Belohradsky BH, Berger M, Borte M, Hagan J, Jolles S, et al. Evaluation of correlation between dose and clinical outcomes in subcutaneous immunoglobulin replacement therapy. Clin Exp Immunol 2012;169:172-81.

[74] Orange JS, Grossman WJ, Navickis RJ, Wilkes MM. Impact of trough IgG on pneumonia incidence in primary immunodeficiency: a meta-analysis of clinical studies. Clin Immunol 2010;137:21-30.

[75] Dichtelmuller HO, Biesert L, Fabbrizzi F, Falbo A, Flechsig E, Groner A, et al. Contribution to safety of immunoglobulin and albumin from virus partitioning and inactivation by cold ethanol fractionation: a data collection from Plasma Protein Therapeutics Association member companies. Transfusion 2011;51: 1412-30.

[76] Orbach H, Katz U, Sherer Y, Shoenfeld Y. Intravenous immunoglobulin: adverse effects and safe administration. Clin Rev Allergy Immunol 2005;29:173-84.

[77] Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., et al. (1993), Cell 72, 279–290.

[78] Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., et al. (1993), Nature 361, 226-233.

[79] Lederman, H. M., and Winkelstein, J. A. (1985), Medicine 64, 145–156.

[80] Farrar, J. E., Rohrer, J., and Conley, M. E. (1996), Clin. Immunol. Immunopathol. 81, 271–276.

[81] Ochs, H. D. and Winkelstein, J. (1996) in Immunologic Disorders in Infants and Children, Stiehm, E. R., eds., W.B. Saunders Company, Philadelphia, pp. 296– 338.

[82] Hermaszewski, R. A. and Webster, A. D. (1993), Q. J. Med. 86, 31–42.

[83]. Conley, M. E. (1999) in The Metabolic and Molecular Bases of Inherited Disease Scriver, C. R., et al., eds. McGraw-Hill, New York, NY (in press).

[84]. Roifman, C. M., Rao, C. P., Lederman, H. M., Lavi, S., Quinn, P., and Gelfand, E. W. (1986), Am. J. Med . 80, 590–594.

[85]. Furr, P. M., Taylor-Robinson, D., and Webster, A. D. (1994), Ann. Rheum. Dis. 53, 183–187.

[86] Wilfert, C. M., Buckley, R. H., Mohanakumar, T., Griffith, J. F., Katz, S. L., Whisnant, J. K., et al. (1977), N. Engl. J. Med. 296, 1485–1489.

[87] Bardelas, J. A., Winkelstein, J. A., Seto, D. S., Tsai, T., and Rogol, A. D. (1977), J. Pediatr. 90, 396–399.

[88] McKinney, Jr., R. E., Katz, S. L., and Wilfert, C. M. (1987), Rev. Infect. Dis. 9, 334– 356.

[89] Conley, M. E. (1985), J. Immunol. 134, 3070–3074.

[90] Mensink, E. J. B. M., Schuurman, R. K. B., Schot, J. D. L., Thompson, A., and Alt, F. W. (1986), Eur. J. Immunol. 16, 963–967.

[91] Anker, R., Conley, M. E., and Pollok, B. A. (1989), J. Exp. Med . 169, 2109–2119.

[92] Timmers, E., Kenter, M., Thompson, A., Kraakman, M. E. M., Berman, J. E., Alt, F. W., and Schuurman, R. K. B. (1991), Eur. J. Immunol. 21, 2355–2366.

[93] Milili, M., Le Deist, F., de Saint-Basile, G., Fischer, A., Fougereau, M., and Schiff, C. (1993), J. Clin. Invest. 91, 1616–1629.

[94] Campana, D., Farrant, J., Inamdar, N., Webster, A. D. B., and Janossy, G. (1990), J. Immunol. 145, 1675–1680

[95] X-Linked Agammaglobulinemia Mary Ellen Conley,Jurg Rohrer and Yoshiyuki Minegishi Clinical Reviews in Allergy and Immunology 19, 183-204 (2000)

[96] Tamagnone L, Lahtinen I, Mustonen T, Virtaneva K, Francis F, et al. 1994. BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TXK family located in chromosome Xp22.2. Oncogene 9:3683–88

[97] Schmidt U, Boucheron N, Unger B, Ellmeier W. 2004. The role of Tec family kinases in myeloid cells. Int. Arch. Allergy Immunol. 134:65–78

[98] Mary Ellen Conley, A. Kerry Dobbs, Dana M. Farmer, Sebnem Kilic, Kenneth Paris, Sofia Grigoriadou, Elaine Coustan-Smith, Vanessa Howard and Dario Campana. Primary B Cell Immunodeficiencies: Comparisons and Contrasts. Annu. Rev. Immunol. 2009.27:199-227.

[99] Conley. M. E. and Cooper, M. D. (1998), Curr. Opin. Immunol. 10, 399–406

[100] Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993), Science 259, 1157–1161.

[101] Rameh, L. E., Arvidsson, A., Carraway, K. L., Couvillon, A. D., Rathbun, G.,Crompton, A., et al. (1997), J. Biol. Chem. 272, 22,059–22,066

[102] Yao, L., Kawakami, Y., and Kawakami, T. (1994), Proc. Natl. Acad. Sci. USA 91,9175–9179.

[103] Bence, K., Ma, W., Kozasa, T., and Huang, X. Y. (1997), Nature 389, 296–299.

[104] Langhans-Rajasekaran, S. A., Wan, Y., and Huang, X. Y. (1995), Proc. Natl. Acad.Sci. USA 92, 8601–8605.

[105] Cheng, G., Ye, Z-S., and Baltimore, D. (1994), Proc. Natl. Acad. Sci. USA 91, 8152–8155

[106] de Weers M, Brouns GS, Hinshelwood S, Kinnon C, Schuurman RKB, et al. 1994. B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. J. Biol. Chem. 269:23857–60

[107] Aoki Y, Isselbacher KJ, Pillai S. 1994. Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells. Proc. Natl. Acad. Sci. USA 91:10606–9

[108] Guo B, Kato RM, Garcia-Lloret M,Wahl MI, Rawlings DJ. 2000. Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. Immunity 13:243–53

[109] Sato S, Katagiri T, Takaki S, Kikuchi Y, Hitoshi Y, et al. 1994. IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. J. Exp. Med. 180:2101–11

[110] Matsuda T, Takahashi-Tezuka M, Fukada T, Okuyama Y, Fujitani Y, et al. 1995. Association and activation of BTK andTec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. Blood 85:627–33

[111] Kawakami Y, Yao L,Miura T, Tsukada S,Witte ON, Kawakami T. 1994. Tyrosine phosphorylation and activation of Bruton tyrosine kinase upon FcγRI cross-linking. Mol. Cell Biol. 14:5108–13

[112] L. Ren, A. Campbell, H. Fang, S. Gautam, S. Elavazhagan, K. Fatehchand, P. Mehta, A. Stiff, BF Reader, X. Mo, JC Byrd, WE Carson, JP Butchar and S. Tridandapani. Analysis of the Effects of the Bruton's tyrosine kinase (BTK) Inhibitor Ibrutinib on Monocyte FcReceptor (FcγR) Function. The journal of biological chemistry Vol.291, n.6, pp. 3043–3052, February 5, 2016

[113] Rawlings DJ, Scharenberg AM, Park H,Wahl MI, Lin S, et al. 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science 271:822–25

[114] Park H,Wahl MI, Afar DE, Turck CW, Rawlings DJ, et al. 1996. Regulation of BTK function by a major autophosphorylation site within the SH3 domain. Immunity 4:515–25

[115] Humphries LA, Dangelmaier C, Sommer K, Kipp K, Kato RM, et al. 2004.Tec kinases mediate sustained calcium influx via site-specific tyrosine phosphorylation of the PLCγ SH2-SH3 linker. J. Biol. Chem. 279:37651–61

[116] Rawlings DJ. 1999. Bruton's tyrosine kinase controls a sustained calcium signal essential for B lineage development and function. Clin Immunol. 91:243–53

[117] Arachiche A, de la Fuente M, Nieman MT (2013) Calcium Mobilization And Protein Kinase C Activation Downstream Of Protease Activated Receptor 4 (PAR4) Is Negatively Regulated By PAR3 In Mouse Platelets. PLoS ONE 8(2)

[118] Yang W, Desiderio S. 1997. BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. Proc. Natl. Acad. Sci. USA 94:604–9

[119] Webb CF, Yamashita Y, Ayers N, Evetts S, Paulin Y, et al. 2000. The transcription factor Bright associates with Bruton's tyrosine kinase, the defective protein in immunodeficiency disease. J. Immunol. 165:6956–65

[120] Conley, M. E., Mathias, D., Treadaway, J., Minegishi, Y., and Rohrer, J. (1998),Am. J. Hum. Genet. 62, 1034–1043.

[121] Holinski-Feder, E., Weiss, M., Brandau, O., Jedele, K. B., Nore, B., Backesjo, C.M., et al. (1998), Pediatrics 101, 276–284.

[122] Yel, L., Minegishi, Y., Coustan-Smith, E., Buckley, R. H., Trubel, H., Pachman, L. M., et al. (1996), N. Engl. J. Med. 335, 1486–1493.

[123] Tedder, T. F., Crain, M. J., Kubagawa, H., Clement, L. T., and Cooper, M. D. (1985), J. Immunol. 135, 1786–1791.

[124] Minegishi, Y., Coustan-Smith, E., Wang, Y-H., Cooper, M. D., Campana, D., and Conley, M. E. (1998), J. Exp. Med . 187, 71–77.

[125] WHO (World Health Organization. (1989), Immunodefic. Rev. 1, 173–205.

[126]. Ziegler-Heitbrock et al.Blood, 21 October 2010

[127] Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C, Geissmann F. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity. 2010;33:375–86.

[128] Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, Kourilsky P, Wong SC. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood. 2011;118:e16–31.

[129] Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, Heine GH. SuperSAGE evidence for $CD14^{++}CD16^{+}$ monocytes as a third monocyte subset. Blood. 2011;118:e50–61.

[130] Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14(bright) $CD16^{+}$ monocyte subset is expanded in rheumatoid arthritis and promotes Th17 expansion. Arthritis Rheum. 2012;64:671–7.

[131] Skrzeczynska-Moncznik J, Bzowska M, Loseke S, Grage-Griebenow E, Zembala M, Pryjma J. Peripheral blood CD14high $CD16^{+}$ monocytes are main producers of IL-10. Scand J Immunol. 2008;67:152–9.

[132] Smedman C, Ernemar T, Gudmundsdotter L, Gille-Johnson P,Somell A, Nihlmark K, Gardlund B, Andersson J, Paulie S. FluoroSpot analysis of TLR-activated monocytes reveals several distinct cytokine secreting subpopulations. Scand J Immunol. 2012;75:249–5

[133] Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T, Ziegler-Heitbrock L. The proinflammatory $CD14^{+}CD16^{+}DR^{++}$ monocytes are a major source of TNF. J Immunol. 2002;168:3536–42.

[134] Grage-Griebenow E, Zawatzky R, Kahlert H, Brade L, Flad H, Ernst M. Identification of a novel dendritic cell-like subset of $CD64^{+}/CD16^{+}$ blood monocytes. Eur J Immunol. 2001;31: 48–56.

[135] Chong SZ, Wong KL, Lin G, Yang CM, Wong SC, Angeli V, Macary PA, Kemeny DM. Human CD8 T cells drive Th1 responses through the differentiation of TNF/iNOS-producing dendritic cells. Eur J Immunol. 2011;41:1639–51.

[136] Evans HG, Gullick NJ, Kelly S, Pitzalis C, Lord GM, Kirkham BW, Taams LS. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. Proc Natl Acad Sci USA. 2009;106:6232–7.

[137] Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler-Heitbrock HW. The novel subset of $CD14^{+}/CD16^{+}$ blood monocytes is expanded in sepsis patients. Blood. 1993;82:3170–6.

[138] Castano D, Garcia LF, Rojas M. Increased frequency and cell death of $CD16^{+}$ monocytes with Mycobacterium tuberculosis infection. Tuberculosis (Edinb). 2011;91:348–60.

[139] Soares G, Barral A, Costa JM, Barral-Netto M, Van Weyenbergh J. $CD16^{+}$ monocytes in human cutaneous leishmaniasis: increased ex vivo levels and correlation with clinical data. J Leukoc Biol. 2006;79:36–9.

[140] Saleh MN, Khazaeli MB, Wheeler RH, Bucy RP, Liu T, Everson MP, Munn DH, Schlom J, LoBuglio AF. Phase II trial of murine monoclonal antibody D612 combined with recombinant human monocyte colony-stimulating factor (rhM-CSF) in patients with metastatic gastrointestinal cancer. Cancer Res. 1995;55:4339 46.

[141] Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, Iwahashi M, Ueno A, Ohmoto Y, Makino H. $CD14^+$, $CD16^+$ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum. 2002;46:2578–86.

[142] Thieblemont N, Haeffner-Cavaillon N, Haeffner A, Cholley B, Weiss L, Kazatchkine MD. Triggering of complement receptors CR1 (CD35) and CR3 (CD11b/CD18) induces nuclear translocation of NF-kappa B (p50/p65) in human monocytes and enhances viral replication in HIV-infected monocytic cells. J Immunol. 1995;155:4861–7.

[143] Nockher WA, Scherberich JE. Expanded $CD14^+CD16^+$ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. Infect Immun. 1998;66:2782–90.

[144] Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. Blood. 1996;87:373–7.

[145] Kok Loon Wong, Wei Hseun Yeap, June Jing Yi Tai, Siew Min Ong, Truong Minh Dang, Siew Cheng Wong. The three human monocyte subsets: implications for health and disease. Immunol Res (2012) 53:41–57. DOI 10.1007/s12026-012-8297-3

[146] Poehlmann H, Schefold JC, Zuckermann-Becker H, Volk HD, Meisel C. Phenotype changes and impaired function of dendritic cell subsets in patients with sepsis: a prospective observational analysis. Crit Care. 2009;13:R119

[147] Sanchez MD, Garcia Y, Montes C, Paris SC, Rojas M, Barrera LF, Arias MA, Garcia LF. Functional and phenotypic changes in monocytes from patients with tuberculosis are reversed with treatment. Microbes Infect. 2006;8:2492–500.

[148] Melo MD, Catchpole IR, Haggar G, Stokes RW. Utilization of CD11b knockout mice to characterize the role of complement receptor 3 (CR3, CD11b/CD18) in the growth of Mycobacterium tuberculosis in macrophages. Cell Immunol. 2000;205:13–23.

[149] Chang ST, Linderman JJ, Kirschner DE. Multiple mechanisms allow Mycobacterium tuberculosis to continuously inhibit MHC class II-mediated antigen presentation by macrophages. Proc Natl Acad Sci USA. 2005;102:4530–5.

[150] Arcila ML, Sanchez MD, Ortiz B, Barrera LF, Garcia LF, Rojas M. Activation of apoptosis, but not necrosis, during Mycobacterium tuberculosis infection correlated with decreased bacterial growth: role of TNF-alpha, IL-10, caspases and phospholipase A2. Cell Immunol. 2007;249:80–93.

[151] Zhang JY, Zou ZS, Huang A, Zhang Z, Fu JL, Xu XS, Chen LM, Li BS, Wang FS. Hyper-activated pro-inflammatory CD16 monocytes correlate with the severity of liver injury and fibrosis in patients with chronic hepatitis B. PLoS One. 2011;6:e17484.

[152] Rodriguez-Munoz Y, Martin-Vilchez S, Lopez-Rodriguez R, Hernandez-Bartolome A, Trapero-Marugan M, Borque MJ, Moreno-Otero R, Sanz-Cameno P. Peripheral blood monocyte subsets predict antiviral response in chronic hepatitis C. Aliment Pharmacol Ther. 2011;34:960–71.

[153] Han J, Wang B, Han N, Zhao Y, Song C, Feng X, Mao Y, Zhang F, Zhao H, Zeng H. CD14(high)CD16(?) rather than $CD14^{low}CD16^{+}$ monocytes correlate with disease progression in chronic HIV-infected patients. J Acquir Immune Defic Syndr. 2009;52:553–9.

[154] Ellery PJ, Tippett E, Chiu YL, Paukovics G, Cameron PU, Solomon A, Lewin SR, Gorry PR, Jaworowski A, Greene WC, Sonza S, Crowe SM. The CD16? monocyte subset is more permissive to infection and preferentially harbors HIV-1 in vivo. J Immunol. 2007;178:6581–9.

[155] Ancuta P, Kunstman KJ, Autissier P, Zaman T, Stone D, Wolinsky SM, Gabuzda D. CD16? monocytes exposed to HIV promote highly efficient viral replication upon differentiation into macrophages and interaction with T cells. Virology. 2006;344:267–76

[156] Crowe S, Zhu T, Muller WA. The contribution of monocyte infection and trafficking to viral persistence, and maintenance of the viral reservoir in HIV infection. J Leukoc Biol. 2003;74: 635–41.

[157] Alexaki A, Wigdahl B. HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination. PLoS Pathog. 2008;4:e1000215.

[158] Giri MS, Nebozyhn M, Raymond A, Gekonge B, Hancock A, Creer S, Nicols C, Yousef M, Foulkes AS, Mounzer K, Shull J, Silvestri G, Kostman J, Collman RG, Showe L, Montaner LJ. Circulating monocytes in HIV-1-infected viremic subjects exhibit an antiapoptosis gene signature and virus- and hostmediated apoptosis resistance. J Immunol. 2009;182:4459–70.

[159] Coquillard G, Patterson BK. Determination of hepatitis C virusinfected, monocyte lineage reservoirs in individuals with or without HIV coinfection. J Infect Dis. 2009;200:947–54.

[160] Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. Science. 1998;282:938– 41.

[161] Cormier EG, Tsamis F, Kajumo F, Durso RJ, Gardner JP, Dragic T. CD81 is an entry coreceptor for hepatitis C virus. Proc Natl Acad Sci USA. 2004;101
:7270–4.

[162] Laskus T, Radkowski M, Piasek A, Nowicki M, Horban A, Cianciara J, Rakela J. Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: evidence of active replication in monocytes/macrophages and lymphocytes. J Infect Dis. 2000;181:442–8

[163] Iwahashi M, Yamamura M, Aita T, Okamoto A, Ueno A, Ogawa N, Akashi S, Miyake K, Godowski PJ, Makino H. Expression of Toll-like receptor 2 on $CD16^{+}$ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. Arthritis Rheum. 2004;50:1457–67.

[164] Baeten D, Boots AM, Steenbakkers PG, Elewaut D, Bos E, Verheijden GF, Berheijden G, Miltenburg AM, Rijnders AW, Veys EM, De Keyser F. Human cartilage gp-39?, CD16? monocytes in peripheral blood and synovium: correlation with joint destruction in rheumatoid arthritis. Arthritis Rheum. 2000;43:1233–43

[165] Koch S, Kucharzik T, Heidemann J, Nusrat A, Luegering A. Investigating the role of proinflammatory $CD16^+$ monocytes in the pathogenesis of inflammatory bowel disease. Clin Exp Immunol. 2010;161:332–41.

[166] Hanai H, Iida T, Takeuchi K, Watanabe F, Yamada M, Kikuyama M, Maruyama Y, Iwaoka Y, Hirayama K, Nagata S, Takai K. Adsorptive depletion of elevated proinflammatory $CD14^+CD16^+DR^{++}$ monocytes in patients with inflammatory bowel disease. Am J Gastroenterol. 2008;103:1210–6.

[167] Grip O, Bredberg A, Lindgren S, Henriksson G. Increased subpopulations of $CD16^+$ and $CD56^+$ blood monocytes in patients with active Crohn's disease. Inflamm Bowel Dis. 2007;13:566–72.

[168] Urra X, Villamor N, Amaro S, Gomez-Choco M, Obach V, Oleaga L, Planas AM, Chamorro A. Monocyte subtypes predict clinical course and prognosis in human stroke. J Cereb Blood Flow Metab. 2009;29:994–1002.

[169] Ulrich C, Heine GH, Seibert E, Fliser D, Girndt M. Circulating monocyte subpopulations with high expression of angiotensin converting enzyme predict mortality in patients with end-stage renal disease. Nephrol Dial Transplant. 2010;25:2265–72.

[170] Rogacev KS, Ziegelin M, Ulrich C, Seiler S, Girndt M, Fliser D, Heine GH. Haemodialysis-induced transient $CD16^+$ monocytopenia and cardiovascular outcome. Nephrol Dial Transplant. 2009;24:3480–6.

[171] Moniuszko M, Bodzenta-Lukaszyk A, Kowal K, Lenczewska D, Dabrowska M. Enhanced frequencies of $CD14^{++}CD16^+$, but not $CD14^+CD16^+$, peripheral blood monocytes in severe asthmatic patients. Clin Immunol. 2009;130:338–46.

[172] Tallone T, Turconi G, Soldati G, Pedrazzini G, Moccetti T, Vassalli G. Heterogeneity of human monocytes: an optimized four-color flow cytometry protocol for analysis of monocyte subsets. J Cardiovasc Transl Res. 2011;4:211–9.

[173] Wu W, Zhang X, Zhang C, Tang T, Ren W, Dai K. Expansion of $CD14^+$ $CD16^+$ peripheral monocytes among patients with aseptic loosening. Inflamm Res. 2009;58:561–70.

[174] Nagasawa T, Kobayashi H, Aramaki M, Kiji M, Oda S, Izumi Y. Expression of CD14, CD16 and CD45RA on monocytes from periodontitis patients. J Periodontal Res. 2004;39:72–8.

[175] Borregaard N. Neutrophils, from marrow to microbes. Immunity 2010;33:657–70.

[176] Dibbert B, Weber M, Nikolaizik WH, Vogt P, Scho¨ni MH, Blaser K, et al. Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation. Proc Natl Acad Sci. 1999;96: 13330.

[177] Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs- Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Investig J Tech Methods Pathol. 2000;80:617–53.

[178] G. Ricevuti, Host tissue damage by phagocytes, Ann. N Y Acad. Sci. 832 (1997) 426–448.

[179] Kang EM, Marciano BE, DeRavin S, Zarember KA, Holland SM, Malech HL. Chronic granulomatous disease: overview and hematopoietic stem cell transplantation. J Allergy Clin Immunol. 2011;127:1319–26 quiz 1327–1328.

[180] Dotta L, Tassone L, Badolato R. Clinical and genetic features of warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome. Curr Mol Med. 2011;11:317–25.

[181] Harris ES, Weyrich AS, Zimmerman GA. Lessons from rare maladies: leukocyte adhesion deficiency syndromes. Curr Opin Hematol. 2013;20:16–25.

[182] Mavroudi I, Papadaki V, Pyrovolaki K, Katonis P, Eliopoulos AG, Papadaki HA. The CD40/CD40 ligand interactions exert pleiotropic effects on bone marrow granulopoiesis. J Leukoc Biol. 2011;89:771–83.

[183] Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Galicier L, Lepelletier Y, Webster D, et al. Common variable immunodeficiency is associated with defective functions of dendritic cells. Blood. 2004;104:2441–3.

[184] Paquin-Proulx D, Santos BAN, Carvalho KI, Toledo-Barros M, Barreto de Oliveira AK, Kokron CM, et al. Dysregulated CD1 profile in myeloid dendritic cells in CVID is normalized by IVIg treatment. Blood. 2013;121:4963–4.

[185] Cham B, Bonilla MA, Winkelstein J. Neutropenia associated with primary immunodeficiency syndromes. Semin Hematol. 2002;39: 107–12.

[186] V. Howard, J.M. Greene, S. Pahwa, J.A. Winkelstein, J.M. Boyle, M. Kocak, M.E. Conley. The health status and quality of life of adults with X-linked agammaglobulinemia Clin. Immunol., 118 (2006), pp. 201–208

[187] J.A. Winkelstein, M.E. Conley, C. James, V. Howard, J. Boyle Adults with X-linked agammaglobulinemia: impact of disease on daily lives, quality of life, educational and socioeconomic status, knowledge of inheritance, and reproductive attitudesMedicine, 87 (2008), pp. 253–258

[188] Thomas U. Marron, Kaileen Rohr, Monica Martinez-Gallo, Joyce Yu, c, Charlotte Cunningham-Rundles TLR signaling and effector functions are intact in XLA neutrophils. Clinical Immunology Volume 137, Issue 1, October 2010, Pages 74–80

[189] J. Kerr, I. Quinti, M. Eibl, H. Chapel, P.J. Späth,W.A. Sewell, A. Salama, I.N. van Schaik, T.W. Kuijpers, H.H. Peter, Is dosing of therapeutic immunoglobulins optimal? A review of a three-decade long debate in Europe, Front. Immunol. 5 (2014) 1–19.

[190] S.V. Kaveri, Intravenous immunoglobulin: exploiting the potential of natural antibodies, Autoimmun. Rev. 11 (2012) 792–794.

[191] S.V. Kaveri,M.S. Maddur, P. Hegde, S. Lacroix-Desmazes, J. Bayry, Intravenous immunoglobulins in immunodeficiencies: more than mere replacement therapy, Clin. Exp. Immunol. 2 (2011) 2–5.

[192] S.Q. Nagelkerke, T.W. Kuijpers, Immunomodulation by IVIg and the role of Fcgamma receptors: classic mechanisms of action after all? Front. Immunol. 5 (2015) 1–13.

[193] S. Casulli, S. Topçu, L. Fattoum, S. von Gunten, H.U. Simon, J.L. Teillaud, J. Bayry, S.V. Kaveri, C. Elbim, A differential concentration-dependent effect of IVIg on neutrophil functions: relevance for anti-microbial and anti-inflammatory mechanisms, PLoS ONE (2011) e26469.

[194] M. Siedlar,M. Strach, K. Bukowska-Strakova, M. Lenart, A. Szaflarska, K.Węglarczyk, M. Rutkowska, M. Baj-Krzyworzeka, A. Pituch-Noworolska, D. Kowalczyk, T. Grodzicki, L. Ziegler-Heitbrock, M. Zembala, Preparations of intravenous immunoglobulins diminish the number and proinflammatory response of $CD14^{+}CD16^{++}$ monocytes in common variable immunodeficiency (CVID) patients, Clin. Immunol. 139 (2011) 122–132.

[195] R. Voltz, F.V. Rosen, T. Yousry, J. Beck, R. Hohlfeld, Reversible encephalopathy with cerebral vasospasm in a Guillain-Barré syndrome patient treated with intravenous immunoglobulin, Neurology 46 (1996) 250–251.

[196] S. Higurashi, Y. Machino, E. Suzuki, M. Suzuki, J. Kohroki, Y. Masuho, Both the Fab and Fc domains of IgG are essential for ROS emission from TNF-a-primed neutrophils by IVIG, Biochem. Biophys. Res. Commun. 417 (2012) 794–799.

[197] J.L. Teeling, E.R. de Groot, A.J.M. Eerenberg, W.K. Bleeker, G. Van Mierlo, L.A. Aarden, C.E. Hack, Human intravenous immunoglobulin (IVIG) preparations degranulate human neutrophils in vitro, Clin. Exp. Immunol. 114 (1998) 264–270.

[198] S. von Gunten, H.U. Simon, Cell death modulation by intravenous immunoglobulin, J. Clin. Immunol. 1 (2010) S24–S30.

[199] H. Tsujimoto, S. Takeshita, K. Nakatani, Y. Kawamura, T. Tokutomi, I. Sekine, Intravenous immunoglobulin therapy induces neutrophil apoptosis in Kawasaki disease, Clin. Immunol. 103 (2002) 161 168.

[200] S.D. Kobayashi, J.M. Voyich, K.R. Braughton, F.R. DeLeo, Down-regulation of proinflammatory capacity during apoptosis in human Polymorphonuclear leukocytes, J. Immunol. 170 (2003) 3357–3368.

[201] S. Aschermann, A. Lux, A. Baerenwaldt, M. Biburger, F. Nimmerjahn, The other side of immunoglobulin G: suppressor of inflammation, Clin. Exp. Immunol. 160 (2010) 161–167.

[202] E. van Mirre, J.L. Teeling, J.W. van der Meer, W.K. Bleeker, C.E. Hack, Monomeric IgG in intravenous Ig preparations is a functional antagonist of FcgammaRII and FcgammaRIIIb, J. Immunol. 173 (2004) 332–339.

[203] I. Pallister, R. Bhatia, G. Katpalli, D. Allison, C. Parker, N. Topley, Alteration of polymorphonuclear neutrophil surface receptor expression and migratory activity after isolation: comparison of whole blood and isolated PMN preparations from normal and post fracture trauma patients, J. Trauma 60 (2006) 844–850.

[204] I.M. Rebecchi, N. Ferreira Novo, Y. Julian, A. Campa, Oxidative metabolism and release of myeloperoxidase from polymorphonuclear leukocytes obtained from blood sedimentation in a Ficoll-Hypaque gradient, Cell Biochem. Funct. 18 (2000) 127–132.

[205] S. François, J. El Benna, P.M. Dang, E. Pedruzzi, M.A. Gougerot-Pocidalo, C. Elbim, Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad, J. Immunol. 174 (2005) 3633–3642.

[206] J. Lim, N.A. Hotchin, Signalling mechanism of the leukocyte integrin αMβ2: current and future perspectives, Biol. Cell. 104 (2012) 631–640.

[207] Smith SL, Eyre S, Yarwood A, Hyrich K, Morgan AW, Wilson AG, Isaacs J; Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate 6, Plant D, Barton A. Investigating CD11c expression as a potential genomic biomarker of response to TNF inhibitor biologics in whole blood rheumatoid arthritis samples. Arthritis Res Ther. 2015 Dec 14;17:359.

[208] Krystufkova O, Mann H, Hulejova H, L Senolt L, Vencovsky J Enhanced expression of CD11c on non-classical CD16+ peripheral blood monocytes in early rheumatoid arthritis Ann Rheum Dis 2014;73:A20-A21.

[209] J.Q. Zhang, G. Nicoll, C. Jones, P.R. Crocker, Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes, J. Biol. Chem. 275 (2000) 22121–22126.

[210] P.R. Crocker, J.C. Paulson, A. Varki, Siglecs and their roles in the immune system, Nat.
Rev. Immunol. 7 (2007) 255–266.

[211] Murphy PM Neutrophil receptors for interleukin-8 and related CXC chemokines Seminars in Hematology [1997, 34(4):311-318]

[212] Kuijpers TW, van der Schoot CE, Hoogerwerf M, Roos D. Cross-linking of the carcinoembryonic antigen-like glycoproteins CD66 and CD67 induces neutrophil aggregation. J Immunol 151:4934, 1993.

[213] Nair KS, Zingde SM: Adhesion of neutrophils to fibronectin: role of the cd66 antigens. Cell Immunol 208:96, 2001.

[214] Yoon Juhan, Terada Akihiko and Kita Hirohito, CD66b Regulates Adhesion and Activation of Human Eosinophils, J Immunol 2007; 179:8454-8462.

[215] Schröder AK, Uciechowski P, Fleischer D, Rink L. Crosslinking of CD66B on peripheral blood neutrophils mediates the release of interleukin-8 from intracellular storage. Hum Immunol. 2006 Sep;67(9):676–682.

[216] S. Ben Mkaddem, M. Aloulou, M. Benhamou, R.C. Monteiro, Role of FcγRIIIA (CD16) in IVIg-mediated anti-inflammatory function, J. Clin. Immunol. 1 (2014) S46–S50.

[217] M. Kocher, M.E. Siegel, J.C. Edberg, R.P. Kimberly, Cross-linking of Fc gamma receptor IIa and Fc gamma receptor IIIb induces different proadhesive phenotypes on human neutrophils, J. Immunol. 159 (1997) 3940–3948.

[218] P. Youinou, V. Durand, Y. Renaudineau, Y.L. Pennec, A. Saraux, C. Jamin, Pathogenic effects of anti-Fc gamma receptor IIIb (CD16) on polymorphonuclear neutrophils in non-organ-specific autoimmune diseases, Autoimmun. Rev. 1 (2002) 13–19

[219] A.A. Bengtsson, Å. Pettersson, S. Wichert, B. Gullstrand, M. Hansson, T. Hellmark, Å.C. Johansson, Low production of reactive oxygen species in granulocytes is associated with organ damage in systemic lupus erythematosus, Arthritis Res. Ther. 16 (2014) R120.

[220] Aukrust P., Frøland S.S., Liabakk N.B., Müller F., Nordøy I., Haug C., Espevik T. Release of cytokines, soluble cytokine receptors, and interleukin-1 receptor antagonist after intravenous immunoglobulin administration in vivo, Blood 84 (1994) 2136–2143.

[221] T. Avril, H. Floyd, F. Lopez, E. Vivier, P.R. Crocker, The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells, J. Immunol. 173 (2004) 6841–6849.

[222] Aaron F. Carlin, Satoshi Uchiyama, Yung-Chi Chang, Amanda L. Lewis, V. Nizet, A. Varki, Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response, Blood 113 (2009) 3333–3336.

[223] Doroshenko T, Chaly Y, Savitskiy V, Maslakova O, Portyanko A, Gorudko I, et al. Phagocytosing neutrophils down-regulate the expression of chemokine receptors CXCR1 and CXCR2. Bloodv2002;100(7):2668-71

[224] R.R. Barbosa, S.P. Silva, S.L. Silva, R. Tendeiro, A.C. Melo, E. Pedro, M.P. Barbosa, M.C. Santos, R.M. Victorino, A.E. Sousa, Monocyte activation is a feature of common variable immunodeficiency irrespective of plasma lipopolysaccharide levels, Clin. Exp. Immunol. 169 (2012) 263–272

[225] A.S. Tjon, R. van Gent, H. Jaadar, P. Martin van Hagen, S. Mancham, L.J. van der Laan, P.A. te Boekhorst, H.J. Metselaar, J. Kwekkeboom, Intravenous immunoglobulin treatment in humans suppresses dendritic cell function via stimulation of IL-4 and IL-13 production, J. Immunol. 192 (2014) 5625–5634.

[226] D. Paquin-Proulx, J.K. Sandberg, Persistent immune activation in CVID and the role of IVIg in its suppression, Front. Immunol. 5 (2014) 637.

[227] D. Paquin-Proulx, B.A. Santos, K.I. Carvalho, M. Toledo-Barros, A.K. Barreto de Oliveira, C.M. Kokron, J. Kalil, M.Moll, E.G. Kallas, J.K. Sandberg, IVIg immune reconstitution treatment alleviates the state of persistent immune activation and suppressed CD4 T cell counts in CVID, PLoS One 8 (2013) 1–9.

[228] L. Fossati-Jimack, G.S. Ling, A. Cortini, M. Szajna, T.H. Malik, J.U. McDonald, M.C. Pickering, H.T. Cook, P.R. Taylor, M. Botto, Phagocytosis is the main CR3-mediated function affected by the lupus-associated variant of CD11b in human myeloid cells, PLoS One 8 (2013) e57082.

[229] P. Aukrust, F.Müller, S.S. Frøland, Enhanced generation of reactive oxygen species in monocytes from patients with common variable immunodeficiency, Clin. Exp. Immunol. 97 (1994) 232–238.

[230] M. Sato, H. Sano, D. Iwaki, K. Kudo, M. Konishi, H. Takahashi, T. Takahashi, H. Imaizumi, Y. Asai, Y. Kuroki, Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are downregulated by lung collectin surfactant protein A, J. Immunol. 171 (2003) 417 425.

[231] P. Sondermann, R. Huber, U. Jacob, Crystal structure of the soluble form of the human fcgamma-receptor IIb: a new member of the immunoglobulin superfamily at 1.7 A resolution, EMBO J. 5 (1999) 1095–1103.

[232] S. Takeshita, H. Tsujimoto, K. Nakatani, Intravenous immunoglobulin preparations promote apoptosis in lipopolysaccharide-stimulated neutrophils via an oxygendependent pathway in vitro, APMIS 113 (2005) 269–277.

[233] Zhao C1, Zhang H, Wong WC, Sem X, Han H, Ong SM, Tan YC, Yeap WH, Gan CS, Ng KQ, Koh MB, Kourilsky P, Sze SK, Wong SC. Identification of novel functional differences in monocyte subsets using proteomic and transcriptomic methods. J Proteome Res. (2009) 4028-38.

[234] S. von Gunten, S. Yousefi, M. Seitz, S.M. Jakob, T. Schaffner, R. Seger, J. Takala, P.M. Villiger, H.U. Simon, Siglec-9 transduces apoptotic and nonapoptotic death signals into neutrophils depending on the proinflammatory cytokine environment, Blood 106 (2005) 1423–1431.

[235] J. Bayry, E.M. Fournier, M.S. Maddur, J. Vani, B. Wootla, S. Sibéril, J.D. Dimitrov, S. Lacroix-Desmazes, M. Berdah, Y. Crabol, E. Oksenhendler, Y. Lévy, L. Mouthon, C. Sautès-Fridman, O. Hermine, S.V. Kaveri, Intravenous immunoglobulin induces proliferation and immunoglobulin synthesis from B cells of patients with common variable immunodeficiency: a mechanism underlying the beneficial effect of IVIg in primary immunodeficiencies, J. Autoimmun. 36 (2011) 9–15.

[236] M. Mitrevski, R. Marrapodi, A. Camponeschi, C. Lazzeri, L. Todi, I. Quinti, M. Fiorilli, M. Visentini, Intravenous immunoglobulin replacement therapy in common variable immunodeficiency induces B cell depletion through differentiation into apoptosis-prone CD21(low) B cells, Immunol. Res. 60 (2014) 330–338.

[237] H. Artac, R. Kara, I. Reisli, In vivo modulation of the expressions of Fas and CD25 by intravenous immunoglobulin in common variable immunodeficiency, Clin. Exp. Med. 10 (2010) 27–31.

[238] J. Bayry, S. Lacroix-Desmazes, O. Hermine, E. Oksenhendler, M.D. Kazatchkine, S.V. Kaveri, Amelioration of differentiation of dendritic cells from CVID patients by intravenous immunoglobulin, Am. J. Med. 118 (2005) 1439–1440.

[239] S. Casulli, H. Coignard-Biehler, K. Amazzough, M. Shoai-Tehrani, J. Bayry, N. Mahlaoui, C. Elbim, S.V. Kaveri, Defective functions of Polymorphonuclear neutrophils in patients with common variable immunodeficiency, Immunol. Res. 60 (2014) 69–76.

[240] M. Siddiqi, Z.C. Garcia, D.S. Stein, T.N. Denny, Z. Spolarics, Relationship between oxidative burst activity and CD11b expression in neutrophils and monocytes from healthy individuals: effects of race and gender, Cytometry 46 (2001) 243–246.

[241] N. Kutukculer, E. Azarsiz, N.E. Karaca, E. Ulusoy, G. Koturoglu, G. Aksu, A clinical and laboratory approach to the evaluation of innate immunity in pediatric CVID patients, Front. Immunol. 6 (2015) 145.

[242] S. Rivas-Fuentes, E. García-García, G. Nieto-Castañeda, C. Rosales, Fcgamma receptors exhibit different phagocytosis potential in human neutrophils, Cell Immunol. 263 (2010) 114–121.

[243] E. García-García, C. Rosales, Signal transduction during Fc receptor-mediated phagocytosis, J. Leuoc. Biol. 72 (2002) 1092–1108.

[244] M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, Y. Nishizuka, Direct activation of calcium-activate, phospholipid-dependent protein kinase by tumor-promoting phorbol esters, J. Biol. Chem. 257 (1982) 7847–7851.

[245] J.E. Niedel, L.J. Kuhn, G.R. Vandenbark, Phorbol diester receptor copurifies with protein kinase C, Proc. Natl. Acad. Sci. USA 80 (1983) 36–40.

[246] F. Nimmerjahn, J.V. Ravetch, Anti-inflammatory actions of intravenous immunoglobulin, Annu. Rev. Immunol. 26 (2008) 513–533.

[247] S. von Gunten, A. Schaub, M. Vogel, B.M. Stadler, S. Miescher, H.U. Simon, Immunologic and functional evidence for anti-Siglec-9 autoantibodies in intravenous immunoglobulin preparations, Blood 108 (2006) 4255–4259.

[248] S. von Gunten, S. Jakob, B. Geering, J. Takala, H.U. Simon, Different patterns of Siglec-9-mediated neutrophil death responses in septic shock, Shock 32 (2009) 386–392.

[249] M.G. Macey, D.A. McCarthy, S. Vordemeier, A.C. Newland, K.A. Brown, Effects of cell purification methods on CD11b and L-selectin expression as well as the adherence and activation of leucocytes, J. Immunol. Methods 181 (1995) 211–219.

[250] G. Hodge, S. Hodge, P. Han, Increased levels of apoptosis of leukocyte subsets in cultured PBMCs compared to whole blood as shown by Annexin V binding: relevance to cytokine production, Cytokine 12 (2000) 1763–1768.

[251] Weineisen, M., Sjoring, U., Fallman, M., Andersson, T. (2004) Streptococcal M5 protein prevents neutrophil phagocytosis by interfering with CD11b/CD18 receptor-mediated association and signaling. J. Immunol. 172, 3798–3807.

[252] Schlesinger, L. S., Horwitz, M. A. (1991) Phagocytosis of Mycobacterium leprae by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-activation inhibits complement receptor function and phagocytosis of this bacterium. J. Immunol. 147, 1983–1994.

[253] Schlesinger, L. S., Horwitz, M. A. (1990) Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. J. Clin. Invest. 85, 1304–1314.

[254] Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R., Horwitz, M. A. (1990) Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. J. Immunol. 144, 2771–2780.

[255] Nunes P, Demaurex N. The role of calcium signaling in phagocytosis, J Leukoc Biol. 2010 Jul;88(1):57-68.

[256] Giogi, C.; Agnoletto, C.; Baldini, C.; Bononi, A.; Bonora, M.; Marchi, S.; Missiroli, S.; Patergnani, S.; Poletti, F.; Rimessi, A.; et al. Redox control of Protein Kinase C: Cell and disease-specific aspects. Antioxid. Redox Signal. 2010, 13, 1051–1085.

[257] Newton A.C., Regulation of the ABC kinases by phosphorylation: Protein kinase C as a paradigm. Biochem. J. 2003, 2, 361–371.

[258] Steinberg S.F., Structural basis of protein kinase C isoform function. Physiol. Rev. 2008, 4, 1341–1378.

[259] Cosentino-Gomes D., Rocco-MachadoN., Meyer-Fernandes J.R., Cell Signaling through Protein Kinase C Oxidation and Activation Int. J. Mol. Sci. 2012, 13, 10697-–10721.

[260] Oancea E., Meyer T., Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell 1998, 3, 307–318.

[261] Gopalakrishna R., Jaken S., Protein kinase C signaling and oxidative stress. Free. Radic. Biol. Med. 2000, 9, 1349–1361.

[262] Choi O. H., Kim J. H., Kinet J. P., Calcium mobilization via sphingosine kinase in signaling by the Fc ε RI antigen receptor. Nature (1996) 380, 634–636.

[263] Rosales, C., Jones, S. L., McCourt, D., Brown, E. J. (1994) Bromophenacyl bromide binding to the actin-bundling protein l-plastin inhibits inositol trisphosphate-independent increase in Ca^{2+} in human neutrophils. Proc. Natl. Acad. Sci. USA 91, 3534–3538.

[264] Rosales, C., Brown E. J., Signal transduction by neutrophil immunoglobulin G Fc receptors. Dissociation of intracytoplasmic calcium concentration rise from inositol 1,4,5-trisphosphate. J. Biol. Chem. (1992) 267, 5265–5271.

[265] McNeil P., L., Swanson J. A., Wright S. D., Silverstein S. C., Taylor D. L., Fc-receptor-mediated phagocytosis occurs in macrophageswithout an increase in average $[Ca^{++}]$i. J. Cell Biol. (1986) 102, 1586–1592.

[266] Di Virgilio, F., Meyer, B. C., Greenberg, S., and Silverstein, S. C. Fc receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca^{2+} levels. J. Cell Biol. (1988) 106, 657–666.

[267] Lennartz, M. R., Lefkowith, J. B., Bromley, F. A., and Brown, E. J. Immunoglobulin G-mediated phagocytosis activates a calcium-independent, phosphatidylethanolamine-specific phospholipase. J. Leukocyte Biol. (1993) 54, 389–398.

[268] Karimi, K., and Lennartz, M. R. Protein kinase C activation precedes arachidonic acid release during IgG-mediated phagocytosis. J. Immunol. (1995) 155, 5786–5794.

[269] Mangla A., Khare A., Vineeth V., Panday N.N., Mukhopadhyay A., Ravindran B., Bal V., George A., Rath S. Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. Blood. 2004 Aug 15;104(4):1191–1197.