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Molecular and Biological aspects of Primary Immunodeficiency

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Abstract

Background: Primary immunodeficiency diseases (PIDs) comprise a genetically heterogeneous group of disorders which affect distinct components of the innate and adaptive immune system. Over 120 distinct genes have been identified, whose abnormalities account for more than 150 different forms of PID. Clinical presentation is highly variable; actually ranging from various patterns of microbial susceptibility to allergy, lymphoproliferation or autoimmune manifestations. The study of patients affected by immunodeficiencies allows to identify new genes involved in the immune response indeed PIDs represent an optimal model to investigate development, function and regulation mechanisms of immune system.

Aim of the study: To define a rational approach to recognize immune deficiencies, with specific emphasis on developing clinical evaluations, understanding the genetic and cellular basis of the disease (Study1 and Study 2), and propose a gene therapy approach as a definitive cure of immunodeficiency due to a defined genic defect (Study3).

study1: We report a patient with dysgammaglobulinemia, and gradual decrease of peripheral B lymphocytes. A new Btk mutation was identified in this patient and protein expression was determined. Although clinical and laboratory history is not totally inconsistent with an atypical variant of XLA, it is unclear if all the clinical presentation/picture is influenced by the new mutation.

Study2: We report for the first time a case of a female patient with combined immunodeficiency characterised by a complete absence of B cells and NK cells, and their progenitors. Surprisingly, she had an absence of monocytes ($CD14^+$) and DC cells. Direct sequencing of genomic and cDNA of Flt3L, Flt3R, and of major genes (Ikaros and of PU.1) regulating the FLT3-FLT3L expression did not show the presence of any mutations. Levels of Flt3R and Flt3L mRNA in peripheral blood resulted significantly higher (10- and 30- fold increase respectively) with respect to healthy control donor, supporting the hypothesis of an inherited imbalance in the expression of Flt3 ligand mRNA.

This finding, in addition to a normal stromal function highlights the possibility of a more specific therapeutic strategy in this child, such as haematopoietic stem cells transplantation.

Study3: Gene therapy could represent a definitive cure for patients for whom conventional HSCT is not available. Several clinical trials on XCGD-gene therapy have been conducted since 1997, but they resulted in low and short-term engraftment of CGD-corrected cells. In this study we develop a new strategy for XCGD gene therapy with stem cells, including Lentiviral Vector (LVV) design and microRNA technology, for a safe and effective treatment of X-CGD.

Key words: Primary Immunodeficiency (PID), Agammaglobulinemia, Chronic Granulomatous Disease (CGD), Gene therapy, Lentiviral Vectors, miRNA

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Doctoral thesis in 'Immunonologia e Biotecnologie Applicate' XXI ciclo

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This thesis is based on the following articles and manuscript:

1) Graziani S, Di Matteo G, Benini L, Di Cesare S, **Chiriaco M**, Chini L, Chianca M, De Iorio F, La Rocca M, Iannini R, Corrente S, Rossi P, Moschese V. *Identification of a Btk mutation in a dysgammaglobulinemic patient with reduced B cells: XLA diagnosis or not?* Clin Immunol. 2008

2) Di Matteo G, Giordani L, Finocchi A, Ventura A, **Chiriaco M**, Blancato J, Sinibaldi C, Plebani A, Soresina A, Pignata C, Dellepiane RM, Trizzino A, Cossu F, Rondelli R, Rossi P, De Mattia D, Martire B; with IPINET (Italian Network for Primary Immunodeficiencies). *Molecular characterization of a large cohort of patients with Chronic Granulomatous Disease and identification of novel CYBB mutations: An Italian multicenter study*. Mol Immunol. 2009 Apr 30. [Epub ahead of print]

3) Finocchi A, Palma P, Di Matteo G, **Chiriaco M**, Lancella L, Simonetti A, Rana I, Livadiotti S, Rossi P. *Visceral leishmaniasis revealing chronic granulomatous disease in a child.* Int J Immunopathol Pharmacol. 2008

4) **Chiriaco M**., Di Matteo G., Sinibaldi C., Giardina E., Folgori L., D'Argenio P., Rossi P. and Finocchi A. *Identification of deletion carriers in X-Linked Chronic Granulomatous Disease by Real Time PCR*. Submitted to Genetic Testing.

Contents

Introduction

Haematopoietic System

The pluripotent haematopoietic stem cells (PHSCs) with its extensive self-renewal potential, regenerates all blood cell types throughout life by differentiating to progenitor cells with gradually restricted developmental potential. The establishment of all haematopoietic lineages during development is regulated by transcription factors and other parameters, such as cytokine receptor signalling, direct cell-cell interactions or other environmental signals. These components have an instructive role and can reprogram a committed cell into another lineage by perturbing their networks, simply changing the balance of two lineage haematopoietic transcription factors (1).

An early step in haematopoiesis is the commitment of short-term haematopoietic stem cells (ST-HSC) to multipotent progenitors (MPPs) which loss the long term self renewal capacity and give rise to early/common lymphoid (ELPs/CLPs) and common myeloid (CMPs) progenitors (2-3) (*Figure 1*).

The loss of the long term self renewal capacity is accompanied by expression of the tyrosine kinase receptor Flt3 (also known as Flk2) in MPP cells (4-5). MPP-Flt3^{low} gives rise to CMP cells, able to development in granulocyte-macrophage progenitor (GMPs) and in megakaryocyte-erothroid progenitors (MEPs). MPP-FLt3^{hi}, called long multipotent progenitors (LMPPs), lacked significant MEPs potential but exhibited B and T lymphoid as well as GM potential (6-7). LMPPs later differentiate to the earliest lymphocyte progenitors (ELPs), which likely generate CLPs in BM and early T-lineage progenitors (ETPs) in the thymus (8-9). CLP cells are lymphoid restricted and can produce B cells, T cells, dendritic cells (DCs) and natural killer cells (NKs), while the progenitor ETP recently was been identified to have T, B, NK and GM potential (10-11).

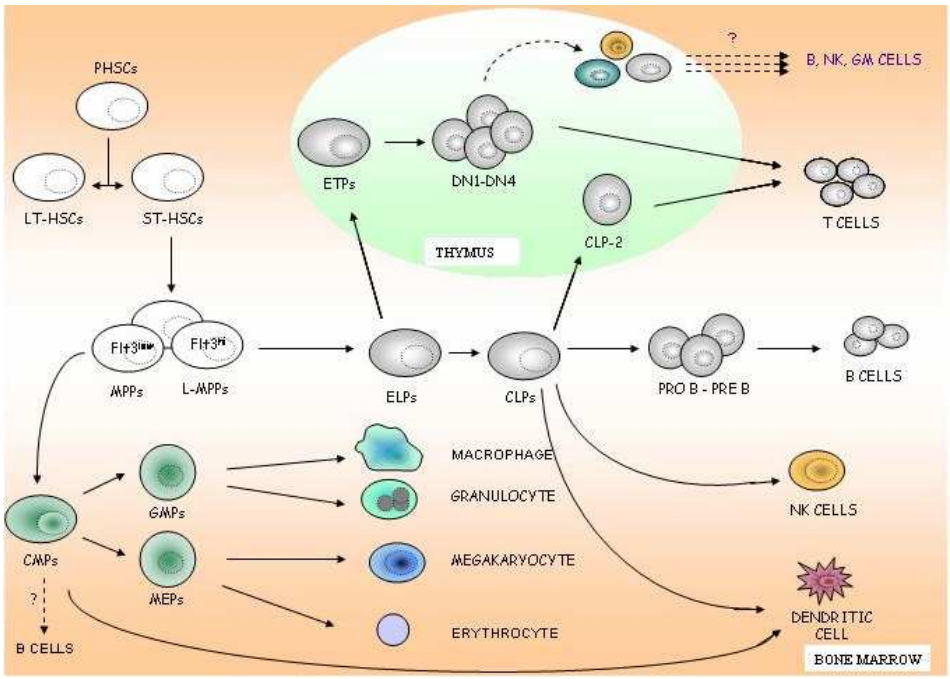


Figure I: Cell fate decision in the early haematopoietic progenitors

Immune System

The primary function of the immune system is to protect the host from infectious microbes. The immune response is able to discriminate self from non self detecting structural features of the pathogens that mark them as distinct from host cell.

The innate response occurs before the contact with microbes and the responses are encoded by genes in the host's germline. The innate response includes barrier mechanisms (eg, epithelial cell and secreted mucus layer), soluble proteins and bioactive small molecules, which are either constitutively present in biologic fluids (eg, complement proteins and defensins) or released from activated cells (including cytokines, chemokines, lipids, bioactive amines and enzymes).

The cellular components of innate immunity are granulocytes, monocytes, macrophages, mast cells and natural killer (NK), which are activated by direct contact of cell surface receptors with various microbial products (eg, via toll-like receptor - LTR). Innate immunity does not confer long-term protection to the host because there is no creation of immunological memory.

The adaptive immune system responds in a much more specific way to particular microbes. This kind of immunity is characterised by the immunological memory that assures the long-term preservation from reinfections. Adaptive responses are based primarily on the antigen-specific receptors expressed on the surfaces of T- and B-lymphocytes. Intact T-cell receptor (TCR) and B-cell antigen receptor (BCR) are assembled by somatic rearrangement of germ line genes. Besides T and B lymphocytes, also antigen presenting cells (APCs) play an essential role in adaptive immunity, serving fight off foreign substances that enter in the body.

Although the innate and adaptive immune responses are fundamentally different in their mechanisms of action, synergy between them is essential for an intact and fully effective immune response.

Primary Immunodeficiency Diseases

Primary immunodeficiency diseases (PIDs) comprise a genetically heterogeneous group of disorders that affect distinct components of the innate and adaptive immune system, such us neutrophils, macrophages, dendritic cells, complement proteins, NK cells, as well as T and B lymphocytes (*Figure 2*). Over 120 distinct genes have been identified, whose abnormalities account for more than 150 different forms of PID (12). PIDs are usually diagnosed in infancy. Even if thought to be rare, currently, the diagnosed cases are increasing because the development of a sophisticated diagnostic testing and prolonged survival of these patients, due to a prompt clinical diagnosis and / or instantly therapy. Clinical presentation is highly variable; actually ranging from various patterns of microbial susceptibility to allergy, lymphoproliferation or autoimmune manifestations (13).

In many cases, through the study of patients with PID, a substantial number of immune genes have been discovered and their role has been clarified. The essential role of them in immune system, suggest us to consider PIDs as optimal models for the investigations of immune system development, function and regulation mechanisms. The complexity of the genetic, immunological and clinical features of PIDs has prompted the need for their classification, with the ultimate goal of facilitating

diagnosis and treatment. The last classification proposed in *The International Union of Immunological Societies (IUIS) Primary Immunodeficiency (PID) Classification Committee-2007* subdivided PIDs in: Combined T and B immunodeficiencies (*Table I*) and predominantly antibody deficiencies (*Table II*).

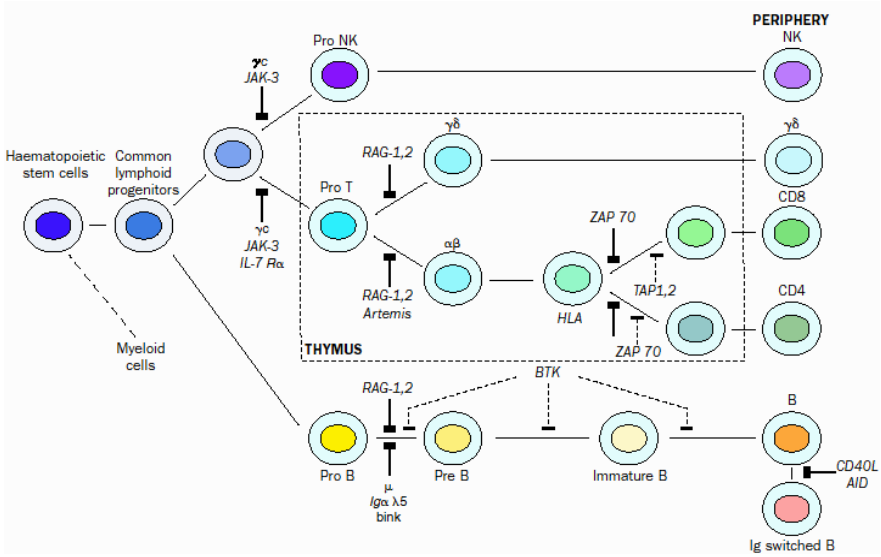


Figure2: PIDs resulting from a block in lymphocyte development

In **Humoral defects** (Agammaglobulinemia or antibody deficiency), in which B cells are unable to produce functional immunoglobulins but T cell are not severe compromised, the infections are caused principally by encapsulated bacteria (*Haemophilus influenza*, *Pneumococcus* spp, *Streptococcus* spp and others), parasites (*Giardia*) and rarely by virus (*enterovirus* and *papillomavirus).*

Patients with **combined T- and B- cells** immunodeficiencies, which have a more severe immunophenotype, are affected not only by recurrent and severe bacterial infections, but also by severe opportunistic, fungal (e.g. *Pneumocystis carinii, Candida, Mycobacteria*,) and severe viral infections (*Cytomegalo-virus, Epstein-Barr virus, Adenovirus, Varicella*).

Defects in phagocyte (neutrophils and monocytes) **function** cause in patients a high incidence of fungal infections (*Aspergillus, Candida*) and catalase positive bacteria (*Staphilococcus aureus*, *Serratia*).

Compared to the other, **defects in the complement** immune system are rare (2%),

Patients' clinical presentations include recurrent infections with pyogenic bacteria (*Neisseria*).

Disease	Circulating T cells	Circulating B cells	Serum Ig	Associated Features	Inheri-tance	Genetic defects/presumed phatogenesis
T^-B^+SCID:						
□c deficiency	↓↓	Normal or ↑	↓	NK cells ↓↓	XL	Defect in γc of receptor for IL-2-4-7-9-15-21
JAK3 deficiency	↓↓	Normal or ↑	↓	NK cells ↓↓	AR	Defect in JAK3 signalling kinase
IL7Rα deficiency	↓↓	Normal or ↑	↓	Normal NK cells	AR	Defect in IL-7 receptor γ chain
CD45 deficiency	↓↓	Normal	↓	Normal γ/δ Tcells	AR	Defect in CD45
CD3δCD3ε CD3ζ deficiency	↓↓	Normal	↓	Normal NK cells	AR	Defect in CD3δCD3εorCD3ζchain of T cell antigen receptor
T^-B^-SCID:						
RAG1/2 deficiency	↓↓	↓↓	↓	Defective VDJ recombination	AR	Complete defect of recombinase activating gene (RAG) 1 or 2
DCLRE1C (Artemis deficiency)	↓↓	↓↓	↓	Defective VDJ recombination, radiation sensitivity	AR	Defect in Artemis DNA recombinase repair protein
ADA deficiency	Absent from birth or progressive ↓	Absent from birth or progressive ↓	progressive ↓	Costochondral junction flaring	AR	Absent ADA, elevated lymphotoxic metabolites (d-ATP,S-adenosyl homocysteine)
Reticular Dysgenesis	↓↓	Normal or ↓	↓	Granulocytopenia, thrombocytopenia	AR	Defective maturation of T, B, and myeloid cells (stem cell defect)

Table I: Combined T and B cell immunodeficiency (↓↓, markedly decreased; ↓, decreased; ↑ increased; AR, autosomal recessive; XL, X-linked form)

Disease	Circulating T cells	Circulating B cells	Serum Ig	Associated Features	Inheri-tance	Genetic defects/presumed phatogenesis
Omenn Syndrome	Present;restricted heterogeneity	Normal or ↓	↓, except IgE ↑	Erythroderma, eosinophilia, adenopathy, hepato-splenomegaly	AR	Missense mutation allowing residual activity,usually in RAG1 or 2 genes, but also in Artemis, IL7Rα and RMRP genes
DNA ligase IV	↓	↓	↓	Microcephaly, facial dystrophy, radiation sensitivity	AR	DNA ligase IV defect, impaired nonhomologous and joining (NHEJ)
Cernunnos / XLF deficiency	↓	↓	↓	Microcephaly, radiation sensitivity	AR	Cernunnos defects, impaired NHEJ
CD40 ligand deficiency	Normal	IgM and IgD B cells present, others absent	↓, except IgM normal or ↑	Neutropenia, thrombo-cytopenia; haemolytic anemia	XL	Defect in CD40 ligand, defective B and dendritic cell signalling
CD40 deficiency	Normal	IgM and IgD B cells present, others isotypes absent	↓, except IgM normal or ↑	Neutropenia, gastrointestinal and liver disease, infections	AR	Defect in CD40, defective B and dendritic cell signalling

Table I: Combined T and B cell immunodeficiency (↓↓, markedly decreased; ↓, decreased; ↑ increased; AR, autosomal recessive; XL, X-linked form)

Disease	Circulating T cells	Circulating B cells	Serum Ig	Associated Features	Inheritance	Genetic defects/presumed phatogenesis
Purine nucleoside phosphorylase deficiency (PNP)	Progressive ↓	Normal	Normal or ↓	Autoimmune haemolytic-anaemia,neurological impairment	AR	Absent PNP, T cell and neurologic defects from elevated toxic metabolites (e.g. d GTP)
CD3γ deficiency	Normal (reduced TCR expression)	Normal	Normal		AR	Defect in CD3γ
CD8 deficiency	Absent CD8, normal CD4 cells	Normal	Normal		AR	Defect in CD8α chain
ZAP-70 deficiency	CD8 ↓, normal CD4 cells	Normal	Normal		AR	Defect in ZAP-70 signalling kinase
Ca^{++} channel deficiency	Normal count, defective TCR mediated activation	Normal count	Normal	Autoimmunity, ectodermicdysplasia, non progressive myopathy	AR	Defect in Orai-1, a Ca^{++} channel component
MHC class I deficiency	normal CD4cells, CD8 ↓	Normal	Normal	Vasculitis	AR	Mutations in TAP1, TAP2 or TAPBP genes giving MHC class I deficiency
MHC class II deficiency	Normal number, CD4 cells ↓	Normal	Normal or ↓		AR	Mutation in transcription factors for MHC class II proteins (*C2TA*, *RFX5*, *RFXAP*, *RFXANK* genes)

Table I: Combined T and B cell immunodeficiency (↓↓, markedly decreased; ↓, decreased; ↑ increased; AR, autosomal recessive; XL, X-linked form)

Disease	Circulating T cells	Circulating B cells	Serum Ig	Associated Features	Inheritance	Genetic defects/presumed phatogenesis
Winged helix deficiency	↓↓	Normal	↓	Alopecia, abnormal thymic epitelium	AR	Defects in forkhead box N1 transcription factor encoded by *FOXN1*, gene mutated in mice
CD25 deficiency	Normal to ↓	Normal	Normal	Lymphoproliferation autoimmunity, (may resemble IPEX syndrome),impaired T-cell proliferation	AR	Defect in IL2Rα chain
STAT5b deficiency	Normal to ↓	Normal	Normal	Growth-hormone insensitive dwarfism, dysmorfic features, eczema, lymphocytic interstitial pneumonitis	AR	Defect of *STAT5B* gene, impaired development and function of γδT-cells, T-reg and NK cells, impaired T cell proliferation

Table I: Combined T and B cell immunodeficiency (↓↓, markedly decreased; ↓, decreased; ↑ increased; AR, autosomal recessive; XL, X-linked form)

Disease	Serum Ig	Associated features	Inheri-tance	Genetic defects/presumed pathogenesis
Severe reduction in all serum immunoglobulin isotype with profoundly decreased or absent B cells:				
Btk deficiency	All ↓	Severe bacterial infections; normal of pro-B cells	XL	Mutation in Btk
μ heavy chain deficiency	All ↓	Severe bacterial infections; normal of pro-B cells	AR	Mutation in μ heavy chain
λ5 deficiency	All ↓	Severe bacterial infections; normal of pro-B cells	AR	Mutation in λ5
Igα deficiency	All ↓	Severe bacterial infections; normal of pro-B cells	AR	Mutation in Igα
Igβ deficiency	All ↓	Severe bacterial infections; normal of pro-B cells	AR	Mutation in Igβ
BLNK deficiency	All ↓	Severe bacterial infections; normal of pro-B cells	AR	Mutation in BLNK
Thymoma with immunodeficiecy	All ↓	Infections; numbers of pro-B cells ↓	None	Unknown
Myelodisplasia	All ↓	Infections; numbers of pro-B cells ↓	Variable	May have monosomy 7, trisomy 8 or dyskeratosis congenita
Severe reduction in serum IgG and IgA with normal, low or very low numbers of B cells:				
Common variable immunodeficiency disorders	IgG & IgA ↓; IgM variable	Clinical phenotype vary:autoimmune, lymphoproliferative, or granulomatous disease	10% AR or AD	Alterations in TACI, BAFFR, Msh5
ICOS deficiency	IgG & IgA ↓; IgM normal		AR	Mutations in ICOS
CD19 deficiency	IgG, IgA,IgM ↓;		AR	Mutations in CD19
XLP1	All may be ↓	Ab deficiency, fulminant Ebv infection and Lymphoma	XL	Mutations in SH2D1A

Table II: Predominantly antibody deficiencies (↓, decreased; ↑ increased; AR, autosomal recessive; AD, autosomal dominant; XL, X-linked form)

Disease	Serum Ig	Associated features	Inheri-tance	Genetic defects/presumed pathogenesis
Severe reduction in serum IgG and IgA with normal, elevated IgM and normal numbers of B cells				
CD40L and CD40 deficiency *Included in Tab:I*				
AID deficiency	IgG & IgA ↓; IgM ↑	Enlarged lymph nodes and germinal centres	AR	Mutations in AICDA gene
UNG deficiency	IgG & IgA ↓; IgM ↑	Enlarged lymph nodes and germinal centres	AR	Mutations in UNG gene
Isotype or light chain deficiencies with normal numbers of B cells				
Ig heavy chain deletions	May be absent of IgG or IgA or IgE	May be asymptomatic	AR	Chromosomal deletion at 14q32
κ chain deficiency	All Ig have λ light chain	Asymptomatic	AR	Mutations in κ constant gene
Isolated IgG subclass deficiency	↓ of IgG subclasses	Usually asymptomatic	variable	unknown
IgA deficiency associated with IgG deficiency	↓ of IgA and IgG subclasses	Recurrent bacterial infenctions in majority	variable	unknown
Selective IgA deficiency	Absent or ↓ of IgA	Usually asymptomatic, may have allergy or autoimmune disease;	variable	unknown
Specific antibody deficiency with normal [Ig] and numbers of B cells	Normal	Inability to make antibodies to specific antigens	variable	unknown
Transient hypogamma globulinemi a of infancy with normal numbers od B cells	IgA and IgG decreased	Recurrent moderate bacterial infections	variable	unknown

Table II: Predominantly antibody deficiencies (↓, decreased; ↑ increased; AR, autosomal recessive; AD, autosomal dominant; XL, X-linked form)

Evaluation for suspected Primary Immunodeficiency Diseases

Efficient early identification of PIDs is important for a correct diagnosis. We can distinguish three fundamental steps to diagnose primary immunodeficiency: 1. Clinical diagnosis, 2. Immunological diagnosis, 3. Molecular diagnosis (14).

Step1: Clinical diagnosis

a) Although the susceptibility to infections is the most frequent sign of PID, some general symptoms strongly suggesting a diagnosis of immunodeficiency are diarrhea, failure to growth and malabsorption. Other manifestations can be autoimmune disease, lymphoproliferative disorders and haematologic disorders (anemia, thrombocytopenia, neutropenia). Specific symptoms associated with immunodeficiency are shown in table below.

CELL MEDIATED IMMUNITY	**HUMORAL IMMUNITY**
Intracellular bacteria (*Salmonella, Mycobacteria, Legionella, Nocardia)* Fungi (*Candida, Cryptococcus, Histoplasma, P. carinii)* Protozoa (*Toxoplasma, Leishmania, Cryptosporidium*) Virus (*Enterovirus, Herpes, Paramyxovirus, Papova virus*)	Encapsulated Bacteria (*Strepto, Staphylo,Haemophilus*) Protozoa (*Giardia, Cryptospor.)* Enterovirus
GENERAL SYMPTOMS Diarrhea, Failure to growth, Malabsorption	
SYMPTOMS ASSOCIATED WIH IMMUNODEFICIENCY	
Specific eczema, Photosensitivity, Partial albinism, Vasculitis, Teleangiectasie, Delayed umbilical cord detachment, Dysmorphic Microcephaly Skeletal abnormalities, Abnormal growth, Ataxia	

b) The **family history** is important for the rapid recognition of genetic disorders, although many mutations may be new and the family history is not necessarily positive. Furthermore, it is necessary investigate about:

- Degrees of consanguinity in the parents
- Unexplained early infant deaths
- Immunodeficiencies cases in family history; familial occurrence of similar symptoms

c) Haemocromo-cytometric exam may quickly reveal, in severe cases, lymphopenia or alterations of other lineages potentially compromised.

Step2: Immunological Diagnosis

The first of laboratory evaluations, in immunological diagnosis, involves:

- **Immunoglobulin analysis and subclass ratio**
- **Vaccine titer levels of recall-antigens** (Tetanus, Diphtheria, Pneumo-coccus and Hepatitis B)
- **Assessment of white cell subsets**: Determine the absolute count of the following lymphocyte subpopulations compared with age-mached reference values. When combined with flow cytometry, the ratio and the type of individual cell population can be determined using a specific mAbs that recognize proteins on the cell surface. In the table below are represented the principal cell subsets with their cell surface markers.

CD3+	T lymphocyte
CD3+/CD4-	Helper- T lymphocyte
CD3+/CD8+	Cytotoxic T lymphocyte
CD3+/HLA-DR+	Activated T lymphocyte
CD3+/CD4-/CD8-	“Double-negative” Tcells
CD3+/TCR-γδ+	Subset T lymphocyte
CD19+ or CD20+	B lymphocyte
CD3-/CD16+ and/or CD56+	NK cells

Functional tests are more elaborated. These investigate about:

- **T cells activation:** Determine the expression of T cell activation markers (CD69 and CD40L) by FACS , after *in vitro* stimulation with PMA/ionomycin

- **T cells proliferation:** Determine the uptake of [^{3}H]-thymidine (or CFSE or activation markers) and compare the results with -preferably- age matched controls after stimulation with:

    - Mitogens (e.g. PHA, PMA + Ionomycin, PWM)
    - Monoclonal antibodies (e.g. CD2± CD28, CD3± CD28)
    - Antigens (e.g. tetanus, after booster vaccination)
    - Allogenic cells

- **Oxidative burst (phagocyte defects) and flow cytometry**

    - Nitroblue tetrazolium test (NBT)
    - Chemoluminescence test
    - Flow cytometry analysis using dihydrorhodamine (DHR)

- **Chemotaxis, granule contents, bacterial killing, phagocytosis**

    - Migration to a chemoattractant (e.g. FMLP)
    - Immunohistochemistry of granule contents, electron microscopy
    - Bacterial killing
    - Phagocytosis

Step3: Molecular diagnosis

When the disease shows a **well characterized clinical phenotype**, mutations in specific disease-gene are sought with molecular analysis. Following, the gene is amplified by polymerase reaction chain (PCR) and then subjected to mutation screening. Even if screening techniques, such us Single Strand Conformation Polymorphism (SSCP) and Denaturing High Performance Liquid Chromatography (dHPLC), are used to rapidly identify fragments where a mutation may exist, only with sequencing is possible to characterize the specific mutation (15)

Conversely, in **patients with atypical clinical manifestation** it is necessary to develop a hypothesis-driven or genome-wide approach to discover 'candidate-gene-disease' (16). The proposed method wants to analyse the genetic basis immunity to infection in human by its Mendelian and complex components. This is illustrated in *Figure 3.*

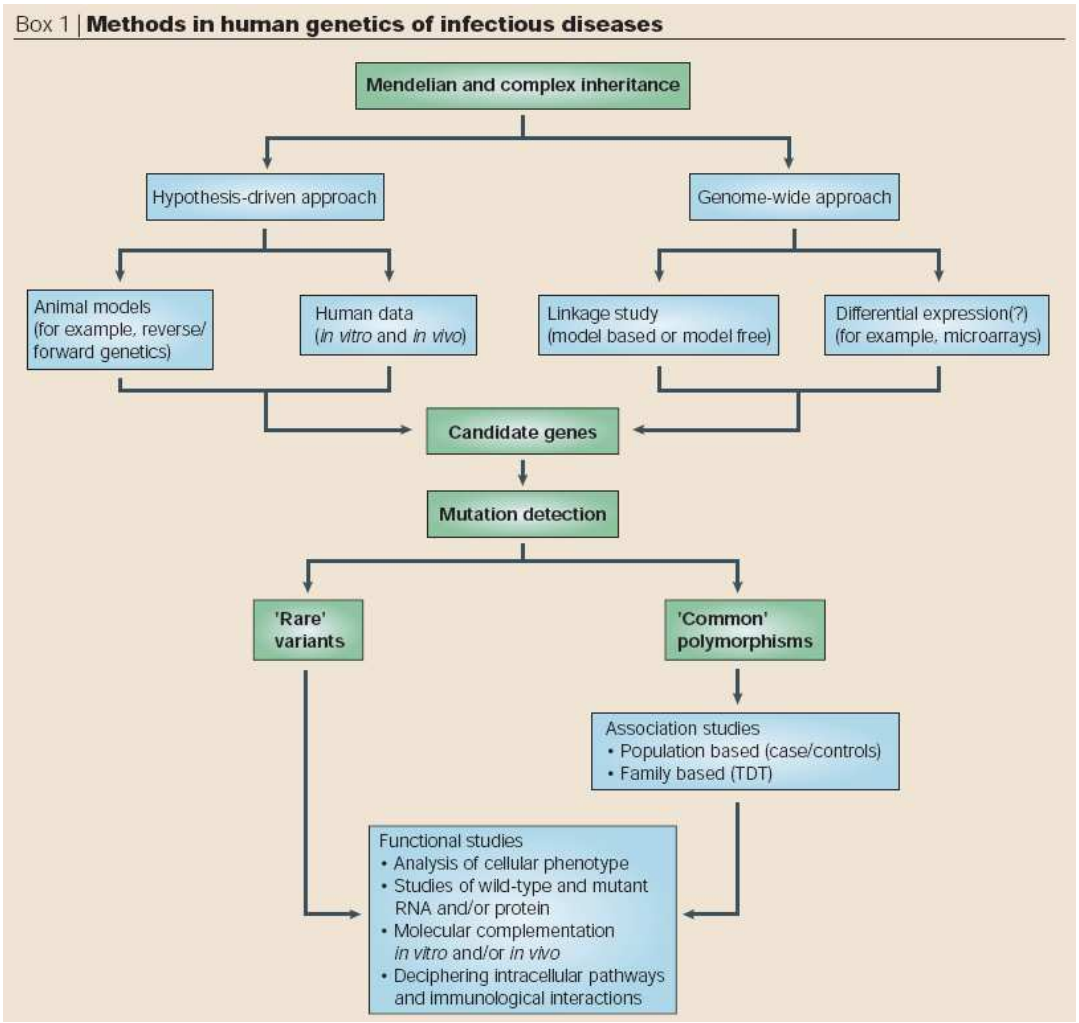


Figure 3: The hypothesis-driven approach selects candidate genes based on data obtained in animal models, on clinical/biological human phenotype (obtained *in vivo* and *in vitro),* or on previous knowledge regarding the disease of interest or a related disease. It can precede using functional or genetic assay. For mendelian traits, rare mutations that have a strong functional impact are generally expected, and causal relationship between the genotype and a cellular phenotype is relatively easy to establish. For complex traits, the role of common mutations in the candidate gene is tested by means of association studies. In this case, functional validation of the polymorphism or of the haplotype encompassing the mutation is difficult to validate immunologically because the effect is expected to be subtle. The genome-

wide approach can be applied to mendelian and complex traits to define candidate genes. These genome screens are carried out by linkage studies (positional cloning), which might based on recessive, dominant and X-linked. However, differential expression analysis and pathway investigation are actually used. Sequencing and association studies are required also to investigate a restricted number of regions/genes selected by hypothesis.
TDT, transmission disequilibrium test.

Genetic aspects of Primary Immunodeficiency Diseases

Although genes essential for immune function are distributed throughout the genome, there is a clear dominance of **X-linked** disorders (e.g. X-linked Agammaglobulinemia-XLA, X linked Chronic Granulomatous Disease-XCGD) caused by mutations in genes, located on x-chromosome, which are important for normal development and functioning of the immuno system. (17). The X linked inheritance pattern is complex and may be recessive or dominant. The mutated gene is always expressed in male patients. Usually, female carriers are asymptomatic caused by X-random inactivation even if, in some cases, skewed X-inactivation in female carriers appears to be associated with significant clinical manifestations (18). In **Autosomal Recessive** immunodeficiency (e.g. SCID), male and female can result equally affected. The patients inherit from both parent two different mutations located in the same gene (19).

Agammaglobulinemia *(Study1 and study2)*

Defects in early B cell development

Defects in early B cell development are characterized by the onset of recurrent bacterial infections in the first 5 years of life, profound hypogammaglobulinemia, markedly reduced or absent B cells in the peripheral circulation, and (in the bone marrow) a severe block in B cell differentiation before the production of surface immunoglobulin-positive B cells. Mutations in *Btk*, the gene responsible for X-linked agammaglobulinemia (XLA), account for approximately 85% of affected patients (20). Approximately half of the remaining patients, have mutations in genes encoding components of the pre-B cell receptor (pre-BCR) or BCR, including μ heavy chain (*IGHM*); the signal transduction molecules Igα (*CD79A*) and Igβ (*CD79B*); and *λ*5 (*IGLL1*), which forms the surrogate light chain with V pre-B (21-24). A small number of patients with defects in *BLNK*, a scaffold protein that assembles signal transduction molecules activated by cross-linking of the BCR, have been reported (25). *Btk* is expressed in myeloid cells and platelets, as well as B cells (26); *BLNK* is expressed in B cells and monocytes (27); and the remaining genes are B cell specific.

X-Linked Agammaglobulinemia (XLA)

In 1952, Bruton (28) reported the case of 8-years old boy with multiple episodes of pneumococcal sepsis associated with the complete absence of the serum globulin fraction as detected by protein electrophoresis. Additional studies described new patients and demonstrated that agammaglobulinemia occurred predominantly in boys and with an X-linked pattern of inheritance (29). By contrast, affected adults were almost equally divided between males and females, and a clear pattern of inheritance was rarely obvious. In the early 1970s, it was shown that patients with XLA had markedly reduced numbers of B cells in the peripheral circulation (30).

In 1993, two groups reported that XLA resulted from mutations in a cytoplasmic tyrosine kinase called *Btk* or Bruton's tyrosine kinase (31).

Btk

Btk is a member of the Tec family of kinases, which participates in several signalling pathways and is essential for early human B cell differentiation. Family members are characterized by a C-terminal kinase domain preceded by SH2 and SH3 domains, a proline rich region, and an NH2-terminal PH (pleckstrin homology) domain.

Btk is activated through cell surface molecules, including, pre-BCR, BCR, IL-5 and IL-6 receptors (on B cells), the high affinity IgE receptor (on mast cells), and the collagen receptor glycoprotein VI (on platelets). Moreover, recent studies, demonstrated a role of Btk in signalling through CXCR4 (on B cells) and Toll-like receptors (TLRs) (on myeloid cells and B cells) (32-34). *Figure* 4 shows a signal transduction through the pre-B cell receptor.

Upon activation, Btk moves to the inner side of the plasma membrane, where it is phosphorylated and partially activated by a src family member (35). Activated Btk and PLCγ2 bind to the scaffold protein BLNK via their SH2 domains, allowing Btk to phosphorylate PLCγ2 (36). This leads to calcium flux and activation of the MAP kinases ERK and JNK. Besides, several studies demonstrated that Btk (37) phosphorylates some transcription factors in the nucleus.

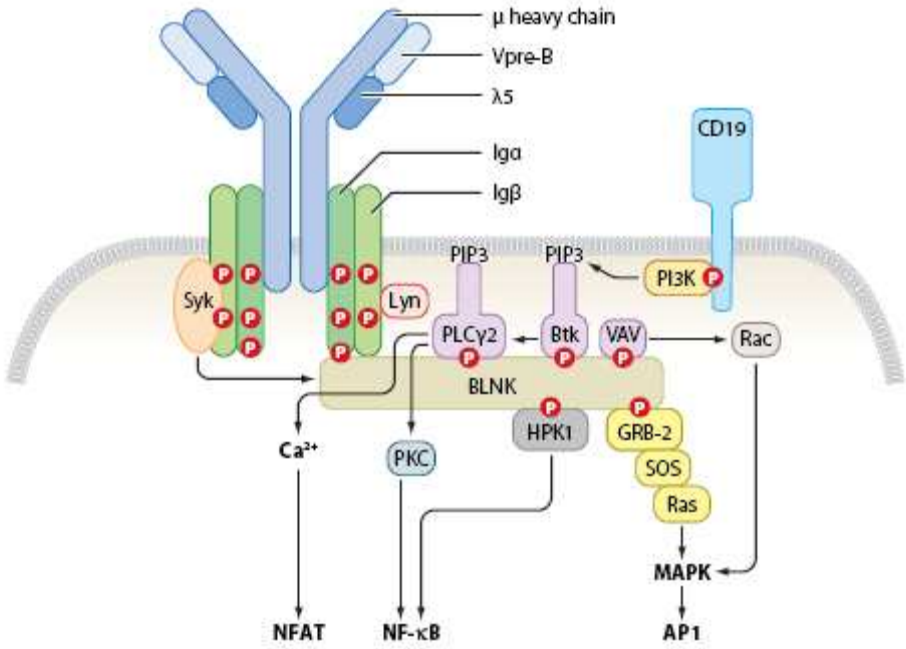


Figure 4: Signal transduction through the pre-B cell receptor.

Clinical Signs and Symptoms in XLA

Mutation of *Btk* gene cause the incomplete differentiation of B cell precursors to mature B cell or the inefficient expansion of pre- B cells into later B cells stages. XLA is characterized by early onset of bacterial infections, profound hypogammaglobulinemia and marked decrease of the peripheral B-lymphocyte population ($CD19^+$ < 2%) (38). Associated infections, particularly bacterial meningitis and pneumonia, are often life threatening. Early diagnosis is critical for proper management of XLA. In several cases, patients can present mild or no clinical symptoms and in the absence of definitive diagnosis by *Btk* sequencing, might commonly receive common variable immunodeficiency (CVID), specific antibody deficiency or IgA deficiency (39-40). XLA shows, as well as other PIDs, a risk of autoimmune disease, such us arthritis, dermatomyositis, autoimmune haemolytic anemia and sclerodermia. Female carriers are usually asymptomatic except for one Japanese female case (41).

Autosomal Recessive Agammaglobulinemia

Starting in the 1970s, several reports described females with a clinical disorder that was indistinguishable from XLA (42). The affected girls had an early onset of disease, profound hypogammaglobulinemia, and less than 1% of the normal number of B cells in the peripheral circulation. This suggested that there were autosomal recessive forms of the disease.

Defects in μ Heavy Chain

In 1996 Yel et al., demonstrated that mutations in μ heavy chain (*IGHM*) cause agammaglobulinemia and a clinical picture that is similar to that seen in XLA (43). Additional patients, with mutations in μ heavy chain, were soon described (44-45). All reported mutations result in the complete absence of CD19+ B cells in the peripheral circulation, with a detection threshold of 0.01%. Although there is considerable overlap, the patients with mutations in μ heavy chain tend to have a more severe phenotype than that seen in patients with mutations in *Btk*. They are recognized to have immunodeficiency at a mean age of 11 months rather than 35 months in patients with XLA, and they have a higher incidence of enteroviral infection and pseudomonas sepsis with neutropenia.

Defects in λ5, Igα, Igβ, and BLNK

A small number of patients with defects in *λ5* (*IGLL1*), *Igα* (*CD79A), Igβ* (*CD79B*), or *BLNK* have been reported. These patients generally have clinical findings that are indistinguishable from those seen in patients with mutations in *Btk*. Similar to patients with defects in μ heavy chain, patients with other forms of autosomal recessive agammaglobulinemia tend to have the onset of severe infections within the first year of life. Significant enteroviral infections and pseudomonas sepsis with neutropenia were seen in patients with *Igα* or *BLNK* deficiency.

Chronic Granulomatous disease *(Study 3)*

Chronic Granulomatous disease (CGD) is an inherited disorder of the NADPH oxidase complex in which phagocytes are defective in generating the superoxide anion and downstream reactive oxidant intermediates (ROIs), hydrogen peroxide (H2O2), hydroxyl anion and hypohalous acid (*Figure 5*) As a result of the defect in this key host defense pathway, CGD patients suffer from recurrent life-threatening bacterial and fungal infections such as *Staphilococcus aureus* and *Aspergillus* species. CGD is normally diagnosed in infancy and the incidence is ~1 in 200,000 births (46).

CGD is also characterized by abnormally exuberant inflammatory responses leading to granuloma formation, such as granulomatous enteritis, genitourinary obstruction, and poor wound healing and dehiscence. Recently, an Italian Multicenter Study (IPINET), demonstrated that pneumonia (47%) and lymphadenitis (45%) are the most common infections, followed by dermatitis (26%), subcutaneous abscess (20%), liver abscess (16%) and osteomyelitis (16%). (47-48).

Genetically, CGD is a heterogeneous disease, caused by mutations in any of the genes encoding the subunits of NADPH oxidase complex. More than two-thirds of all cases are X-linked recessive and result from defects in the *Cybb* gene that encodes the gp91*phox* subunit; the remaining cases are autosomal recessive and caused by defects in *Cyba*, *Ncf-1* and *Ncf-2* which encoding p22*phox*, p47*phox* and p67*phox* respectively. To date, there are no reports of CGD caused by defects in the gene for a fifth oxidase subunit, p40*phox* (49-50).

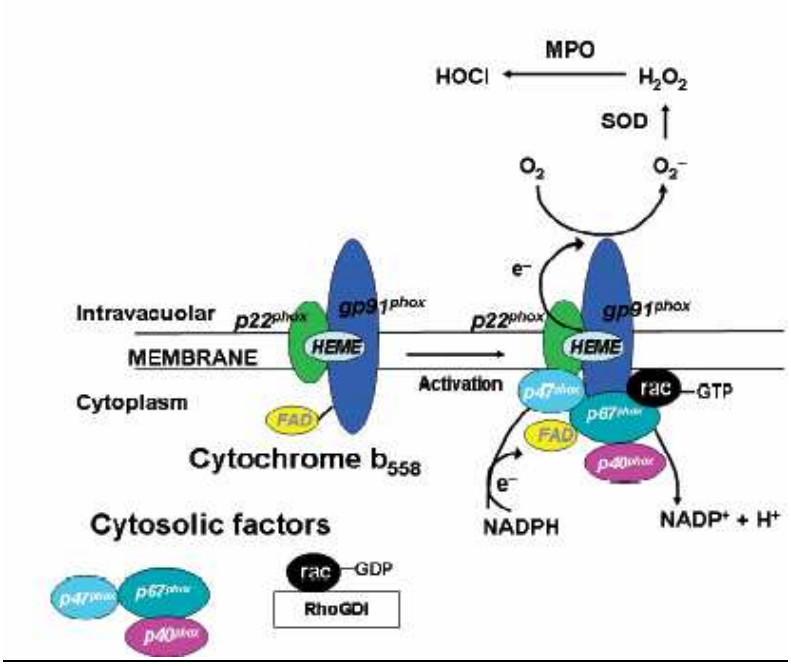


Figure 5: In unstimulated cells, the NADPH oxidase components are segregated into membrane and cytosolic compartment. P40*phox*, p47*phox* and p67*phox* are associated (1:1:1) in the cytosol. Rac2 is complexed in the cytoplasm with Rho-guanine nucleotide dissociation inhibitor. Upon activation, a series of protein-protein and protein-lipid interactions occur. Both p47*phox* and p67phox are phosphorilated and translocated with p40phox to membrane-bound cytochrome gp91*phox*. Rac2 binds GTP and migrates to the membrane independently of the p67*phox*-p47*phox* complex. In its complexed activated form, NADPH oxidase is able to transfer electrons from cytosolic NADPH to external molecular oxygen.

X linked CGD

The description of a patient who suffered from CGD and also from Duchenne muscular dystrophy and retinitis pigmentosa made it possible to localize the cybb gene (OMIM number 306400) on short arm of chromosome X. (51). The mutation responsible was a microdeletion in the Xp21 locus, affecting all genes involved on these diseases.

Additional patients from European, American and Japanese groups were soon described (52-54). According to Human Gene Mutation Database (HGMD), single-nucleotide substitutions account for 58% of the defects; small deletions, insertions and insertions-deletions account for 26% and large deletions and insertions for 14%. The remaining cases are caused by mutations in the regulatory region of the *Cybb* promoter (1.5%) and complex rearrangements (0.5%).

Generally, mutations in the *Cybb* gene lead to a lack of gp91*phox* expression generating a complete absence of the gene (X91°) phenotype, even if normal level ($X91^+$) and reduced level ($X91^-$) are reported too. NADPH oxidase activity is always totally abolished in X91° and $X91^+$ CGD, while in $X91^-$ CGD neutrophils, this activity can be reduced.

X linked CGD patients have a more severe clinical complications and higher mortality rates (5%) than patients with Ncf1 mutated (2%). (55). In general, XCGD-gp91° female carriers, with 10% normal granulocytes, are asymptomatic; in rare cases, female carriers with the same or a higher proportion of normal circulating neutrophils, may have a clinical manifestation of host defence defect.

Clinical managements of CGD

Lifelong antimicrobial prophylaxis with trimethoprim-sulfametho-xazole (or dicloxacillin in CGD patients who are allergic to sulfa) and antifungal prophylaxis with itraconazolo are indicated to decreased the rate of infection in CGD patients (56). Although the prophylaxis with antiinfectious drugs reduces the incidence of infection in CGD patients, the overall annual mortality is still high (2-5%).

Because CGD results from defect in myeloid lineage cells, bone marrow transplantation (BMT) is a potentially curative option for CGD patients when an HLA-mached donor is available. A survey of the European experience (1985-2000) showed that the overall success rate of unmodified haemopoietic allograft combined with myeloablative conditioning for those with an HLA-identical donor

is 81% with an overall mortality of 15% (57). The transplantation appears to be most successful if performed in infancy or early childhood. Graft-versus-host disease (GVHD) and inflammatory flare-up at infectious site are the major risks associated with BMT. Currently, there is a 10% of mortality.

Gene therapy represents a definitive cure for patients for whom conventional HSCT is not possible. Several evidences indicate that even a modest correction (> 10%) of NADPH oxidase in a fraction of myelomonocytes could be sufficient to restore the defective enzyme activity and provide clinical benefit (58). Gene therapy for CGD has been applied in cellular level studies, murine CGD models. Moreover several clinical trials on XCGD-gene therapy have been conducted since 1997, but resulted in low and short-term engraftment of CGD-corrected cells (59).

Rationale and Aims of the study

The human immune system is responsible for protecting the host against infections caused by bacteria, viruses, fungi and parasites. This protection is achieved through cells, organs and tissues arranged in a dynamic interactive network that form innate (non specific) and adaptive (specific) immune response. A defect in either of these components leads to disease and may ultimately result in death of the patient. This is particularly true in primary immunodeficiency disease (PIDs), which are caused by congenital defects of the immune system.

The diagnosis of PIDs is complemented through a combination of cellular-, immunochemical-, and molecular based approaches. While cellular based assay facilitate an assessment of the phenotype manifested, a molecular approach provides a definitive result to the origin of the immunodeficiency. Moreover, characterization of the molecular defect has important implications to better understand the pathophysiology of the disease. Several treatments exist in order to favour the reconstitution of immune system in immune-compromised patients, and new therapeutic approaches are constantly under investigation.

The aim of this project is to define a rational approach to recognize immune deficiencies, with specific emphasis on developing clinical evaluations, understanding the genetic and cellular basis of the disease (study 1 and 2) and proposing gene therapy as definitive cure of immunodeficiency when the mutated gene is known (study 3).

In particular we investigated:

1) Identification of a Btk mutation in a dysgammaglobulinemic patient with reduced B cells: XLA diagnosis or not?

2) Clinical, haematological and molecular characterization of an atypical case of Agammaglobulinemia with B, NK, DC and monocyte deficiency.

3) Chronic Granulomatous Disease (CGD): Gene Therapy with Stem Cells.

Materials & Methods

All subjects gave their informed consent to perform immunological and genetic analysis. The studies were performed at the 'Pediatric Immunology and Biotechnology Laboratory' Tor Vergata University, Rome and in 'Telethon Institute for Gene Therapy' San Raffaele, Milan.

Molecular Investigations

DNA extraction

Genomic DNA, isolated from the PBMC and/or MNC cells of patients, relatives and healthy controls donor (QIAamp DNA Blood kit, QIAGEN GmbH, Hilden, Germany). Purified suspensions of PBMC and MNC cells were obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

RNA extraction and RT-PCR

Total RNA was isolated from PBMCs and MNC cells using TRIZOL Reagent procedure (Invitrogen-Life Technologies, Milan - Italy). Isolated total RNA was analyzed by spectrophotometer (OD 260) and 500 ng was used for cDNA synthesis carried out with random primers and SuperScript TM III First-Strand Synthesis SuperMix (Invitrogen-Life Technologies, Milan - Italy) in a total volume of 20 ul. Reverse transcription was in according to the manufacturer's instructions.

PCR Reaction

DNA and cDNA was amplified using primers flanking the specific genes' exons. The primer pairs were designed using IDT SciTools Primer Quest SM software (www.idtdna.com) to generate amplicons between 300 and 400 bp . PCR reaction were carried out in a volume of 50 μl containing 100 ng of genomic DNA, 200 μM of each dNTP, 0.4 μM of each primer and 1.25 U GoTaq DNA polymerase (Promega, Madison WI, USA). The samples were denaturated at 95 °C for 5 min

followed by 40 cycles at 95 °C for 30 s, at exons specific annealing temperature for 30 s, extension at 72 °C for 30 s with a final extension at 72 °C for 5 min (*TableIII*)

Denaturing High Performance Liquid Chromatography (DHPLC)

DHPLC used as a screening method to analyze Btk and Flt3R genes, was performed on a WAVE analysis system (Transgenomic, Omaha, NE) using different temperature conditions (*Table III*). PCR products with abnormal chromatography were sequenced.

Sequencing

Sequencing was performed after PCR products purification with QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Both strands were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3130 automatedsequencer (Applied Biosystems, Foster City, CA, USA).

Vectors construction

We subcloned gp91*phox* sequence (from M.Grez) into *NcoI* - *SalI* sites of Blue Script 'BGI-10.mCherry' and then we transferred them in backbones to obtain four different transfer vectors. Transfer vectors have boxes (LTRs) at both end, splice donor and acceptor site (SD and SA), encapsidation signal including the 5'portion of the gag gene (ψ-GA), Rev-response element (RRE), two different promoters (PGK and SFFV), woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), miRNA target (miRT) sequence.

Mature miRNA sequences were retrieved from the miRNA registry (http://microrna.sanger.ac.uk/). miRNA target sequences (miRT) were synthesized as oligonucleotides (Primm SRL, Milan, Italy).

Expression investigations

Real Time PCR-SYBR Green

The expression study was performed using 96-well optical reaction plate using an ABI Prism 7900HT Sequence Detection System (Applied Biosystem, Foster City, CA) with Real Master Mix (Eppendorf AG – Hamburg - Germany). PCR conditions were in according to the manufacturer's instructions. PCR were set up in a total volume of 25 ul containing 2.5x Real Master Mix/20Xsybr Solution, 200 nM one primer-pair, 5ul of cDNA adjusted to 10 ng. We chose GNB2L1 gene as genomic reference. The primers were designed using IDT SciTools Primer Quest SM software (www.idtdna.com) to generate amplicons between 300 and 400 bp. (*Table III*) The PCR program was initiated at 95°C for 4 min, followed by 40 cycles of 30 seconds at 94°C, 30 seconds annealing at exon specific temperature and 30 seconds at 72°C. Absolute quantification of target amplicon in the patient was performed by interpolation of the threshold cycle number (Ct) against the corresponding standard curve. The control was normalized to value 1.

Immunoblot Analysis

Total cells were lysed on ice cold JS lysis buffer (50mM Tris/HCl pH 8, 150mM NaCl, 1.5mMMgCl2, 5mMEGTA, 1% Triton-X, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM DTT) for 20 min on ice and then clarified by centrifugation at 12000 rpm for 10 min at 4 °C. 20 μg of total cell lysate were size-fractionated by SDS-PAGE gel and then transferred to nitrocellulosemembrane (Protran, Schleicher & Schuell-Bioscience GmbH, and Dassel, Germany). Membranes were blocked in 5% milk for 1 h at room temperature and then incubated with a primary antibody (anti-Btk C20, Santa Cruz Biotechnology Inc., CA, USA), 1:300 dilution, for 1 h at room temperature. Specific proteins were visualised by ECL (LiteAblot, Euroclone SpA, Switzerland).

TCR Spectratyping

PBMC were fractionated into $CD4^+$ and $CD8^+$ subsets by the use of anti-CD4+ or anti-CD8+ monoclonal antibody-coupled magnetic beads (Dynal AS, OSLO, Norway) following manufacturer's instructions. Total RNA was extracted by TRIzol® (Gibco-BRL/Life Technologies) in accordance with the manufacturer' s instructions. PCR were performed using SuperscriptTM One-Step RT-PCR with PLATINUM Taq (Invitrogen) in according to manifacture's instruction. *Table IV* showed the primers sequences. PCR products were diluted in a stop buffer (deionised formamide and TE pH 7.5) and denatured at 95° C for 4 min. 9 µl of each sample were loaded in wells of a 6% polyacrylamide gel (Pharmacia Ready Mix Gel). The sample run in a DNA Automatic Fluorescence Sequencer (Pharmacia ALFDNA Sequencer, Sweden), and finally analysed by specific software (Pharmacia DNA Fragment Manager 2.0). Data interpretation was done following the visual scoring method. Based on this method three main pattern profiles of distribution were identified: Polyclonal/Gaussian profiles (p) with 5-9 peaks per TCRBV family and a Gaussian "bell shaped distribution"; Polyclonal altered profiles (pa) with 5 or more peaks with a non Gaussian-like distribution pattern; skewed/perturbed profiles (sk) with a number of peak comprised in a range from 1 to 4 peaks or with a multipeak pattern having one solitary peak >50% of the total peak area or one or more deleted peaks. Polyclonal and Polyclonal altered profiles were considered normal.

Vector Copy Number Analysis

Cells were cultured for at least 14 days after transduction in order to get rid of non-integrated vector forms. Genomic DNA was extracted by using "Maxwell 16 Cell DNA Purification Kit" (Promega, Madison/WI, USA), according to manufacturer's instructions. Vector copy numbers per genome (VCN) were quantified by quantitative Real Time PCR using HIV primer and probe set against the primer binding site (PBS) region of LV. Endogenous DNA amount was quantified by a

primer/probe set against the human telomerase gene (Telo) and TAMRA-Probe, or the murine ß-actin gene (ß-Act) and VIC-probe. Copies per genome were calculated by the formula: (ng LV/ng endogenous DNA) x (n° of LV integrations in the standard curve). The standard curve was generated by using a CEM cell line stably carrying 4 vector integrants, which was previously determined by Southern blot analysis. All reactions were carried in out in triplicate in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) (*Table IVa*).

Functional Investigations

T-cell proliferation assay

$2x10^5$ PBMCs were triple cultured in microtiter plates for 3 and 6 days with mitogens and antigens respectively at 37° C with CO2 0,5% in complete culture medium consisting of RPMI 1640 with L-glutamine (Sigma, St. Louis, MO) supplemented with 10% AB Human serum (Sigma), 50 IU/ml penicillin and 50 µg/ml of streptomycin (Sigma). Mitogenic stimuli used were phytoaemmaglutinin (PHA) 5µg/ml, pokeweed mitogen (PWM) 0.35 µg/ml (Sigma) and OKT3 3µl (stock supernatant obtained from OKT3 cell line, ATCC-Sigma, MI, Italy). Common antigens used were antigen solutions of Candida Albicans, Cytomegalovirus (CMV) and Herpes virus 1 (HSV-1) 15 µl/well (AID GmbH-Strasberg-Germany). PBMC from the same samples were cultured for 3 and 6 days without any stimulus as negative controls in complete culture medium. To analyse T-cell proliferation, all the cultures were then pulsed with 0,5 µCi 3H-thymidine for additional 6 hours and harvested onto glass fibre filters (Packard, MI, Italy) and analysed by B-counter scintillator (Canberra Packard Instrument Company, Meriden, CT, and U.S.A.). Data were expressed as count per minute (cpm) and elaborated as stimulation index (SI), defined as the ratio between the average counts of stimulated and unstimulated PBMC. SI = proliferation of PBMC with

mitogen, or antigenSpontaneous proliferation of PBMC Lymphoproliferations towards mitogens, was considered positive in relation to the sequent scheme:
PHA > 35.000 cpm; PWM > 18.000 cpm; OKT3 > 25.000 cpm
Lymphoproliferations towards common antigens (Tetanus, Candida) were considered positive if cpm > 5.000.

MLR

Allogeneic PBMCs were used as responders. Two different healthy donors were used to avoid the possibility that a low proliferation was due to HLA compatibility. Allogeneic cells were added to each well with a ratio Stimulators / responders of 1:10. Negative controls PBMCs alone; positive controls consisted in PMBCs stimulated with PHA (5μg/ml). Patients' PBMCs were co-cultured with healthy donors allogeneic PBMCs for 6 days. Then 0,5 μCi ^{3}HThymidine was added and the cells were cultured for additional 6 hours. Finally cells were harvested onto glass fibre filters (Packard, MI, Italy) and analysed by B-counter scintillator (Canberra Packard Instrument Company, Meriden, CT, and U.S.A.).

DHR

Granulocyte oxidative burst was determined quantitatively with Burst test Kit (ORPEGEN Pharma, Germany). PLB 985 cells before and after transduction, and transduced HSPC murine cells, were put in PBS and then in ice for 15 min. Then, four testing tubes were fi lled with 100 μL of cells each and 2×107 unlabeled opsonized bacteria *E. coli*, 20 μL of substrate solution (negative control), 20 μL fMLP (peptide *N*-formyl-MetLeuPhe) as chemotactic low physiological stimulus (low control) and 20. μL phorbol 12-myristate 13-acetate (PMA), a strong non-receptor activator (high control). All the samples were incubated for 10 min at 37.0 °C in a water bath, dihydrorhodamine (DHR) 123 as a fluorogenic substrate was added and incubated again in the same conditions. The oxidative burst occurred with the production of reactive oxygen substrates (ROS) (superoxide anion,

hydrogen peroxide) in granulocytes stimulated *in vitro*. In ROS-stimulated granulocytes, nonfluorescent DHR 123 underwent conversion to fluorescent rhodamine (R) 123 registered in the flow cytometer.

Facs analysis

Studio1

Flow cytometric analysis of Btk phosphorylation Lymphoblastoid cell lines (10×106 cells) were collected by centrifugation and stimulated by hydrogen peroxide (10 mM) at 37 °C for 5 min.Collected cells were fixed for 10 min at 37 °C and then permeabilized by adding 1 ml of BD Phosflow buffer III for 30 min on ice according to BD Phosflow protocol 3 (BD Biosciences Pharmigen, San Diego, CA, USA). Pelleted cells were resuspended in BD Pharmigen Stain buffer containing PE conjugated anti-phosphoY551-Btk (BD Biosciences Pharmigen, San Diego, CA, USA) at room temperature and incubated a minimum of 1 h. The analysis was carried out on FACScan using CellQuest software (BD Biosciences Pharmigen, San Diego, CA, USA).

Studio2

All antibodies and appropriate isotype controls are human specific and are from BD/Pharmingen (San Diego, CA, USA). Bone marrow cells were stained with: PC5-CD34, FITC-TdT, PE-cKit, PE-IL3R, FITC-CD19, PE-CD79a, PE-IL7R, PE-CXCR4, PE-Flt3R, Tc-CD19, FITC-CD15 and PE-CD56. Peripheral blood cells were staining with: PE-Flt3R, APC-CD8, PerCP-CD4, FITC-CD15, APC-CD14 FITC-CD3, PerCP- CD3 and APC-CD11c. Cells were stained for 20 min at 4 °C, and washed. Cells were resuspended in PBS containing 10 ng/ml 7aminoa-ctinomycin D (7-AAD) to exclude dead cells. Cells were analysed on FACS Canto cytometer and analysed by FCS express software.

Studio3

Cells grown in suspension were washed and resuspended in PBS containing 2 % fetal bovine serum (FBS). Cells transduced or not, were stained with human PE-labeled Anti-Flavocytochrome b_{558} (7D5) (MBL Medical and Biological Laboratories), human APC-CD11b (BD Pharmingen) and mouse FITC-cKit (BD Pharmingen). Only mouse cells before the classic procedure for immunostaining were blocked in PBS, 5 % rat serum, 2 % FBS for 15 min at 4 °C. For human immunostaining this is not necessary. After cells were stained for 20 min at 4 °C, and washed. Cells were resuspended in PBS containing 10 ng/ml 7 aminoa-ctinomycin D (7-AAD) to exclude dead cells. Cells were analysed on FACS Calibur and analysed with CellQuest Software (Becton-Dickinson).

Cell lines and Cell Investigations

Ebv-Cell lines

EBV-transformed lymphoblastoid cell lines (LCL) were obtained by incubating about 5×106 PBMC with 2 ml of supernatant from the EBV-secreting cell line B95-8 for 1 h at 37 °C. Infected cells were then washed and cultured at 1×106 cells/ml in RPMI 1640 containing 25 mM Hepes, 20% fetal bovine serum from U.S., 2 mM L-glutamine and 1 μg/ ml of cyclosporin A in 24-well culture plates. Clumps of cells were observed at the end of the first or the second week and stable EBV lymphoblastoid cell lines were established by the end of 4 weeks.

293T-Cell lines

293T cells grown in IMDM medium supplemented with 2Mm glutamine, 10% FBS, Penicillin (100unit/ml), and Streptomycin (100ug/ml) in humidified incubator at 37°C under atmosphere of 5% CO2/95% air.

PLB-Cell lines

PLB-985 cells (obtained from Dinauer MC) grown in RPMI1640 medium supplemented with 2Mm glutamine, 10% FBS, Penicillin (100unit/ml), and Streptomycin (100ug/ml) in humidified incubator at 37°C under atmosphere of 5% CO2/95% air. For granulocytic differentiation cells at a density of $1x10^{\wedge 6}$ cells/ml were exposed to 1,2% DMSO for 5-6 days. Under these conditions ~ 80 % of wt and transduced cells acquired respiratory-burst activity

Clonogenic assay

At days 0, 4 and 8 both fresh CD34 cells and cultured cells were suspended in 1ml of complete methylcellulose medium (MethoCult, Stem Cell, Canada) supplemented with 30% FBS, SCF (50 ng/ml), IL-3 (10 ng/ ml), GM-CSF (10 ng/ml) and EPO (3 U/ml) and plated induplicate at the concentration of 1 ´ 103/ml in 35mm tissue culture dishes. Cultures were incubated at 37°C in a humidi®ed atmosphere of 5% CO2. Colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit erythroid (BFU-E) and colony-forming unit mixed (CFUGEMM) were enumerated using an inverted microscope at 10 and 14 d. Single colonies were plated in 0´1 ml of methylcellulose culture medium supplemented with growth factors in 96-well plates. Cultures were incubated at 37°C in a humidi®ed atmosphere of 5% CO2. After 14 d, secondary CFU-GM, BFU-E and CFU-GEMM were scored and enumerated under an inverted microscope.

In vitro assay for B/NK cell progenitors

Fresh purified $CD34^+$ cells ($CD34^+$ Microbeads; Miltenyi Biotec) were plated at 10 cells/well on flat-bottom 96-well plates preseeded with a semiconfluent layer of the MS5 stromal cell line. Culture medium was the same for all the $CD34^+$ cells plated, independent of the cytokines used during transduction, and consisted of RPMI (Euroclone, UK) supplemented with 10% human serum from healthy blood donors, 5% FCS, lglutamine, penicillin/streptomycin, and the recombinant human cytokinesTPO, Flt3-ligand, SCF, IL-7, and IL-15 (R&D) (20 ng/ml) and 250 IU/

ml IL-2 (Roche, Milan, Italy). Half the medium was replaced weekly. At weeks 3–4, wells with visible cell proliferation were selected and cells collected to be analyzed by FACS for the presence of B cells ($CD19^+$) and NK cells ($CD56^+$).

Vectors Production and Titration

VSV-pseudotyped third-generation Lentiviral vectors were produced by Ca PO transfection into 293Tcells, as described in Gentner B et al., Nature Method 2008. Briefly, supernatants were collected, passed through a 0.22 μm filter, and purified by ultracentrifugation as described. Expression titer was estimated on 293T cells – which do not express high levels of the miRNA tested in this study - by limiting dilution. Vector particles were measured by HIV-1 gag p24 antigen immunocapture (NEN Life Science Products).

PLB985 Transduction

PLBmut were trasduced with serially diluted LVVs (from 10^-2 to 10^-6) containing gp91*phox* cDNA at different multiplicity of infection (MOI). (*Table IX* in preliminary result study 3).

Murine Transduction

day1: 1x10^6 Lin- cells were plated in 1.5 ml of StemSpamSerum with Pen/Strep, Glu and pre-stimulated over night with SCF (100ng/ml), Flt3L (100 ng/ml), IL3 (20ng/ml) and TPO (50 ng/ml). day 2: Pre-stimulated cells are transduced with LVVs at different MOI: 10,5 (LV.PGK.gp91), 17,5 (LV.PGK.gp91_126T), 75 (LV.PGK.gp91-130T) and 13,5 (LV.SFFV.gp91). day 4: Liquid and MethoCult Culture were performed.

Liquid Culture

We plated tranduced murine Lin- cells ($5x10^{\wedge 5}$ cells) in StemSpan serum (StemCell) with cytokines and growth factors cocktail (SCF, Flt3L, IL3, TPO) and huG-CSF (100ng/ml). After 2 days we washed them and new medium replaced with hu-G-

CSF and SCF. On day 8 we used some of the cells for cytospin analysis and the remaining were plated in fresh medium with hu-G-CSF to induce granulocytes differentiation.

Methocult Culture

Transduced cells were plated in methylcellulosa medium at concentration of 2500cells/plate/ml medium. Medium conteined a mix of growth factors (StemCell) and after 14 days colonies were collected, washed and analysed by FACS Calibur.

Table III

DNA sequence	**primer name**
AATCAGGAACTTGTGCTGGC	cDNA PU.1-1F
GTTTGGCGTTGGTATAGATCCG	cDNA PU.1-1R
GCTGGATGTTACAGGCGT	cDNA PU.1-2F
TGAAGTTGTTCTCGGCGAAG	cDNA PU.1-2R
GACAGGCAGCAAGAAGAAGA	cDNA PU.1-4F
CTGAACTGGTAGGTGAGCTT	cDNA PU.1-4R
CTTCGCCGAGAACAACTTCA	cDNA PU.1-3F
AGGTCCAACAGGAACTGGTA	cDNA PU.1-3R
CAACCGCAAGAAGATGACCT	cDNA PU.1-5F
TTGGGAGGAGGTTAATGGGT	cDNA PU.1-5R
GCAGGCATTTGTTGGGTTAGAGCA	PU.1 GEN-1F
AGGAAACCCTGACTTCCCACTGAT	PU.1 GEN-1R

Table III

primer name	**DNA sequence**
IKAROS 1F	CCA AGT TAG CAG GAC ACT CT
IKAROS 1R	ACC AGG GTC TAC CAA CCT TA
IKAROS 2F	TGT GCC AGT CTG ATA CTC CAG CAA
IKAROS 2R	ACA GGT GAA GGA AAC ACT CAG GCA
IKAROS 3F	GCC CGA GAC TCA CAC TTC TTC TTT
IKAROS 3R	TGC CAG TTG AGG GAA CAC AAT GGA
IKAROS 4F	GAA TGG GCG TGC CTG TGA AAT GAA
IKAROS 4R	ATG CAC CTT CTC CAG GCC ACT AAA
IKAROS 5F	AGC GTT AAG GAG CTG GCA GGT TTA
IKAROS 5R	AGG GTT AGC CAG CAA GGA CAC AAT
IKAROS 6F	CCC CTT ACA CAG AAG GCT GGC ATT
IKAROS 6R	AAG GCT CTG CTC CTA AGG CTG CAT
IKAROS 7F	TAA CAT TGG ACG CGA CTG AAC CCT
IKAROS 7R	TCC CTT TCT TCC ACC CTC AAC TCA
IKAROS 8F1	AGA TTT CAG CTG TTG CTG CCA GAC
IKAROS 8R1	TGT GGT TGG TCA GGT AGA TGA GAC
IKAROS 8F2	GTC TCA TCT ACC TGA CCA ACC ACA
IKAROS 8R2	GTA GCC GCA CAT GTT GCA CTC AAA
IKAROS cDNA 2F	GCG ACG CAC AAA TCC ACA TAA CCT
IKAROS cDNA 4R	TTC ATT TCA CAG GCA CGC CCA TTC
IKAROS cDNA 5F	ATG GGC GTG CCT GTG AAA TGA ATG
IKAROS cDNA 5R	AGT TGC AGA GGT GGC ATT TGA AGG
IKAROS cDNA 6F	GCC CAA TGT GCT CAT GGT TCA CAA
IKAROS cDNA 6R	TTG GTC CAT CAC GTG GGA CTT CAT
IKAROS cDNA 7F	CCA AGC CAT CAA CAA CGC CAT CAA
IKAROS cDNA 8F	TCA TCT ACC TGA CCA ACC ACA TCG
IKAROS cDNA 8R	TTG TCT GGT CCA GTC CAG TCT ATG

Table III

primer name	**DNA sequence**
FLT3L 1F	CAG TGA TAA AGC TAG TGC TAG GGC
FLT3L 1R	CTC CAC TTC TAA GAA CCT CGA C
FLT3L 2F	CAG ACG TCG AGG TTC TTA GAA GTG
FLT3L 2R	TCT ATC TCC CTC TCC TCT ATC TCC
FLT3L 3F	ATG CAA ACT GGA CAG CAT TGG ACC
FLT3L 3R	AGT TCA AGA CCA CCC TGG GAA ACA
FLT3L 4F	CAA GGT TCT GTG GCT TCT TCT G
FLT3L 4R	AGG TCC CTC CCA ACA TGA CTT A
FLT3L 5F	TGC AGG ACG TAA GTC ATG TTG GGA
FLT3L 5R	GGA TTT CAT CAT GTT GGC CAG GCT
FLT3L 6F	AAA CTC TGA GAG CCA GAG CTC ACT
FLT3L 6R	GGT AAA CTG AGC TCC AGA AAG AGG
FLT3L 7-8 F	AGA TGA GCA AAC TGA GGC AC
FLT3L 7-8 R	AAA GTG CTG GGA TTA CAG GC
FLT3L -01 1F	TCT CTG GCT GTC ACC CGG CTT
FLT3L-01 1R	CGG TGA CTG GGT AAT CTT GAA GCA
FLT3L-01 2F	AAC AAC CTA TCT CCT CCT GCT GCT
FLT3L-01 2R	GGC TGA AAG GCA CAT TTG GTG ACA
FLT3L-01 3F	AGA CTG TCG CTG GGT CCA AGA T
FLT3L-01 3R	TTG AGG AGT CGG GCT GAC ACT
FLT3L-01 4F	TGG ATC ACT CGC CAG AAC TTC TCC
FLT3L-01 4R	GTT GTT TAA GGC TCC GCA GGA TGA
FLT3L-01 5F	TCA TCC TGC GGA GCC TTA AAC AAC
FLT3L-01 5R	ACA AGG GCT TTG TAC AGA GTT GGG
FLT3-02 1F	AGG GTC CGA GAC TTG TTC TTC TGT
FLT3L-02 1R	TTT GAC AGC GAA GTC GGA GGA GAT
FLT3L-02 2F	ATC TCC TCC GAC TTC GCT GTC AAA
FLT3L-02 2F BIS	GCG CGT GAA CAC GGA GAT ACA CTT
FLT3L-02 2RBIS	AGC AGC AGT AGG AGG AGC AGA
FLT3L-03 1F	CCG GCC GAA ATG ACA GTG CT
FLT3L-03 1R	ACG GTG ACT GGG TAA TCT TGA AGC
FLT3L-03 3F	TTC GCT TCG TCC AGA CCA ACA TCT
FLT3L-03 3R	AGG TCA GTG CTC CAC AAG CA
FLT3L-03 4F	AGT GTC AGC CCG ACT CCT CAA
FLT3L-03 4R	TCT AGC CAA CTT CCT CTG TGT CCA
FLT3L-04 1F	CCC TTC CAC ACC CAA CTG
FLT3L-04 1R	AAA CAG GAT CAG GCC TGG CA
FLT3L-04 4R	TCT GTG TGC CTC AGT ATC CTC CC

Table IV

Sequences of primers for Spectratyping Analysis
Vβ1: CAGTTCCCTGACTTGCACTC
Vβ2: GCTTCTACATCTGCAGGTGC
Vβ3: GAGAGAAGAAGGAGCGCTTC
Vβ4: GCAGCATATATCTCTGCAGC
Vβ5.1: CTCGGCCCTTTATCTTTGCG
Vβ5.3: CCCTAACTATAGCTCGAGC
Vβ6.1: GATCCAGCGCACACAGC
Vβ6.2: GATCCAGCGCACAGAGC
Vβ7: CCTGAATGCCCCAACAGC
Vβ8: GAACCCAGGGACTCAGCTG
Vβ9: GGAGCTTGGTGACTCTGCTG
Vβ11: CAGGCCCTCACATACCTCTCA
Vβ12: CAAAGACAGAGGATTTCCTCC
Vβ13: GTCGGCTGCTCCCTCCC
Vβ14: GTCTCTCGAAAAGAGAAGAGG
Vβ15: GTCTCTCGACAGGCACAGGC
Vβ16: GAACTGGAGGATTCTGGAGTT
Vβ17: CCAAAAGAACCCGACAGCTTTC
Vβ18: GTGCGAGGAGATTCGGCAGC
Vβ20: CCTCCTCAGTGACTCTGGC
Vβ21: GGCTCAAAGGAGTAGACTCC
Vβ22: GTTGAAAGGCCTGATGGATC
Vβ23: CAGTTCAGTGACTATCATTCTG
Vβ24: GGGGACGCAGCCATGTACC

Table IVa

DNA sequence	**primer name**
fwd: 5'-TACTGACGCTCTCGCACC-3' rev: 5'TCTCGACGCAGGACTCG-3' FAM 5'-ATCTCTCTCCTTCTAGCCTC-3'	HIV HIV Probe
fwd: 5'-GGCACACGTGGCTTTTCG-3' rev: 5'-GGTGAACCTCGTAAGTTTATGCAA-3' TAMRA 5'-TCAGGACGTCGAGTGGACACGGTG-3	telomerase telomerase Probe
murine fwd: 5'-AGAGGGAAATCGTGCGTGAC-3' murine rev 5'CAATAGTGATGACCTGGCCGT-3' VIC 5' CACTGCCGCATCCTCTTCCTCCC-3'	beta actina Probe

Study1: **Identification of a Btk mutation in a dysgammaglobulinemic patient with reduced B cells: XLA diagnosis or not?**

In this study we present clinical, haematological and molecular analysis of a 31 years old male with a gradual decline of peripheral B cells, recurrent respiratory infections and with a novel mutation in Btk gene. Generally, defect in peripheral blood B lymphocytes (CD19+<2%) and mutation in Btk confirm a diagnosis of XLA. Patient's mother, maternal aunts and a maternal female cousin are heterozygote for the same mutation and although XLA female carriers are usually asymptomatic, they suffer from a mild to severe pulmonary emphysema.
We report a puzzling case where the patient's clinical history and laboratory findings divorce from molecular genetics.

Case Report

Medical history of the 31 year-old male patient, revealed recurrent episodes of enteritis associated to failure to thrive since his first months of life. At age of 3 months he developed measles complicated with pneumonia and pulmonary abscess. In the following years he suffered from recurrent episodes of *Staphilococcus aureus'* pneumonia, urinary tract infections and diffuse pyodermitis with cutaneous abscess. Moreover,data of first decade of his life reported a dysgammaglobulinemia (normal IgG with low IgA and IgM serum concentrations) and normal total count of lymphocyte with an inverted $CD4^+/CD8^+$ ratio and 8% of B lymphocytes, suggesting a diagnosis of common variable immunodeficiency. At that time severe chronic obstructive lung disease was observed and pulmonary Computed Tomography (CT) proved the presence of pulmonary interstitial fibrosis.
At 28 years of age he was hospitalized for weight loss, jaundice and abdominal pain. An Endoscopic Retrograde Cholangio-Pancreatography (ERCP) revealed a dilatation of both intrahepatic bile ducts and Wirsung's Duct with a clinical

diagnosis of sclerosing cholangitis and a chronic obstructive pancreatitis. Enteral nutrition, antibiotics and substitutive pancreatic enzyme together with steroid therapy were started with a partial clinical response.

Clinical, immunological and molecular investigation was extended to maternal family members, and mutation in *Btk* gene was found in patients, his mother, maternal aunts and a maternal female cousin (*Figure 6*).

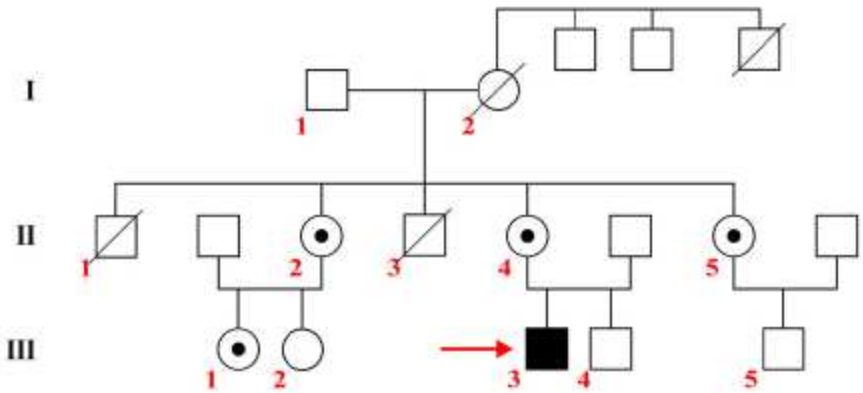


Figure 6: Genealogic tree of our XLA patient

Results

Immunological investigations

Immunological data of the patient, showed a dysgammaglobulinemia due to low IgA and IgM with normal IgG, low value of $CD19^+$ B cells (percentage value in repeat studies ranging from 1.98 to 2.8%) and inverted $CD4^+/CD8^+$ ratio were confirmed. CD3+ lymphocyte count and proliferative lymphocyte response to mytogens (data not shown) were normal. Post-vaccination antibody titers to Tetanus toxoid and *Haemophilus influenzae* were normal but he had a low response to Streptococcus pneumoniae. His mother (II4), two maternal aunts (II2;II5) and one female cousin (III3) reported recurrent respiratory infections during childhood and suffered from pulmonary emphysema which was very severe in II5. As shown

in *table V*, immunological values are normal, circulating B cells ranged from 4% to 7.9% with a normal distribution of transitional, mature and memory B cells.

	III3	**II2**	**II4**	**II5**	**range**
Lymphocytes	1230/ul	1400/ul	1380/ul	1380/ul	
$CD3^+$ (%)	89	82	80	84	61-84
$CD4^+$ (%)	39	55	60	54	32-60
$CD8^+$ (%)	46	28	21	29	13-40
$CD19^+$ (%)	2	4.7	7.9	4.2	10-31
$CD19^+$ $CD27^+IgD^+IgM^+$ (%)	10	19	29	nt	13.7
$CD19^+$ $CD27^+IgD^-IgM^-$ (%)	13	30	28	nt	13.8
$CD24^{high}$ (%)	6	2.7	2.6	nt	2.06±0.27%
IgM (mg/dl)	6	65	71	62	40-230
IgG (mg/dl)	833	856	987	843	700-1600
IgA (mg/dl)	32	99	118	80	70-400

Table V: Immunological data of our patient and female carriers. nt, not tested

Molecular investigations

Genomic DNA was analyzed for mutation in *SAP*, *CD40L* and *Btk* genes. Sequencing of *SAP* and *CD40L* genes detected no mutations. On the contrary, *Btk* gene molecular analysis, performed by DHPLC and sequencing, revealed a new missense substitution in exon 11 (c.1078 a > g, sequ.X58957) causing threonine change to alanine in position 316 of SH2 protein domain (*Figure 7*).

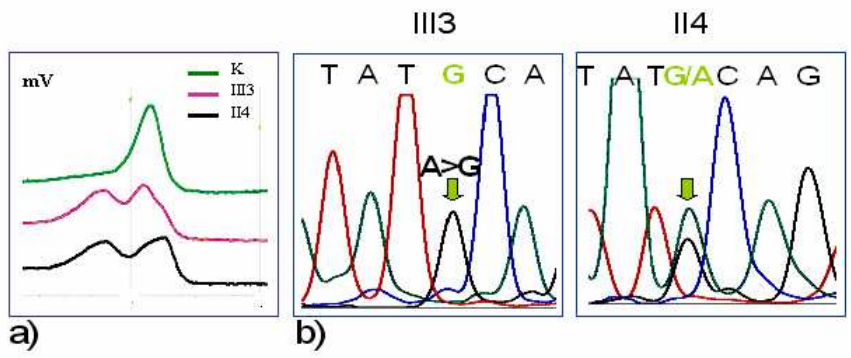


Figure 7: Molecular analysis of *Btk* gene in patient (III3) and his mother (II4)

Functional investigations

Btk protein was detected in total lymphoblastoid cells line (LCL) lysates by immuno-blotting analysis. As shown in *figure 8* the 77 kD Btk protein, detected in patient (III3) and his mother (II4), is similar to healthy control but different respect on XLA1 and XLA2.

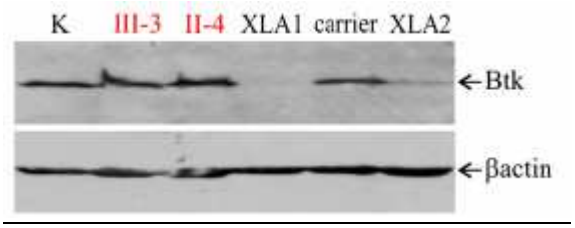


Figure 8: Btk expression by immunoblotting

The presence of a Btk phosphorylated active form was evaluated by FACS analysis LCL from the patient (III3) preserved the ability to phosphorylate Btk after hydrogen peroxide stimulation compared to LCL from a healthy control. Conversely, the Btk phosphorilated form was absent in LCL from XLA1 and XLA2 patients (*Figure 9*).

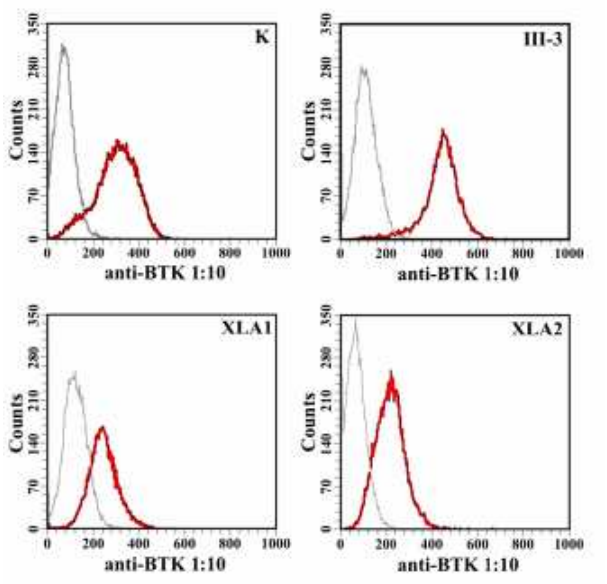


Figure 9: FACS analysis of Btk phosphorilation in LCL stimulated by H_2O_2

Discussion

We report the case of a PID patient with dysgammaglobulinemia, a gradual decrease of peripheral B lymphocytes and a clinical history of recurrent infections complicated with a severe sclerosing cholangites and chronic obstructive pancreatitis. Although, liver involvement in primary hypogammaglobulinemia has been reported mainly to consist of nodular regenerative hyperplasia with an autoimmune origin not yet elucidated (60), in our patient, the lack of pancreatic clinical response to steroid treatment, seems to support an obstructive pancreatitis rather than an autoimmune one (61). Moreover, this patient, who was initially classified as having CVID, has a mutation in Btk gene, which is the genetic hallmark of XLA.

Molecular investigations of *Btk* gene, revealed a new missense substitution in exon 11 (c.1078 A>G) causing threonine change to alanine in position 316 of SH2 protein domain. This mutation does not affect protein expression and kinase activity, even if impairment of phosphotyrosine-binding capacity cannot be excluded as previously reported for other Btk SH2 mutations (63-64). Clinical investigations reveal a normal level of IgG, normal titers to Tetanus and *Haemophilus Influenzae* vaccines, but low IgA, low IgM and poor response to Streptococcus pneumonie immunization. These immunological findings might be consistent with a less severe form of XLA, but this is in contrast with his poor clinical outcome. Recent studies showed that mutation in *Btk* gene is not always associated to a clinical disease (65).

Although XLA carriers are usually asymptomatic, caused preferential selection of the non-mutated X chromosome as the active allele in B cell precursors, female members with the same patient' mutation, suffer from a mild to severe pulmonary emphysema. Skewed pattern of X- chromosome inactivation were analyzed at HUMARA locus and PGK-1 gene, but they resulted not informative (data not shown). To conclude, patient's clinical and laboratory history is not totally inconsistent with an atypical variant of XLA. However more functional studies are

required to elucidate the effects of this *Btk* mutation and the mechanisms responsible for the clinical and immunological phenotype. Conversely, the occurrence that the T316A change in Btk SH2 domain is a novel non-pathogenic mutation, suggest us to considerate other gene potentially involved.

Study 2: **Clinical, haematological and molecular characterization of an atypical case of Agammaglobulinemia with B, NK, DC and monocyte deficiency.**

In this study we present clinical and haematological evaluations of an 8-years-old female child with combined immunodeficiency characterized by a complete absence of B cells, NK cells and their progenitors, surprisingly by the absence of monocyte lineage ($CD14^+$) and DC cells. Moreover, the common origin of these lineages from early haematopoietic stem cell progenitors (HSCs) suggested us a block in early stages of haematopoiesis. Mice mutated in *PU.1* and *Ikaros* transcription factors and cytokine receptor *Flt3R* and its ligand (*Flt3L*) generated a clinical phenotype similar to our patient (66-68). For this reason we analyzed these factors with molecular analysis. Besides, we investigated alteration in the bone morrow (BM) microenvironment and HSCs clonogenic/differentiation capacity by proliferation and differentiation cellular tests.

Background

The establishment of all haematopoietic lineages during development is mediated by transcription factors and other parameters such as cytokine receptor signalling, direct cell-cell interactions or other environmental signals that have an instructive role to build lineage-specific networks. Transcription factors can reprogram a committed cell into another lineage by perturbing its transcription factor network; thus simply changing the balance of two lineage haematopoietic transcription factors is possible to the reversible reprogramming of committed cells.

Trascription factors PU.1 and Ikaros

Nuclear factors *PU.1* and *Ikaros* are broadly expressed in the hematopoietic system, including HSCs, early lymphoid progenitors such as CLPs and various myeloid lineages. *PU.1*$^{-/-}$ mouse, present a disrupts B cells differentiation from uncommitted progenitors as well as defects in T cells and DCs differentiation, and some of the broad effects of PU.1 deletion can be attributed to a loss of CMPs and CLPs (69-71).

Ikaros with Helios and Aiolos constitutes a family of Kruppel-type zinc finger DNA-binding factors involved in haematopoietic cell fate decisions (72). The lack of Ikaros, in mice knock-out, does not prevent the development of LMPPs although changes their potential of differentiation (73).The hypomorfic *Ikaros* ^{null} mutant mice lacked all B cells, NK, fetal T cells and led to a strong reduction of DCs, whereas development of the early T-lineage progenitor (ETP) was normal (74).

Flt3-receptor and Flt3-ligand

Another key element of the haematopoietic system regulated by Ikaros and PU.1 is a member of the class III receptor tyrosine kinases Flt3 receptor (Flt3R) that is expressed primarily at very early stages of haematopoiesis. Ikaros ^{null} mice lack Flt3R that, with its cognate ligand (Flt3L), are involved in signalling pathways regulating the proliferation and differentiation of immature haematopoietic cells (75). *Flt3L*$^{-/-}$ mice have significantly reduced numbers of myeloid and lymphoid progenitors in the bone marrow (BM) and reduced numbers of DC and NK cells in the lymphoid organs, although *Flt3L*$^{-/-}$ have a normal thymic cellularity (76).

Case Report

A female infant was hospitalized in 'Atri Hospital' at the age of 3 months for bronchiolitis and at age of 16 months for pneumonia and diarrhea. A diagnosis of agammaglobulinemia was made and she was transferred to Children Hospital Bambino Gesù in Rome (IgG 15 mg/dl, IgM 17 mg/dl, IgA 23 mg/dl). Since then, immunoglobulins substitute therapy and antibiotic prophylaxis was started. In six years of follow up, she presented recurrent and persistent upper and lower respiratory infections, partially responsive to anti inflammatory and antimicrobial therapy. The pulmonary clinical picture has been worsening in the last two years bronchiectasis and pulmonary opacity previously not reported, were detected by Chest Computerized Tomography scan.

Different bacterial microrganisms (Haemophilus, Pseudomonas) as in chronic bronchopneumopaty were repeatedly isolated in sputum and bronchoalveolar lavage.(BAL). Cytological examination of BAL fluid showed a marked presence of increase of inflammatory elements (92% of neutrophils; 1% of lymphocytes; 8% of macrophages), rare ciliated cells and red blood cells, negative for virus, and mycobacteria. In 2008, patient had chickenpox without complications. However since then she has a worsening of general condition and a severe weight loss.

Blood cells count revealed leucocytosis, absence of monocytes and an occasionally thrombocytopenia. An increased of reticolocytes was repetitively documented in absence of anemia and signs of hemolitic anemia. Peripheral blood smear confirmed morphologically absence of monocytes and large NK cells. No platelets aggregates, but 2-4 giant platelets were found. Neutrophils (13%), lymphocytes (77%) and stimulated lymphocytes (10%) were detected. Since, duodenal mucosal histology showed mild degree of villous atrophy with intraepithelial lymphocytosis, she was treated with a gluten-free diet with a mild improvement. Currently the patient is treated with enteral nutrition therapy.

Immunological investigations

Immunological data showed an Agammaglobulinemia with absence of B cells ($CD19^+CD20^+$), NK ($CD16^+CD56^+$) and monocyte cells ($CD14^+$). Blood cell count (A) and cellular immunity (B) results are reported at different medical time points (*Table VI*). Moreover, dendritic cell subsets (myeloid type 1-mDC1, plasma-cytoid-pDC) in peripheral blood were absent.

	2005	***2006***	***2007***	***2008***	***2009***
GB (mcL)	11460	7030	12480	4510	7470
NEUT (mcL/%)	7002 (61,1%)	3530 (50,2%)	7520 (60,2%)	2800 (62.1%)	4310 (57.7%)
LINF (mcL/%)	3976 (34,7%)	2850 (40%)	3690 (29,5%)	1290 (28.5%)	1770 (23.7%)
MON(mcL/%)	11 (0,1%)	160 (2,3%)	280 (2,2%)	90 (2%)	130 (1.7%)
EOS (mcL/%)	412 (3,6%)	380 (5,4%)	790 (6,3%)	260(5.8%)	1080(14.4)
GR (/mcL)	4310000	4410000	4560000	3890000	4220000
Hb (g/dl)	12,8	13,6	13,3	12.1	13.3
Hct (%)	37,3	38,5	37,1	34.9	37.5
MCV/MCH fL/pg	85,1/29,7	87,3 / 30,8	81,4/29,1	89.8 / 31.1	88.8 / 31.5
RET (mcL/%)	668900 (15,5%)	644200 (14,6%)	--	--	513000 (12.15%)
PLT (mcL)	147000	113000	273000	132000	239000

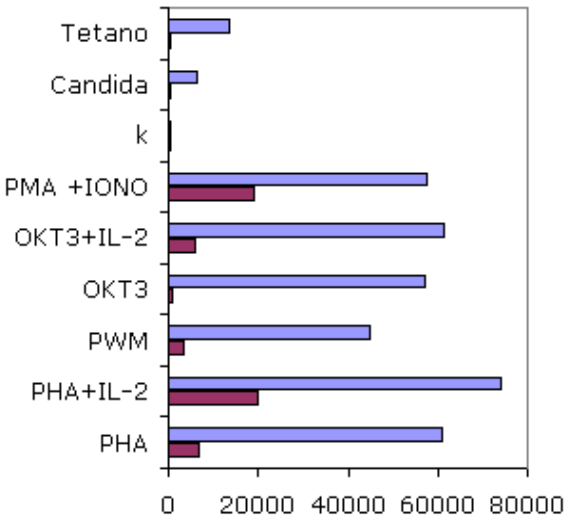
Table VI A: Blood cell count in Peripheral Blood

	2005	*2006*	*2007*	*2008*	*2009*
LINF	5020	3270	3690	1290	1770
CD3	4769 (95%)	3237 (99%)	99%	1264 (98%)	1761 (99.5%)
CD4	1706 (34%)	981 (30%)	72%	374 (29%)	508 (28.7%)
CD8	2861 (57%)	2289 (70%)	25%	890 (69%)	1255 (70.9%)
CD16/56	50 (1%)	49 (1,5%)	0	n.v.	35 (0.2%)
CD19	50 (1%)	n.v.	0	n.v.	0 (0.0%)
CD4-CD45Ro	5%	38 (3,9%)	4	129 (10%)	
CD4-CD45RA	24%	232 (23,7%)	21	232 (18%)	
CD8-CD45Ro	32%	60 (26,7%)	33	361 (28%)	
CD8CD45RA	35%	92 (40%)	37	516 (40%)	
CD33CD14		n.v.			n.v.
IgG (mg/dl)	622	509	699	606	507
IgA (mg/dl)	< 3	7	16	<5	<5
IgM (mg/dl)	4	3	< 3	<10	<10

TableVI B: Cellular Immunity and immunoglobulin ratio analysis in Peripheral Blood

The lymphocyte proliferation assay measured after mitogen and antigen stimulation by ^{3}H –thymidine incorporation, revealing a markedly decreased response to polyclonal mitogens (Phyto-Hemgglutinin-PHA, PokeWeed Mitogen -PWM), monoclonal antibody (OKT3) and to recall-antigens (Candida and Tetanus). PHA and OKT3 responses were partially restored by addition of IL-2. *Figure10* shows a representative result of ten different lymphocyte proliferation experiments.

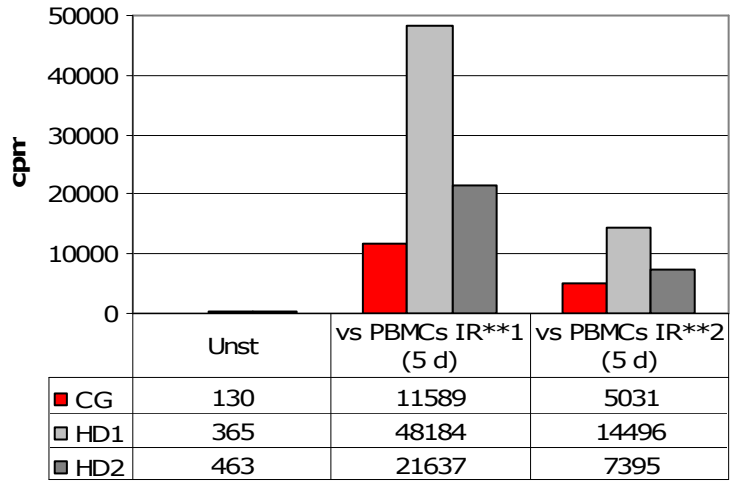
Moreover, the expression of T cell activation markers, (CD69 and CD40L) evaluated after *in vitro* stimulation with PMA/ionomycin by FACS, resulted normal (data not shown).



	CG	HD
k	483	892
PHA	6909	60993
PHA+IL-2	19906	73946
PWM	3381	44911
OKT3	722	57234
OKT3+IL-2	5740	61416
PMA +IONO	19130	57776
k	432	270
Candida	460	6439
Tetano	365	13626

Figure 10: Lymphocyte proliferation assay.

The in vitro mixed lymphocyte reaction (MLR), that study alloresponsiveness to histocompatibility antigens, was normal in our patient (*Figure 11*). Analysis of T cell receptor (TCR) βV distribution on CD4+ and CD8+ cells revealed a polyclonal profile (data not shown).



	SI vs 1	SI vs 2
CG	3,71	38,70
HD1	132,00	39,70
HD2	46,70	15,90

Figure 11: Mixed lymphocyte reaction. cpm -count per minut

Virological and Microbiological investigations

BAL immunophenotype showed the presence of macrophage/monocytes: CD3+ (96%), CD4+ (53%), CD8+ (39%), CD16 (2%), CD45+ [1, 9 % (80% CD33+CD14+CD45+)]. Interestingly, were revealed $CD68^{+}$ and CD163 $^{+/-}$ macrophages.

All virological and microbiological investigation performed, resulted negative, as show in table below.

PCR peripheral blood	EBV and CMV	negative
PCR rhinopharyngeal aspirate	Chlamydia pn and Mycoplasma pn	negative
Pharyngeal aspirate cultural examination		negative
Bronchial aspirate	Pneumocystis Carinii	negative
Gastric aspirate colture	Mycobacterium	negative
PCR Bronchial washing	Adenovirus, CMV,EBV, Chlamydia Pneumonie, Mycoplasma	negative
Bone Marrow Biopsy	Mycobacterium	negative

Molecular investigations

Genomic DNA of our patient was analysed for mutations in *μ-chain*, *λ-5* and *Igκλ* genes, which are mutated in recessive Agammaglobulinaemia. Direct sequence of them did not detect the presence of mutations.

Bone morrow microenvironment and HSCs investigations

The haematopoietic microenvironment or stroma plays a decisive role in proliferation and differentiation of haematopoietic cells. We studied if bone marrow cells from patient are altered in their ability to form adherent stromal layer and if $CD34^{+}$ HSCs preserved their clonal differentiation capacity and self-renewal potential.

Semisolid Culture

Haematopoietic cells cultured in semisolid media showed a quantity deficit of granulopoietic and multipotent progenitors evaluated in Colony-Forming Unit-Granulocyte-Macrophage (CFU-GM) and in Colony-Forming Unit- Granulocyte Erythroid-Macrophage-Megakariocyte (CFU-GEMM). Erythropoietic progenitor cells (Colony Forming Unit- Erythroid, CFU-E and Burst Forming Unit- Erythroid, BFU-E), were normally represented in quantity, but revealed a low proliferation capacity restored only after cytokine stimulation (Table below)

	Value obtained	Normal range
Granulopoietic colture		
Clusters	95.5	1002.6± 447.4
Colony	0.5	105.2 ± 75.1
Erythropoietic colture		
CFU-E	8.5	151.2 ± 79.5
BFU-E	9.5	88.5 ± 48.9
Methocult Culture		
CFU-E	47	155.3 ± 105.0
BFU-E	103.5	159.4 ± 73.2
CFU-GEMM	6	38.0 ± 23.0

Stromal Culture

The stromal culture analysis performed in 2009, revealed a good capacity of patient's bone marrow cells to form adherent stromal layer.

Bone Marrow Analysis

Bone Marrow biopsy and aspirate revealed rich cellularity with well represented erythroblastic and granuloblasti series. Moreover, no evidence of atypical immature populations was found by cytomorphology analysis. B cells ($CD19^+$), NK ($CD16^+$) and Monocyte ($CD33^+CD14^+$) progenitors resulted absent (data not shown).

We determined the ratio and the type of individual cell population in patient's bone marrow and in a healthy control, by FACS, using specific mAbs that recognize proteins on the cell surface. FACS analysis results showed a general low expression of surface marker in our patient respect on healthy control donor. In particular analysis revealed an absence of $CD34^+TdT^+$ population in patient respect on control and an inverted ratio $CD34^-CXCR4^+$ with $CD34^-CXCR4^+$ in patient's bone marrow respect on healthy control donor (*Figure 12*)

To exclude Myelodysplastic Syndromes cells arrested in metaphase are examined microscopically. The analysis reveals in CG a normal karyotype without chromosome aberrations including monosomy 7 and trisomy 8 (data not shown).

In addition, assuming a case of Dyskeratosis Congenita (DKC) we performed radiosensibility analysed the telomerase function. No anomaly was detected.

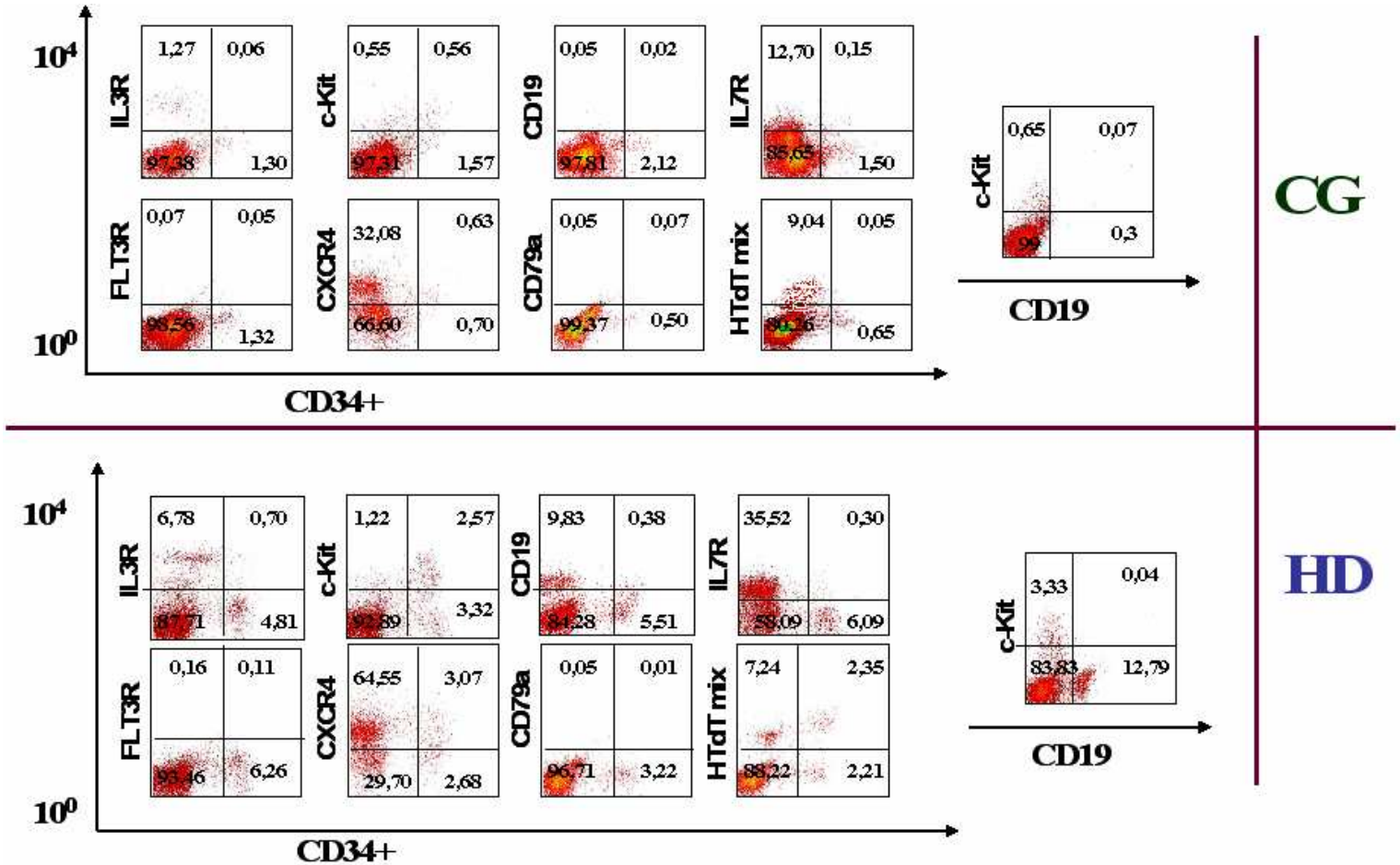


Figure 12: Bone Marrow FACS analysis in patient (CG) and healthy control donor (HD)

Results

CD34$^+$ proliferation test

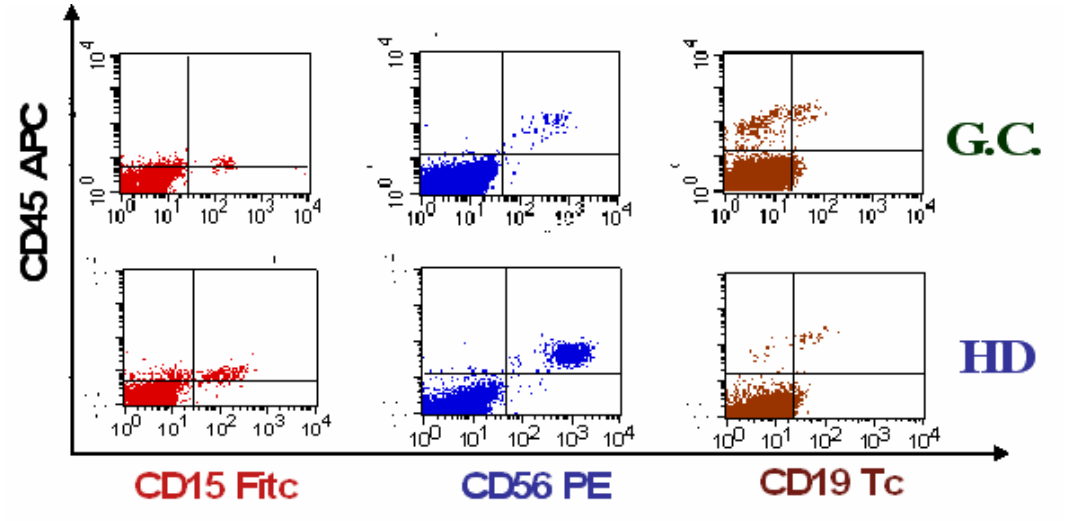
CD34$^+$ cells were plated with a single stimulus (Tpo, SCF, Flt3L, IL3, IL6, GM-CSF) or in presence of a mix of stimuli (Tpo, Flt3L, SCF, IL3). The results revealed that CD34$^+$ cells of patient are able to proliferate after stimuli, although at lower frequency than HD. However, when CD34$^+$ cells of CG were stimulated with Flt3L alone they are unable to proliferate (*Table VII*).

CG	mean	SD	SI
Cells without stimulus	34	19,8	1,0
TPO	79	46,7	2,3
Flt3L	34	14,1	**1,0**
SCF	58	2,8	1,7
GM-CSF	199	18,4	5,9
IL3	819	298,4	24,1
IL6	39	9,9	1,1
Mix of stimuli	2137	1656,0	62,9

HD	mean	SD	SI
Cells without stimulus	2433,5	119,5	1,0
TPO	4313,5	1393,7	1,8
Flt3L	21879,5	58,7	**9,0**
SCF	8353	840,0	3,4
GM-CSF	26222	1072,0	10,8
IL3	44201,5	3382,1	18,2
IL6	2131	1036,6	0,9
Mix of stimuli	181836,5	10540,8	74,7

In vitro B-and NK-cell differentiation

Potential differentiation of $CD34^+$ cells from patient and healthy donor, plated on murine stromal layer in presence of a combination of cytokines and growth factors, were analyzed for the presence of NK cells, B-cell and granulocytes. As show in *Figure 13* the cells of our patients are able to differentiate in NK cells ($CD56^+$) and B cells ($CD19^+$), although at lower frequency than the healthy donor.



positive wells/wells totali (%)

	GC	HD
CD15+	8.2%	9.2%
CD56+	3.8%	3.8%
CD19+	2.2%	7.1%

Figure13: B-and NK-cell differentiation *in vitro*: $CD34^+$ cells were plated on murine stromal cell line (MS-5) with human serum, IL2, IL7, IL15, TPO, SCF, and Flt3L.

Molecular investigations

Direct sequencing genomic DNA and cDNA of Ikaros and Flt3 ligand resulted wild type, while PU.1 and Flt3 receptor analysis is still under investigation because we were unable to amplify the fifth and first exon of *PU.1* and *Flt3R* genes.

Functional investigations

We studied the expression of Ikaros, Flt3R and Flt3L mRNA by Real-time PCR using RNA extracted from $CD34^+$ and $CD34^-$ cells in bone marrow (BM) and PBMCs of peripheral blood (PB) (*Figure 14*).

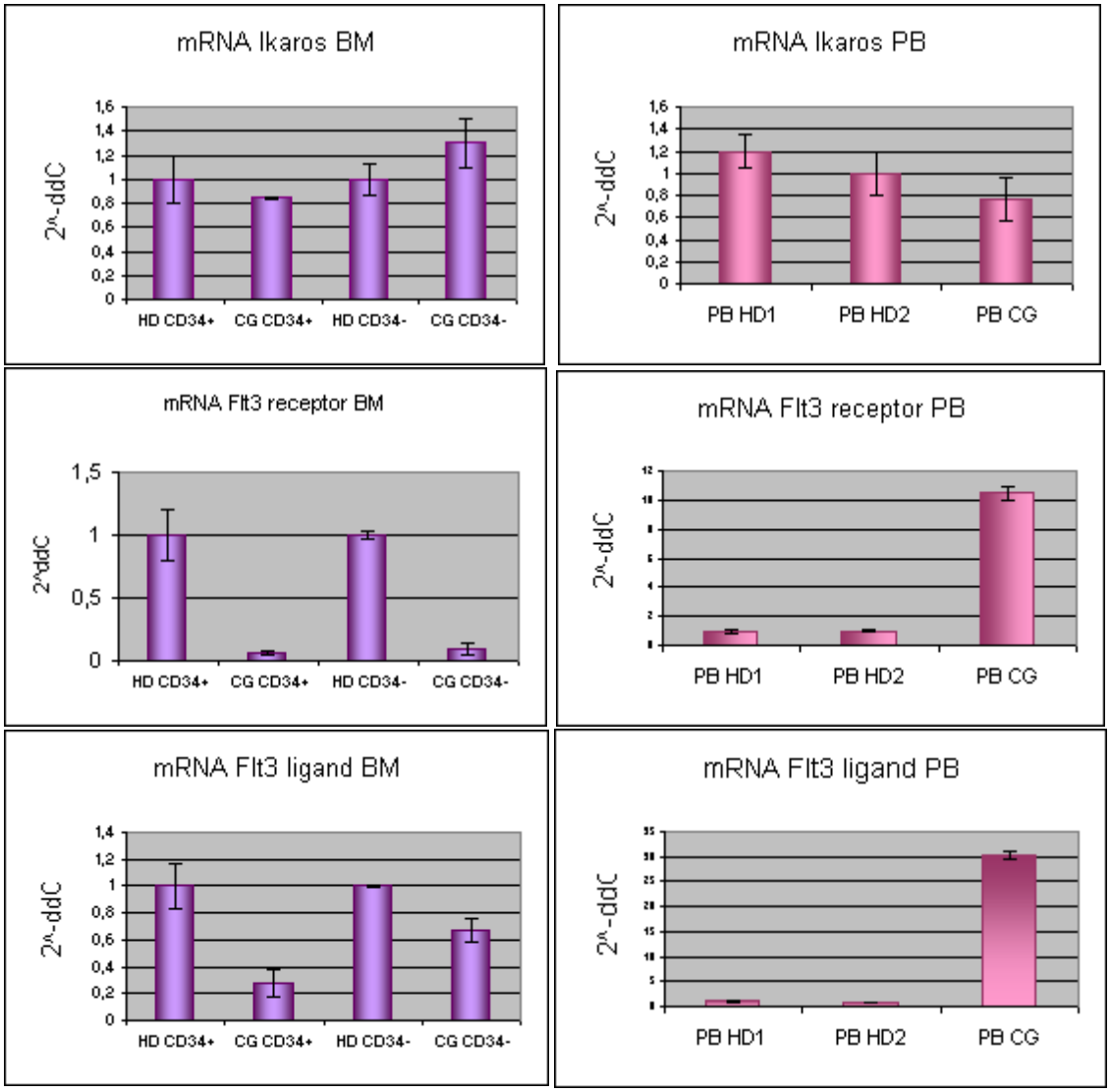


Figure 14: Absolute Quantitative Real-Time PCR. Summary of 3 independent experiments are represented.

The expression of Ikaros mRNA in patient's $CD34^+$ and $CD34^-$ cells is similar to the healthy controls; conversely we get a lower expression of Flt3L mRNA and a nearly total absence of Flt3R. In addition, peripheral blood analysis shows a normal expression of Ikaros, but 10- and 30- fold increase of Flt3R and Flt3L, (respectivetly) expression. A high expression of Flt3R was confirmed also by FACS analysis on peripheral T lymphocyte ($CD3^+CD4^+$ and $CD3^+CD8^+$) and granulocyte cells (CD11c and CD15) (*Figure 15*).

A new Real Time PCR study of Flt3L mRNA was performed including patient's parent. As shown in *Figure 16* the expression of mRNA of CG is ~ 30 fold increase compared to healthy control donor, while patients' parents mRNA is 10-14 fold increase.

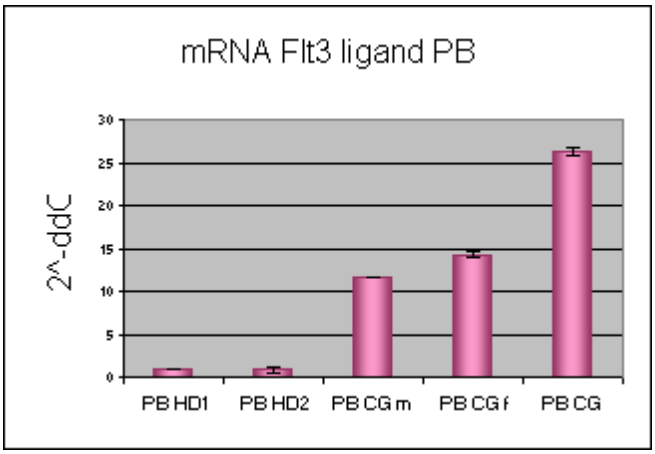


Figure16: Absolute Quantitative Real-Time PCR. Summary of 3 independent experiments are represented.

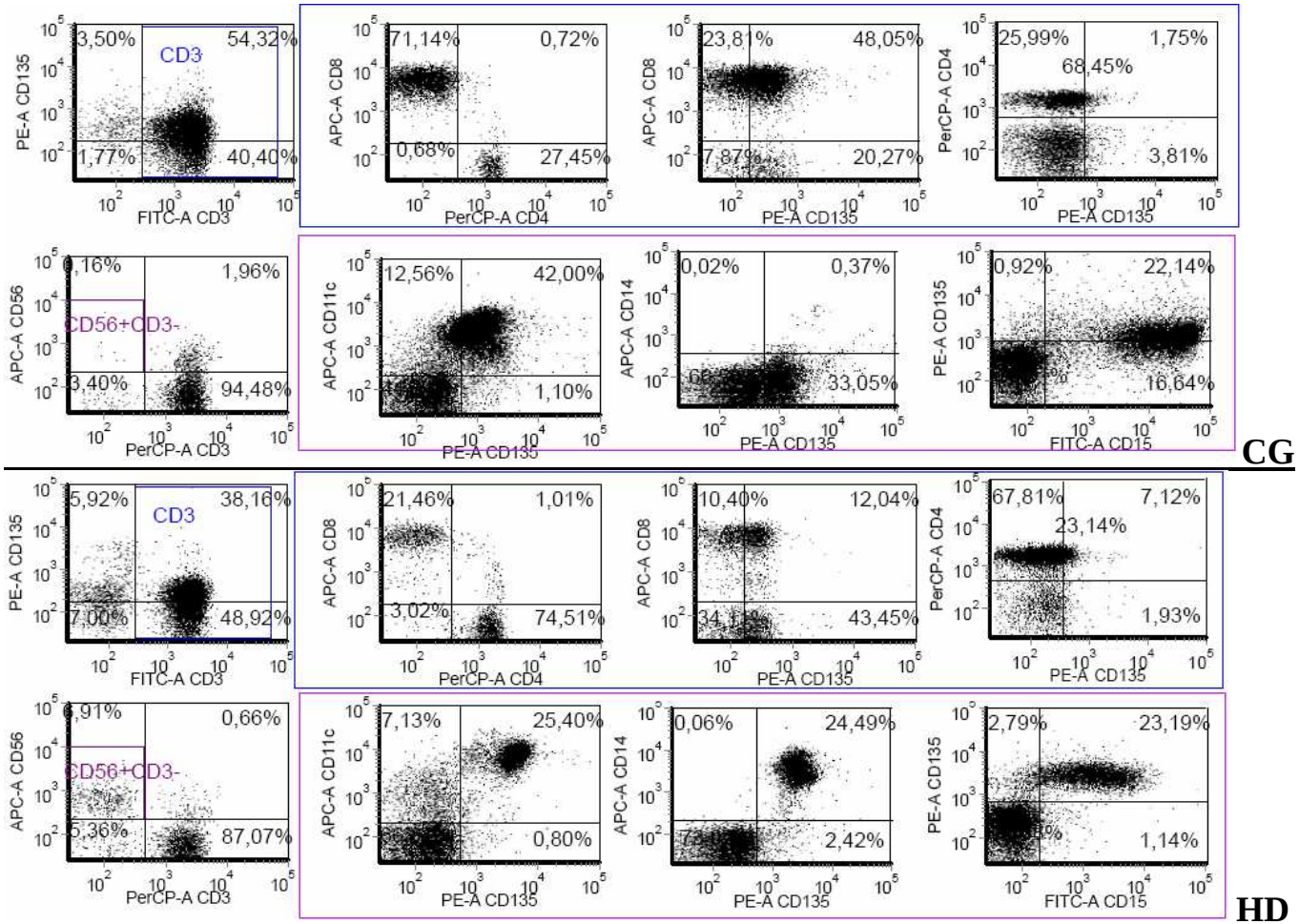


Figure 15: FACS analysis on patient's (CG) and healthy control donor's (HD) peripheral blood

Discussion

We report for the first time a case of a female patient with combined immunodeficiency characterised by a complete absence of B cells and NK cells, and their progenitors. Surprisingly, she had no monocytes ($CD14^+$) and DC cells. Altered function and decreased numbers of monocyte lineage have been well described since 1996, in congenital defects of phagocytes or associated to combined immunodeficiency (77-79). However, the complete absence of $CD14^+$ monocyte cells in association with agammaglobulinemia, has never been reported thus far. Recessive Agammaglobulinemia and myeolodisplasia were excluded, as well, Dyskeratosis Congenita was ruled out by radiosensibility and telomerase function.

Our patient presented a deficit in many terminal cellular lineages of immune/ haematological system, suggesting a block in the early stages of haematopoietic commitment. In patient's bone marrow, only 0, 2 % of $CD34^+$ cells were detected. These cells showed a common low expression of cell surface markers IL3R, cKit, IL7R, TdT, Flt3R and CD79a suggesting decrease of several cell'precursors. Levels of granulopoietic, erythropoietic and multipotent progenitors resulted significantly lower compared to healthy control. This block could be caused either by alteration in environmental signals or, by defects in molecules regulating the specific lineage commitment. Stromal cell culture analysis revealed a normal microenviroment partially excluding, our first hypothesis.

Patient's $CD34^+$ cells stimulated only with Flt3L are unable to proliferate whereas react in presence of several different stimuli (mix of stimuli or single stimulus).

These evidences strongly support an alteration in Flt3R/Flt3L pathway.

Up to date, direct sequencing of genomic and cDNA of Flt3L, Flt3R, and of major genes (Ikaros and of PU.1) regulating the FLT3-FLT3L did not detect the presence of any mutations. Flt3R exon and one PU.1 exon are under investigation (80). Noteworthy, patient's $CD34^+$ and $CD34^-$ cells present a lower expression of Flt3L-mRNA associated with a nearly total absence of Flt3R-mRNA.

Although these data can be partially influenced by the decrease number of $CD34^+$ cellular population observed in this child, levels of Flt3R and Flt3L mRNA in peripheral blood resulted significantly higher (10- and 30- fold increase respectively). This data can be partially explained by the pathological expansion of CD8+ T-cell subsets expressing higher levels of Flt3R mRNA (see Fig.15). However, dosage of Flt3L mRNA in patient' parents resulted 10-14 fold increase compared with healthy donors even in presence of a normal cell immunophenotype, strongly supporting the hypothesis of an inherited imbalance in the expression of Flt3 ligand mRNA**.**

To elucidate the mechanism regulating FLT3/FLT3L pathway, more functional studies are required. We are currently investigating the Flt3 receptor and Flt3 ligand expression in purified $CD8^+$ and $CD4^+$ cells population (Real Time PCR and Western Blotting).

In deeper study of the Flt3R/Flt3L pathway may suggest further analysis of other genes potentially compromised in this patient.

Remarkable, the analysis Flt3R/Flt3L pathway might fully unravel its crucial role in the development of common haematopoietic progenitors. These findings, in addition to a normal stromal function (experiments *ex vivo),* highlights the possibility of a more specific therapeutic strategy in this child, such as haematopoietic stem cells transplantation.

Study 3: **Chronic Granulomatous Disease (CGD): Gene Therapy with Stem Cells**

Different strategies have been adopted to functionally correct the defective gene of interest. Replication-incompetent retroviral vectors are, presently, the best delivery tools of a therapeutic transgene to haematopoietic stem cells. Retrovirus vectors enter into the target cell, reverse transcribe and integrate the therapeutic gene at non-specific sites in the host genome from where it is expressed. To selectively target HSC with the viral vector and avoid transduction of other tissues, standard gene therapy uses HSC isolated either from cytokine mobilised aphaeresis products, cord blood or bone marrow samples. The cells are then transduced *ex vivo*.

HSCs are heterogeneous with respect to their susceptibility to retrovirus transduction. The transduction efficiency mainly depends on the type of retrovirus (γ-, lenti-, and foamy-virus), the virus vector envelope (pseudotyping) and the cell cycle status of the HSC (80).Unlike γ-retroviruses, which are dependent on cell division for integration, lentiviruses and spuma-(foamy) viruses are able to transverse the intact nuclear membrane of a non-dividing HSC but are still more efficient at entering the nucleus during mitosis (81).

Stringent regulation of transgene expression is required to increase the safety and efficacy of gene therapy. To control vector expression, transcriptional targeting by tissue specific promoters has been used. This kind of vectors can be limited by insufficient level of expression or the lack of fine specificity. Recently, a natural system to regulate gene expression was discovered acting at the post-transcriptional level, by small molecules of RNA, called microRNA (miRNA). The use of microRNA targets expressed in specific stages of haematopoietic differentiation could provide an additional level of regulation of expression of the therapeutic gene by allowing its expression in differentiated cells but not in the *stem* cell

compartment. (82). The aim of this study is to develop a new strategy for XCGD gene therapy with stem cells, including Lentiviral Vector (LVV) design and microRNA technology for a safe and effective treatment of X-CGD.

Background

XCGD gene therapy with γ-retroviral vectors

The first gene therapy approaches with retroviral vectors have been performed in the US. They were unsuccessful because of the low engraftment of gene corrected cells in the absence of conditioning. (83). The report on successful gene marking up to 10% of myeloid cells gene therapy for severe combined immunodeficiency due to adenosine deaminase deficiency (84) prompted the use of a similar conditioning regimen (busulfan i.v.) for the treatment of X-CGD patients.

Two adult CGD patients, treated with a submyeloablative dose of busulfan, received an infusion of autologous $CD34^+$ cells transduced with a murine retroviral vector encoding gp91*phox*. Both patients showed efficient engraftment and expression in the myeloid series, with functional correction and clinical benefits. However, molecular analyses revealed a clonal dominance of vector insertions in a region close to the protoncogene *MDS1/Evi1*, which then evolved into myelo-dysplastic syndrome in both patients. (85). The risk of insertional mutagenesis and transactivation of proto-oncogenes from retrovirus-mediated gene therapy with unknown long-term consequences revealed in this recent trial clearly points to the necessity of developing a next generation vectors with better safety. In addition, indirect evidence suggest that the ectopic gp91*phox* in HSC could lead to the production of reactive oxygen species that may cause DNA damage or alterations in cell growth, or inducing apoptosis (86).

XCGD gene therapy with lentivirus vectors (LVV)

Self-inactivating HIV-based lentiviral vectors are the best candidates since they can transduce with high efficiency primitive HSC (87) and carry a safer profile of integration with respect to retroviral vectors (88).
Self-inactivating (SIN) vectors lacking the potent retroviral enhancer elements within the long terminal repeats (LTR) show much less transactivation potential than conventional LTR-driven vectors (89). Transgene expression in SIN vectors is driven by an internal cellular promoter, further reducing the probability of oncogene activation at the stem cell level. These vectors have been already tested successfully in vitro and in the NOD/SCID preclinical model for CGD (90-91) using a constitutive viral promoter to drive expression of gp91*phox*.

MicroRNA

MicroRNAs (miRNA) are single stranded RNA molecules of 21-23 nucleotides in leght, which regulate gene expression. miRNAs are encoded by genes, but they are not translated into protein (non-coding RNA). Each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional miRNA.(*Figure 17*).

Stable knockdown of microRNA in vivo by lentiviral vectors

Recently, miRNA inhibition had been reported in cells over-expressing miRNA target (miRT) sequences complementary to an miRNA seed region, suggesting that miRT sequences can act as miRNA ‘decoys’(94). In fact, miRT sequences can be incorporated into lentiviral vectors to regulate transgene expression in a cell type- and differentiation state-dependent manner. Moreover, no evidence for saturation of miRNA activity in cell trasnduced with such miRNA-regulated lentiviral vectors was found (95), and the over expressing miRT specifically affects the targeted miRNA rather than saturating the effector pathway.

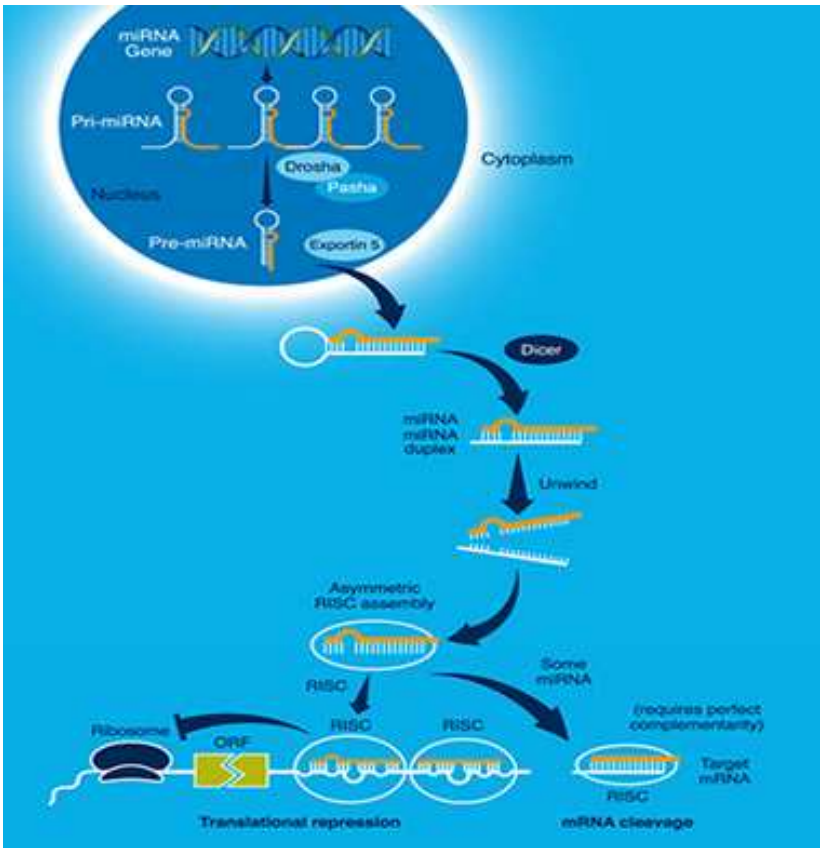


Figure 17: MicroRNA biogenesis and mechanism of action. When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and inhibit translation or sometimes induce mRNA degradation by argonaute proteins, the catalytically active members of the RISC complex. (92-93).

Preliminary Results

Human Cell lines

In our study we used myelomonocytic cell line (from M.C.Dinauer & M.Grez) PLB985 (wt) and XCGD PLB985 (mutated in *Cybb* gene). In preliminary experiments we confirmed that only PLB985 wt, but not PLB mut, had gp91*phox* protein expressed on cell surface and a functional NADPH oxidase (data not shown).

Production of Lentiviral Vectors

In collaboration with the group of Prof. Naldini (TIGET-San Raffaele di Milano), we selected mir126 and mir130 which are expressed in HSPC and down-regulated during granulocyte differentiation (data not published). We subcloned gp91*phox* sequence (from M.Grez) into *NcoI* - *SalI* sites of Blue Script 'BGI-10.mCherry' and then we transferred them in backbones to obtain four different transfer vectors (*Figure 18*). VSV-pseudotyped third-generation LV were efficiently produced following transient transfection of 293T cells.

Initially the titer of vectors was evaluated by FACS on the first PLB transduced cells (data not shown), and after recalculated by quantitative Real Time PCR on 293T. The titer and infectivity value of concentrated vector supernatant are reported in *table VIII*.

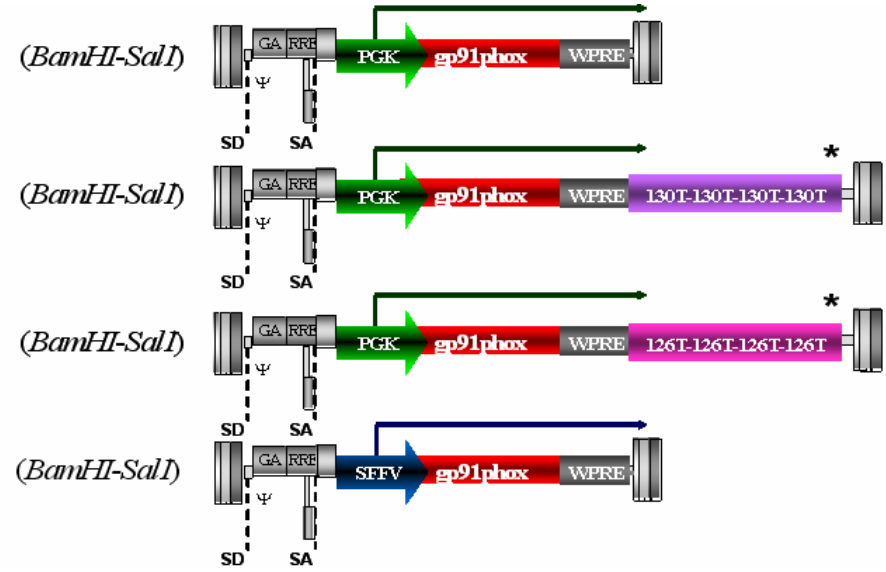


Figure 18: Transfer vectors. Boxes at both end shown **LTRs**; **SD** and **SA**, splice donor and acceptor site;**ψ** encapsidation signal including the 5'portion of the gag gene **(GA)**; **RRE**, Rev-response element; **PGK** and **SFFV**, promoters; **WPRE**, woodchuck hepatitis virus post-transcriptional regulatory element; miRNA target (miRT) sequence.

	TITER after 15 days (TU/ml)	**INFECTIVITY (TU/ug)**
LV.PGK.gp91	3.5*10^8	3.5*10^8
LV.PGK.gp91_126T	7*10^8	7*10^8
LV.PGK.gp91_130T	2.5*10^8	2.5*10^8
LV.SFVV.gp91	5.4*10^8	5.4*10^8

Table VIII: Titer and infectivity of vectors. Infectious particle were determined on 293T cells by limiting dilution. Vector particles were measured by HIV-1 gag p24 antigen immunocapture assay.

Transduction of human PLB985 X-CGD cell line

PLBmut were trasduced with serially diluted LVVs (from 10^-2 to 10^-6) containing gp91*phox* cDNA at different multiplicity of infection (MOI) *Table IX* below.

	Multiplicity of Infection (MOI)*				
LV.PGK.gp91	18	1.8	0.18	0.018	0.0018
LV.PGK.gp91_126T	35	3.5	0.35	0.035	0.0035
LV.PGK.gp91_130T	120	12	1.2	0.12	0.012
LV.SFVV.gp91	27	2.7	0.27	0.027	0.0027

* Parameter used for the prediction of gene transfer events

X-CGD PLB985 cells transduced with the LVVs expressing the gp91*phox* cDNA, showed increased cell surface expression of the protein as indicated by FACS analysis *Figure 19.* Activity of NADPH oxidase was evaluated, in transduced cells, by DHR test after dimethylsulfoxide (DMSO)-induced granulocytic differentiation (*Figure 20*). The results revealed a restored functional activity of the NADPH oxidase.

Vector Copy number (VCN) was estimated by Taqman PCR on transduced cells. *Table* below shows VCN of selected transduced cells.

	VCN
PLB.PGK.gp91 (0,18 MOI)	0,3
PLB.PGK.gp91 (1,8 MOI)	4,8
PLB.PGK.gp91_126T (0,35 MOI)	0,36
PLB.PGK.gp91_126T (3,5 MOI)	4,8
PLB.PGK.gp91_130T (1,2 MOI)	0,38
PLB.PGK.gp91_130T (12 MOI)	2,24
PLB.SFVV.gp91 (0,27 MOI)	0,8
PLB.SFVV.gp91 (2,7 MOI)	7,15

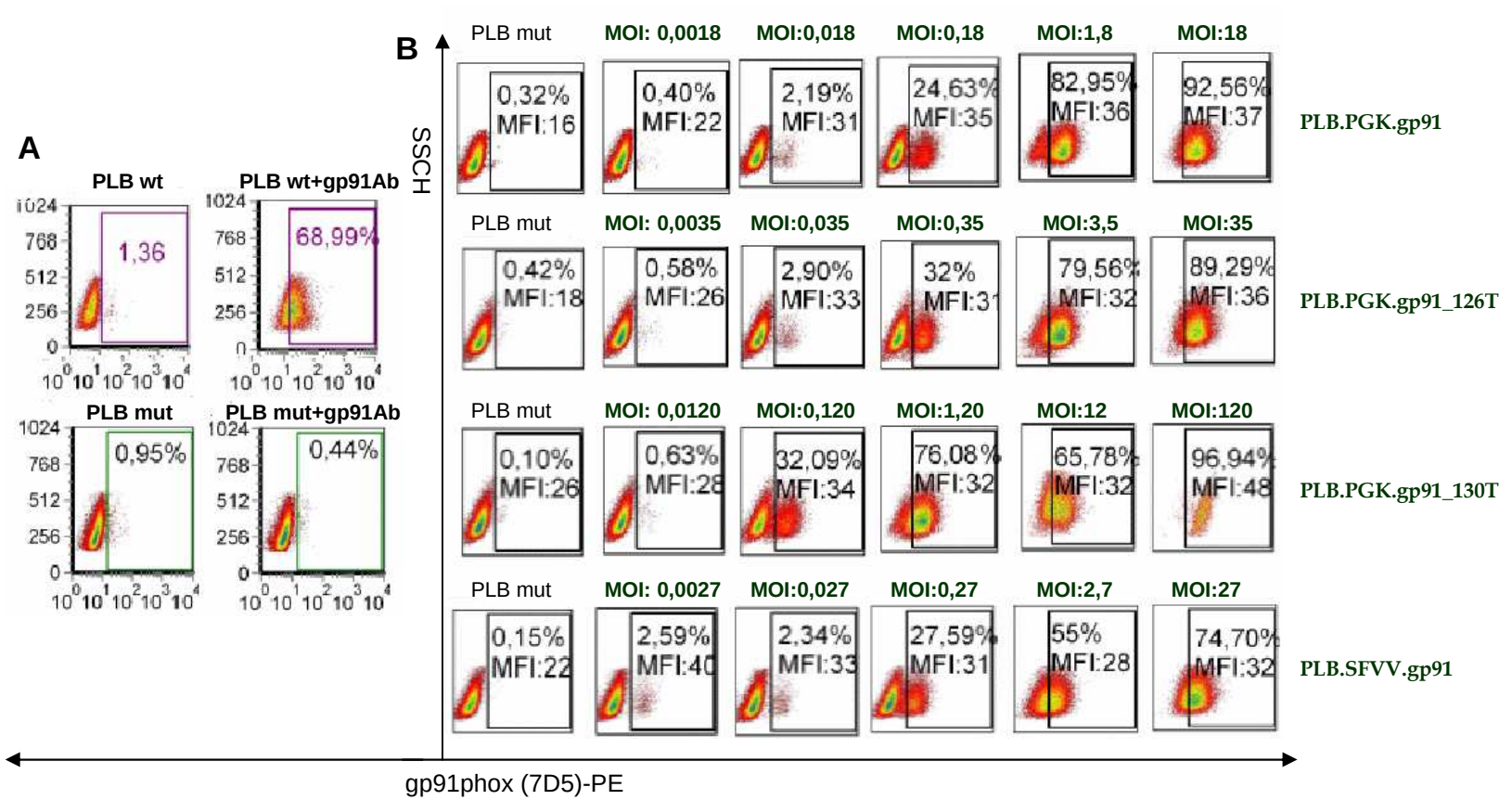


Figure 19: FACS analysis of cell surface expression of human gp91phox (7D5) in PLB985 wt, PLB985 mut (X-CGD) **(A)** and PLB985 mut (X-CGD) transduced with serially diluted LVVs **(B).** The analysis was made after 6 days from transduction.

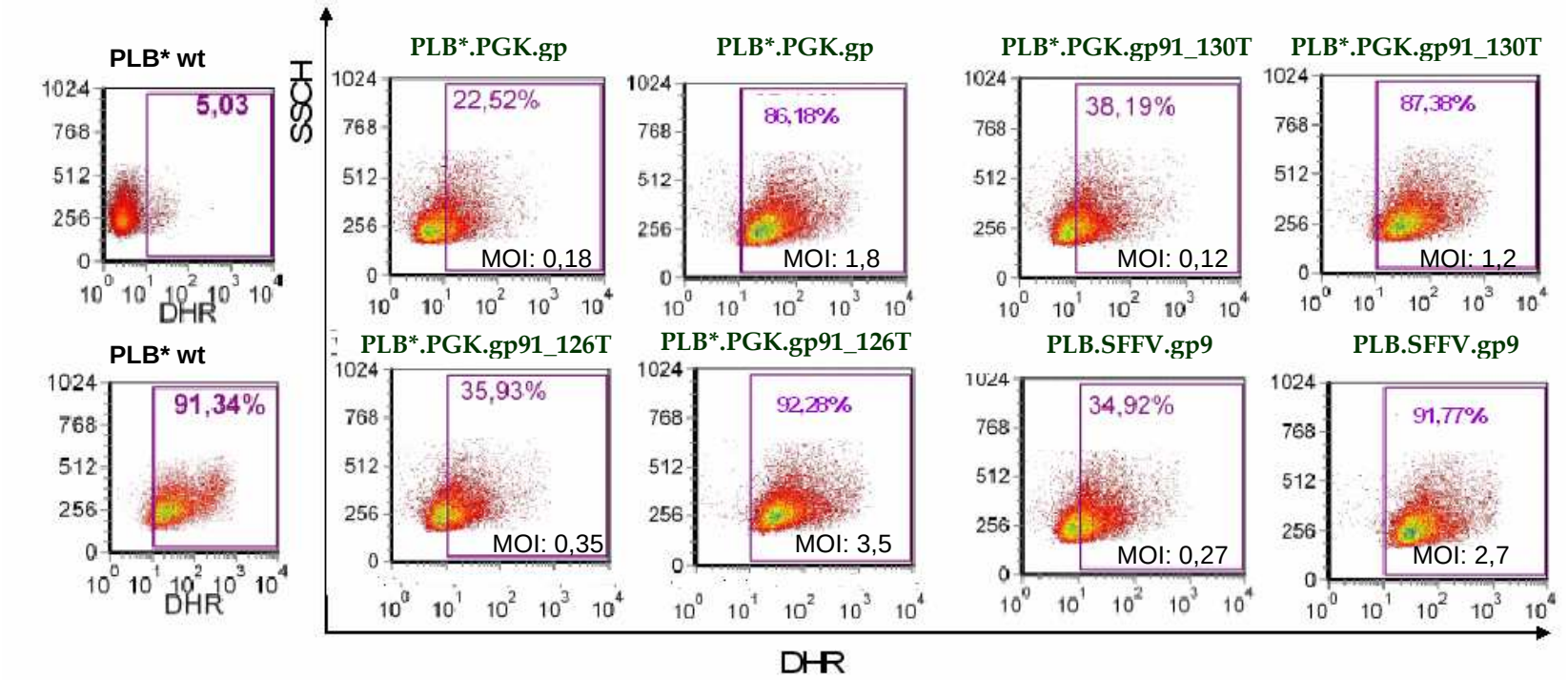


Figure 20: Functional NADPH oxidase activity by DHR test. We show representative dot-plots of PLB985 mut (X-CGD) trasduced with LVVs after dimethylsulfoxide (DMSO)-induced granulocytic differentiation. PLB differentiated (*)

To determine whether there is a specificity of PLB.PGK.gp91_126T knockdown by LV.mir-126 we superinfected transduced cells with a LVV co-expressing mir-126 and the ‘orange fluorescent protein’ at MOI 0.45, 4.5 and 45. As show in *figure 21* the expression of miR-126 target sequence (PLB.PGK.gp91_126T) results in the block of gp91*phox* expression in cells expressing miR-126. Conversely, in cells that do not express miR-126 the gp91phox expression is maintained. As a consequence, of the block of gp91*phox* protein expression there is a failure of NADPH oxidase activity (*Figure 22*).

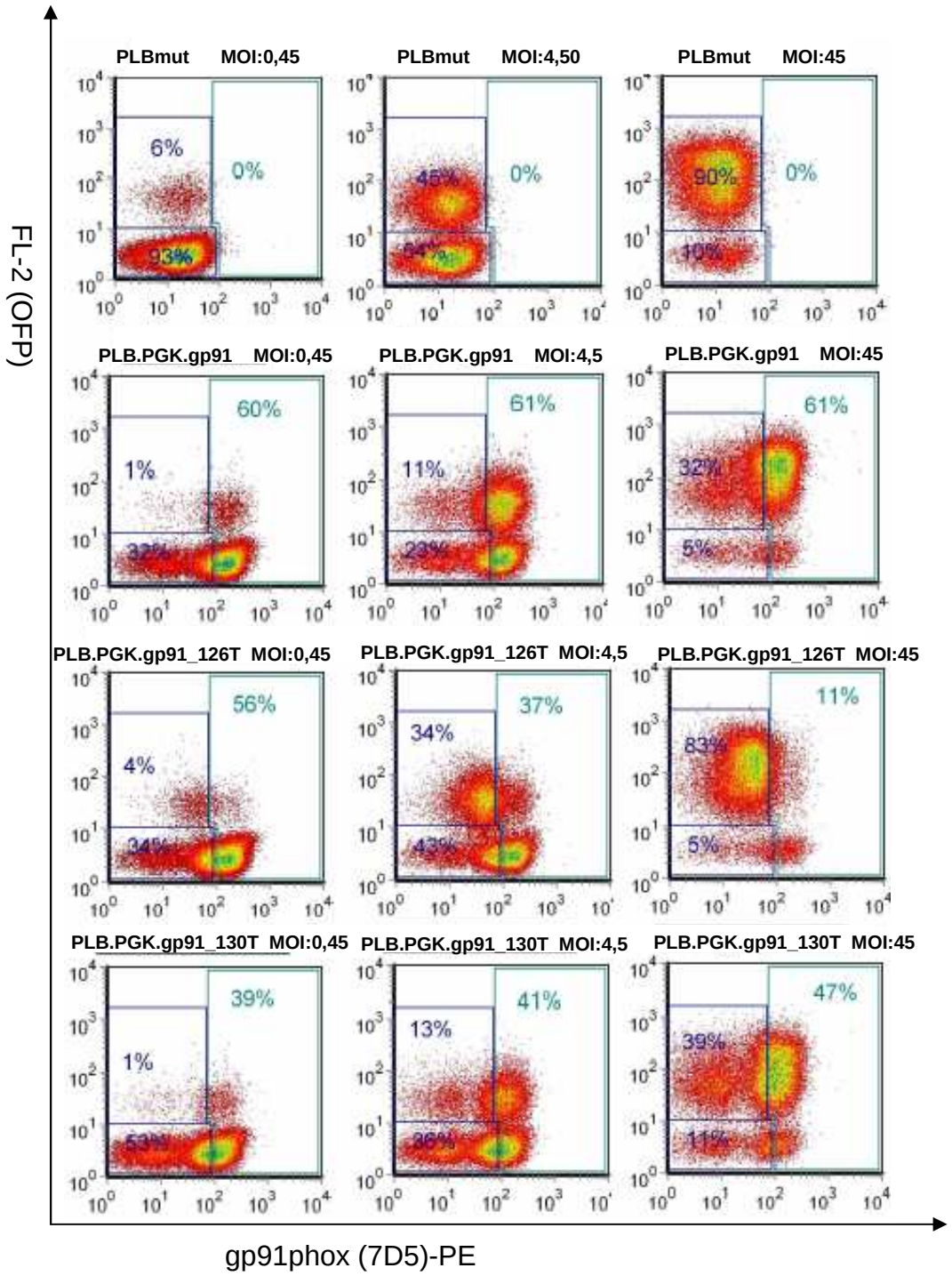


Figure 21: FACS analysis of cell surface expression of human gp91phox (7D5) in PLB985 transduced with LVVs (containing gp91*phox*) and superinfected with LVV coexpressing mir-126 and orange fluorescent protein (OFP).

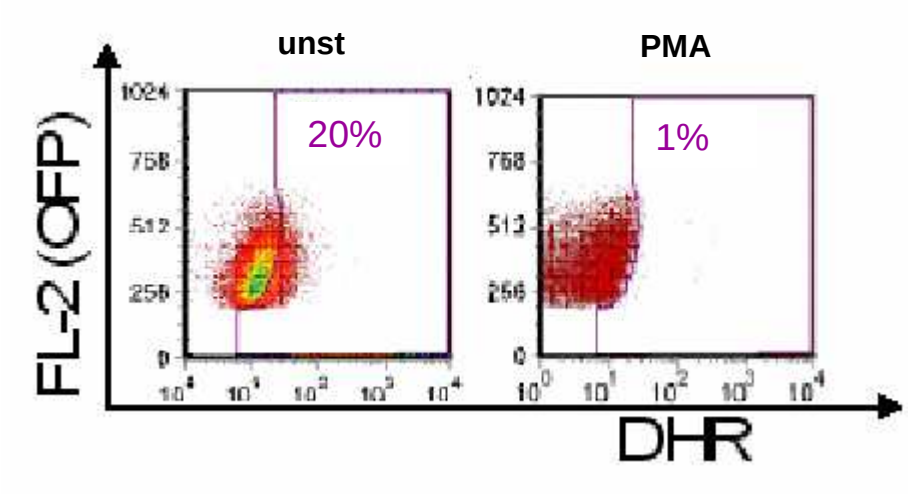


Figure 22: NADPH oxidase activity, after PMA stimulation, in PLB.PGK.gp91_126T superinfected with LVV coexpressing mir-126 and the LNGFR marker gene.

Transduction of murine Haematopoietic Stem Cell Progenitor (HSPC)

HSPC cells were harvested from the femurs and tibias of 4 KO-XCGD mice (from M.Grez) at 5 weeks of age. Lin- cells were isolated (the purity was analyzed by FACS-data not shown), and transduced at specific MOI for each LVVs (*Table* below). Two days after transduction, we performed Liquid and MethoCult culture.

	MOI
LV.PGK.gp91	10.5
LV.PGK.gp91_126T	17.5
LV.PGK.gp91_130T	75
LV.SFVV.gp91	13.5

Liquid Culture

We plated tranduced murine Lin- cells in StemSpan serum in presence of cytokines and growth factors cocktail (SCF, Flt3L, IL3, TPO, huG-CSF). After 2 days we washed them and new medium replaced with hu-G-CSF and SCF. On day 8 we used some of the cells for cytospin analysis (*figure 23*) and the remaining were plated in fresh medium with hu-G-CSF to induce granulocytes differentiation.

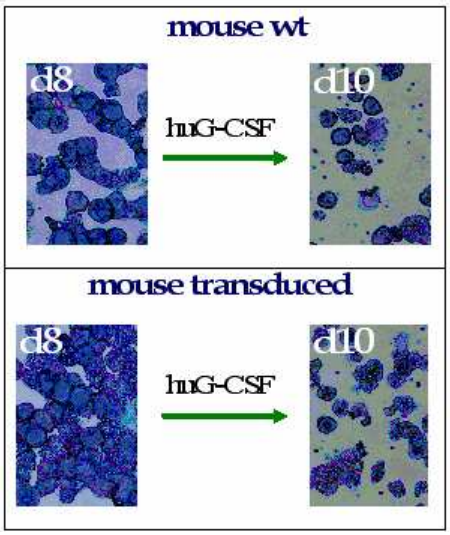


Figure 23: Morphological analysis (CYTOSPIN) before and after huG-CSF.

Facs analysis, performed on day 10, showed increased cell surface expression of gp91phox protein. Additionally CD11b (granulocyte) and CD117 (haematopoietic stem cell) markers were investigated to assess the state of cellular differentiation (*Figure 24*). Moreover, the results revealed a restored functional activity of the NADPH oxidase (*Figure 25*). Vector Copy number (VCN) was estimated by Taqman PCR on transduced cells. *Table* below shows VCN of selected transduced cells.

	VCN
LV.PGK.gp91 (10,5 MOI)	9,8
LV.PGK.gp91_126T (17,5 MOI)	9,06
LV.PGK.gp91_130T (75 MOI)	--
LV.SFVV.gp91 (13,5 MOI)	10,63

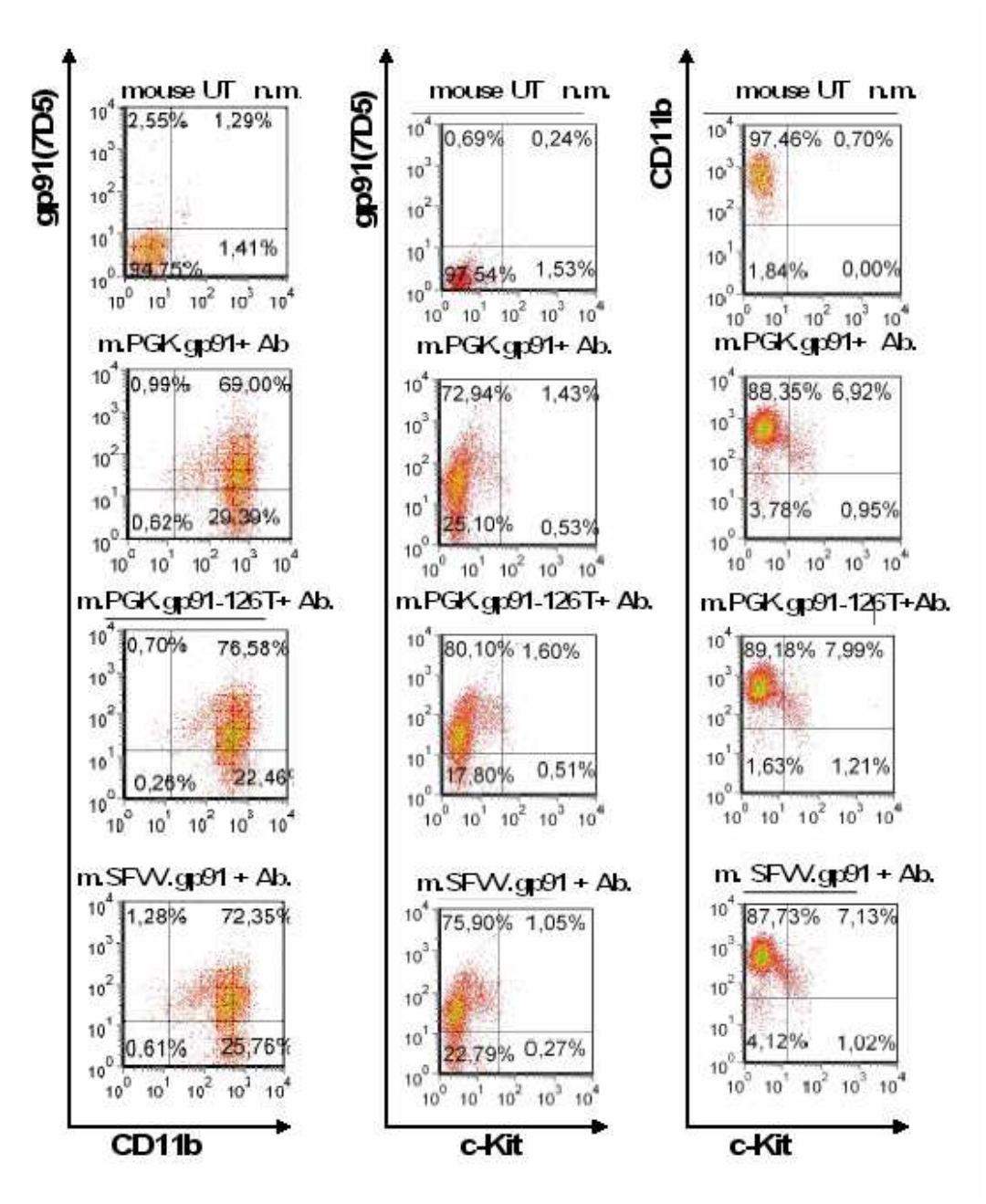


Figure 24: FACS analysis showed the expression of gp91*phox* and the differentiation status of the cells (c-Kit^{low}/CD11b^{+}). Staining with 7AAD identified live or dead cells (data not shown).

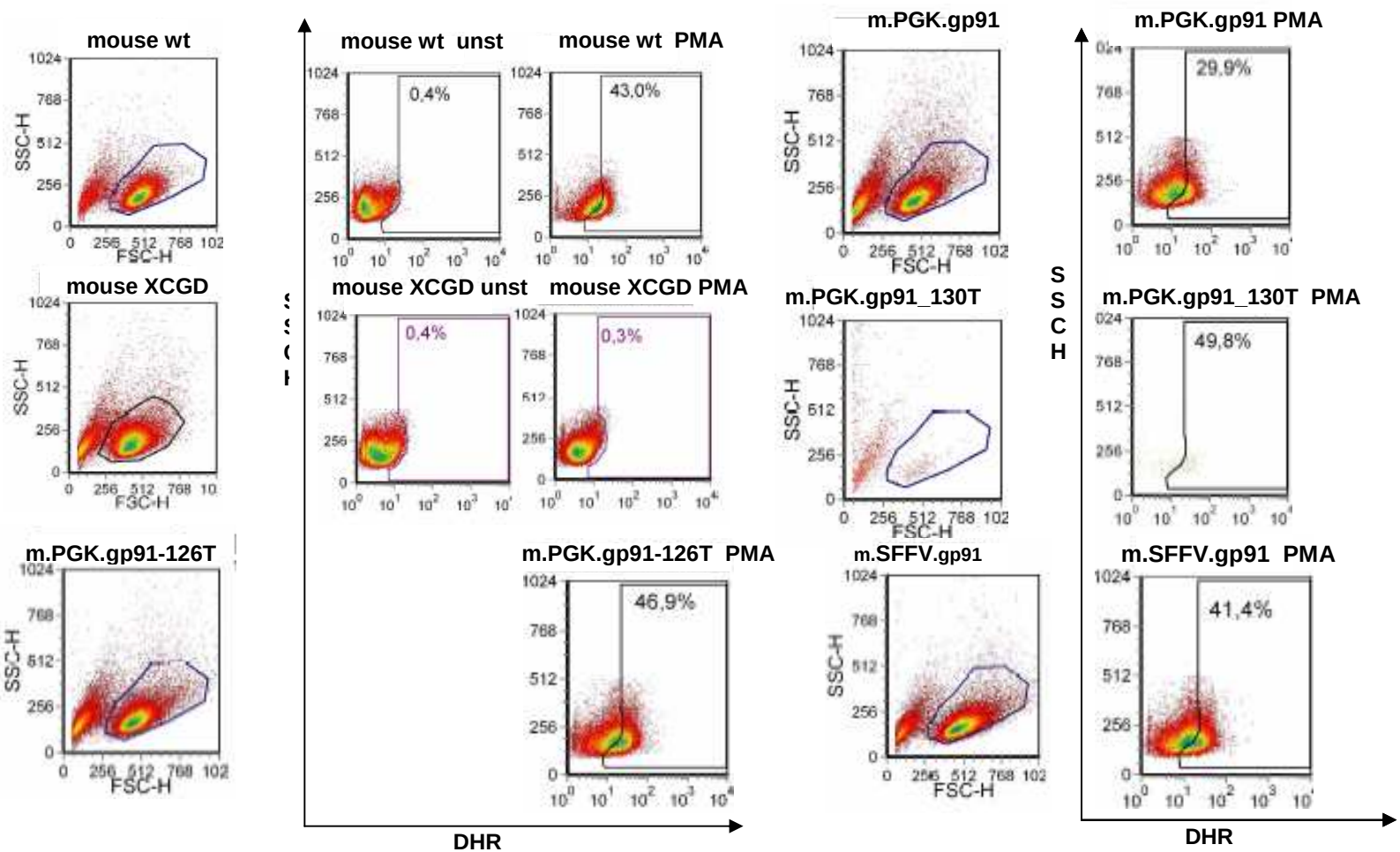


Figure 25: Functional NADPH oxidase activity in HSPC of mouse XCGD transduced, after PMA activation, by oxidation of DHR 123 after G-CSF-induced granulocytic differentiation

MethoCult Culture

Transduced cells were plated in methylcellulosa medium containing a mix of growth factors and after 14 days colonies were collected and washed. Facs analysis showed a presence of gp91phox and CD11b surface markers (*Figure 26*).

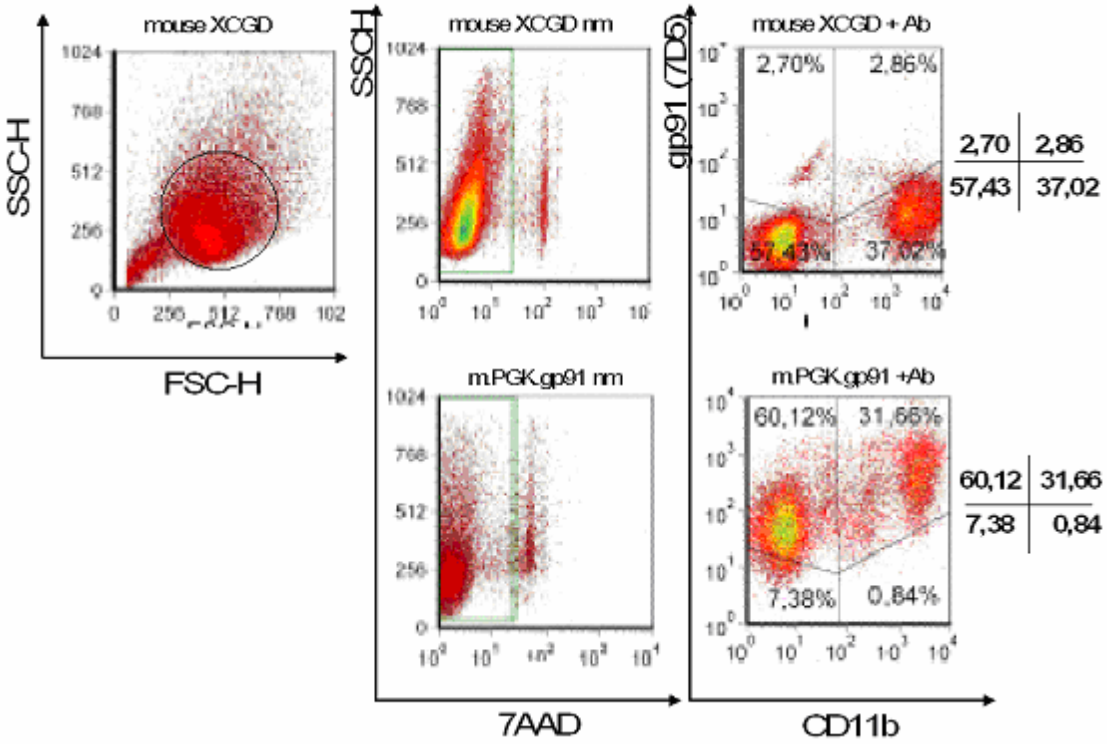


Figure 26: FACS analysis showed the expression of gp91phox and CD11b in differentiated myeloid colonies

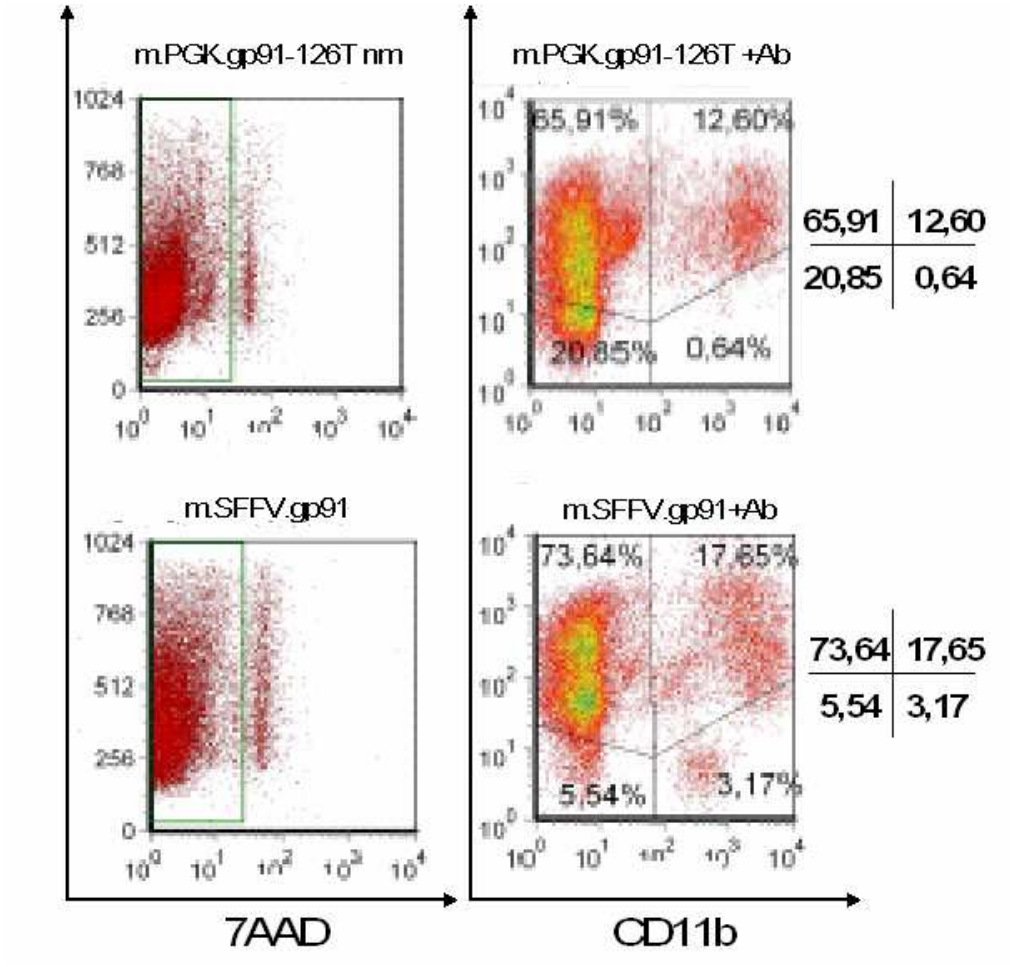


Figure 26: FACS analysis showed the expression of gp91phox and CD11b in differentiated myeloid colonies

Discussion

In the current study, we propose a new strategy for XCGD gene therapy with stem cells, including Lentiviral Vector (LVV) design and microRNA technology for a safe and effective treatment of X-CGD. Most of the actual vectors used so far in in gene therapy trials are long terminal repeat driven vectors and were derived from murine leukemia virus (MLV). Because their innate property to integrate into the genome of the host cells, the risk of insertional mutagenesis and transactivation of proto-oncogenes, from retrovirus-mediated gene therapy, is high (96). Currently, self-inactivating HIV-based lentiviral vectors represent the best candidates since they can transduce with high efficiency primitive HSC and carry a safer profile (97). In this study we produced four different VSV-pseudotyped third-generations lentivirus vectors (LVVs) encoding gp91phox, using the strong promoter SFVV and the ubiquitous promoter PGK.

Currently, to control transgene expression, transcriptional targeting by tissue specific promoters has been used. In our study we introduced miRNA technology to regulate gene expression at post-transcriptional level. miRT target sequences are express in specific differentiated cells and stages of haematopoiesis (98). We introduced in each of two PGK LVVs, respectively, four tandem repeat of mir126 (LV.PGK.gp91.126_T) and four tandem repeat of mir130 target sequence (LV.PGK.gp91.130_T). We selected them because 126- and 130- miRNA are expressed in HSPC (down-regulate LVV expression in stem cell compartment) and are down-regulated during granulocyte differentiation (permit LVV expression in the correct specific cellular type).

Human X CGD PLB985 cell line, mutated in *Cybb* gene encoding gp91*phox*, transduced with our LVVs showed a restoration of their capacity to express gp91 phox protein on cellular surface (25-83%); moreover, functional activity of the NADPH oxidase was re-established after DMSO-induced granulocytic differentiation (23-92%).

Our results demonstrated that in presence of mir126 target (containing in LV.mir-126), lentiviral vector containing a specific miRT sequence (LV.PGK.gp91_126T) is downregulated resulting in the block of gp91phox expression and NADPH functional activity. We demonstrated also that the downregulation by LV.mir-126 is extremely specific, because did not influence the gp91phox expression in other lentiviral vectors.

We obtained good transduction results also in experiments performed in vitro with mouse stem cells. Lin- cells isolated from KO –XCGD (without gp91*phox* protein) transduced with our four LVVs restored the expression of gp91*phox* protein (Liquid Culture and Methocult) and the functionality of NADPH oxidase (Liquid Culture).

In conclusion, our preliminary results indicate an efficient transduction capacity of all four lentiviral vectors. Both promoters used, SFFV and PGK, are able to induce a good expression of gp91*phox* protein and to restore NADPH oxidase activity. Moreover the vectors containing miRT express the transgene only in differentiated human cell lines.

In the future we want to:

- Transduce murine HSC and human HSC from patient
- Verify the specificity of miRNA regulation. These sequences should revert transgene expression on HSC, but not in differentiated cells.
- Produce a new LentiViral Vector with myeloid-specific promoters and tested its on human cell lines (X CGD PLB) and on murine HSC (KO-CGD Lin-)
- Transduction of XCGD patient’s monocyte
- Choose vector with the best safety profile which is efficient in correcting the disease

- Compare efficacy of restoration of oxidase activity by the different vector constructs → Efficacy
- Evaluate toxicity caused by gp91phox over expression in transduced HSC → Safety

Conclusion

In this study we have demonstrated that a deep clinical, immunological and molecular evaluation is necessary to obtain a clear picture of a patient case. Through the analysis of our patients we have delineated a rational approch to investigate patients with immunodeficiency considering PIDs as an optimal model to investigate development, function and regulation mechanisms of immune system. In order to favour the reconstitution of immune system in patients with X-linked Granulomatous Chronic Disease, we propose a new strategy of gene therapy with stem cells, including Lentiviral Vectors design and microRNA technology. Although we have already obtained good preliminary results, many investigations remain to be done in the future.

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