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T-CELL LARGE GRANULAR LYMPHOCYTE LEUKEMIA: PATHOGENESIS AND MOLECULAR DEVELOPMENT

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ABBREVIATIONS

Ac	Antibody
AcMo	Monoclonal Antibody
ADAM 17	ADAM metallopeptidase domain 17
Ag	Antigen
Ag490	2-cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide
AICD	Activation-induced cell death
ARMS	Amplification Refractory Mutation System
Bak	Bcl-2 homologous antagonist killer
BaX	Bcl-2-associated protein X
Bcl-2	B-cell lymphoma-2
BM	Bone Marrow
BSA	Bovine Serum Albumine
BSF	B-cell stimulatory factor 2
CBM	Cytokine Binding Module
C1P	Ceramide 1-phosphate
cDNA	Complementary DNA
CLPD-NK	Chronic Lymphoproliferative Disorder of NK cells
CMV	Citomegalovirus
Ct	Threshold Cycle
CTL	Cytotoxic T cell
DAC	Demethylation agent 5-aza-2'-deoxycytidine
DISC	Death-inducing signaling complex
EBV	Epstein Barr Virus
ECL	ChemiLuminescence
EDTA	Ethylenediaminetetraacetic acid
ESA	Erytropoietin Stimulating Agents
F/H	Ficoll/Hipaque
FACS	Fluorescence Activated Cell Sorting
FasL	Fas Ligand
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FNIII	Fibronectin type 3-like
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HRP	Horseradish peroxidase
HTLV	Human T-Lymphotrophic Virus
Ig	Immunoglobulin
IL-6	Interleukin-6
IL-6Rα	Interleukin-6 Receptor alfa
IL-2	Interleukin-2

IL-15	Interleukin-15
JAK	Janus Kinase
LGL	Large Granular Lymphocyte
LGLL	Large Granular Lymphocyte Leukemia
Mcl-1	Myeloid cell leukemia sequence-1
mgp130	Membrane gp130
mIL-6Rα	Membrane IL-6 Receptor alfa
MHC	Major Histocompatibility Complex
MSC	Mesenchimal Stem Cell
MTX	Methotrexate
MSP	Methylation Specific PCR
PAGE	Polyacrylamide gel electrophoresis
PB	Peripheral blood
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polimerase Chain Reaction
PI	Propidium iodide
PS	Phosphatidylserine
Pt	Patient
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RT	Retro Transcriptase
RT-PCR	Real Time -polymerase chain reaction
RTK	Receptor tirosine kinase
S1P	Sphingosine 1-phosphate
SDS	Sodium Dodecil Solfate
sgp130	Soluble gp130
sIL-6Rα	Soluble IL-6 receptor alfa
SH2	Src Homology 2
SOCS	Suppressor of cytokine signaling
STAT3	Signal Transducer And Activator of Transcription 3
T	Thymidine
TAD	Trans activation domain
TCR	T cell receptor
T-LGLL	T-Large Granular Lymphocyte Leukemia
U	Uracil
UNG	Uracil-DNA glycosylase
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

ABSTRACT

Large granular lymphocyte leukemia (LGLL) is a rare and heterogeneous lymphoproliferative disorder characterized by the chronic proliferation of clonal large granular lymphocytes (LGLs) with cytotoxic activity. Two subtypes of LGL proliferations are distinguished: the most common type (~85% of cases), T-LGL Leukemia (T-LGLL), is sustained by T-cells and the rarer type (~15% of cases), Chronic Lymphoproliferative Disorder of Natural Killer cells (CLPD-NK), sustained by NK-cells. This PhD will focus on the T-LGL Leukemia.

The etiology of T-LGLL still remains matter of debate. The main hypothesis suggests an antigenic stimulation as initial step activating LGLs, which undergo a clonal expansion then maintained by the abnormal release of cytokines (mainly IL-6 and IL-15) and by an impairment of the apoptotic machinery due to the activation of several survival pathways.

The project developed in this PhD was aimed to better define this etiopathogenetic hypothesis and to find new therapeutic targets.

First of all, we have taken into account the JAK/STAT pathway, reported to be deregulated in T-LGLL. We focused on STAT3, constitutively activated in LGLs, and its specific inhibitor SOCS3, that we found to be down-expressed and unresponsive to IL-6 triggering. The pathophysiology of this mechanism is unclear, since we found no methylation in SOCS3 promoter region; anyway we still hypothesize an epigenetic mechanism, since the use of a demethylating agent restored SOCS3 responsiveness.

We then focused our attention on the main activator of STAT3/SOCS3 axis, IL-6, and we observed that it enhances LGL survival through STAT3 phosphorylation. We also provided evidence that IL-6 is mainly expressed by LGLs-depleted PBMC population of T-LGLL patients characterized by low levels of circulating LGLs (<55%). The involvement of IL-6 in leukemic LGL survival was confirmed by the observation that incubation with anti-IL-6 and anti-IL-6Rα (IL-6 receptor) induced apoptosis in LGLs, likely related to a reduction of STAT3 phosphorylation.

The next step was the analysis of IL-6 receptor system that is composed of two functionally different chains: IL-6Rα and gpl30. Our data suggested that IL-6 acts through a trans-signaling mechanism in T-LGLL, being IL-6Rα highly detected in patients' plasma and poorly expressed by LGLs. Interestingly, trans-signaling is peculiarly implicated in inflammatory disease.

Among STAT3 target genes, CCL5 is a chemotactic agent reported to be over-expressed in LGL leukemia. In our lab we found that its expression is responsive to IL-6 stimulus. Since

the BM of patients is frequently infiltrated by LGLs and mesenchymal stromal cells (BM-MSCs) are reported to be a putative source of IL-6, we studied their role in T-LGLL and demonstrated that they promote LGL survival and may trigger CCL5 expression in LGLs, through IL-6 release.

We then focused on IL-15, a T-LGLL key cytokine mainly expressed by T-LGLL patients' dendritic cells (DC), these latter being considered the responsible for LGL activation through antigen presentation. We studied IL-15 effects on IL-6 signaling and we found that this cytokine promotes the trans-signaling mechanism through an enhanced IL-6 expression by patients' PBMCs and the inhibition of IL-6Rα expression by LGLs.

Finally, recent data in the literature reported mutational hot spots in SH2 domain of STAT3, suggesting that STAT3 constitutive activation may result from these acquired genetic mutations. Accordingly, we analyzed whether patients' LGLs showed any of STAT3 hot spot mutations up to now quoted. We observed that two mutations, D661Y and Y640F, were detected in nearly 20% of cases and more specifically in patients characterized by a high percentage of circulating LGLs (>55%), accounting for 40% of all LGL cohort.

RIASSUNTO

La malattia linfoproliferativa dei linfociti granulati (LGLL) è una malattia rara caratterizzata da una linfocitosi cronica dei grandi linfociti granulati (LGL) con attività citotossica. Da un punto di vista immunologico, si distinguono due forme di LGLL: La T-LGLL, caratterizzata dalla proliferazione di LGL di tipo T, nonché forma più frequente della patologia (~85% dei casi) e la NK-CLPD, più rara (~15% dei casi) in cui la linfocitosi viene sostenuta da cellule di tipo NK.

L'eziopatogenesi della T-LGLL risulta essere ancora materia di dibattito. L'ipotesi più accreditata indica la stimolazione antigenica come evento in grado di attivare gli LGL; queste cellule intraprendono dunque un'espansione clonale, che viene mantenuta in un secondo tempo dal consistente rilascio di citochine (prevalentemente IL-6 ed IL-15) e da un equilibrio vita/morte cellulare alterato, generato quest'ultimo dalla contemporanea attivazione di numerose vie di sopravvivenza cellulare.

Nel nostro laboratorio abbiamo seguito differenti linee di ricerca, con l'obiettivo di verificare tale ipotesi eziopatogenica e trovare nuovi target terapeutici.

Abbiamo preso prima di tutto in considerazione la via di segnale JAK/STAT, che si riporta essere alterata nella T-LGLL. Ci siamo soffermati in particolare sulla proteina STAT3, che risulta essere costitutivamente attivata, e su SOCS3, suo specifico inibitore, emerso essere, dai nostri studi, *down*-espresso e non responsivo all'IL-6 nella T-LGLL.

Le basi fisiologiche determinanti il mancato meccanismo a *feed-back* negativo di SOCS3 non risultano ancora definite, dal momento che il promotore di SOCS3 non è risultato essere regolato tramite metilazione; è possibile tuttavia che un meccanismo epigenetico sia comunque responsabile dell'alterata regolazione della via di segnale JAK/STAT, in quanto abbiamo osservato che l'uso di un agente demetilante è in grado di ripristinare l'attività inibitoria di SOCS3.

Abbiamo quindi analizzato il principale attivatore dell'asse STAT3/SOCS3, ovvero l'interleuchina 6 (IL-6), evidenziando come tramite l'attivazione di STAT3 essa sia in grado di promuovere la sopravvivenza degli LGL. Abbiamo inoltre rilevato che tale citochina è espressa in particolar modo dai PBMC non LGL di pazienti caratterizzati da bassi livelli di LGL circolanti (<55%).

Il coinvolgimento di IL-6 nella sopravvivenza degli LGL è stato confermato da un'induzione dell'apoptosi in seguito a coltura con anticorpi bloccanti anti-IL-6 ed anti-IL-6Rα (recettore α dell'IL-6), dovuta ad una riduzione dei livelli di STAT3 attivata.

Passaggio successivo è stato analizzare il sistema recettoriale di IL-6, composto da due differenti catene: IL-6Rα e gpl30. I dati ottenuti suggeriscono un meccanismo di *trans-signaling*, particolarmente coinvolto nei processi di tipo infiammatorio, alla base dell'azione di IL-6 nella T-LGLL; IL-6Rα è presente infatti ad alti livelli nel plasma dei pazienti, pur venendo scarsamente espresso dagli LGL.

Tra i geni *target* di STAT3 vi è CLL5, un potente agente chemotattico che si riporta essere *over*-espresso nella T-LGLL; nel nostro laboratorio abbiamo osservato inoltre che la sua espressione è responsiva allo stimolo con IL-6. Dal momento che il midollo osseo dei pazienti risulta essere frequentemente infiltrato dagli LGL e che le cellule mesenchimali stromali (BM-MSCs) risultano essere una presunta fonte di IL-6, abbiamo analizzato il ruolo di queste cellule nella T-LGLL e dimostrato che esse sono in grado di promuovere la sopravvivenza degli LGL e probabilmente stimolare l'espressione di CCL5 da parte degli LGL, tramite il rilascio di IL-6.

Abbiamo studiato l'interleuchina 15 (IL-15), una citochina chiave della T-LGLL espressa in particolar modo dalle cellule dendritiche (DC) dei pazienti, considerate responsabili dell'attivazione degli LGL tramite presentazione dell'antigene.

Abbiamo analizzato gli effetti di IL-15 sul *signaling* di IL-6 ed abbiamo evidenziato come tale citochina sia in grado di promuovere il meccanismo di *trans-signaling* attraverso l'induzione d'espressione di IL-6 nei PBMC dei pazienti e l'inibizione dell'espressione di IL-6Rα negli LGL.

Dati recenti di letteratura riportano, infine, *hot spot* mutazionali nel dominio SH2 di STAT3; è stato ipotizzato che la costitutiva attivazione di STAT3 sia dovuta a tali mutazioni, per tale motivo abbiamo indagato, nella nostra coorte di pazienti, la presenza delle mutazioni descritte finora riportate in letteratura. Abbiamo riscontrato le due mutazioni, D661Y e Y640F, sono presenti in circa 20% dei casi e specificamente in pazienti caratterizzati da un'alta percentuale di LGL circolanti (>55%), che costituiscono il 40% dei casi con LGLL.

INTRODUCTION

1. LARGE GRANULAR LYMPHOCYTES

Large granular lymphocytes (LGLs) represent approximately 10-15% of peripheral blood mononuclear cells in adult and are involved in cell-based cytotoxicity. Normally present in peripheral blood with values ranging from 0.2 and $0.4x10^9$ LGL/L (200-400 LGL/mm^3), these cells have well defined morphology and phenotype[1]. LGLs are large cells (15-18 μm) with abundant cytoplasm containing typical azurophilic granules and reniform or round shaped nucleus (Figure 1)[2].

Basing on immunophenotype we can separate two different classes of LGLs, which are: Natural Killer cell line (CD3 negative), representing 85% of total LGL population and T cell line (CD3 positive), which constitutes the remaining 15% and needs an activation step before acquisition of cytotoxic properties[3].

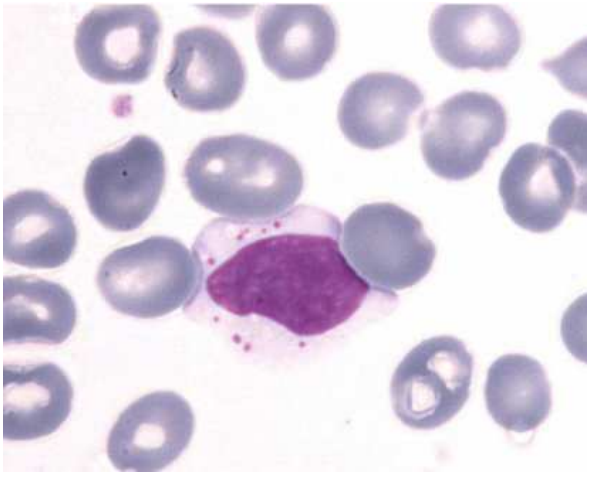


Figure 1: Peripheral blood large granular lymphocyte. Reniform nucleus and cytoplasmatic azurophilic granules are clearly visible.

Well defined functional characteristics give LGLs a fundamental role in the progression of immune response: both T and NK LGLs have cytoplasmatic granules containing perforins and granzymes B[4], and have therefore a central role in cell-mediated immune response, being able to exert a cytotoxic activity.

NK-LGLs lack of T-Cell Receptor (TCR), show a MHC (Major Histocompatibility Complex) -non-restricted cytotoxicity and belong to innate immune system, T-LGLs on the other hand show a TCR rearranging during lymphocyte maturation, moreover are able to exert a MHC-

restricted citotoxicity and belong to adaptive immune system[4]. T-LGLs therefore represent cytotoxic lymphocytes *in vivo* (CTLs) and are activated by antigen[5,6].

1.1 PROPERTIES AND FUNCTIONS OF T-LGLs

Thanks to TCR, T lymphocytes can specifically recognize antigens. This receptor is an heterodimer of two trans membrane polypeptide chains (chain α and chain β) covalently bound by sulphur bridge. The formation of TCR takes place during T-lymphocytes maturation process in thymus, after V(D)J recombination of TCR. A small subpopulation of T-lymphocytes show another TCR, that contains two different polypeptide chains, γ and δ, and characterize γδ T-lymphocyte class. Extracellular peptides can be recognized by TCR only if presented by MHC; TCR binding causes transition from double positive cells (CD4+ CD8+) to two different cell lines: a regulatory cell type, called lymphocytes T helper (Th) CD4+ CD8-, that recognizes antigen presented by MHC class II, and CTL CD8+ CD4-, a cytotoxic cell line that recognizes antigen presented by MHC class I[7].

Phenotypically, CTLs express CD3, CD8 and CD57 (adhesion molecules shown by completely differentiated effector CTLs), while are CD27 and CD28 negative. Effector proteins, like granzymes, perforins and NKG2D receptor, are up-regulated in CTLs, while homing receptor like CCR7 and CD62L are down regulated[6].

Following activation, CTLs undergo a strong expansion in order to deal with prolonged or quantitatively relevant antigenic stimuli. Since they can specifically recognize antigen on the surface of infected cells, CTLs have a key role in cell mediated immunity; this interaction initiates a chain of events leading to cell lysis, a mechanism also known as “cell mediated cytotoxicity”.

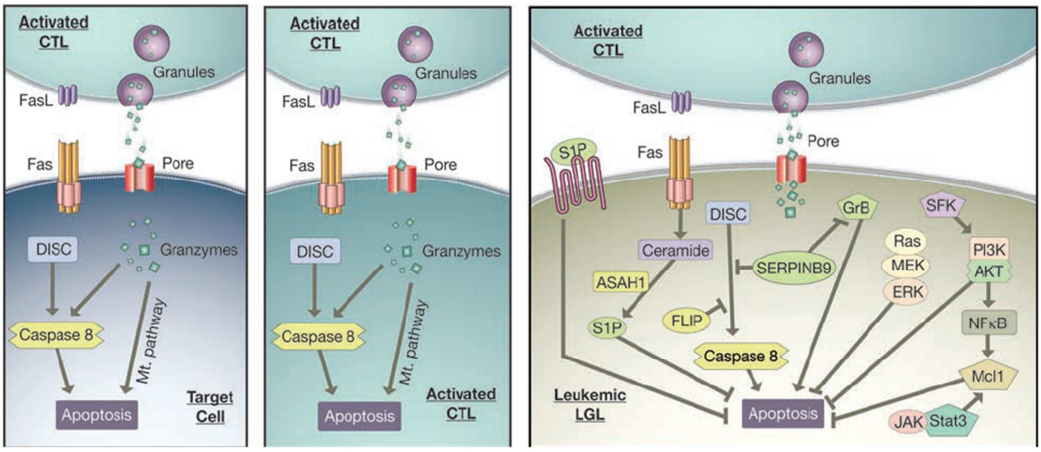
We can identify two major pathways through which LGLs can kill their targets: granule-mediated pathway and receptor-mediated pathway[8].

Granule-mediated pathway depends on the exocytosis of cytolytic granules, morphologically characterizing LGLs, to induce lysis and apoptosis of target cells. Once target is recognized, granules are polarized towards the immune synapse where LGLs interact with target and, after that, they can release a variety of cytotoxins, such the pore-forming protein perforin and a family of serine proteases-granzymes, like granzyme B (GrB)[9]. Perforin, in addition to directly inducing osmotic lysis by forming pores on target cell membrane, is necessary for granzyme delivery within target cells[8].

Granzymes, by their protease activity, potentiate the death of target cells by cleaving and activating the effector caspases as well as pro-apoptotic Bcl-2 family members (Figure 2, panel A)[9].

The receptor-mediated pathway relies on the engagement and activation of "death receptor", such as Fas (CD95), a member of tumor necrosis factor receptor family, by their ligands (FasL or CD95L). Death receptors are universally expressed on several cell types while, the corresponding ligands are frequently found on activated CTLs. Receptor-mediated pathway is not specific for the immune system and the death receptor-ligand interaction is not MHC-restricted.

Instead, LGL-target interaction brings target cells to the proximity of LGL and upon ligation, death receptors cluster and induce the formation of death inducing signaling complex (DISC) which involves the same signaling molecules induced by granzymes (Figure 2, panel A)[9].



Panel A Panel B Panel C

Figure 2: (A) Receptor- and granules-mediated cytotoxicity, through Fas or granzymes containing granules, respectively; **(B)** AICD (activation-induced cell death) induction within CTL once the immunity activity is completed. **(C)** Leukemic LGLs show resistance to AICD, due to the involvement of other signaling pathways that oppose to AICD.

Together, granule-mediated pathway delivers an instant and potent cytolytic potential, while the receptor-mediated pathway acts as a moderate but persistent supply. Both pathways contribute to LGL clearance after activation in order to prevent an enhanced activity of these cells that may alter the immunity homeostasis. This "death pathway" is termed activation-induced cell death (AICD): both granule- and receptor-mediated pathways can act on activated LGLs through endocellular or extracellular granule leakage which triggers similar signaling pathways as in target cells. After activation, LGLs express elevated death receptors, making themselves sensitive to the death ligands causing AICD (Figure 2 panel B)[9].

Thus, the resistance to Fas-mediated apoptosis and, consequently, the failure to undergo AICD is the main reason causing abnormal clonal expansion of LGLs with the final result of an accumulation of these cells in peripheral blood that remain competent in the long term (Figure 2, panel C). It is well assumed that this event represents the hallmark of LGLL disorders[10].

2. LARGE GRANULAR LYMPHOCYTE DISORDERS

LGL disorder is a rare disease characterized by a clonal and chronic proliferation of cytotoxic lymphocytes. In the last years, the understanding of natural history, immunophenotype, pathophysiology and treatment of this disease has greatly expanded and LGL disorder is now recognized as a well-defined clinical entity. Indeed, on 2008 the World Health Organization (WHO) classified this disorder as belonging to T- and NK-cell mature neoplasias.

The classification holds for three categories[11]:

- T-cell large granular lymphocytes leukemia (T-LGLL)
- Aggressive NK-cell leukemia (ANKL)
- Chronic lymphoproliferative disorder of NK cells (CLPD-NK).

Two subtypes of LGL proliferations therefore are distinguished: in the first one, LGL clone is sustained by T-cells and it is the most common type of LGL proliferation (~85% of cases)[3], while in the second LGL clone is sustained by NK-cells and it represents ~15% of cases[11].

2.1 T-CELL LARGE GRANULAR LYMPHOCYTE LEUKEMIA

T-LGLL is the most frequent LGL disorder in Western countries[3]. Is a rare disease of the elderly, with a median age at diagnosis of 60 years, however it has been described in all age groups, including in the pediatric population. Males and females are affected equally[4].

2.1.1 Clinical presentation

T-LGLL typically has an indolent clinical behavior with a median survival >10 years. Diagnosis generally follows an incidental finding of cytopenia and about a third of patients

results asymptomatic. About 60% of patients will become symptomatic during disease course[3,4]. Recurrent infection and fatigue may occur in association with neutropenia and anemia. The liver is often involved histologically but may not be enlarged. The presence of lymphadenopathy would be considered an uncommon disease manifestation[4].

Diagnosis of LGL leukemia is established by documentation of an increased number of clonal LGLs; initially, a circulating LGL count $2x10^9$/L was considered as mandatory (normal number of LGLs in the peripheral blood is $0.3x10^9$/L), but now a lower count (range, 0.4-$2x10^9$L) can be compatible with the diagnosis[12].

Eighty-five percent of patients develop neutropenia during the disease course, that is severe (0.5×10^9/L)[3,4], in 50%. Anemia and thrombocytopenia are less common and observed in approximately 50% and 20% of patients, respectively[3,4].

Possible mechanisms for neutropenia found in LGLL include deregulated Fas/FasL-induced apoptosis of myeloid cells, immune complex or antibody-mediated neutrophil destruction, hypersplenism or direct inhibition of myeloid maturation by cytokines[3,4]. Which of these mechanisms is dominant remains unclear and may vary among patients.

T-LGLL is associated with a wide spectrum of autoimmune disorders, most of them involving connective tissue. In particular, among arthropathies associated with LGLL, rheumatoid arthritis (RA) is the most common autoimmune disease, occuring in 25%-35% of patients[3,4]. Other autoimmune disorders, including systemic lupus erythematous and Hashimoto's thyroiditis, can occur in patients with this disorder, but less frequently[4].

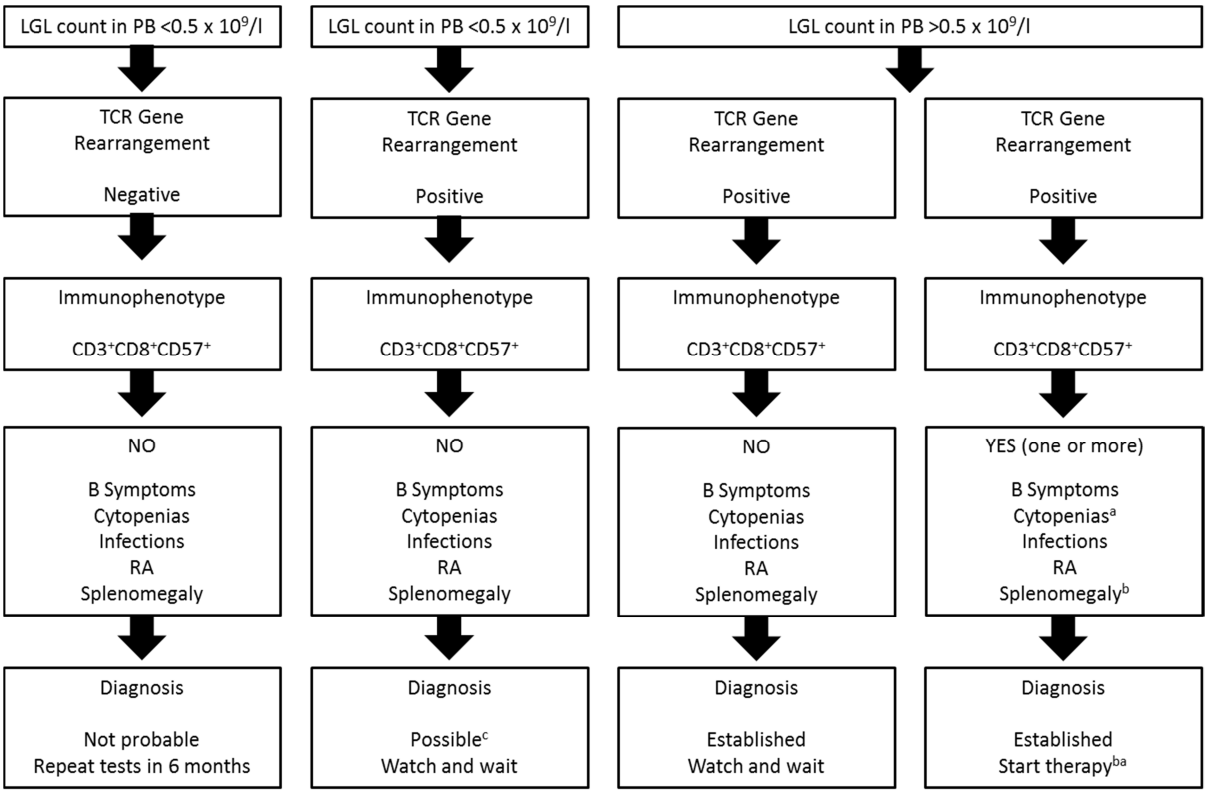
2.1.2 Diagnosis

The disease should be suspected in all patients with an increased number of LGLs in peripheral blood and unexplained cytopenias. A careful morphologic examination of LGLs in peripheral blood and/or a flow cytometry analysis of mature T-cell immunophenotyping panel (including CD3, CD5, CD7, CD8, CD16, CD25, CD56, CD57, TCRαβ and γδ) and TCR gene rearrangement are required. Bone marrow trephine biopsy is usually necessary to confirm the diagnosis, particularly if patient requires treatment.

Main features in T-LGLL diagnosis, is an abnormal proliferation of CD8+ T cells and the evidence of clonality, established by gene rearrangement studies (Table 1). Indeed, as T cells rearrange TCR genes during their normal development, all the cells arising from a malignant, transformed T cell will have the same sequence of TCR genes. This can be demonstrated either by Southern Blotting or by Polymerase Chain Reaction (PCR) analysis. However, PCR

is currently the technique most widely used in clinical setting. Flow cytometry using monoclonal antibodies against TCR variable region family gene segments can also aid in the establishment of clonality in T-cell disorders.

Table 1. Algorithm of the diagnosis of T-LGLL:



[a] Moderate to severe or symptomatic cytopenias. [b] Symptomatic splenomegaly. [c] Small oligoclonal/clonal populations of circulating LGLs of unknown significance are detected in elderly individuals and after organ transplantation; bone marrow biopsy can be valuable in this situation; clinical correlation is recommended. Abbreviations: PB, peripheral blood; RA, rheumatoid arthritis; TCR, T-cell receptor.

Diagnosis of T-LGLL therefore requires three criteria[3]:

- Cytopenia (neutropenia and/or thrombocytopenia)
- Evidence of expansion of CD3+CD8+CD57+ T cells
- Clonal TCR gene rearrangement

T-LGLL is an heterogeneous disease, with different clinical manifestations; therefore treatment standardization is difficult to achieve[13]. The disease is often asymptomatic and up to half of patients may not require therapy.

In these cases regular follow-up required to establish the disease course and to identify any need for treatment[4], usually indicated with significant symptomatic anemia (<9g/dL) and/or

need for transfusion, severe neutropenia (<0.5 x 10^9/L), severe thrombocytopenia (<50 x 10^9/L), or any combination of these symptoms[3]. Other indications for treatment include recurrent infections and massive splenomegaly[14].

The main treatment involves immunosuppressive therapy rather than chemotherapy. Immunosuppressive therapy includes methotrexate (MTX) or cyclophosphamide (both at low doses) or cyclosporine A.

Therapy with low doses *per os* MTX (10 mg/m^2 per week), a drug employed as an effective anti-inflammatory and immunosuppressive agent[3,15] with the ability to alleviate RA[15], demonstrated complete clinical remission through normalization of blood counts[15] in about only 50% of patients with T-LGLL[4,16]. Short dosing of corticosteroids in addition to MTX treatment may help to alleviate B symptoms and improve the blood cell count[4] however, because of adverse effects, administration of high-dose steroids for more than 1 month is usually not recommended.

Corticosteroids such as prednisone can be used to hasten the clinical response. Alone, this compound has been shown to improve neutrophil count but doesn't result in a long-lasting remission[3,13], because LGL clone continues to persist. Prophylactic antibiotics therapy is recommended for patients under corticosteroids therapy, presenting severe symptomatic neutropenia[3].

Administration of growth factors, as ESA (*Erytropoietin Stimulating Agents*) and GM-CSF (*Granulocyte-Macrophage Colony-Stimulating Factor*), may help to treat cytopenias, even if for short periods.

Treatment with chemotherapic agents should be considered for patients refractory or relapsing after immunosuppressive therapy[17] and for young individuals with a significative medullary infiltration.

Treatment with purine analogs like Pentostatine, Fludarabine and 2-chlorodeoxyadenosine (2CDA) gave promising results, even if in a low percentage of patients[10,18,19].

Monoclonal antibodies like Alemtuzumab (anti-CD52), Humanized MiK-β-1(anti-CD122) and Siplizumab (anti-CD2), antithymocyte globulin or splenectomy are considered treatment options in the case of failure with the first-line treatment[3].

2.1.3 Etiology

The etiology of T-LGLL still remains matter of debate, due to the fact that no single, specific agent but a series of events can finally trigger LGLs proliferation. Some reports strongly

support the role of chronic antigenic stimulation by exogenous antigens, such as human T-cell lymphotrophic virus (HTLV) or putative endogenous autoantigens as the initial stimulus inducing the activation and clonal expansion of effector CD8+ LGLs[4]. Later, LGLs expansion may be sustained by the action of cytokines, like interleukin-2 (IL-2) and interleukin-15 (IL-15)[2] (Figure 3).

The exact role of retroviral infection as etiologic agent has not been entirely established[4], it has been described that most patients with T-LGLL are not infected with prototypical HTLV, but they showed serum reactivity against a small peptide derived from the HTLV-I envelope protein p21e[16].

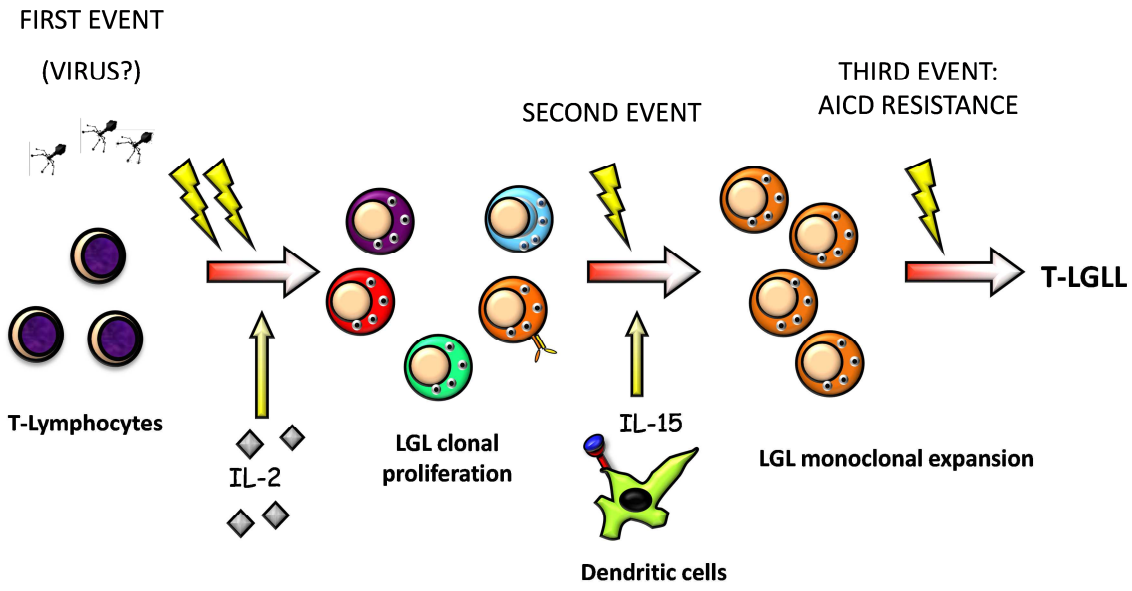


Figure 3: T-LGLL Etiopathogenetic hypothesis. Abbreviations: IL-2, Interleukin 2; IL-15, Interleukin 15; AICD, activation-induced cell death.

Data from our lab[20] have shown a possible role for dendritic cells (DC) in the pathogenesis of LGLL. These cells belong to the antigen-presenting cell (APC) family, characterized by the ability to recognize antigens and later to present the same antigens and adhesion molecules on their surface to T cells, inducing their activation.

DCs can be involved in LGLs proliferation; after the recognition of a specific antigen they may maintain proliferation by releasing cytokines like IL-2, IL-15 and IL-18. Immunohistochemical analysis performed on T-LGLL patients osteomedullary biopsies have shown the presence of direct contact between LGL and DC, in contrast to healthy controls in which cells have a random distribution. It has been hypothesized that the medullary environment represents the place where pathological proliferation starts and thus DCs represent viral infection target cells[20].

Moreover, proliferating LGLs are characterized by an impaired apoptotic machinery, due to the activation of several survival pathways, which results in LGL aberrant outliving and number increase.

3. CYTOKINES INVOLVED IN T-LGLL

3.1 INTERLEUKIN 6

IL-6-type cytokines form a subfamily of helix bundle cytokines. All IL-6-type cytokines comprise four long α-helices termed A, B, C and D (Figure 4) which are arranged in a way that leads to up-up-down-down topology.

IL-6 is produced and released by a wide variety of cell types, e.g. monocytes/macrophages, fibroblasts, endothelial cells, and astrocytes in response to infections or injuries.

IL-6 exerts pleiotropic functions by acting in endocrine, paracrine and autocrine manner on different target cells. It plays important roles in the regulation of immune system and is the main mediator of the acute-phase response which is characterized by the synthesis and secretion of acute-phase plasma proteins by the liver, elevated serum glucorticoid levels and often fever. It is known that IL-6 leads to the differentiation of both B and T lymphocytes, acts as a differentiation factor in hematopoiesis and in general, regulates differentiation, proliferation and survival of many other cell types[21].

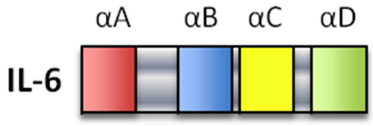


Figure 4: The four domains A, B, C and D, that form á-helices, are highlighted in different colors.

This cytokine exerts its biological activities through two molecules: a non-signaling α-receptor termed IL-6 receptor α chain (IL-6Rα), also known as glycoprotein 80 (GP80) or CD126 and the signal transducing receptor glycoprotein 130 (gp130), also referred to as IL-6Rβ or CD130. The ectodomains of the IL-6 receptors contains an array of Fibronectin type III (FNIII)-like and Ig-like domains (Figure 5). Each receptor contain at least a cytokine binding module (CBM) domain that consists of two FNIII domains. CBM is characterized by conserved structural features, such as a WSXWS motif in the C-terminal domain and a

distinct pattern of cysteine residues in the N-terminal domain. An Ig-like domain is also located N-terminally to the membrane-proximal CBM[22].

To induce cascade signaling, IL-6 first binds to the trans-membrane IL-6Rα (mIL-6Rα), that is not determinant for signal transduction having a small cytoplasmic domain. On the contrary gp130 has three additional membrane-proximal FNIII domains[22] and its cytoplasmic domain contains several potential motifs for intracellular signaling, such as YSTV sequence for SHP2 (Src homology domain-containing protein tyrosine phosphatase-2) recruitment and YXXQ motifs (where X is any aminoacid) for STAT activation.

gp130 does not have any intrinsic kinase domain but, like other cytokine receptors, its cytoplasmic domain contains regions for the association with a non-receptor tyrosine kinase, like JAK, and can initiate downstream signaling cascades.

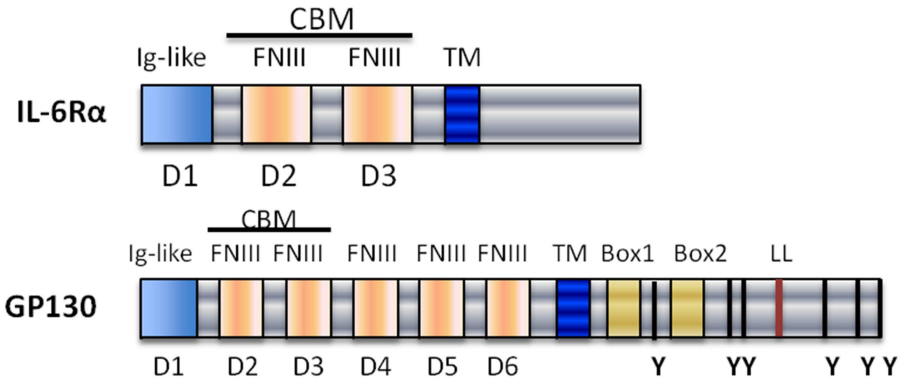


Figure 5: Structural organization of IL-6Rα and gp130. Tyrosine residue of gp130 that become phosphorylated are indicated (Y). For gp130 the box1 and box2 regions, as well as the dileucine motif (LL) are showed. Abbreviations: CBM, cytokine binding module; FNIII, Fibronectin type III; TM, transmembrane; Ig-like, immunoglobuline-like.

Neither IL-6 nor mIL-6Rα alone binds or activates gp130; IL-6 first binds to mIL-6Rα and the IL-6/mIL-6Rα complex is able to recruit gp130 receptor and triggers its homodimerization. A high affinity functional receptor complex of IL-6, mIL-6Rα and gp130 is formed; this IL-6 system is called "classical signaling"[23] (Figure 6).

Whereas membrane-bound gp130 (mgp130) is ubiquitously expressed, mIL-6Rα expression is restricted mainly to hepatocytes, neutrophils, monocytes and macrophages; anyway, many cells not expressing mIL-6Rα are reported to be responsive to IL-6.

The cytokine has been found to use an alternative pathway to activate target cells lacking mIL-6Rα, called trans-signalling[23] (Figure 6), which acts through the soluble agonistic form of the IL-6Rα (sIL-6Rα), generated by both alternative splicing of mRNA and limited proteolysis of the membranous form by ADAM (a disintegrine and metalloproteinase)-17[22].

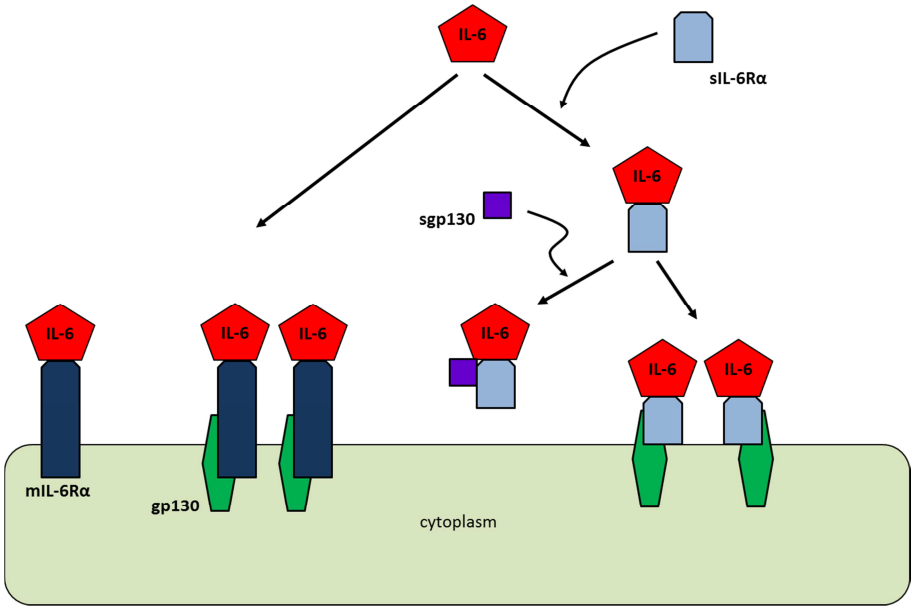


Figure 6: Representation of IL-6 classical signaling on the left and IL-6 trans-signaling on the right.

One important difference between classical and trans-signaling is that classical signaling is correlated with anti-inflammatory and regenerative activities, while trans-signaling contributes to the pro-inflammatory activities of IL-6, e.g. by controlling leukocyte infiltration, inhibition of T-cell apoptosis and Treg differentiation[24].

It has been shown that in chronic inflammatory diseases such as inflammatory bowel disease, peritonitis, asthma, RA and in colon cancer, IL-6 trans-signaling is critically involved in the maintenance of the disease state, due to the high levels of IL-6 and sIL-6Rα detected.

Also a soluble form of gp130 (sgp130) has been discovered, which acts as the natural inhibitor of IL-6/sIL-6Rα complex and selectively inhibits IL-6 trans-signaling without affecting classic signaling[25].

3.2 INTERLEUKIN 15

IL-15 is an inflammatory cytokine, belonging to a subfamily of four helix cytokines, that stimulates T and NK cells activity. There are two isoforms for this 14-15 kDa glycoprotein encoded by interleukin 15 gene on chromosome 4: one of 48 aminoacids (IL-15 LSP) that can be bounded to cytoplasmatic membrane or secreted, and another one with 21 aminoacids (IL-15 SSP), which can be nuclear or cytoplasmatic and has a role in cell cycle regulation.

Interleukin 15 is constitutively expressed by a large number of cells, among which macrophages, dendritic cells, keratinocytes, fibroblasts, but only few cell types, as monocytes and dendritic cells, can translate mRNA[26].

Main interleukin 15 functions include[26,27]:

- Stimulus of cytotoxic T lymphocytes proliferation
- NK cells differentiation from hematopoietic progenitors in bone marrow
- Induction of NK cells activation
- Promotion of normal B lymphocytes proliferation and activation
- Activation of CD3+ and CD3- large granular lymphocytes
- Attraction of neutrophils and monocytes in sites of infection.
- Regulation of pro-inflammatory cytokines production by macrophages
- Induction of IL-2 expression by dendritic cells

Expression of IL-15 and its specific receptor IL-15R is upregulated in many infective, inflammatory and immunological diseases, among which T-LGLL.

The IL-15 specific receptor consists of:

- a specific subunit, IL-15Rα
- IL-15Rβ subunit (CD122) founded also in IL-2 receptor
- Common γ chain (γc)

In most cases IL-15 binds IL-15Rα with high affinity in the endoplasmatic reticulum and the resulting complex is carried on cell surface where, together with IL-15Rβ, it triggers the trasductional cascade (Figure 7).

An uncommon mechanism requires instead the transfer of IL-15Rα on cell surface, where it binds secreted IL-15 (Figure 7). Different IL-15Rα isoforms, derived from alternative splicing, have been identified, but it’s not clear yet if they have an agonist or antagonist role in the signaling pathway activation[28].

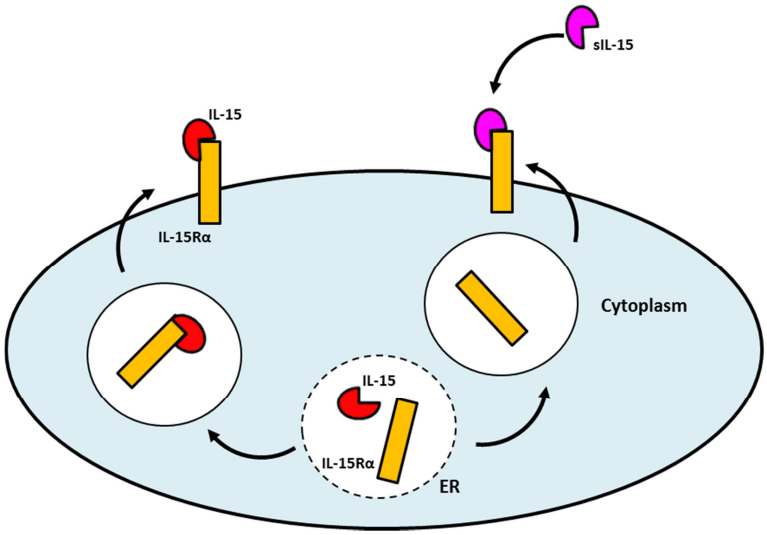


Figure 7: Binding mechanisms of IL-15 and IL-15Rα.

Once IL-15/IL-15Rα complex is formed, it is exposed on cell surface, where it contacts IL-15Rβ and common chain γ and allows IL-15 to exert its functions. This mechanism could happens through two ways (Figure 8):

- Trans-presentation: IL-15/IL15Rα complex of a cell, generally monocyte or dendritic cell, binds to IL15Rβ/γ_c of another cell type, like CD8 T lymphocytes and NK cells. From recent studies has emerged that this mechanism of IL-15 signaling pathway is the most common[28,29].

Cis-presentation: the IL-15/IL-15Rα complex binds to a IL-2/IL-15Rβ/γ_c complex located on the same cell surface. Activation of several signaling pathways, including JAK/STAT and RAS/MEK/ERK, follows IL-15 binding to its receptor.

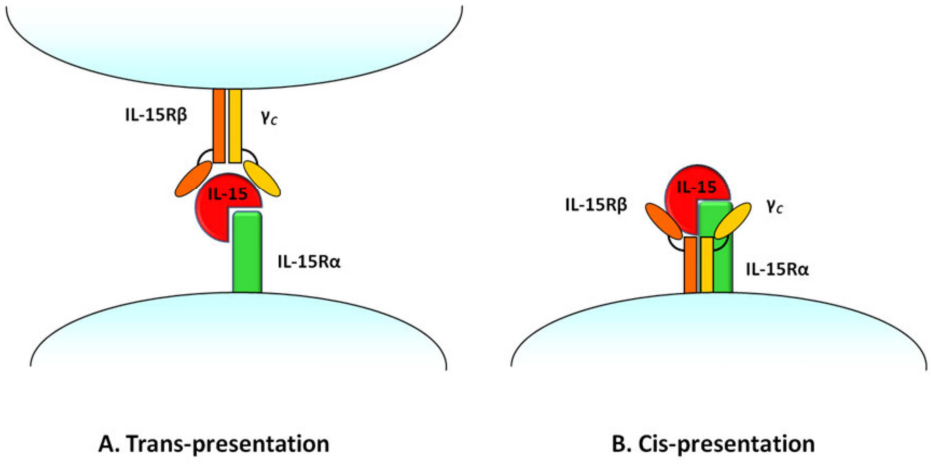


Figure 8: IL-15 signaling transduction mechanisms; Panel A: Trans-presentation. Panel B: Cis- presentation.

3.3 CCL5

CCL5 (also known as RANTES) is a potent chemotactic agent for memory T lymphocytes and monocytes that belongs to the CC chemokine subfamily and is expressed after cellular activation in fibroblasts, T cells, monocytes, endothelial cells and certain epithelial cells[30].

This chemokine is reported to be strongly expressed by peripheral blood mononuclear cells of LGLL patients, together with other inflammatory cytokines, as MIP-1β, IL-18, IL-6, IL-15 and to a lesser extent IL-8 and IL-1Ra[31].

This pattern of cytokine up-regulation characterizes LGLL and can be found also in some chronic infections or autoimmune diseases, sustaining the hypothesis for which LGLL is characterized by a chronic inflammatory background.

Moreover, literature data report that among various human T-cell lines (those infected with HTLV-I) preferentially express CCL5, supporting the etiopathogenic hypothesis of a chronic antigenic stimulation by exogenous antigens, and that this expression is mediated in part by a transcriptional trans-activator of HTLV-1, Tax, via the activation of Nuclear factor-kB (NF-kB)[30].

Yang and colleagues described another mechanism of CCL5 production by NF-kB activation[32]: These Authors found that unphosphorylated STAT3 (USTAT3) binds to the NF-kB dimers p65/p50 in competition with IkB. The USTAT3/NF-kB complex translocates into the nucleus, binds to DNA, and activates NF-kB-regulated genes, among which CCL5. Therefore, CLL5 over-expression seen in patients' plasma could be explained by higher expression levels of USTAT3 in T-LGLL patients, due to the constitutively activated JAK/STAT axis.

4. SIGNALING PATHWAYS ACTIVATED IN LEUKEMIC LGLs

Signaling cascades serve several purposes: to carry a message from the cell surface into the nucleus, to amplify the signal and to regulate the cell in order to adapt to the environment. Doing so, signaling pathways do not work isolated, instead they are involved in dynamic and complex interactions.

As mentioned above, while normal activated CTLs readily undergo Fas-FasL-mediated apoptosis, leukemic LGLs are resistant to FasL-mediated apoptosis or AICD. This raises from two possible causes: (1) Fas-FasL apoptotic machinery is deficient in leukemic LGLs, or (2)

some constitutively survival signals keep leukemic LGLs alive despite of Fas-mediated death signals.

Starting from the actual knowledge that no mutations in Fas or FasL genes were found in LGLL[4], we can consider that some survival signals keep leukemic LGLs away from death.

There are several intracellular signaling pathways, having a cross-talk with the Fas-FasL pathway, that could be responsible for AICD escaping.

Concerning Fas-FasL pathway alteration, it was reported an abnormal DISC formation; indeed, higher basal levels of c-FLIP protein, a DISC inhibitor, were detected that contributes to the Fas-resistant phenotype in leukemic LGLs[4].

Many signaling pathways are deregulated in LGL leukemia (Figure 9)[9,33,34]:

• RAS/MEK/ERK: a constitutive active form of Ras (H-Ras-GTP) is expressed in PBMCs, promoting cell survival by directly inducing transcription of FLIP and myeloid cell leukemia sequence-1 (Mcl-1) genes;

• NF-kB: LGLs show constitutively active NF-κB, that induces the expression of Mcl-1 and c-Rel genes;

• PI3K/Akt: SFK maintains PI3K in its constitutively activated form, with consequent constitutive Erk activity. The result is abnormal DISC formation and Fas-resistance;

• JAK/STAT: STAT1 and STAT3 are constitutively activated and STAT3 induces the expression of Mcl-1 gene;

• Sphingolipid Rheostat: LGLs express abundant anti-apoptotic sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) and low levels of proapoptotic ceramide and sphingosine.

The signaling network model proposed by Zhang *et al*[34] suggests that the persistence of the cytokine IL-15 and platelet-derived growth factor (PDGF) is sufficient to reproduce all known deregulations in leukemic T-LGLs.

IL-15 alters the expression of Bcl-2 family members, i.e. Bcl-2, Bcl-XL, Bim, Noxa, and Mcl-1 and many of these genes result deregulated in T-LGLL, such as Bcl-2 related X gene (Bax), that is down-regulated, and Mcl-1, which is up-regulated (Figure 9)[34]. The deregulation of T-cell homeostasis due to the alteration of pro- and anti-apoptotic factors could be responsible for leukemic LGLs survival. In the present study a particular attention has been addressed to the deregulation of JAK/STAT pathway.

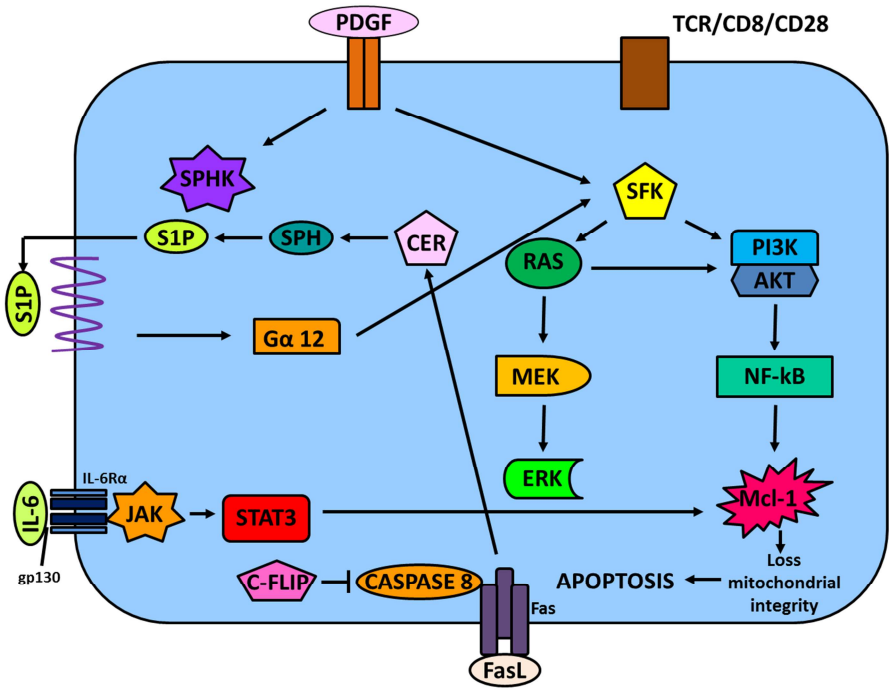


Figure 9: Various survival signaling pathways interact and cross-regulate each other at various levels. The dynamic interaction leads to survival of leukemic large granular lymphocytes.

4.1 THE JAK/STAT PATHWAY

JAK/STAT pathway is one of the few pleiotropic cascades used to transduce a multitude of signals for cell development and homeostasis. In mammals, JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors.

JAK activation stimulates cell proliferation, differentiation, migration and apoptosis, events that are critical to hematopoiesis, immune development, and other processes. Predictably, mutations that involve pathway activity affect these processes[35], for example, mutations that constitutively activate or fail to regulate JAK signaling properly, cause inflammatory disease, erythrocytosis and different forms of leukemia.

Following T-cell activation, the association of two receptor subunits' cytoplasmic domains with JAK tyrosine kinases is required (Figure 10).

Two kinase-homologous domains at the C-terminus characterize JAK kinases: one is a non-catalytic regulatory domain, whereas the other has tyrosine kinase activity.

Once ligand-receptor complex is formed, it triggers its own dimerization and recruits two JAKs, which are therefore brought into close proximity; this step allows their trans-phosphorylation and activation.

Once JAK kinases are activated, they phosphorylate both receptors and the main substrates, represented by STAT proteins, a family of transcription factors, which in turn can form homo- or heterodimers[9]. These proteins reside as latent transcription factors in the cytoplasm and once activated through phosphorylation and dimerization, they translocate into the nucleus and induce gene transcription (Figure 10).

In T-LGLL STAT3 is constitutively activated[36,37]. The activated form of STAT3 binds to the enhancer regions of target genes that are involved in apoptosis and cell proliferation, such as Bcl-xl, Mcl-1, IAP family, cell-cycle regulator cyclin D1, c-Myc, and vascular endothelial growth factor (VEGF)[38].

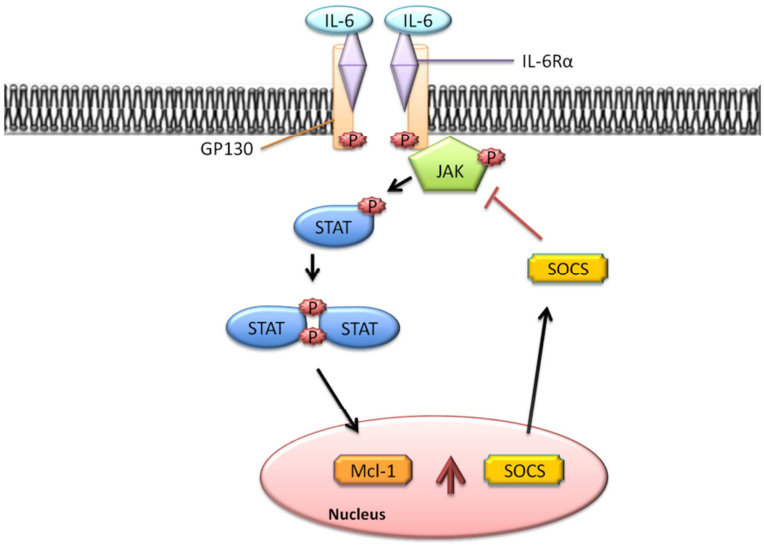


Figure 10: The IL-6/JAK2/STAT3 pathway. gp130 activation through binding of IL-6/IL-6Rα complex, mediates the activation of JAK protein that in turn phosphorylates STAT3. Once STAT3 is activated, it dimerizes and translocates into the nucleus to induce transcription of target genes like Mcl-1 and suppressor of cytokine signaling 3 (SOCS3).

The key role of STAT3 target genes requires a refined regulation mechanism, mainly carried on by SOCS proteins, a family of at least eight members containing a SH2 domain (phosphotyrosine binding), a SOCS box at the C-terminus (ubiquitin ligase binding) and a small kinase inhibitory (KI) region located N-terminal to the SH2 domain.

SOCS proteins complete a negative feedback loop in the JAK/STAT pathway: activated STATs stimulate transcription of SOCS genes and resulting SOCS proteins can affect their negative regulation by different means in order to turn off the pathway:

- by binding phosphotyrosines on the receptors, SOCS inhibits the recruitment of signal transducers, such as STAT proteins, to the receptor;
- SOCS can bind directly to JAKs or to the receptors to specifically inhibit JAK kinase activity.

The constitutive expression and activation of STAT1 and STAT3 by leukemic LGLs could be responsible of their survival; as a matter of fact, 2-cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide (AG490), an inhibitor of JAK2/3 and consequently of STAT1/3, can restore apoptosis and Fas sensitivity in LGLs.

In our lab we focused our attention on STAT3 protein. This protein contains about 750-850 amino acids and the domain structure comprises from N- to C-terminus: an oligomerization domain, the so-called coiled-coil domain, the DNA-binding domain in the center of the molecule, the linker domain, the SH2 domain that binds to a specific phosphotyrosine in a cytokine receptor's cytosolic domain and the trans-activation domain (TAD) with critical tyrosine and serine residues (Figure 11).

Unphosphorylated STAT3 represents the inactive form, that resides in the cytoplasm until activation occurs by transient association of STAT3 with cytokine receptors[39].

STAT3 recruitment to the activated JAKs has been shown to be mediated by SH2 domain and requires the phosphorylation of receptor tyrosine motifs, specifically tyrosine 705[39]. The phosphorylation activates STAT3, that can form homodimers or heterodimers and translocates to the nucleus, where it induces the expression of target genes.

After cytokine stimulation STAT3 is phosphorylated also on serine residue 727 within the trans-activation domain, although it is not clear what is the serine kinase involved in the process. Serine residue 727 phosphorylation seems unable to interfere with STAT3 tyrosine phosphorylation, while it regulates STAT3 transcriptional activity[40].

STAT3 specific inhibition, which could be performed by antisense oligonucleotides, has been shown to induce significant apoptosis and to restore Fas-sensitivity in leukemic LGLs. In particular, STAT3 inhibition correlates with a Mcl-1 decrease, indicating a role of this anti-apoptotic protein in LGLs survival[37].

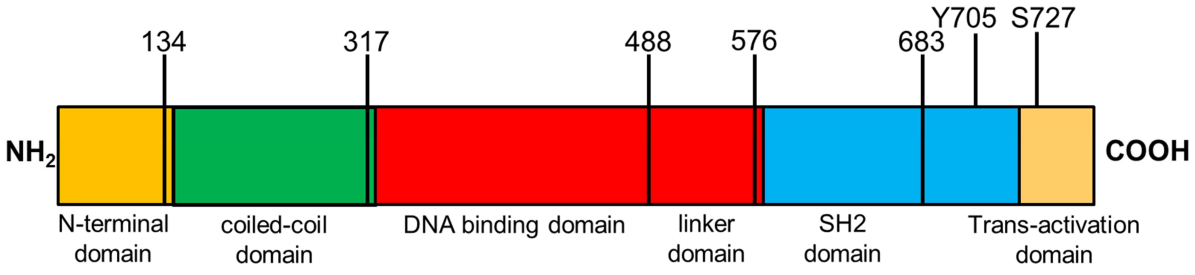


Figure 11: STAT protein structure. Abbreviations: Y, tyrosine; S, serine.

Recent data in the literature report mutational hot spots in SH2 domain of STAT3; it has been hypothesized that constitutive activation may result from increased stabilization of STAT3 homodimers or heterodimers through enhanced hydrophobic attraction between STAT monomers caused by STAT3 mutations[41,42]. We therefore analyzed whether patients' LGLs showed any of the STAT3 hot spot mutations above quoted.

AIM OF THE STUDY

The PhD project carried on different research lines with the aim to better understand the pathogenesis and the molecular mechanisms characterizing the development of T-LGLL.

T-LGLL is characterized by a chronic and clonal expansion of LGLs, sustained by a continuous cytokine stimulus and an impairment in the apoptotic machinery. This latter is due to the activation of several survival signaling pathways.

In our lab we focused on JAK/STAT pathway, since it is reported to be deregulated in T-LGLL, with a constitutive activation and over-expression of the transcription factor STAT3.

In order to explain this impairment we studied:

- SOCS3 expression and promoter region. This protein is STAT3 specific inhibitor, normally acting through a negative feedback mechanism. Being among STAT3 target genes, it is expected to be, in turn, over-expressed in T-LGLL.
- IL-6 levels and its receptor system to evaluate its involvement in the development of T-LGLL, considering that it is reported to be the main activator of JAK/STAT pathway.
- IL-15 levels and its effects on IL-6 signaling. IL-15, as IL-6, is an inflammatory cytokine and has a well defined key role in T-LGLL. It is mainly secreted by patients' cells belonging to the monocyte/macrophage lineage, among which DC, that are considered the responsible of LGLs antigen activation.
- STAT3 mutations in SH2 domain, since in some patients affected by T-LGLL, STAT3 hot spot mutations, inducing STAT3 activation, have been recently demonstrated.

Being bone marrow of patients frequently infiltrated by LGLs, we studied also:

- The role of CLL5 in LGL BM infiltration. CCL5 is induced by STAT3, is a strong chemotactic agent and an important mediator of acute and chronic inflammation, and is reported to be over-expressed in LGLL.
- BM-MSCs involvement in the survival maintenance and compartmentalization of leukemic LGLs, since these cells are considered a source of IL-6, the main activator of STAT3 signaling.

MATHERIALS AND METHODS

1. PATIENTS AND CONTROL DONORS

Sixty patients affected by T-LGLL were studied. Chronic peripheral blood lymphocytosis (lasting more than 6 months) was sustained by at least 2,000 LGLs/mm^3 (range: 25%-95% of lymphocyte pool)[43], with the phenotype CD3+CD57+CD8+CD16±CD56±. At the time of the study no patients had received treatment, with a follow up ranging from 1 to 16 years. In all patients clonality was demonstrated by molecular analysis of TCR gene rearrangement and often a prevalent expression of a discrete TCR Vβ region was established, according to the methods previously reported[44]. Twenty healthy donors were used as controls in all experiments performed, which were approved by the Institutional Review Board. All enrolled patients and controls provided written informed consent in accordance with the Declaration of Helsinki.

2. LYMPHOCYTES SEPARATION FROM PERIPHERAL BLOOD

Lymphocytes were isolated from peripheral blood of T-LGLL patients and healthy individuals. Mononuclear cells were obtained from samples of heparinized venous blood by *Ficoll-Hypaque* (F/H) (Amersham Biosciences; San Francisco, CA) density gradient centrifugation.

This is a simple and rapid method of purifying PBMCs that takes advantage of the density differences between mononuclear cells and other elements of the blood. Mononuclear cells and platelets collect on top of the F/H layer because they have a lower density. In contrast, red blood cells (RBC) and granulocytes have a higher density than F/H and collect at the bottom of the F/H layer.

Peripheral blood was first diluted in 1:3 ratio with 0.9% sodium chloride (saline) at room temperature, gently agitated, and later layered slowly over F/H solution. We proceeded with a centrifugation at 900g for 20 min at 20°C, without brake. The ring of mononuclear cells formed at F/H interface was aspirated and subjected to two subsequent washes with saline by centrifugation at 400g for 10 minutes at 20°C (Figure 13). The pellet was resuspended in an adequate amount of saline buffer; the cells were counted in a Burker chamber and used as desired.

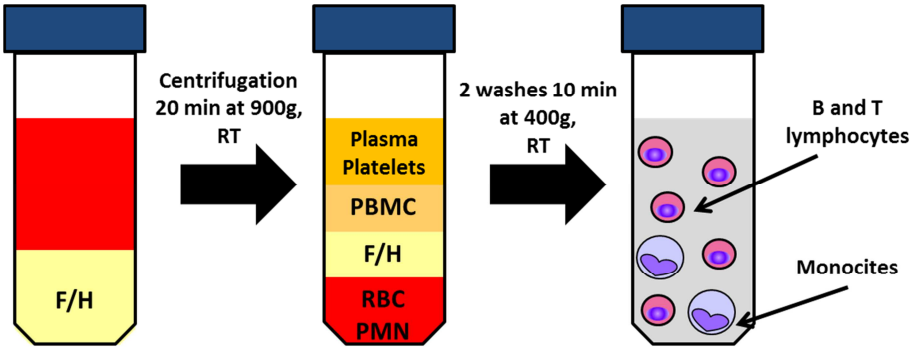


Figure 13: Isolation of mononuclear cells from peripheral blood by stratification on Ficoll/Hipaque. By centrifugation on F/H, mononuclear cells were isolated from peripheral blood. Mononuclear cells and platelets were concentrated above the layer of F/H because they have lower density; on the contrary, the red blood cells (RBC) and granulocytes (PMN) have a higher density than the F/H and collect on the bottom of the tube. RT: room temperature.

3. T-LGLs SORTING AND FLOW CYTOMETRY

The immunophenotypic analysis on lymphocytes obtained from peripheral blood of T-LGLL patients was performed through flow cytometry. This technique allows a multiparametric evaluation of antigenic characteristic of single cells by the analysis of visible and fluorescent light they emit when flow through a liquid medium.

The immunophenotyping is based on the identification of surface and intracellular Ag using monoclonal antibodies (moAbs) conjugated with fluorochromes. The presence of a certain Ag is revealed and used as an indicator of belonging to a cell line and of cell level maturation.

Target cell population used in this thesis was detected by fluorochromes-conjugated moAbs (Becton Dickinson, Sunnyvale, CA, USA) anti-CD3, anti-CD16, anti-CD56, anti-CD57 and anti-CD8.

Analysis was performed by flow cytometer FACSCalibur (Becton Dickinson) and data obtained were processed by Macintosh CELL Quest software program (Becton Dickinson). For each analysis at least $1x10^4$ events were acquired and analysis was performed overlapping samples' histograms (obtained with different antibodies) with isotypic control (fluorescein isothiocyanate (FITC)-conjugated IgG1 antibody) histogram.

LGLs from patients and CTLs from controls (the putative normal counterpart of pathologic LGLs, represented by CD8+CD57+ cells) were obtained by sorting process, based on recognition of specific surface antigens.

An antibody conjugated with FITC fluorochrome was incubated with a mononuclear cells quantity between 30 and $125x10^6$ and diluted in phosphate buffered saline (PBS) for 20 minutes at 4°C. IgG1 FITC-conjugated antibody was used as isotypic control.

Antibody treated cells were then subjected to two washes and resuspended in PBS-EDTA (Ethylenediaminetetraacetic acid). Antibody conjugated cells were then isolated from total cell population by FACSAria cell sorter (BD Biosciences, San Jose CA, USA), with a large band-pass filter at 530 nm and Argon laser (488 nm, 100mW) that excited FITC fluorochrome. For each analyzed sample 5,000 events/sec had been acquired.

In order to minimize the possibility of nozzle clogs, the sample had to be put through a *40 micron* cell strainer before sorting. After filtering, the cells should be kept on ice and protected from light. To avoid clumps formation, for really sticky cells another filtering step before sorting was required. Purity and vitality of obtained cells were then analyzed, and resulted always higher than 95%.

Cells were then employed for total RNA extraction.

4. T-LGLs PURIFICATION BY IMMUNOMAGNETIC BEADS

Alternatively to sorting technique, especially for low cellularity samples, T-LGLs from patients were obtained using magnetic separations over columns (MACS; Miltenyi Biotec, Auburn, CA). This technique employs superparamagnetic particles coupled to highly specific monoclonal antibodies, in our specific case CD57, which magnetically labels target cell population.

Thanks to their small size, approximately 50 nm, microbeads, which are also biodegradable, do not activate cells and do not saturate cell surface epitopes.

In detail, PBMCs obtained from F/H separation were centrifuged at 300g for 10 minutes; after removal of supernatant, pellet was resuspended with purification buffer (PBS 1X, BSA (Bovine Serum Albumine) 0.5%, EDTA 2 mM). MACS microbeads were added to cell suspension (80 µl buffer and 20 µl of microbeads for 10^7 cells) and incubation at 4-8 °C was performed for 15 minutes. A washing step was performed by adding 1-2 mL of buffer per 10^7 cells and after a centrifuge step at 300g for 10 minutes, cells were placed onto specific magnetic columns (MACS) placed on a MACS magnetic separator; this amplifies the magnetic field by 10,000-fold, therefore inducing a high gradient within the column.

Magnetically labeled cells were retained within the column, unlabeled cells flown through. After a washing step, the column was removed from the magnetic field of the separator, and the target cells were eluted from the column, washed with physiologic solution, counted and used for experiments.

5. CELL CULTURES

LGLs purified from patients or CTLs from controls were cultured at $2x10^6$ cells/ml in RPMI-1640 medium (Euroclone) containing L-glutamine (2mM) and HEPES (25mM) (Gibco Laboratories, Grand Island, NY, USA) added with FCS (Fetal Calf Serum) at a concentration of 0,5%. Penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco Laboratories, Grand Island, NY, USA) were added to the medium at a concentration of 1%.

Cells were then incubated at 37°C with humidity and 5% CO_2.

All cell treatments were performed under a laminar flow cabinet (Biohazard). We stimulated LGLs and PBMCs from patients with IL-6 (10 ng/ml) and IL-15 (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) and we evaluated STAT3, pSTAT3, levels at 24 and 48 hours.

The effect of IL-6 triggering on LGLs or CTLs culture was studied by incubation with 10 ng/ml IL-6 to observe STAT3 activation, SOCS3 and CCL5 expression after 1 hour, Mcl-1 expression up to 6 hours and LGL apoptosis up to 7 days. Except for CCL5 the same analysis were performed following 96 hours cells treatment with DAC (5 μM).

To inhibit STAT3 phosphorylation LGLs were cultured with AG490 (50 μM) (Calbiochem, Darmstadt, Germany), a specific inhibitor of Jak2.

To neutralize IL-6 action, PBMCs were cultured in the presence of a anti-IL-6 or anti-IL-6Rα (2 μg/ml) antibodies; a mouse IgG1 antibody was used as a control (2 μg/ml).

Finally we used IL-6 (10 ng/ml) and IL-15 (10 ng/ml) on CD57- cells from patients and PBMCs from controls to observe IL-6 and IL-15 expression at 24 h and 48h, and we use these cytokines also on patients' CD57+ cells and controls' CTLs to observe IL6Rα expression at 24h and 48h.

6. MOLECULAR ANALYSIS

6.1 TOTAL RNA EXTRACTION

Total RNA was extracted from peripheral blood T lymphocytes by "Rneasy Mini" kit (QIAGEN, Hilden, Germany) according to manufacturer's instruction.

Lymphocytes were lysed using a solution containing β-mercaptoethanol and lysis buffer; 70% ethanol (usually 350 μl or 600 μl) then was added according to product user manual, and samples were transferred to a purification column (supplied by the kit) and then centrifuged at 10,000 rpm for 15 seconds, followed by elute removal.

After the addition of 350µl of RW1 buffer (supplied by the kit), samples were centrifuged again at 10,000 rpm for 15 seconds and the elutes were discarded. Then we proceeded with 20 minutes of incubation with 80µl of a DNase I solution (QIAGEN). After having washed with 350µl of RW1 buffer, followed by centrifugation at 10,000 rpm for 15 seconds, the collector tube was replaced. 500µl of RPE buffer (supplied by the kit) were added, the samples were centrifuged at 10,000 rpm for 15 seconds, and the elutes were discarded. Again, 500µl of RPE buffer were added and a centrifugation at 15,000g for 2 minutes was carried out. Columns were then transferred to 1.5ml tubes, 30µl of H_2O- Rnase free (supplied by the kit) were added on the membrane, and purification was performed by centrifugation at 10,000 rpm for 1 minute in order to dissociate the RNA from the membranes and bring it into solution.

The extracted RNA was then quantified using a spectrophotometer Ultrospec 1100 pro (Amersham Biosciences). RNA samples were diluted 1:70, their absorbance (A) at wavelengths of 260nm (λ nucleotides) and 280nm (λ proteins) was determined, and A260/A280 ratio was calculated. Values less than 2 indicate a protein contamination of RNA preparation.

RNA concentration was finally calculated using the following formula:

A260 x dilution factor x 40 = RNA concentration in µg/ml.

The value of 40 corresponds to the concentration of RNA, expressed in µg/ml, when obtained A260 is 1.

6.2 cDNA SYNTHESIS

The reverse transcription from mRNA to cDNA (complementary DNA) uses a reverse transcriptase (RT), which generates a cDNA strand from a template of mRNA.

In this thesis, total RNA has been used as template and the reaction was performed in the presence of an oligo-dT primer, which hybridizes to the poly(A) tail of mRNA. The RT used is the Myeloblastoma Avian Virus (AMV) polymerase (Promega, Madison, WI, USA); this enzyme has 5'→3' activity (with RNA or DNA as a template) and a 3'→5' RNase H activity, which degrades the RNA filament of the double helix DNA-RNA, produced during cDNA synthesis. This latter was performed by the "Reverse Trascription System" kit (Promega Corporation; Madison, WI).

The reaction mixture is composed of:

- 4 µl of 25mM $MgCl_2$
- 2 µl of 10X RT buffer

- 2 µl of the mixture of the 4 deoxynucleosides triphosphates (dNTP mix) 10mM
- 0.5 µl of an enzyme inhibitor of RNase 40 µg/µl
- 0.6 µl of AMV RT 25 µg/µl
- 1 µl of primer Oligo (dT) 0.5 mg/ml
- 1 µg of RNA
- RNase-free H_2O to final volume of 20 µl

Before the additon of RT reaction mixture, tubes containing template RNA were placed into a pre-heated 70°C heat block for 5 minutes. After this initial step, mixture containing RNA was incubated at 42°C for 15 minutes, then placed at 95°C for 5 minutes (to stop the RT reaction) and, finally, put on ice for a few minutes. Thus, the cDNA obtained was stored at -80°C or used immediately to set up the amplification reaction by means of Real Time -polymerase chain reaction (RT-PCR).

6.3 REAL TIME PCR

RT-PCR is a technique commonly used in molecular biology to detect the amplification of cDNA and therefore RNA expression levels. It's based on the direct correlation between fluorescence emission and cDNA amplification.

In our lab, each sample was amplified in duplicate and genes of interest were STAT3, IL-6, IL-6Rα, IL-15, CCL5, Mcl-1 and gp130 besides the housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), which is expressed constitutively in all cells and acts as normalizer. For each gene, RT-PCR was also performed on a negative control.

The amplification of the genes of interest were obtained using ABI PRISM 7000 sequence detection system (Applied Biosystems; Foster City, CA) in a volume of 15µl. For each sample a reaction mix was used, made with:

- 1,5 µl cDNA
- 4,2 µl H_2O without RNAse
- 0,9 µl primer Forward (Fw) 5 µM (Sigma)
- 0,9 µl primer Reverse (Rev) 5 µM (Sigma)
- 7,5 µl SYBR Green PCR Master Mix 2X (Applied Biosystems), which contains Taq polymerase, A,C,G,U nucleotides, reaction buffer, magnesium chloride, uracil-N-glycosylase (UNG) and SYBR Green (Applied Biosystems).

The primers used for amplification of gene of interest are reported in Table 2 and were designed in our lab, except for STAT3, for which we used the primers as described in Haider *et al*[45].

For the reaction, an initial step at 50°C for 2 minutes is required in order to activate UNG enzyme, which breaks down possible contamination derived from previous reactions, that show uracil (U) in place of thymidine (T)

After this step, reaction conditions were:

- Denaturation at 95°C for 10 minutes
- Annealing at 60°C for 15 seconds
- Extension at 72°C for 1 second

} For 45 cycles

A dissociation curve to distinguish specific from nonspecific amplification was also generated.

Threshold cycle (Ct) was acquired for each sample and starting quantities (Co) of each sample were obtained with Ct method, which is based on the following equation:

$$Co=2^{-\Delta\Delta Ct}$$

where ΔCt represents the difference between target gene Ct and housekeeping gene Ct, and ΔΔCt is obtained by subtracting sample ΔCt from calibrator ΔCt.

Calibrator is a sample used as reference which is included in every reaction plate in order to compare different plates' results.

Table 2: Primers sequences for RT-PCR.

PRIMER	SEQUENCE
IL-6 Fw	5'-GGCACTGGCAGAAAACAACCTG-3'
IL-6 Rev	5'-TCACCAGGCAAGTCTCCTCATTGAAT-3'
SOCS3 Fw	5'-CAGCTCCAAGAGCGAGTACCA-3'
SOCS3 Rev	5'-AGAAGCCGCTCTCCTGCAG-3'
STAT3 Fw	5'-AGGAGGAGGCATTCGGAAA-3'
STAT3 Rev	5'-AGCGCCTGGGTCAGCTT-3'
IL-6Rα Fw	5'-GGCTGAACGGTCAAAGACATTC-3'
IL-6Rα Rev	5'-CGTCGTGGATGACACAGTGA-3'
gp130 Fw	5'-GTACATGGTACGAATGGCAGCATAC-3'
gp130 Rev	5'-TCCTTGAGCAAACTTTGGGGTAG-3'
GAPDH Fw	5'-AATGGAAATCCCATCACCATCT-3'
GAPDH Rev	5'-CGCCCCACTTGATTTTGG-3'
IL-15 Fw	5'-AGTGATGTTCACCCCAGTTGC-3'
IL-15 Rev	5'-TGCATCCAGATTCTGTTACATTCCC-3'
CCL5 Fw	5'-TCTGCCTCCCCATATTCCTCGG-3'
CCL5 Rev	5'-GGCGGTTCTTTCGGGTGACAAAG-3'
Mcl-1 Fw	5'-GAAAGTATCACAGACGTTCTCGTAAGG-3'
Mcl-1 Rev	5'-AACCCATCCCAGCCTCTTTG-3'

6.4 DNA EXTRACTION

Genomic DNA was extracted by "Genomic DNA Purification kit" (Gentra, MN, USA), from at least $1x10^6$ to $20x10^6$ PBMCs or CD57+ cells, obtained from peripheral blood.

According to manufacturer's instruction, cells were lysed using the Cell Lysis Solution. RNAse A solution was added in order to eliminate RNA eventually contained in the sample; an incubation at 37°C for 15-60 minutes was then performed.

Hence RNase A treated cell lysate was cooled to room temperature by placing on ice and Precipitation Solution was added. After a centrifugation step the precipitated proteins formed a tight pellet.

The supernatant containing the DNA was poured into a clean tube containing 100% isopropanol (2-propanol) and the solution was mixed by inversion in order to facilitate DNA precipitation. After a centrifugation step the DNA was visible as a small while pellet.

Surnatants had to be poured off and 70% Ethanol was added to the tube to wash the DNA pellet.

After a centrifugation step ethanol was poured off and DNA Hydratation Solution was added to rehydrate DNA.

Rehydrated DNA was then store at 4°C, or at -20°C/-80°C for long-term storage

Quantification by spectrometer was finally required in order to obtain DNA concentration and purity.

6.5 DNA MODIFICATION AND METHYLATION-SPECIFIC PCR (MSP)

DNA extracted was then modified by sodium bisulfite, converting only unmethylated cytosines to uracil, as described by Herman *et al*[46].

The "Methylamp™ DNA Modification Kit" has been used in order to perform bisulfite conversion and converted DNA was used as template for MSP as described[47], using either a methylation- or unmethylation-specific primer set.

The sequence amplified was from nucleotides –525 to –384 of the SOCS3 promoter region, defining the start codon ATG as +1.

Methylation- and unmethylation-specific primers are reported in Table 3.

Table 3: Primers sequences for MSP.

PRIMER	SEQUENCE
Methylation-specific Fw	5'-GGAGATTTTAGGTTTTCGGAATATTTC-3'
Methylation-specific Rev	5'-CCCCCGAAACTACCTAAACGCCG-3'
unmethylation-specific Fw	5'-GTTGGAGATTTTAGGTTTTTGGAATATTTT-3'
unmethylation-specific Rev	5'-AAACCCCCAAAACTACCTAAACACCA-3'

PCR products were analyzed in 2% agarose gel stained with Syber Safe (Invitrogen, Paisley, UK) and visualized under ultraviolet illumination in order to detect DNA samples containing a methylated SOCS3 promoter region.

6.6 BISULFITE SEQUENCING

Bisulfite-modified DNA was amplified using primers recognizing SOCS3 promoter region from nucleotide –704 to –186 (forward: 5'-GATTTGAGGGGGTTTAGTTTTAAGGA-3' and reverse 5'-CCACTACCCCAAAAACCCTCTCCTAA-3') as reported by Isomoto *et al*[48].

The PCR products were cloned into the PCR II vector in accordance with manufacturer's instruction employing the "TOPO-TA cloning kit" (Invitrogen).

Plasmid DNA purified from 5 randomly picked clones using the “Plasmid miniprep kit” (Bio-Rad Laboratories, Hercules, CA) were obtained and prepared for automated DNA sequencing analysis. The reaction conditions were as follows: 96°C 10 seconds, 50°C 5 seconds, 60°C 4 minutes, for 25 cycles.
DNA was sequenced using dye terminator technology and an ABI 3130 sequencer (Applied Biosystems), which is based on Sanger sequencing

6.7 SCREENING OF SOCS3 AND STAT3 MUTATIONS

To sequence SOCS3 gene we used primers designed to cover the entire coding region (678 bp) as reported by Cho-Vega *et al*.[49]:
upstream primer 5'-CATGCCCTTTGCGCCCTT
downstream primer 5'-AGATCCACGCTGGCTCCGT,
For the screening of STAT3 mutations D661V, D661Y, D661H, Y640F, N647I and K658N, recently described by Koskela *et al*.[41], we constructed the same set of primers to amplify the exon 21, where all the mutations are located. Template DNA was obtained from patients’ purified LGLs and remaining autologous PBMCs. As healthy control we used DNA obtained both from CD8+CD57+ cells and PBMCs of buffy coats.
DNA was sequenced as described in the previous paragraph.
The presence of D661Y and Y640F mutations, non-detectable by direct sequencing due to the low sensitivity of the method (reaching 25% of positive cells) was also analyzed by a DNA tetra-primer amplification refractory mutation system (ARMS) assay, as reported by Jerez *et al*[42].

6.7.1 Sanger Sequencing

Sanger Sequencing is a multi-step process, consisting of:

- PCR of STAT3 gene exon 21, located on chromosome 17
- Control PCR employing an agarose gel at 2%
- Clonewell based purification/ purification from gel
- Cycle sequencing, using dye terminator technology
- Column-based purification of cycle sequencing products
- Sanger-sequencing.

- **Exon 21 PCR**

STAT3 gene exon 21 PCR produces high amounts of exon 21 copies that can be used for mutation analysis. For each sample a reaction mix was used, made with:

- 27.5 µl H_2O
- 5 µl Buffer 10X
- 3 µl $MgCl_2$ 25mM
- 4 µl dNTPmix 10mM
- 4 µl Primer Fw 20pM
- 4 µl Primer Rev 20pM
- 0,5 µl TaqGold 1U/ul

Mix were split in aliquots of 48µl that were distributed in PCR test tubes. 2µl of sample's concentrated DNA were then addicted.

Test tubes were finally centrifugated at 4°C in a mini-centrifuge and put in thermal cycler.

The reaction conditions were as follows:

- Denaturation at 94°C for 10 minutes
- Denaturation at 94°C for 30 seconds
- Annealing at 60°C for 1minute and 30 seconds
- Extension at 72°C for 1 minute

} For 35 cycles

- Extension at 72°C for 10 minute
- Rapid thermal ramp to 12 °C and hold

- **Control of PCR products employing an agarose gel**

PCR products were analyzed with a 2% agarose gel, addicted of 5% Syber Safe.

5µl of each sample and 2µl of molecular weight (100 bp) were loaded on gel and electrophoretic run was performed. Finally gel analysis and acquisition were performed with ChemiDoc (Bio-Rad,USA) strumentation and software.

- **Clonewell based purification**

Exon 21 PCR products were purificated by Clonewell procedure, that requires E-Gel® CloneWell™ 0.8% SYBR Safe™ gels (Invitrogen life technologies, California, USA) and the

E-Gel® iBase™ Power System (Invitrogen). This system allows the extraction of DNA bands of interest from the gel without any additional purification steps.

- **Purification from gel**

Alternatively to Clonewell, purification of Exon 21 PCR products can be performed by purification from gel.
QIAquick Gel Extraction Kit (QIAGEN Duesseldorf, Germany) was used. This protocol is designed to extract and purify DNA of 70 bp to 10 kb; up to 400 mg agarose can be processed per spin column.
Once agarose gel was placed on trans illuminator, the band of interest became visible and could be excised with a clean, sharp scalpel; the gel slice was putted inside a sterile and colorless 1.5 ml tube and weighed. According to manufacturer's instructions, 3 volumes of Buffer QG were added to 1 volume of gel (100 mg ~ 100 µl), followed by incubation at 50°C for 10 min (or until the gel slice has completely dissolved). The tube was vortexed every 2–3 min to help dissolve gel.
To increase the yield of DNA fragments <500 bp and >4 kb, 1 gel volume of isopropanol was added to the sample, and the resulting mix was applied to a QIAquick spin column placed in a provided 2 ml collection tube; followed a centrifugation step for 1 minute.
Flow-through was discarded and QIAquick column was placed back in the same collection tube. A wash step was performed adding 750 µl of PE Buffer and performing centrifuge for 1 minute.
After flow-through elimination an additional centrifuge was performed for 1 minute. Column was the applied to a 1.5 ml collection tube and 30 µl of EB Buffer (10 mM Tris-Cl, pH 8.5) were added in the middle of the column. After an incubation of 1 minute at room temperature, 1 minute centrifuge was performed and column eliminated; the remained tube containing purificated DNA sequence was conserved at 4°C.

- **Cycle Sequencing**

Cycle sequencing protocol allows the separation of already purified amplified DNA fragments for sequencing process. This method exploits the repetition of heat denaturation, alignment and extension cycles, to increase the signal level starting from low DNA template quantities.

The technique, based on Sanger sequencing, employs a PCR reaction that synthesizes new strands of DNA complementary to a single-stranded template. The template DNA is supplied with a mixture of all four deoxynucleotides, four dideoxynucleotides (ddNTPs)-each labeled with a different color fluorescent tag, and DNA polymerase. Beacause of this feature, this sequencing technique is called dye terminator sequencing.

Dideoxynucleotides are chain-terminating inhibitors of DNA polymerase; the absence of the 3'-hydroxyl group (-OH) means that, after being added by a DNA polymerase to a growing nucleotide chain, no further nucleotides can be added as no phosphodiester bond can be created, leading to the termination of the DNA sequence.

During PCR, as all four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a dideoxynucleotide. The result is a new set of DNA chains all of different lengths. The fragments are then separated by size using gel electrophoresis.

As each labeled DNA fragment passes a detector at the bottom of the gel, the color is recorded. The DNA sequence is then reconstructed from the pattern of colors representing each nucleotide in the sequence.

Two reaction mix, one with primer Forward (Fw) and one with primer Reverse (Rev), were set up for sequencing template forward and reverse strands respectively. Each mix contains:

- Terminator Ready MIX 8 µl
- Primer Fw o Primer Rev (3,2pM/ul) 1 µl
- H_2O sterile 8 µl

Kit ABI PRISM BigDye terminator v1.1 Cycle Sequencing Kits (Applied Biosystems) provides both Terminator Ready MIX and Primers.

Terminator Ready MIX contains: 4 different BigDye Terminator (labeled ddNTPs), DNA polymerase AmpliTaq, $MgCl_2$, Tris-HCl Buffer (pH 9.0).

Employed enzyme is a variant of DNA polymerase Taq; indeed a point mutation is located in the active site of the enzyme and determines a less effective discrimination towards ddNTPs.

Moreover the enzyme has another mutation in the amino terminus domain that apparently eliminates the 5'→3' exonuclease activity of AmpliTaq DNA polymerase.

Two 0.2 ml collection tubes were provided for each samples, one tube for Forward strand and the other tube for Reverse strand. In each tube, 9 µl of MIX and 11 µl of diluted sample DNA were applied.

DNA is diluted according to the intensity of bands during CloneWell or gel excision.

The more visible and therefore concentrated is the band, the less DNA is required; indeed, high DNA concentrations interfere with sequencing process.

Collection tube with diluted DNA were finally placed in a thermal cycler, set up for the cycle-sequencing.

The reaction conditions were as follows:

- Denaturation at 96°C for 1 minute
- Denaturation at 96°C per 10 seconds
- Annealing at 50°C for 5 seconds
- Extension at 60°C for 4 minutes

} For 25 cycles

- Rapid thermal ramp to 4 °C and hold until ready to purify.

- **Column purification**

After Cycle Sequencing reaction, fast and easy removal of unincorporated dye terminators and primer dimers is performed; therefore reaction products could be analyzed with a DNA sequencer.

The DyeEx 2.0 Spin Kit (QIAGEN) was employed. Spin columns supplied by the kit use gel-filtration technology.

To resuspend the resin, the columns were gently vortexed. The caps of the columns were losen a quarter turn to avoid a vacuum inside the spin columns.

The bottom closures of the columns then were snapped off and each column was placed in a 2 ml collection tube (supplied by the kit). Followed a centrifugation step at 700 x g for 3 minutes.

Each spin column was carefully transferred to a 1.5 ml clean centrifuge tube and the sequencing reaction was slowly applied directly onto the center of slanted gel bed surface. Followed another centrifuge step at 700 x g for 3 minutes, and spin columns were removed from the microcentrifuge tubes. The eluates contained the purified DNA, ready for the sequencer.

- **Sanger Sequencing**

Sanger sequencing is the first automated method of DNA sequencing based on the selective incorporation of chain-terminating ddNTPs by DNA polymerase during *in vitro* DNA replication. The method produces a DNA “ladder” of fragments that differ in length by one base and bear a specific ddNTP chain terminator, each of which emit light at different wavelengths.

Starting from low quantities of DNA, the method can sequence DNA fragments 400-1,000 bp long, sorting fragment by length and detecting the fluorescence signal.
Cycle Sequencing reaction products were loaded in the sequencer ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), by following these steps:

- 0.5 ml capless tubes were labeled with numbers assigned and 25 µl of TSR (template suppression reagent) were added to each DNA sample. Tubes were vortexed and pulsed down briefly.
- Samples were placed in the thermocycler at 95°C for 2 minutes to obtain DNA denaturation, then were placed immediately on ice until their use.

TSR is used to prevent clogging of the capillary with DNA template during injection.
The sequencer employs a 16-capillary electrophoresis and is provided with a laser that allows multiple colour detection of laser-induced fluorescence.
Samples were applied in capillaries filled with a polymer (POP7, Applied Biosystems) and after an electrophoretic run they are separated according to their molecular weight.
Light emitted by labeled ddNTPs is collected and, according to wavelenght, separated by a spectrophotometer. Obtained data were analyzed by "sequencing analysis" software, which elaborates fluorescence signals collected by the sequencer and creates electropherograms.

6.7.2 ARMS-PCR

The amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base changes or small deletions. ARMS is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. The method allows the identification of sequencing refractory mutations, which are mutations "invisible" at Sanger sequencing analysis.
In our lab we performed tetra-primer ARMS-PCR for the detection of D661Y and Y640F mutations, reported to be the more frequent mutations in T-LGLL.
Two PCR reaction tubes containing the same DNA sample were respectively added with primers designed for the amplification of mutated DNA in one reaction tube and for the amplification of non mutated DNA in the other reaction tube.
DNA dilution was requested before PCR in order to obtain the final DNA concentration of 100 ng/µl. Finally, different reaction mix were set up for each mutation of interest (Table 4), and reaction conditions were optimized.

Table 4: Reagents' quantities in each ARMS-PCR mix.

	Mix for D661Y wt	Mix for D66Y mut	Mix for Y640F wt	Mix for Y640F mut
Sterile H_20	11.5 µl	12.75 µl	11.5 µl	12.75 µl
10X Buffer	2.5 µl	2.5 µl	2.5 µl	2.5 µl
$MgCl_2$ 25 mM	1.5 µl	1.5 µl	1.5 µl	1.5 µl
dNTPs mix 10 mM	2 µl	2 µl	2 µl	2 µl
Fw outer	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Rev outer	2.5 µl	-	2.5 µl	-
Fw inner	1.25 µl	-	1.25 µl	-
Rev inner	-	2.5 µl	-	2.5 µl
TaqGold 1U/µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl

24 µl of each mix and 1 µl of diluted DNA were added to sterile reaction tubes to obtain a final volume of 25 µl. After a brief pulse down, samples are loaded in the thermocycler. Polymerization programs differ, according to searched mutations, for the annealing temperature, which depends on nucleotide composition and on primers length (Table 5).

Thermocycler amplification programs were the following:

D661Y:

- Denaturation at 95°C for 5 minutes
- Denaturation at 94°C for 30 seconds
- Annealing at 58°C for 30 seconds
- Extension at 72°C for 1 minute

} For 35 cycles

- Extension at 72°C for 10 minutes
- Rapid thermal ramp to 4 °C and hold

Y640F:

- Denaturation at 95°C for 5 minutes
- Denaturation at 94°C for 30 seconds
- Annealing at 58°C for 30 seconds
- Extension at 72°C for 1 minute

} For 35 cycles

- Extension at 72°C for 10 minutes
- Rapid thermal ramp to 4 °C and hold

Amplifications products then were loaded on an 3% w/v agarose gel with 5% v/v Syber Safe. Electrophoretic run was performed at a gel length dependent voltage for 1 hour and a half. 2 µl of molecular weight (Invitrogen, Life Technologies) were employed.

Gel imaging and analysis then were performed with ChemiDoc XRS (Bio-Rad).

Table 5: Primers sequences for D661Y and Y640F mutations' ARMS-PCR

PRIMER	SEQUENCE
Primer D661Y Fw outer	5'-CCTAGCTGTAGGTTCCATGATCTTTCCT-3'
Primer D661Y Rev outer	5'-AAAATTAAATGCCAGGAACATGGAAAAT-3'
Primer D661Y Fw inner	5'-AAATCATCATGGGCTATAAGATCACGG-3'
Primer D661Y Rev inner	5'-GGAGACACCAGGATATTGGTAGCGTA-3'
Primer Y640F Fw outer	5'-AAAAAATGGGCAGTTTCTCTGAGATGACC-3'
Primer Y640F Rev outer	5'-CCAGTGGAGACACCAGGATATTGGTAGC-3'
Primer Y640F Fw inner	5'-ACCCAGATCCAGTCCGTGGAACCTTA-3'
Primer Y640F Rev inner	5'-ACATGTTGTTCAGCTGCTGCTTTGAGA-3'

Tetra-primer ARMS PCR uses in a single PCR reaction four primers, one pair of inner allele-specific primers and one pair of outer standard primers.

While the forward and reverse outer standard primers bind the DNA template at 3'- and 5'-terminal, the forward and reverse inner primers selectively bind the wild-type and the mutant allele, respectively.

The specificity of inner primers is conferred by the identity of the terminal 3' nucleotide with either the wild-type or the mutant allele, and is increased by the introduction of a deliberate mismatch at position -1 from the 3'-terminus.

Reverse inner primer can bind the template only when mutation occurs, while forward inner primer binds only to wild type sequence; so, when the mutation occurs, a percentage of forward outer primer in employed by PCR reaction with reverse inner primer, while when template shows the wild type sequence, a percentage of inner outer primer is employed in concert with forward inner primers. Tetra-primer PCR therefore produce not only amplicons with outer primers, but also mutation-specific amplicons.

By positioning the two outer primers at different distances from the polymorphic nucleotide, the PCR products differ in length (Table 6), allowing them to be discriminated by gel electrophoresis.

Table 6: Primers combinations and amplicons length for D661Y and Y640F tetra-primer ARMS-PCR.

MUTATION	SEQUENCE	PRIMERS	AMPLICON LENGHT
D661Y	Wt/mut	Fw outer D661Y + Rev outer D661Y	290bp
	wt	Fw inner D661Y + Rev outer D661Y	182bp
	mut	Rev inner D661Y + Fw outer D661Y	161bp
Y640F	Wt/mut	Fw outer Y640F + Rev outer Y640F	197bp
	wt	Fw inner Y640F + Rev outer Y640F	119bp
	mut	Rev inner Y640F + Fw outer Y640F	131bp

7. PROTEIC ANALYSIS (WESTERN BLOT)

7.1 PREPARATION OF CELL LYSATES

For each sample aliquots of 250,000 and 500,000 LGLs or PBMCs from patients and CTLs from controls were lysed in 50µl of the following buffer:

- 50mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) pH 6.8;
- 5mM Ethylenediaminetetraacetic acid (EDTA);
- 10% glycerol;
- 2% SDS (sodium dodecyl sulphate);
- 1% β-mercaptoethanol;
- Dye (Bromophenol blue or pyronin) (Sigma-Aldrich; Milan, IT).

Subsequently, the lysates were vortex, boiled at of 100°C for 5 minutes and then subjected to SDS-PAGE (polyacrylamide gel electrophoresis).

7.2 POLYACRYLAMIDE GEL ELECTROPHORESIS IN SDS (SDS-PAGE)

The polyacrylamide gel electrophoresis in SDS is one of the methods used to separate a mixture of proteins on the basis of their molecular weight.

SDS is a ionic detergent that binds tightly to proteins causing their denaturation; in the presence of an excess of SDS, approximately 1.4 g of detergent will bind to each gram of protein, providing a constant amount of negative charge per unit mass. Therefore, during electrophoresis, all protein-SDS complexes move toward the anode, and thanks to the molecular sieve properties of the gel, their mobility is inversely proportional to their molecular weight. By the migration of standard proteins of known molecular weight simultaneously to samples, it is possible to determine the protein sample weights.

SDS polyacrylamide gel was prepared following Laemmli method, for which the electrophoretic plate consists of two types of gel:

- *Stacking gel* at pH 6.8, which concentrates the protein samples so that they are all aligned at the start of electrophoresis;
- *Running gel* at pH 8.8, in which the real separation of proteins occurs.

The plate size of 10×8 cm was fixed in the Hoefer Mighty Small-If 250 Scientific Instruments machine (Amersham Biosciences). The electrophoresis was run for about 2 hours at 25mA.

7.3 WESTERN BLOTTING

The western blotting (WB) or immunoblotting is an immunoassay able to detect traces of a specific protein in a heterogeneous mixture, combining the high resolving power of gel electrophoresis with the specificity of the antibodies. The WB is a technique with high sensitivity, able to detect quantities of protein in the order of nanogram.

After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane by the action of an electric field, obtained by applying the appropriate current of 350 mA for 2 hours and 30 minutes. The buffer used for the transfer consisted of: 25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS with a final pH of 8.0 (Buffer A). After the transfer, the membrane was left overnight in the saturation buffer consisting of 50mM Tris-HCl, pH 7.5, 150mM NaCl and 5% bovine serum albumin (BSA) (BufferB), for nonspecific sites saturation. This step was followed by incubation for 2 hours and 30 min at room temperature of the primary antibodies (Abs), diluted in: 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% BSA (buffer C).

For our study we used the following Abs: rabbit monoclonal anti-P-STAT3 Tyr XP (Cell Signaling; Beverly, Massachusetts); rabbit monoclonal anti-total STAT3 (Cell Signaling; Beverly, Massachusetts); anti Mcl-1 (Cell Signaling Beverly, Massachusetts), rabbit monoclonal anti-P-ERK (Cell Signaling; Beverly, Massachusetts), rabbit monoclonal anti-total ERK (Cell Signaling; Beverly, Massachusetts), monoclonal mouse anti-SOCS3 Ab (AbD Serotec Oxford, UK) and mouse monoclonal anti-β-actin (Sigma-Aldrich; Milan, Italy). Three washes of 10 min, each at room temperature, were subsequently performed, using buffer C supplemented with 0.1% Tween. Membranes were then incubated for 30 minutes with a secondary anti-IgG Ab, obtained against the animal species immunized for the primary Ab. The secondary Ab is conjugated with horseradish peroxidase (Amersham International Biotechnology; Buckingamshire, UK) and diluted in buffer C.

After three additional washes, the membrane was subjected to the detection antibody with the enhanced ChemiLuminescence system (ECL) (Pierce; Rockford, Illinois): the membrane is incubated for 1 min with 1ml of luminol and 1 ml of H_2O_2, which in contact with the peroxidase and, as a result, with the Ag-Ab complex, gives rise to an oxidation reaction with light emission. The blots were acquired with ImageQuant LAS 500 imager (Amersham Biosciences) and densitometry analysis was performed with the software ImageQuantTL (Amersham Biosciences), provided with the instrument. Quantization on target protein/β-actin, expressed as arbitrary units, were normalized on Jurkat cell line.

8. ELISA TEST

Enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to quantify the protein concentration in organic liquids. We used Instant ELISA® (eBioscience) for the analysis of IL-6, sIL-6Rα and CLL5 concentration in cells cultures plasma and supernatant.

Samples were stored frozen at -20°C to avoid loss of bioactive human IL-6/sIL-6Rα/CCL5. Before starting the number of microwell strips required to test were determined counting the desired number of samples plus microwell strips for blanks and standards (coloured). As suggested by test protocol, each sample, standard, blank and control sample should be assayed in duplicate.

Protocol is reported in user manual attached to test kit. As general principles an anti-human coating antibody is adsorbed onto microwells and the human antigen of interest present in the sample or standard binds to antibodies adsorbed to the microwells. Then a biotin-conjugated anti-antibody is added and binds to the human protein of interest captured by the first antibody. As final step, streptavidin-HRP (Horseradish peroxidase) is added and binds to the biotin conjugated anti-human antibody.

Unbound biotin conjugated anti-human IL-6/ sIL-6Rα/ CCL5 and Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of soluble human protein present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IL-6/ sIL-6Rα/ CCL5 standard dilutions and sample concentration determined.

9. APOPTOSIS ANALYSIS BY FLOW CYTOMETRY

Apoptosis was assessed by annexin V/Propidium Iodide (PI) staining (BD Pharmingen).

Annexin V/PI staining was performed on PBMCs or LGLs after culture in RPMI 0.5% FCS, with or without the following agents: human recombinant IL-6, anti-IL6, anti-IL-6Rα antibodies (R&D Systems, Minneapolis, MN) demethylating agent 5-aza-2'-deoxycytidine (DAC) (Sigma Aldrich St. Louis, MO) and AG490 (Calbiochem, Darmstadt, Germany). A staining with anti-CD57-FITC was used to identify leukemic LGLs from PBMCs.

During the early stages of apoptosis the plasma membrane undergoes profound changes: phosphatidylserine (PS), a negatively charged aminofosfolipide normally expressed only on the inner side of the plasma membrane, is exposed on the outer surface. Annexin V is a protein that, in presence of high concentrations of Ca^{2+}, recognizes and binds selectively the PS, and therefore is useful for the identification of apoptotic cells that expose the phospholipid on their surface.

Aliquots of $2x10^5$ cells were harvested, washed, and incubated for 10 min in the dark at room temperature with: 100µl of binding buffer, a Ca^{2+}-rich solution that optimizes the binding of Annexin V to the PS, 5µl of Annexin V-FITC, and 10µl of PI, provided by the kit (1µl/ml final concentration).

After the incubation, 100µl of binding buffer were added and cells, at the final concentration of 1×10^6cells/ml, were analyzed by flow cytometer FACScan. For each sample 20,000 events were acquired and the number of apoptotic cells was expressed as percentage of Annexin V positive cells in the total cells analyzed.

10. CHEMOTAXIS ANALYSIS BY FLOW CYTOMETRY

Neoplastic LGL motility in presence/absence of MSC conditioned medium, was analyzed by adding complete medium and MSC conditioned medium to a multiple well plate, step followed by application of Transwell inserts (Corning, NY, USA) and by the addiction of complete medium and $2x10^6$/ml cells to their inside compartment (Figure 14).

Employed medium volumes consider the transwell insert diameter and are suggested by manufacturer's instructions. After 24 hours of incubation at 37°C and 5% CO_2, transwell inserts were remowed from the multiple well plate, and media from the lower wells were transferred into FACS tubes.

Software CellQuest Pro (BD Biosciences) was used for the analysis and for the set up of the instrument. For chemotaxis analysis of collected media we set the acquisition time at 60 seconds. Each sample was read three times.

Known the suspension volume drawn by the cytometer and number of acquired events, total number of migrated cells could be easily determined. This value in normalized on the number of cells migrated in alone condition (well containing only complete medium and not MSC conditioned stimulus) and Migration Index (MI) was obtained.

MI= number of cells migrated with stimulus/ number of cells migrated without stimulus

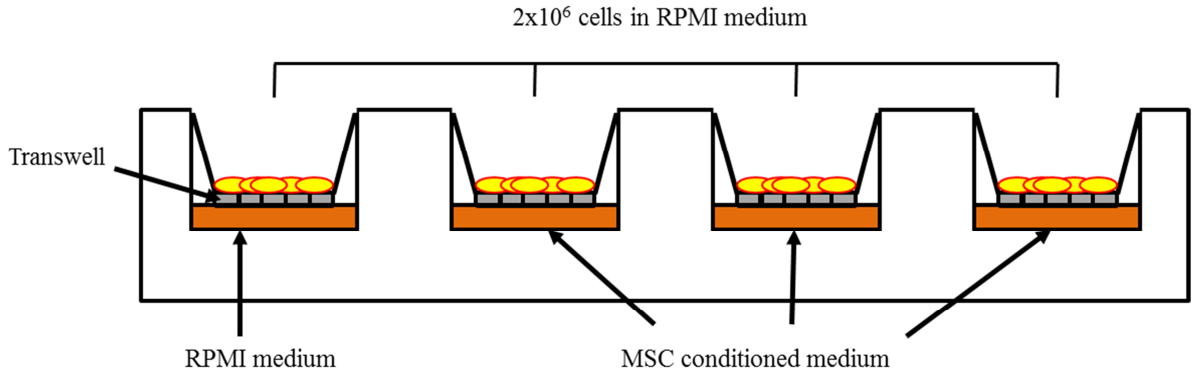


Figure 14: representative model of chemotaxis analysis.

11. STATISTICAL ANALYSIS

Data are expressed as mean plus or minus standard error (SE), and statistical analysis was performed by Student's t-test. A value of P less than .05 was accepted as significant.

RESULTS

1. LEUKEMIC LGLs DISPLAY HIGH EXPRESSION AND ACTIVATION OF STAT3

According to previously published data[37], RT-PCR results showed that STAT3 is over-expressed in purified leukemic T-LGLs as compared to normal CTLs, that are considered the normal counterpart of LGLs in healthy individuals (mRNA arbitrary units: 1.16 ± 0.12 and 0.65 ± 0.18 respectively, P=.0028) (Figure 15 A). Moreover, by purifying specific LGL subsets, we observed that the highest expression of STAT3 was specifically detected on CD3+CD57+ clonal LGLs, while autologous LGL-depleted PBMCs were consistently shown to display significantly lower amounts of STAT3 both by RT-PCR and by Western blot analysis (Figure 15 B, mRNA arbitrary units: 1.33 ± 0.11 and 0.99 ± 0.10 respectively, n = 10, P<.05; Figure 15 C, Western blot arbitrary densitometry: 1.14 ± 0.12 in patients and 0.45 ± 0.10 in controls, P=.014). Moreover, since STAT3 phosphorylation in tyrosine residue confers the ability to STAT3 to induce the transcription of several anti-apoptotic genes, we analyzed tyrosine 705 phosphorylation status of STAT3 in patients' LGLs as compared to controls. We observed that leukemic LGLs showed increased, although variable, amounts of activated STAT3 which were very low or even absent in controls (Figure 15 C).

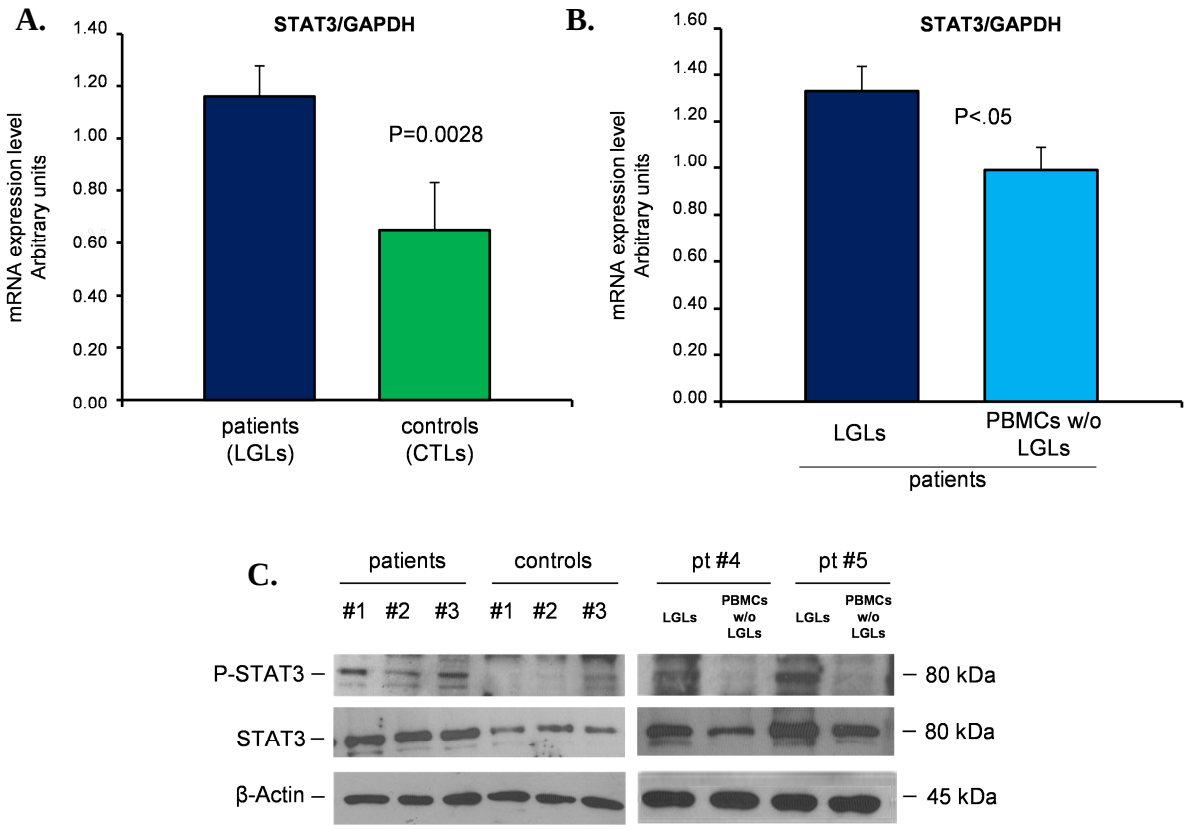


Figure 15: (A) STAT3 mRNA expression levels in LGLs from patients and in CTLs from controls. Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH. **(B)** STAT3 mRNA expression levels in LGLs from patients and in patients' LGLs depleted PBMC population. Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH. **(C)** Western blotting results of P-Y705 STAT3 and total STAT3 in PBMC from patients, PBMCs from controls, patients' LGLs and PBMC depleted population. The housekeeping β-Actin was used as calibrator.

2. SOCS3 IS DOWN-EXPRESSED AND UNRESPONSIVE IN PATHOLOGIC LGLS

STAT3 phosphorylation in tyrosine residue confers the ability to STAT3 to induce the transcription of several anti-apoptotic genes and of its specific suppressor, SOCS3.

Although STAT3 was highly expressed and activated, using RT-PCR we surprisingly observed that SOCS3 mRNA median value was significantly lower, almost undetectable, in patients' LGLs as compared with controls' CTLs (Figure 16 A, mRNA arbitrary units: 0.26 ± 0.04 and 0.63 ± 0.17 respectively, P=.003; Figure 16 B, Western blot: 0.59 ± 0.05 and 0.85 ± 0.10 respectively, P=.014).

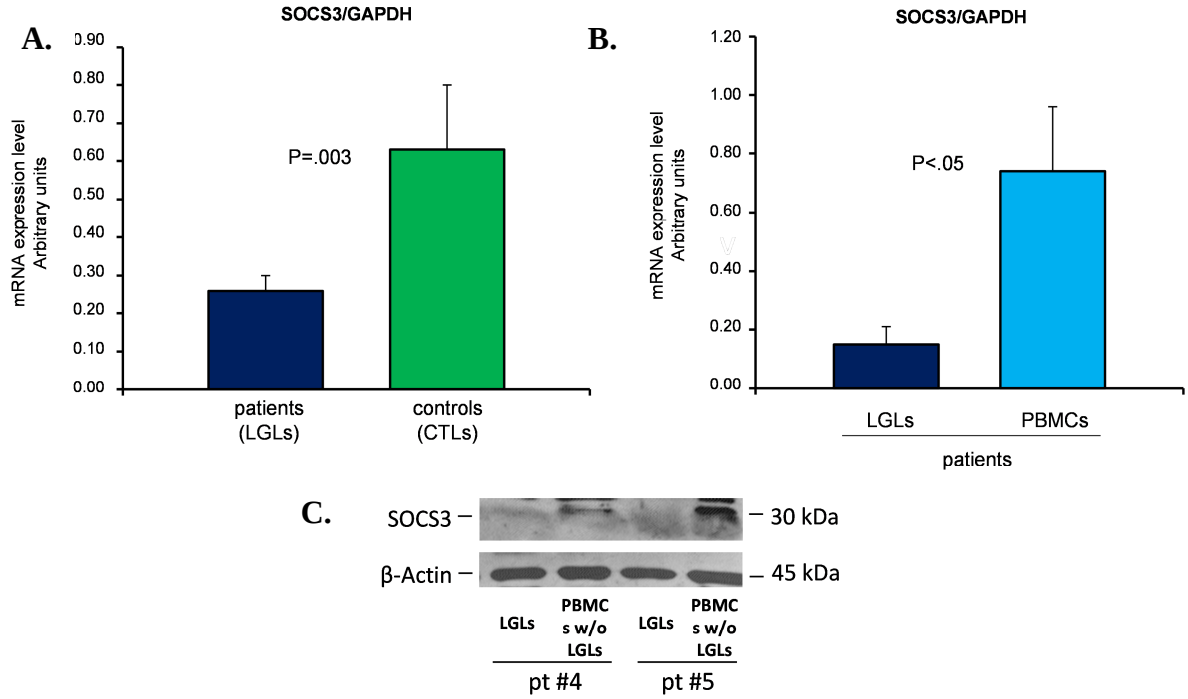


Figure 17: (A) SOCS3 mRNA expression levels in patients' LGLs and LGLs depleted PBMC population. Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH; **(B)** patients' LGLs and LGLs depleted PBMC population are analyzed by Western Blot. Proteins examined are SOCS3 and the housekeeping β-Actin. This latter is used as normalizator.

To evaluate whether, in patients, SOCS3 is responsive to STAT3 phosphorylation, we stimulated *in vitro* both patients' LGLs and controls' CTLs with IL-6. This latter is considered the main activator of STAT3/SOCS3 axis and its role in STAT3 up-regulation is well known[50].

By Western blot and RT-PCR analysis, we observed that, while STAT3 is physiologically able to respond to the IL-6 trigger both in patients and controls, SOCS3 expression markedly increased only in healthy individuals, whereas in patients' LGLs it remained quantitatively unchanged at near undetectable levels (Figure 18 A; mRNA arbitrary units: 1 hour alone condition, 0.12 ± 0.04 in patients' LGLs and 0.31 ± 0.08 in controls' CTLs; 1 hour IL-6 stimulus, 0.16 ± 0.04 in patients' LGLs and 1.40 ± 0.38 in controls' CTLs; Figure 18 B, Western blot: 0.59 ± 0.05 in patients and 0.85 ± 0.10 in controls, P=.014).

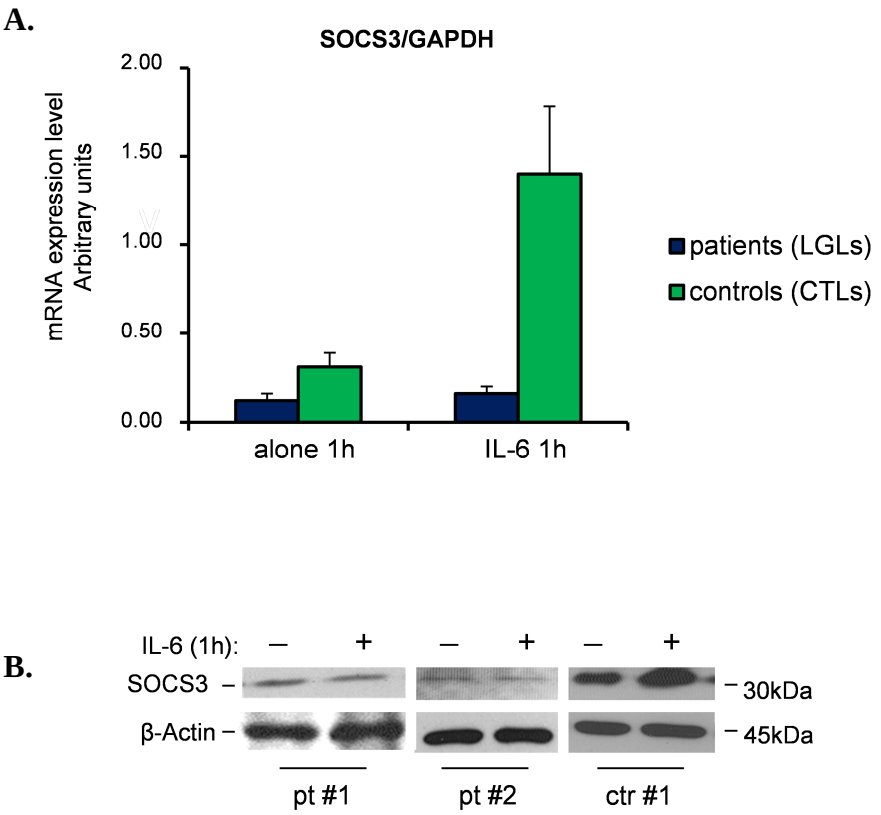


Figure 18: (A) SOCS3 mRNA expression levels in LGLs from patients and CTLs from controls at 1 hour, with or without IL-6 stimulus. Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH; **(B)** after 1 hour IL-6 stimulus, LGLs from patients and CTLs from controls are analyzed by Western Blot. Proteins examined are SOCS3 and the housekeeping β-Actin. This latter is used as normalizator.

To determine whether SOCS3 gene mutation was present, the SOCS3 coding region was sequenced and neither mutation nor alternative splicing of the SOCS3 gene were detected in any of the 27 patients studied.

3. THE DEMETHYLATING AGENT 5-AZA-2'-DEOXYCYTIDINE (DAC) RESTORES IL-6 MEDIATED SOCS3 EXPRESSION IN LGLs FROM PATIENTS

In several tumors where JAK/STAT pathway is involved, SOCS3 was occasionally found silenced through an epigenetic mechanism[47,48,51,52]. To evaluate whether an epigenetic mechanism accounts for SOCS3 inhibition, leukemic LGLs were incubated with DAC, a hypomethylating agent that hypomethylates DNA by inhibiting DNA methyltransferase. Following 96 hours incubation with this agent, IL-6 treated LGLs showed an increase of SOCS3 expression both at transcription and protein level (Figures 19 A-B), progressively fading in the next hours (data not shown) since SOCS3 is short-living. On the contrary, LGLs

untreated with DAC maintained SOCS3 down-modulated independently from IL-6 stimulation.

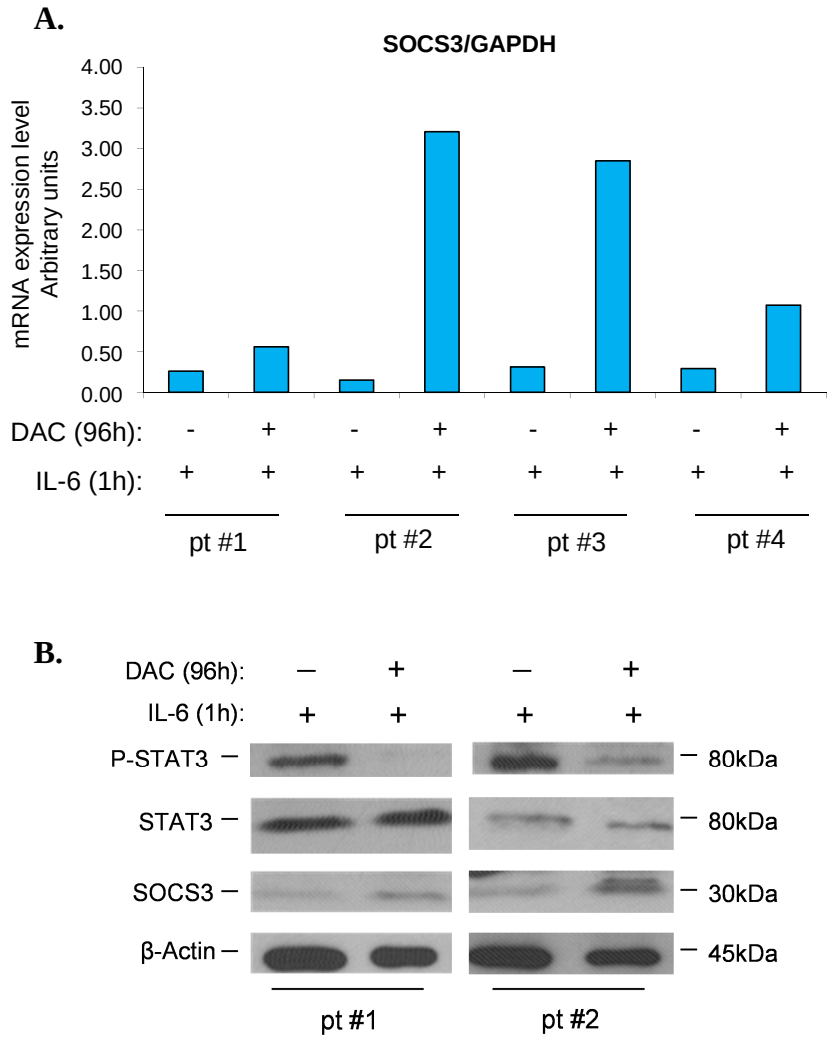


Figure 19: Results obtained by LGL cultures treated for 96 hours with or without DAC and then stimulated with IL-6 for one hour. **(A)** SOCS3 expression level in LGLs obtained by RT-PCR and normalized on GAPDH. The results of four representative patients are reported. **(B)** Western blot analysis of LGL extracts for phosphorylated STAT3, total STAT3 and SOCS3, β-Actin served as a loading control. Two representative cases of patients are shown.

4. SOCS3 RESTORATION LEADS TO P-STAT3 AND Mcl-1 REDUCTION AND LGL APOPTOSIS

To investigate whether in leukemic LGLs JAK/STAT signaling was re-established as a consequence of SOCS3 restoration, we tested the modulation of P-STAT3 and Mcl-1 after 1, 3 and 6 hours LGL culture.

We observed that SOCS3, once re-established by DAC and triggered by IL-6, led to a decrease of P-STAT3 already after one hour (Figure 19 B) and to a progressive decrease of Mcl-1, one of the most important pro-apoptotic target genes induced by P-STAT3 in LGLL (Figure 20 A-B).

On the other hand, cells not pre-treated with DAC maintained stable or even increased Mcl-1 levels. These data were observed both at mRNA and protein level.

Moreover, in these experiments, we showed that LGL apoptosis increased after DAC incubation with a pattern consistent to the addition in the culture of the inhibitor of STAT3 signaling AG490 (Figure 20 C).

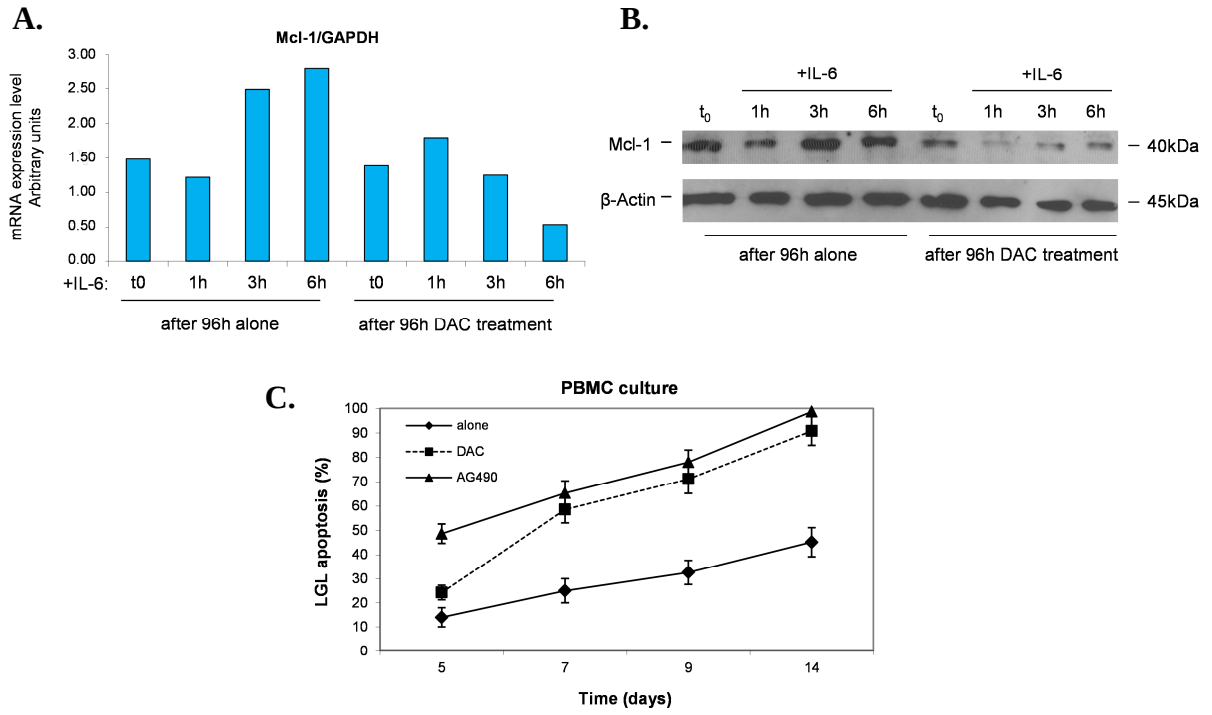


Figure 20: Results obtained by LGL cultures treated for 96 hours with or without DAC and then stimulated with IL-6 for one hour. **(A)** Mcl-1 expression level obtained by RT-PCR and **(B)** by Western blot analysis of LGLs from one representative LGLL patient. **(C)** Annexin V/PI assay measuring LGL mortality percentage (± SE) in PBMC culture after DAC treatment (4 days of incubation) or with AG490, inhibiting STAT3 signaling. A staining with anti-CD57-FITC was used to identify leukemic LGLs from PBMCs.

5. METHYLATION ANALYSIS OF SOCS3 PROMOTER IN LEUKEMIC LGLs

To determine whether methylation of SOCS3 promoter was involved in its expression in leukemic LGLs, methylation specific PCR (MSP) of SOCS3 promoter was performed using genomic DNA isolated from leukemic LGLs and autologous LGL-depleted populations. Once total DNA was modified, in order to discriminate the methylated from the unmethylated

promoter sequence, a total of 20 samples from patients and additional 6 samples from healthy individuals were analyzed using MSP of the SOCS3 promoter region, comprising nucleotides -525 to -384.

PCR product from LGLs of patients under study identified only unmethylated sequences in all cases studied. Consistent results were obtained also from the autologous LGL-depleted PBMCs population and in control CTLs. Data on two representative patients are shown in Figure 21 A.

These results were confirmed by sequencing assays on 10 patients and 5 controls (Figure 21 B). A total of 44 CpG sites located between nucleotides -678 and -216 of the SOCS3 promoter were tested by bisulfite sequencing. We found that methylation was not increased in SOCS3 promoter CpG island of any LGLL patient with respect to controls.

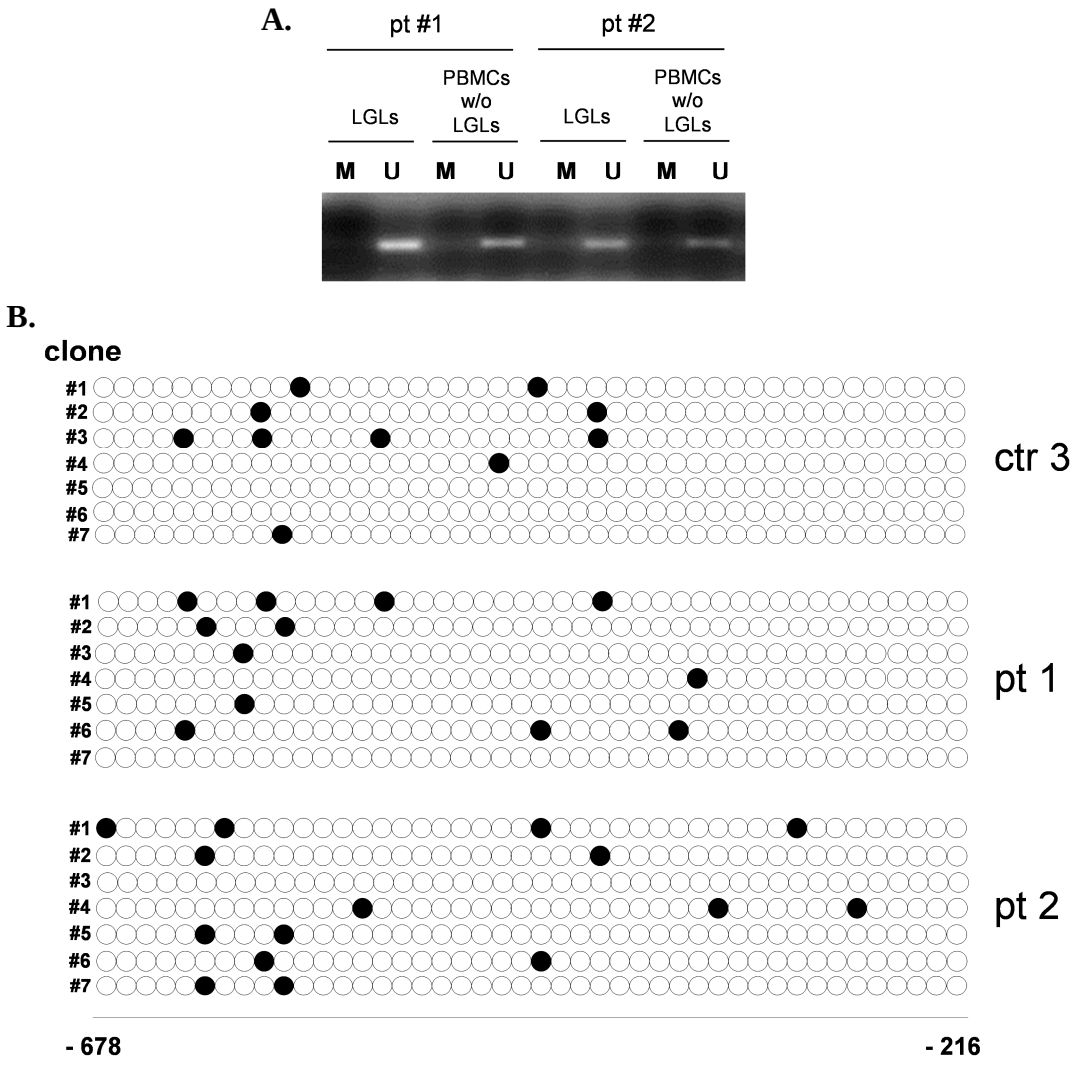


Figure 21: (A) Methylation specific PCR of SOCS3 promoter in LGLs and the autologous LGL-depleted PBMCs of two representative patients. M: methylated band and U: unmethylated band. **(B)** Methylation of CpG

dinucleotides. The figure reports the methylation pattern in the promoter region of the SOCS3 gene for one representative control and two representative patients. The filled circles indicate methylated CpG dinucleotides, the empty circles unmethylated ones. The numbers indicate the CpG dinucleotide position before the start codon. The analysis of controls was performed in the sorted CTLs; instead in patients the analysis was performed on sorted LGLs. The methylation frequency, for each sequenced clone, was calculated as the number of methylated CpG dinucleotides over the total CpG dinucleotides present in the analyzed sequence. The difference of median methylation frequency methylation was similar in the patients with respect to the controls.

6. MONONUCLEAR CELLS OF PATIENTS PRODUCED IL-6 AND THE SOLUBLE FORM OF IL-6-Rα

Literature data report high IL-6 level in acute and chronic inflammatory conditions, where it activates cell survival and proliferation pathways[53,54]. With this as a background we studied IL-6 expression levels in T-LGLL patients and we observed by Elisa Test high quantity of the cytokine in patients' plasma (Figure 22, 29.67 ± 8.91 pg/ml and 0.67 ± 1.37 pg/ml respectively, P<.05).

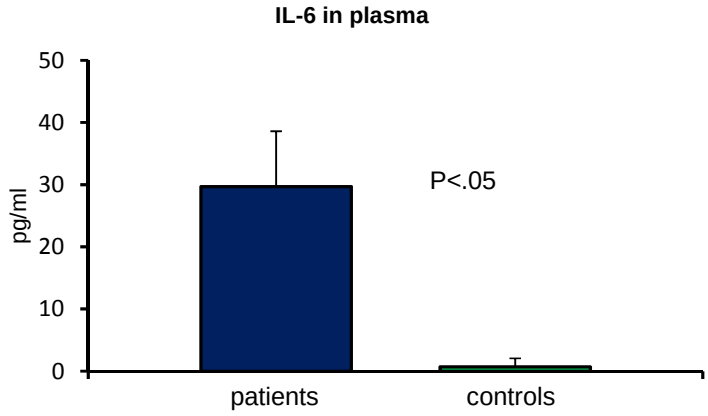


Figure 22: Histograms of IL-6 level in plasma of patients and controls measured by ELISA test.

Furthermore, we observed that patients' cells expressed higher mRNA level of IL-6 as compared to controls. This increased expression of IL-6 mRNA was restricted to the LGL-depleted PBMC population (Figure 23 A, P<.01). Consistent results were obtained by ELISA test, analyzing IL-6 levels in the supernatants of cell culture after 24 hours incubation in RPMI 0.5% FCS medium.

Patients' PBMCs depleted of LGLs released high levels of IL-6 as compared to autologous purified leukemic LGLs and to control PBMCs (Figure 23 B, P<.01).

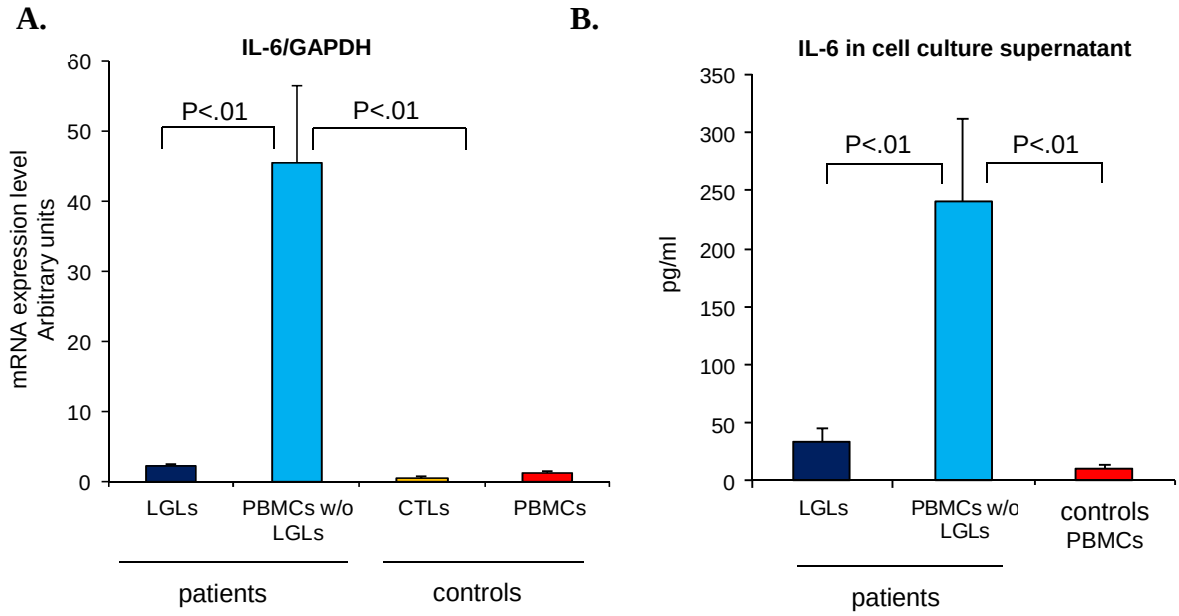


Figure 23: (A) IL-6 mRNA expression and **(B)** secretion level in patients (LGLs and LGL-depleted PBMCs, n = 27) and in controls (CTLs and PBMC, n = 18).

Recently, it has been found that IL-6 acts not only through the "classical signaling" but also by an alternative pathway called trans-signaling[23]. According to the classical signaling, IL-6 first binds to the membrane IL-6 receptor alpha (mIL-6Rα), then the complex associates with the signal transducing membrane protein gp130. In the alternative signaling, IL-6 binds to the soluble agonistic form of IL-6Rα (sIL-6Rα) and can activate also cells lacking mIL-6Rα.

Therefore, we analyzed IL-6Rα expression levels and by RT-PCR we found that IL-6Rα was poorly expressed by LGLs of patients as compared to PBMCs (mRNA arbitrary units: 3.76 ± 1.44 vs 78.72 ± 28.33 respectively; P<.01) and to CTLs and PBMC from controls (mRNA arbitrary units: 73.57 ± 10.61 and 70.86 ± 10.85, respectively; P<.01 for each comparison) (Figure 24 A).

By Elisa test we showed that the loss of IL-6Rα by leukemic LGLs is balanced by a significant higher level of sIL-6Rα in plasma of patients as compared with controls (Figure 24 B: 84.32 ± 6.38 ng/ml and 51.22 ± 16.65 ng/ml, respectively; P<.05), suggesting that IL-6 activation is mediated by a trans-signaling process.

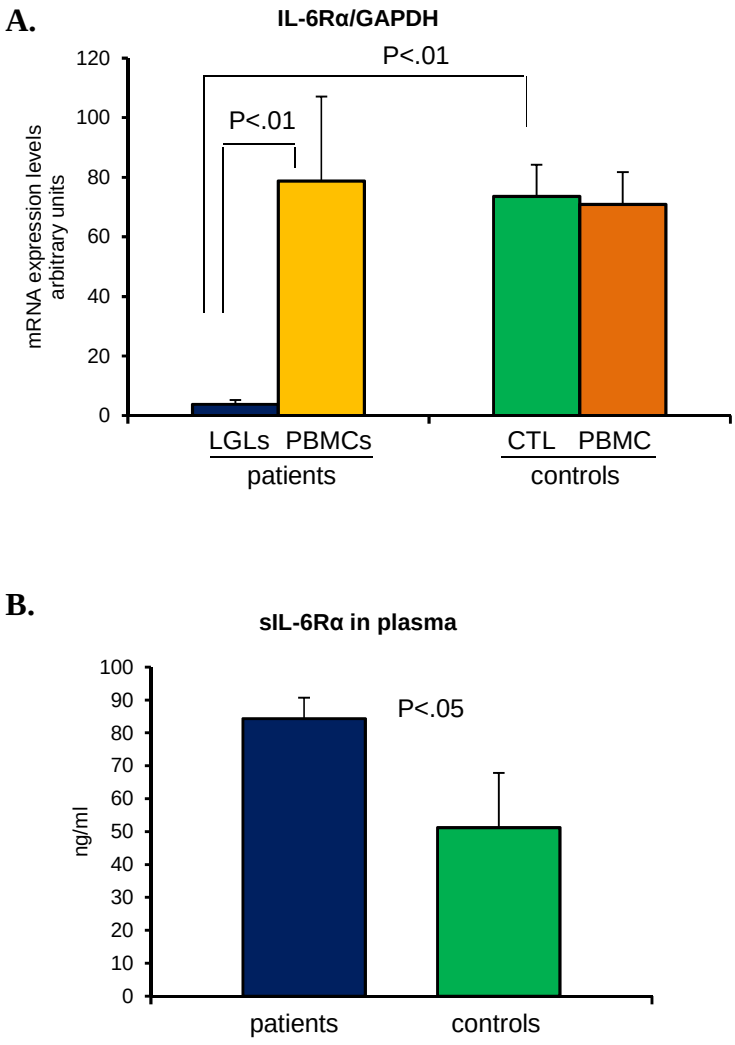


Figure 24: (A) Histograms of IL-6Rα mRNA expression level in patients (LGLs and PBMCs) and in controls (CTLs and PBMCs). **(B)** Histograms of IL-6Rα levels in plasma of patients and controls, measured by ELISA.

7. IL-6 EXPRESSION IS INVERSELY CORRELATED TO THE AMOUNT OF CIRCULATING LGLs IN T-LGLL PATIENTS

Due to the heterogeneity detected in the plasma levels of secreted IL-6, we tried to correlate the amount of IL-6 to the percentage of circulating LGLs in PBMCs (LGL range in our cohort of patients: 25%-95%).

Interestingly, we observed that lower IL-6 levels were detected in patients presenting higher number of leukemic LGLs, on the contrary, patients with higher IL-6 levels associate to patients characterized by low circulating LGLs (Figure 25).

The results indicate that IL-6 secretion was inversely correlated to LGL percentage. We established at 55% the threshold of LGL percentage that better distinguishes patients

characterized by high IL-6 plasma levels from patients with lower IL-6 levels. Considering that IL-6 is a mediator of inflammation, we suggest that low-burden disease patients are mostly characterized by an active inflammatory background.

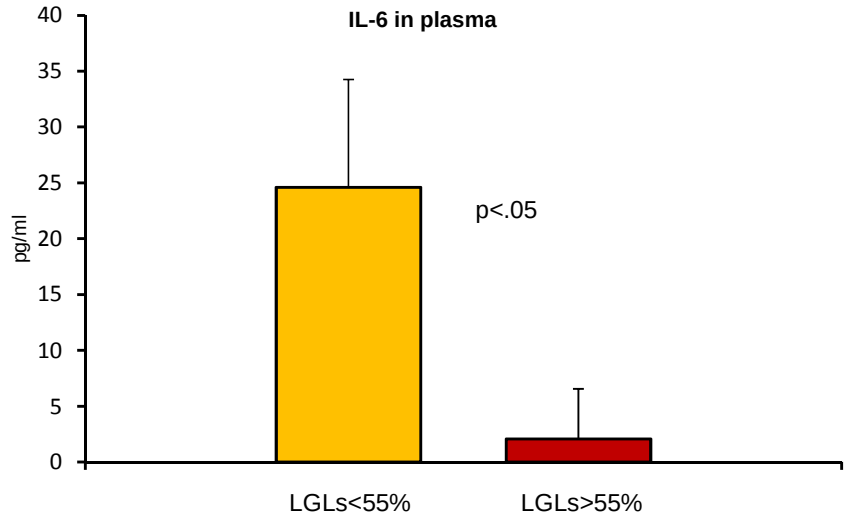


Figure 25: Histograms of IL-6 level in plasma of patients characterized by circulating LGLs < 55% and in plasma of patients with circulating LGLs > 55%, measured by ELISA. IL-6 levels in LGLs < 55% group: 24.61 ± 9.64 pg/ml; IL-6 levels in LGLs > 55% group: 2.05 ± 4.50 pg/ml; P<0.05.

8. IL-6 IS INVOLVED IN SURVIVAL CELL MAINTENANCE AND IN STAT3 PHOSPHORYLATION STATUS

As reported in Figure 26 A, freshly purified LGLs demonstrated a better survival when in culture with the residual autologous PBMCs or alone in the presence of exogenous IL-6 or with autologous plasma (10%).

To evaluate whether LGL survival and STAT3 phosphorylation are dependent on IL-6 released by patients' PBMCs, these latters were incubated in the presence of blocking antibodies to IL-6 or IL-6Rα and both P-STAT3 and apoptosis level after 1, 2, 4 and 7 days of culture were analyzed.

Our results indicated that LGL apoptosis statistically increased (Figure 26 B) with a concomitant P-STAT3 decrease (Figure 26 C) following incubation with anti-IL-6 or anti-IL-6Rα.

Moreover, we observed that the survival rescue obtained in LGL culture with autologous plasma is abrogated with the addition of the neutralizing antibodies, thus confirming that

autologous plasma IL-6 mediates LGL survival (Figure 26 D). These results suggest that IL-6 is necessary to maintain STAT3 activation thereby supporting leukemic LGL survival.

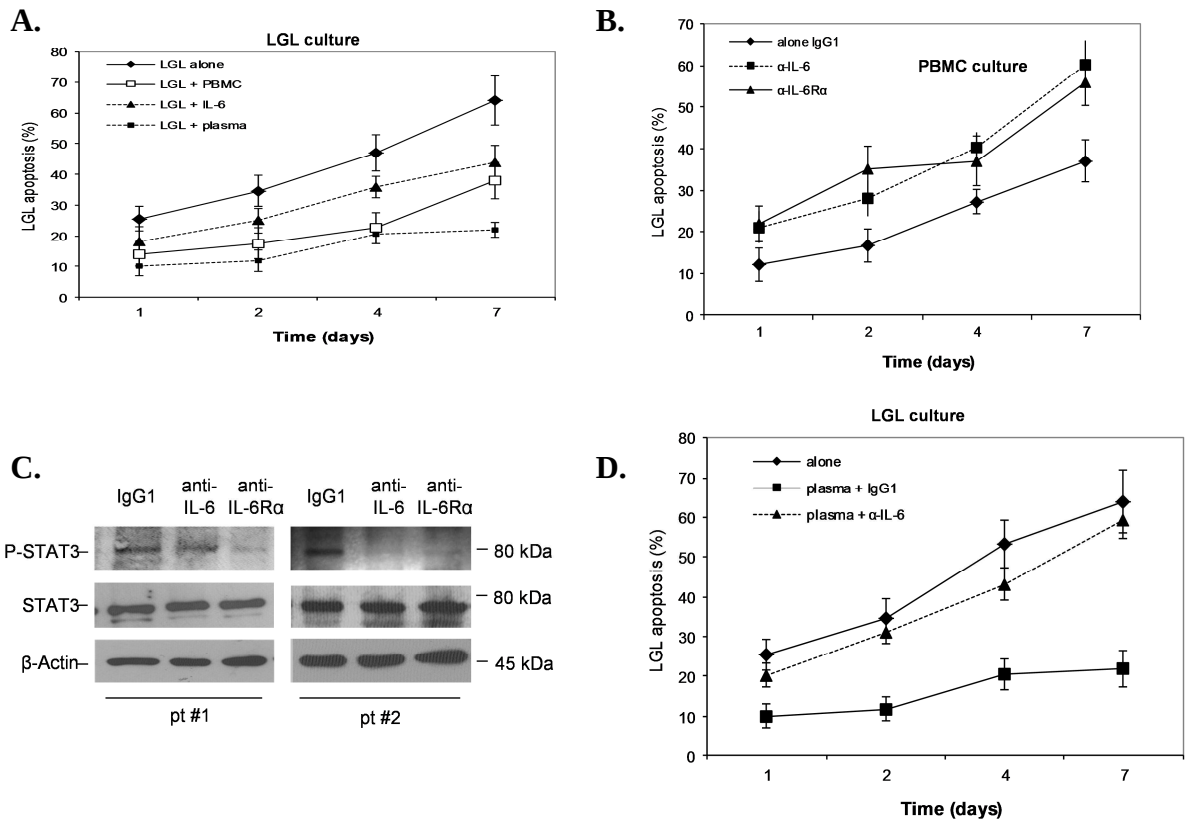


Figure 26: Apoptosis was measured after 1, 2, 4 and 7 days of culture in RPMI 0.5% FCS by Annexin V/PI. A staining with anti-CD57-FITC was used to identify leukemic LGLs from PBMCs. **(A)** Purified LGL apoptosis was analyzed in cells cultured alone or with autologous PBMCs or with IL-6 or with autologous plasma 10%. **(B)** LGL apoptosis time course and **(C)** WB analysis of P-STAT3 and STAT3 in PBMC at 4 hours of culture (two representative patients) with control IgG1, with anti-IL6 and anti- IL-6Rα Abs. β-Actin served as a loading control. **(D)** LGL apoptosis in culture alone and with autologous plasma in the presence of isotype control antibody and of anti-IL6 and anti-IL-6Rα Abs. The apoptosis histograms show the mean results (± SE) from 4 individual experiments.

9. IL-6 CAN BE PRODUCED BY MSCs AND MODULATE CCL5 EXPRESSION THROUGH STAT3 SIGNALING CASCADE

Through STAT3, IL-6 activates the transcription of some target genes, among which CCL5[32]. This cytokine, strongly expressed by PBMCs of T-LGLL patients[31], is an important mediator of acute and chronic inflammation. Since IL-6/STAT3 axis is constitutively activated in T-LGLL patients and CCL5 is over-expressed, we investigated whether IL-6/STAT3 axis could modulate CCL5 expression. By RT-PCR we found that IL-6 stimulation increased CCL5

mRNA levels in T-LGLL patients, while in controls no significative increase has been detected (Figure 27).

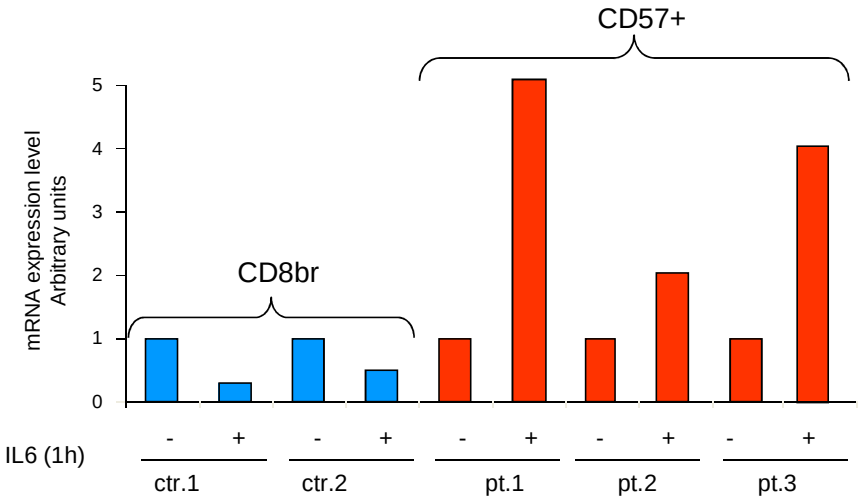


Figure 27: (A) CCL5 mRNA expression levels in patients' LGLs and controls' CTLs stimulated with IL-6 for one hour. Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH.

Moreover, by transwell migration assay, we detected a high motility of LGLs in presence of medium with CCL5, which in turn acts as a chemo-attractant in T-LGLL (Figure 28).

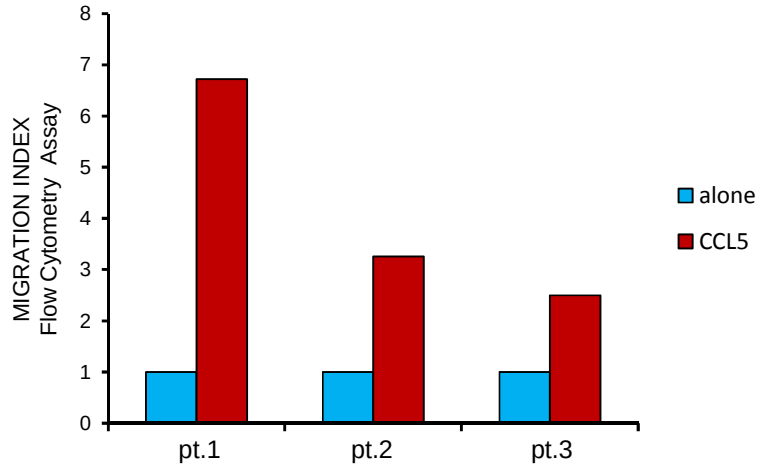


Figure 28: Chemotaxis analysis for the evaluation of neoplastic LGL motility in presence/absence of CCL5 stimulus for 24 hours.

Being bone marrow mesenchymal stem cells (BM-MSCs) reported to be a source of IL-6, and considering that bone marrow has been frequently demonstrated to represent a privileged site of LGLs infiltration[20], we investigated the role of BM-MSCs in CCL5 upregulation, compartmentalization and survival of leukemic LGLs through IL-6 release.

By apoptosis analysis we found an increased survival of T-LGLs after incubation with MSCs' medium, while treatment with IL-6 neutralizing antibodies decreased survival sustained by MSC conditioned medium (Figure 29). This result confirms the hypothesis that MSCs promote LGLs survival through IL-6 release.

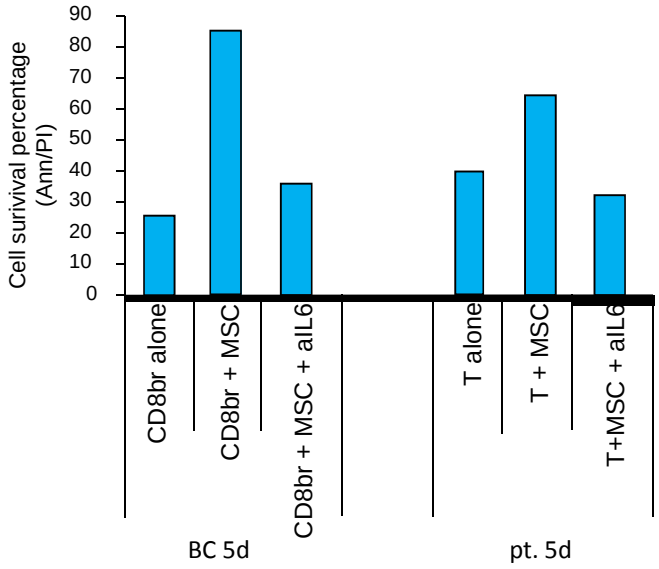


Figure 29: Apoptosis assay by Annexin V/PI on CTLs from controls and LGLs from patients. Results were obtained by cell cultures treated for 5 days with MSC medium in the presence of isotypic control, and with MSC medium added with anti-IL6 and anti-IL-6Rα Abs (in the histogram indicated with the general word "aIL-6").

By Transwell assay we evaluated the ability of neoplastic cells to migrate in presence of neoplastic MSC conditioned medium. We expressed results as Migration Index (MI= number of migrated cells in presence of MSC conditioned medium / number of migrated cells in presence of medium alone).

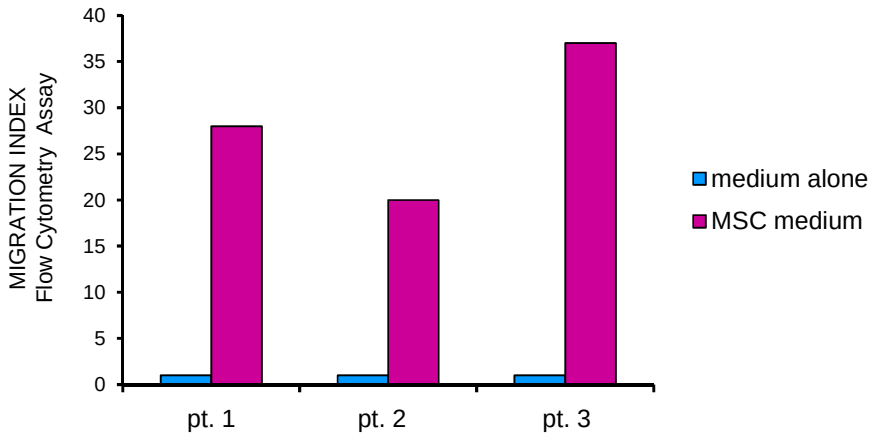


Figure 30: Chemotaxis analysis for the evaluation of neoplastic LGL motility in presence/absence of MSC conditioned medium for 24 hours.

We detected a high motility of LGLs in presence of MSC medium (Figure 30), therefore it could be hypothesized that MSCs, triggering CCL5 expression in LGLs through IL-6 release, could promote the recruitment of neoplastic cells in the bone marrow.

10. IL-6 AND IL-6Rα EXPRESSION IS RESPONSIVE TO IL-15 STIMULUS

Considering the role of IL-15 in LGLL pathogenesis we evaluated IL-15 effect on IL-6 signaling. By RT-PCR and Elisa Test we observed that IL-15 strongly induced IL-6 expression and secretion in patients' PBMCs after 24 hours culture (Figure 31 A and B, respectively).

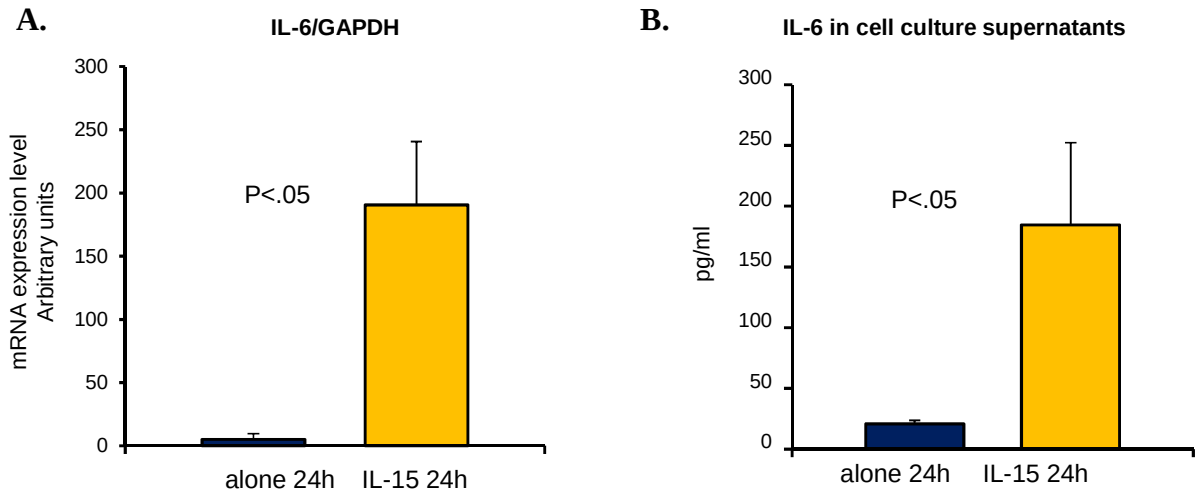


Figure 31: (A) IL-6 mRNA expression levels in patients and controls' PBMCs in culture after 24 hours alone (mRNA arbitrary units: 5.073 ± 4.56 in patients' PBMC and 1.47 ± 0.61 in PBMC from controls) and after 24 hours with IL-15 (190.6 ± 127.56 in patients' PBMC and 103.88 ± 21.01 in PBMC from controls. The increase of IL-6 expression after IL-15 stimulation is statistically significant (P<.01). Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH. **(B)** Histograms of IL-6 level in cell culture supernatants of patients' PBMC, measured by ELISA test (alone 24h condition: 20.91 ± 2.91 pg/ml; IL-15 24h condition: 184.65 ± 67.69 pg/ml, P<.05).

Moreover, by RT-PCR we observed that IL-15 can inhibit IL-6Rα expression in LGLs (Figure 32). Therefore this cytokine could account for the down-expression of IL-6Rα observed in LGLs, ultimately promoting the trans-signaling process.

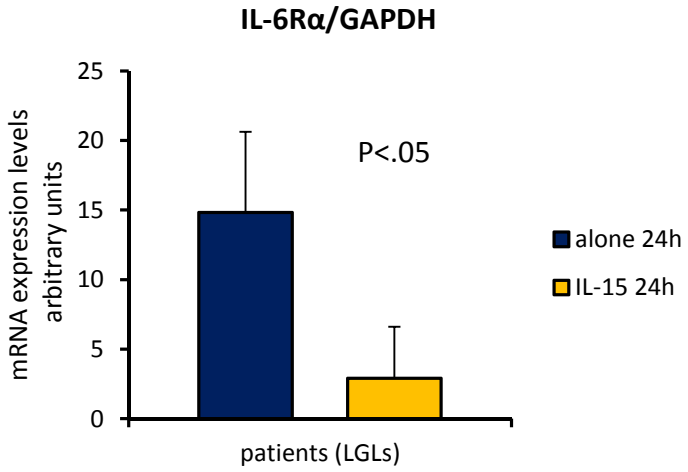


Figure 32: Hystograms of IL-6Rα mRNA expression level in patients' LGLs in culture after 24 hours alone (mRNA arbitrary units: 14.83 ± 5.8) and after 24 hours with IL-15 (2.91 ± 3.7). Gene expression data are normalized to the expression levels of the housekeeping gene GAPDH.

11. BOTH IL-6 AND IL-15 PROMOTE LGLs SURVIVAL

IL-6 is the main activator of STAT3 and IL-15 seems to promote IL-6 trans-signaling pathway in T-LGLL. Therefore, next step was the analysis of IL-6 and IL-15 effects on STAT3 activation in leukemic LGLs.

By Western Blot analysis we observed that both 1 hour IL-6 stimulus and 24 hours IL-15 stimulus strongly increased STAT3 phosphorylation levels (Figure 33 A-B), indicating that STAT3 protein in LGLs is responsive to these cytokines.

These mechanisms therefore contribute to LGLs survival.

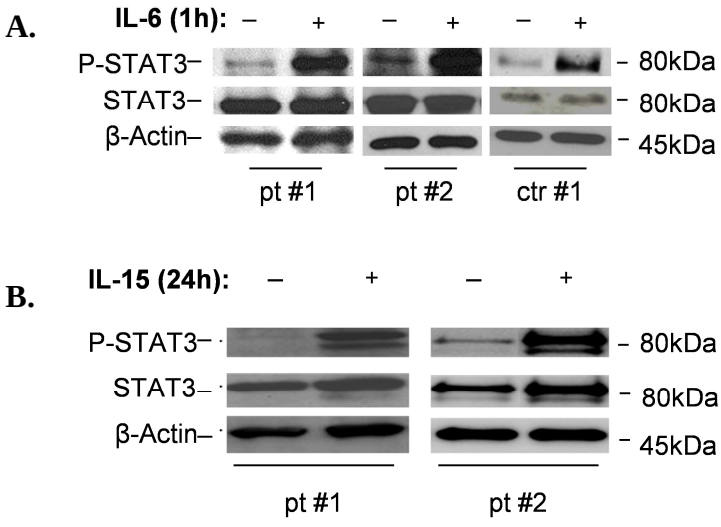


Figure 33: WB analysis of P-STAT3 and STAT3 in patients' LGLs at **(A)** 1 hours stimulus with IL-6 and at **(B)** 24 hours stimulus with IL-15. β-Actin served as a loading control.

12. PATIENTS WITH A HIGH PERCENTAGE OF CIRCULATING LGLS MAY SHOW MISSENSE MUTATION IN STAT3 SH2 DOMAIN

Recently, it has been discovered that mutational hot spots in the SH2 domain of STAT3 are present in a percentage of patients with T-LGLL[41,42]. Y640F and D661Y are reported to be the more frequent mutations (90% of cases) (Figure 35 A). Consistently, in our cohort of patients, by Sanger sequencing and PCR ARMS we also found only these mutations. We found 11 mutated out of 60 patients affected by T-LGLL (18%).

12.1 MUTATIONAL ANALYSIS BY SANGER SEQUENCING

Sixty T-LGLL patients were analyzed by Sanger Sequencing for their LGL and LGL-depleted PBMC population and mutations were reported in 9 patients (15%): 6 patients showed Y640F mutation and 3 patients showed D661Y mutation. Interestingly, when a patient is mutated, the mutation is found only in LGLs and not in remaining PBMCs. Moreover, we observed that:

- not all LGLs are mutated at the same time, but STAT3 mutations can be found only in a subpopulation of LGLs;
- in all the 17 healthy controls analyzed CTLs and PBMCs resulted unmutated in STAT3;
- mutations were found only in patients with a high percentage of circulating LGLs. Considering the arbitrary cut off point proposed for IL-6 expression levels, mutations were found only inside the group of patients characterized by circulating LGLs >55% and low IL-6 level.

12.2 MUTATIONAL ANALYSIS BY ARMS-PCR

In order to confirm the results obtained by Sanger Sequencing, we performed also ARMS-PCR method, which allows the identification of sequencing refractory mutations, for the detection of D661Y and Y640F mutations.

Out of 60 T-LGLL patients analyzed 11 resulted mutated (18%): 8 patients showed Y640F mutation and 3 patients were characterized by D661Y mutation.

Among these 11 patients, 9 were already found mutated by Sanger Sequencing. ARMS-PCR method therefore added 2 new mutated cases to our analysis.

12.3 PHENOTYPE OF LGL CLONE IN MUTATED PATIENTS

Immunophenotype analysis showed a high heterogeneity in the group of patients (table 6). Among T-LGLL patients, the majority of cases displayed a double positive CD3/CD8 leukemic clone and a low percentage of cases displayed a double positive CD4/CD8 or were CD4+/CD8- T clone.

In the majority of cases, the leukemic clone is characterized by the expression of CD57. Only a subpopulation of LGLs expressed CD16, which can be coexpressed or not with CD57.

Inside leukemic T cell population some cases showed the expression restriction for one or more (2-3) Vβ at the same time.

By observing the phenotype of mutated as compared to the unmutated patients, we showed that STAT3 mutation not only identifies patients with >55% of PBMCs but is associated with a specific phenotype:

- CD3/CD8 positivity
- CD57 positivity
- CD16 positivity
- CD56 negativity

Differently, patients with an LGL percentage >55% but negative for the mutation are phenotypically characterized by the negativity for CD16 and the positivity for CD56.

Table 7: T-LGLL patients characterized by a percentage of circulating LGLs >55% on total PBMCs.

# Pt.	DISEASE	AGE/ SEX	% LGL	IMMUNOPHENOTYPE	Vβ / KIR	MUTATION
1	T-LGLL	M74	85%	**CD3/CD57/CD16**	Vβ5.2	**Y640F**
2	T-LGLL	M41	71%	**CD3/CD57/CD16**	-	**Y640F**
3	T-LGLL	F62	55%	**CD3/CD57/CD16**	Vβ2	**D661Y**
4	T-LGLL	F73	62%	**CD3/CD57/16**	Vβ7.1	**Y640F**
5	T-LGLL	F76	73%	**CD3/CD57/CD16**	Vβ21	**Y640F**
6	T-LGLL	M52	89%	**CD3/CD57/CD16**	KIR2D2/3	**Y640F**
7	T-LGLL	M53	86%	**CD3/CD57/CD16**	KIR2D2/3	**Y640F**
8	T-LGLL	M54	72%	**CD3/CD57/CD16**	-	**Y640F**
9	T-LGLL	F65	57%	**CD3/CD16**	Vβ14	**Y640F**
10	T-LGLL	F53	67%	**CD3/CD57/CD16**	Vβ9 KIR2D2/3	**D661Y**
11	T-LGLL	F51	55%	**CD3/CD16/CD57**	Vβ14	**D661Y**
12	T-LGLL	F78	65%	**CD3/CD57/CD16**	-	-
13	T-LGLL	M48	70%	**CD3/CD57/CD56**	Vβ5.1	-
14	T-LGLL	F73	65%	**CD3/CD57/CD56**	-	-
15	T-LGLL	M85	78%	**CD3/CD57/CD56**	Vβ13.6	-
16	T-LGLL	M66	73%	**CD3/CD57/CD56**	Vβ1	-
20	T-LGLL	M40	95%	**CD3/γδ/CD56/CD16**	KIR2D2/3	-
21	T-LGLL	F73	66%	**CD3/CD57/CD56**	Vβ2	-
22	T-LGLL	F75	79%	**CD3/CD57/CD16**	-	-
23	T-LGLL	M79	55%	**CD3/CD57**	Vβ17 KIR2D1	-

Patients' immunophenotypes, Vβ and KIR restriction and the results of mutational analysis by Sanger Sequencing and ARMS-PCR, are reported.

DISCUSSION

In this PhD project, we provide evidence that two different mechanisms account for the constitutive STAT3 activation in patients with T-LGLL: 1) the high production of IL-6, released by autologous LGL-depleted population of patients' PBMC, and in the bone marrow MSCs, and 2) the down modulation of SOCS3, which is responsible for the lack of the physiological negative feedback mechanism controlling STAT3 activation. Interestingly, we demonstrated that in leukemic LGLs the constitutive activation of STAT3 may be down-modulated by DAC, suggesting a putative role of demethylating agents in LGLL treatment strategies. Moreover, we demonstrated that the subset of patients characterized by STAT3 mutations were specifically included within the high LGL burden patients' group, which seems to be independent by cytokine stimulation.

The constitutive activation of JAK/STAT pathway has been claimed to be involved in the development of several human cancers, including hematologic neoplasms (acute myeloid leukemias, Sezary syndrome, multiple myeloma)[40,55,56]. In T-LGLL, Epling-Burnette *et al* reported that LGLs from patients constitutively express high levels of activated STAT3 as compared to PBMC of healthy individuals[37]. They also showed that STAT3 activation contributes to Fas resistance resulting in abnormal survival of leukemic LGL and is also responsible of the over-expression of the anti-apoptotic protein Mcl-1[37].

During this PhD, we confirmed that over-expression and activation of STAT3 in T-LGLL is a common feature for all T-LGLL patients and demonstrated that patients' PBMCs release high amounts of IL-6 as compared to healthy individuals, suggesting that the activation of STAT3 may result from a persistent stimulation of this cytokine, which is central in inflammatory processes and has been demonstrated to play a role in multiple myeloma and in other neoplastic disorders where the JAK/STAT pathway is involved[40,57,58].

Furthermore, we provided evidence that IL-6 is secreted through a paracrine mechanism, since its production is not mediated by leukemic LGLs but by the residual normal autologous LGL-depleted PBMCs, likely cells belonging to the monocyte/macrophage lineage. Also MSCs seem to play a key role in the cytokine secretion in the bone marrow where patients' LGLs show high frequency of infiltration[20]. Through the release of IL-6, our results indicate that MSCs are able to stimulate CCL5 secretion by LGLs, triggering the chemotactic environment of the bone marrow, since we demontrated the ability of CCL5 to induce the migration of T LGLs.

Taken together, these data highlight the importance of tumor environment[59] and point out that additional cells, different from the malignant LGL clone, contribute to the disease development and maintenance.

We also provided evidence that patients' PBMCs release *in vivo* high amount of soluble IL-6Rα allowing LGLs, that not express membrane-bound IL-6Rα but only gp130, to be responsive to IL-6 through the trans-signaling process[60]. This mechanism has been demonstrated in many chronic inflammatory diseases, including chronic inflammatory bowel disease, peritonitis, rheumatoid arthritis, asthma, as well as colon cancer, where IL-6 trans-signaling is critically involved in the maintenance of a disease state, by promoting transition from acute to chronic inflammation[60-62]. Our results also indicate that IL-15, a well described cytokine crucial in LGLL pathogenesis, can sustain IL-6 trans-signaling, then contributing to maintain the inflammatory background of the disease.

Both IL-6 and IL-15 stimuli strongly enhance the phosphorylation of STAT3, confirming their central role in maintaining LGL survival in LGLL, demonstrated also by the relevant inhibition of STAT3 phosphorylation in the presence of antibodies against IL-6 or IL-6Rα.

Interestingly, even if STAT3 is constitutively expressed and activated in T-LGLL, we found a very low expression of its specific inhibitor, SOCS3, in patients' LGLs as compared to the CTLs of controls. Consistently, the feedback loop sustained by this protein which is crucial in controlling the level of STAT3 activation, can be inhibited in some tumors characterized by P-STAT3 over-expression[47,48,52].

Comparison between pathological LGLs and the remaining autologous PBMCs indicate that the signaling pattern defined by high P-STAT3 and low SOCS3 expression was a discrete characteristic of leukemic LGLs. This mechanism in the pathophysiology of T-LGLL lacks a clear explanation yet. In fact, sequencing the entire coding region of 27 patients, we ruled out the presence of SOCS3 gene mutations, and also showed that the promoter was not inactivated via CpG island methylation, as demonstrated in other neoplastic clinical conditions[47,48,52].

Even if SOCS3 promoter in T-LGLL patients showed no methylation, by treating leukemic LGLs with the demethylating agent DAC, SOCS3 expression was restored, with reappearance of P-STAT3 suppression and inhibition of downstream signals, in particular Mcl-1 expression[63]. Although the fine mechanisms tuning out SOCS3 expression has not up to now clarified, for the first time our results demonstrated a link between constitutive STAT3 activation, ultimately resulting in LGL survival, and epigenetic regulatory mechanisms. More interestingly, our data provided evidence of the reversibility of epigenetic inhibition using

hypomethylating agents, thus opening the possibility that an epigenetic approach to re-express SOCS3 may be exploited for the treatment of T-LGLL[64].

Recently, recurrent somatic missense mutations (more often Y640F and D661Y) in the SH2 domain of STAT3 have been reported in nearly one third of patients affected by LGL disorders[41]. It has been hypothesized that STAT3 mutations can enhance its constitutive activation by an increased stabilization of STAT3 homodimers or heterodimers through enhanced hydrophobic attraction between STAT monomers. However, this putative role of STAT3 mutations is still controversial. As a matter of fact, whereas STAT3 activation is a constitutive mechanism in all T-LGLL patients[37,41], STAT3 mutations are reported only in a fraction of cases, despite that the presence of monoclonal rearrangement of TCR was shown in all patients[41,42].

In our series of patients we found 11 cases with STAT3 mutations (6 with Y640F and 2 with D661Y, 18%) out of 60 monoclonal patients analyzed. Interestingly, we demonstrated that the group of patients accounting for STAT3 mutations in T-LGLL is characterized by cases with high percentage of circulating LGLs (more than 55% LGLs)[41,42], and it is worth of notice that previously published results about STAT3 mutations considered only patients with very high LGL clonal expansions. To compare our results with literature data by taking into account only patients with clonal and >55% LGLs, we found an incidence of mutations (11 out 29 cases, 38%) more consistent with literature data.

The finding that mutated patients affected by T-LGLL show a particular and distinctive phenotype of LGL clone (CD8+/CD57+/CD16+) further suggests that patients bearing STAT3 mutations might be distinguished as a different subgroup of patients.

Another interesting feature of STAT3 mutated patients was that they were characterized by a very low level of IL-6 in their plasma. These results suggest that an initial step along the development of disease still characterized by low lymphocytosis (LGLs <55% of total PBMCs), is mostly sustained by extrinsic factors contributing to the relevant inflammatory background. A subsequent step can be recognized by a high lymphocytosis (LGLs >55% of total PBMCs) in which LGL disease likely goes on independently from exogenous stimuli, becoming self-maintaining due to the contribution of the emerging STAT3 mutations. It is suggested that these phases can represent two sequential steps in the progression of disease.

Taking together, these results indicate that genomic mutations might only marginally account for *in vivo* activation of STAT3 in LGLL patients and further emphasize the heterogeneity of mechanisms sustaining LGLL. Among these the tumor environment play a key role, being

responsible for the activation of LGLs by antigene presentation, and for progression of the disease by the secretion of important mediators of inflammation.

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PUBLICATIONS

1. Teramo A, Gattazzo C, **Passeri F**, Lico A, Tasca G, Cabrelle A, Martini V, Frezzato F, Trimarco V, Ave E, Boscaro E, Piazza F, Facco M, Trentin L, Semenzato G, Zambello R. Intrinsic and extrinsic mechanisms contribute to maintain the JAK/STAT pathway aberrantly activated in T-type large granular lymphocyte leukemia. Blood;121(19):3843-3854, S3841.