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Characterization Of The Bone Marrow Microenvironment That Provides Survival Signals Contributing To Therapy Failure In Patients With Leukemia.

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RIASSUNTO

Le patologie ematologiche sono disordini clonali generati dalla trasformazione neoplastica di cellule progenitrici ematopoietiche. La progressione leucemica delle cellule tumorali è indotta da eventi intrinseci come l'attivazione di oncogeni e la perdita di geni soppressori. Sebbene i difetti cellulari intrinseci causativi della trasformazione neoplastica siano stati studiati estensivamente nel campo delle leucemie, i fattori del microambiente midollare coinvolti negli eventi della tumorigenesi non sono stati ancora chiaramente definiti. In particolare, le cellule staminali ematopoietiche risiedono nel midollo osseo ed interagiscono con un microambiente altamente organizzato composto da diverse sotto-popolazioni di cellule stromali e una matrice extracellulare ricca di fibronectina, collagene e vari proteoglicani. L'interazione tra le cellule staminali e il microambiente midollare è critica nella regolazione dei processi di migrazione, "self-renewal", proliferazione e differenziazione cellulare. Similmente alla loro controparte normale, i progenitori cellulari ematopoietici leucemici restano dipendenti dai segnali provenienti dal microambiente per sopravvivere e proliferare durante la progressione neoplastica. In particolare, la cellula staminale ematopoietica normale o tumorale, risiede all'interno di aree del midollo osseo altamente specializzate, definite nicchie del microambiente midollare: la nicchia endosteale (o osteoblastica) e la nicchia vascolare. La nicchia osteoblastica, localizzata nella superficie interna della cavità dell'osso, fornisce stimoli di quiescenza per le cellule staminali long-term (cosi definite poiché cellule indifferenziate con elevata capacità di generare cellule a loro volta immature e toti/multi potenti a lungo termine) coinvolte nell'emopoiesi. Invece, le nicchie vascolari, che consistono in cellule sinusoidali endoteliali ricoprenti il vaso sanguigno, promuovono la proliferazione e la differenziazione di cellule ematopoietiche staminali short-term (ovvero cellule staminali più differenziate con capacità di generare cellule a loro volta immature a breve termine). L'interazione delle cellule stromali e della matrice extracellulare con i blasti leucemici controlla anche i segnali anti-apoptotici che contribuiscono alla progressione neoplastica e alla persistenza del clone neoplastico residuo dopo trattamento.
La LMC è una neoplasia mieloproliferativa che colpisce le cellule staminali del midollo emopoietico da cui derivano i globuli bianchi (leucociti), le piastrine ed i globuli rossi. Alla base della malattia c'è un evento genetico che porta alla formazione del cromosoma Philadelphia (Ph)**,** così chiamato dal nome della città in cui è stato scoperto nel 1960.
La malattia rappresenta circa il 15% di tutti i casi di leucemia e la prevalenza è stimata intorno a uno-due casi ogni 100.000 persone/anno. L'età media alla diagnosi è circa 60 anni e raramente inferiore a 20 anni.
Un ruolo centrale nella patogenesi della CML è la formzione del gene di fusione tra il gene "Abelson murine leukemia" (ABL) sul cromosoma 9 e il gene "breakpoint cluster region" (BCR) sul cromosoma 22. Il gene di fusione BCR-ABL codifica per l'oncoproteina BCR-ABL.
Il gene ABL appartiene alla categoria delle tirosino-chinasi (TK), enzimi che fosforilano i substrati a livello di residui di tirosina. mediante la sua attività TKI, abl è in grado di attivare una "cascata" di altre proteine intracellulari che innescano i meccanismi della proliferazione cellulare.
Nella t(9;22) il gene ABL viene pressoché interamente traslocato sul cromosoma 22 e la proteina ibrida derivata dal gene ibrido BCR-ABL mantiene una attività TK

aumentata e non più sottoposta a normali meccanismi di controllo. Da ciò deriva il potenziale leucemogenico della proteina ibrida, per le conseguenti interferenze a livello dell'attività proliferativa della cellula neoplastica, dei meccanismi di adesione e della responsività ai fattori regolanti la proliferazione, e dei meccanismi di morte cellulare (apoptosi). Quindi la proteina ibrida BCR-ABL è una TK costitutivamente attiva che promuove la crescita e la replicazione cellulare attivando una serie di cascate a valle come RAS, RAF, la JUN chinasi, MYC e STAT.
Nella storia naturale della LMC si distinguono tre fasi successive:

Fase cronica

I pazienti presentano un elevato numero di globuli bianchi in tutti gli stadi di maturazione e, meno del 10% di cellule indifferenziate (definite blastiche), nel sangue periferico e nel midollo osseo. Nella evoluzione naturale della LMC la fase cronica può durare 5 o 6 anni prima di progredire verso la fase accelerata.

Fase accelerata

La fase accelerata è una fase intermedia nella storia della malattia in cui si comincia ad evidenziare una certa resistenza alle terapie. È caratterizzata dalla presenza nel sangue periferico o nel midollo osseo del 10-30% di cellule indifferenziate. I sintomi peggiorano e comprendono febbre di origine sconosciuta, dolore osseo, sintomi correlati all'ingrossamento della milza o del fegato, come nausea e dolori addominali. Possono manifestarsi diminuzione delle piastrine e anemia progressiva. Si osservano anche nuove e multiple anomalie cromosomiche. La fase accelerata naturalemnete dura in media da 6 a 9 mesi.

L'Organizzazione Mondiale della Sanità (OMS) definisce questo stadio della LMC in base alla presenza di uno o più dei seguenti condizioni:

a) percentuale di blasti (cellule immature) nel midollo osseo o nel sangue periferico pari al 10-19%;

b) percentuale di basofili nel sangue periferico pari o superiore al 20%;

c) conta piastrinica persistentemente inferiore a 100 x 10^9/l o superiore a >1000 x 10^9/l, indipendentemente dalla terapia;

d) aumento di dimensioni della milza e aumento del numero dei globuli bianchi nel sangue periferico, indipendentemente dalla terapia;

e) segni di evoluzione clonale (cioè comparsa di un'anomalia genetica aggiuntiva che non era presente al momento della diagnosi). Le alterazioni citogenetiche associate più frequentemente all'evoluzione della malattia sono un cromosoma Philadelphia aggiuntivo, la trisomia 8 (presenza di tre copie del cromosoma 8 invece di due), l'isocromosoma 17q (cromosoma anomalo formato dalla duplicazione di uno dei due bracci del cromosoma, che sostituisce l'altro) e la trisomia 19.

Crisi blastica

In circa il 25% dei pazienti la malattia passa dalla fase cronica direttamente alla fase blastica, saltando la fase accelerata.

La crisi blastica, che rappresenta lo stadio terminale della malattia, è caratterizzata dalla presenza di oltre il 30% di blasti nel sangue periferico o nel midollo osseo e da un aumento dei sintomi legato al progressivo e grave scompenso della funzione midollare: facile affaticabilità legata alla anemia, complicanze emorragiche legate alla carenza di piastrine e complicanze infettive legate alla progressiva riduzione/scomparsa di globuli bianchi maturi.
La crisi blastica è una situazione molto difficile e non esiste una forma di terapia veramente soddisfacente, incluso il trapianto. I nuovi farmaci, in una percentuale minoritaria di pazienti, sono in grado di riportare condizioni di stabilità (che spesso non sono durature).

L'OMS definisce la crisi blastica in base alla presenza di uno o più dei seguenti condizioni:

a. percentuale di blasti nel midollo osseo o nel sangue periferico pari o superiore al 20%;

b. presenza di ampi aggregati o gruppi di blasti alla biopsia del midollo osseo;

c. sviluppo di blasti al di fuori del midollo osseo. In circa un terzo dei pazienti i blasti hanno un aspetto linfoide (cioè somigliante a quello dei progenitori dei linfociti), mentre nei due terzi rimanenti hanno un aspetto mieloide (cioè somigliante a quello dei progenitori di tutti gli altri globuli bianchi) o indifferenziato (cioè senza caratteristiche distintive particolari). Le sedi extramidollari più frequentemente interessate dalla BC sono la pelle, i linfonodi, la milza, le ossa e il sistema nervoso centrale, ma la fase blastica può coinvolgere qualunque altro distretto corporeo.

La malattia viene generalmente diagnosticata in fase cronica, spesso in occasione di esami di routine quando il paziente è asintomatico. Molto raramente alla diagnosi il paziente si presenta in fase accelerata o blastica.
Fino a poco più di dieci anni fa, la terapia per la LMC era limitata ad alcuni agenti terapeutici non specifici come l'agente alchilante ciclo cellulare non-specifico busulfan, idrossiurea ed interferone alfa.
L'inibitore della sintesi del DNA I idrossiurea fu introdotto negli anni '70 e sostituì il busulfan per la sua maggior maneggevolezza nel controllo della soppressione midollare e per la mancanza di tossicità polmonare. Negli anni '80 fu sperimentato nei pazienti affetti da LMC l'uso dell'INF-α. Questo farmaco induce una risposta ematologica completa nell' 50-70% dei pazienti ed una risposta citogenetica (definita come percentuale di cellule midollari Ph+ inferiore al 35%) nel 10-20% dei casi. Purtroppo la terapia con INF-α induce alcuni effetti collaterali importanti: sintomi simil influenzali, calo ponderale e, meno frequentemente, diarrea e neurotossicità che si presenta con depressione e deficit anamnestici, cioè disturbi della memoria. L'unico intervento curativo è il trapianto allogenico di cellule staminali (AlloSCT); una scelta terapeutica associata ad un elevato rischio di morbidità e mortalità.
La terapia della LMC è stata profondamente modificata negli ultimi dieci anni dalla introduzione di una classe di farmaci noti come "inibitori delle tirosino-chinasi**"** (TKI). Il primo di questi farmaci ad affermarsi nel trattamento della LMC è stato l'Imatinib mesilato, cui hanno fatto seguito il Nilotinib e il Dasatinib. I farmaci TKI sono una

classe di antineoplastici che appartengono ad una categoria di nuovi farmaci detti “a bersaglio molecolare (target) ” o “biologici ” o ancora “intelligenti ”. Caratteristiche peculiari e vantaggiose di questi nuovi farmaci sono:

1. l’azione selettiva su definiti substrati delle cellule tumorali,
2. la modesta insorgenza di effetti indesiderati,
3. la possibilità di essere somministrati, in alcuni casi (come nel caso dell’Imatinib), per via orale,
4. la possibilità di utilizzo in associazione con terapie tradizionali.

L’introduzione di una terapia “a bersaglio molecolare” nella pratica clinica ha migliorato drammaticamente la storia naturale della malattia, incrementando la sopravvivenza globale dei pazienti con LMC a dieci anni dal 20% all’ 80-90%.
Nonostante i notevoli progressi ottenuti nella terapia della LMC con i TKI, numerose evidenze sperimentali hanno dimostrato chiaramente che inibire l’attività chinasica BCR-ABL non è sufficiente ad eliminare la malattia minima residua, la cui persistenza è dovuta ad una mancata eliminazione delle cellule staminali leucemiche nel tessuto midollare. Pertanto una strategia curativa, necessariamente deve prevedere la combinazione di un farmaco TKI con agenti che specificamente agiscono sulla cellula staminale leucemica all’interno della nicchia midollare.
Il progetto sviluppato ha inizialmente valutato il ruolo del microambiente tumorale nella determinazione degli eventi di leucemogenesi, di progressione e di induzione della resistenza al trattamento sia su cellule neoplastiche isolate da pazienti affetti da LMC in trattamento con TKI (Farmaci inibitori della tirosin chinasi: Imatinib, Dasatinib o Nilotinib) sia su linee cellulari Ph+ immortalizzate.
A tale scopo le cellule leucemiche derivate dai pazienti in trattamento con i TKI, sono state testate per le loro proprietà di adesione e interazione con il mocroambiente midollare, con il fine di identificare gli eventuali fattori peculiari che li legano alla nicchia osteoblastica midollare. In particolare sono state raccolte le cellule Ph+ isolate dai campioni di BM (sangue midollare) e dal PB (sangue periferico) di paziente affetti da LMC in pretrattamento e/o durante il trattamento con TKI (in particolare al terzo e sesto mese di trattamento).
In particolare abbiamo ricevuto 41 campioni midollari e 41 campioni periferici in pre-trattamento (Visita 1) e 40 campioni (sia midollari che periferici per ogni singolo paziente) alla Visita 2 (alla fine del terzo mese) e alla Visita 3 (fine del sesto mese). Dai 41campioni midollari e/o periferici in pre-trattamento abbiamo selezionato la popolazione cellulare CD34+ e 14 popolazioni di cellule stromali MSCA+ . Dai 40 campioni biologici arrivati alla visita successiva non abbiamo ricavato un numero sufficiente di cellule da poter selezionare le cellule CD34+ o MSCA+.
L’antigene CD34 si ritrova sulle cellule progenitrici ematopoietiche di tutte le linee così come la maggior parte delle cellule staminali primitive totipotenti. L’antigene CD34 ha la sua massima espressione sulla maggior parte delle cellule staminali primitive e viene gradualmente perso quando le cellule progenitrici predestinate della linea si differenziano. L’antigene CD34 é anche presente sulle cellule endoteliali capillari e sulle cellule stromali del midollo osseo. L’antigene CD34 é espresso in circa il 60% dei casi di leucemia acuta, sia linfoide che mieloide.
Nelle CML, l’aumento del numero di CD34+ può essere un indice rilevante della progressione a fase accelerata o blastica.

Le cellule CD34+ sono state selezionate dalle cellule mononucleate totali dopo separazione immunomagnetica usando il kit MiniMACS per le cellule CD34 (Miltenyi Biotec), secondo le istruzioni della ditta produttrice.
Le cellule staminali si distinguono in totipotenti, capaci di trasformarsi in qualsiasi tipo di tessuto; pluripotenti, che si trasformano solo in alcuni tipi di tessuti; unipotenti, che possono dar luogo soltanto ad un tipo cellulare. Le cellule staminali mesenchimali sono una popolazione cellulare pluripotente.
Le "cellule staminali mesenchimali" (MSCs), dotate di capacità di automantenimento e differenziativa in senso osteoblastico, condrocitario, adipocitario, mioblastico e fibroblastico. Sono anche denominate "cellule stromali midollari", data la loro capacità di generare le cellule del microambiente midollare. Infatti, le cellule staminali mesenchimali sono contenute all'interno dello stroma midollare.
L' anti-MSCA (W8B2) è un anticorpo sviluppato per l'isolamento delle cellule stromali mesenchimali (MSC) dall'aspirato midollare. L'espressione dell'antigene MSCA-1 è ristretto, nella popolazione cellulare midollare, alla frazione $CD271^{bright}$. Le cellule MSC $CD271^{bright}CD45^{dim}$ hanno un'altissima capacità clonogenica comparata alla frazione midollare $CD271^{+}CD45^{+}$.
Le cellule MSCA+ sono state selezionate dalle cellule mononucleate totali dopo separazione immunomagnetica, secondo le istruzioni della ditta produttrice.
Nella prima parte del lavoro scientifico abbiamo osservato sperimentalmente che sia le linee Ph+ (K562, KT1 o BV173) che cellule staminali CD34+Ph+ sono significativamente protette dalla mortalità cellulare TKI-dipendente (Imatinib, Nilotinib o Dasatinib) quando incubate in presenza (direttamente o separate da una membrana che lascia passare solo i fattori solubili) della linea cellulare stromale HS5 o di linee stromali MSCA+ ottenute ex-vivo da campioni midollari isolati da pazienti affetti da LMC alla diagnosi.
Inoltre, ho osservato nel mio studio sperimentale che la resistenza è BCR-ABL indipendente, mediata presumibilmente dalla attivazione della cascata JAK-STAT3. Infatti, il co-trattamento delle linee tumorali Ph+ con uno dei TKI e un inibitore specifico della tirosin chinasi non recettoriale JAK2 è in grado di superare questa resistenza. Inoltre le due categorie di farmaci hanno mostrato un significativo effetto sinergico nell'azione terapeutica.
Nella parte finale del progetto, con il fine di determinare il ruolo esatto dei differenti fattori di crescita e delle citochine nella sopravvivenza delle cellule leucemiche, abbiamo determinato mediante il saggio ELISA assay BioPlex il livello di espressione contemporaneamente di 20 differenti citochine prodotte dalle cellule stromali isolate da pazienti affetti da LMC alla diagnosi o dalla linea immortalizzata HS5. I fattori solubili valutati sono stati: IL1a, IL1b, IL3, IL6, IL7, IL8, IL10, IL12, IL15, G-CSF, M-CSF, SCF, SDF1, TRAIL, HGF, PDGFbb, GM-CSF, MIP-1a, TNFa e VEGF. Questi fattori sono noti avere un ruolo chiave nell'interazione delle cellule staminali Ph+ con il microambiente midollare.
Quindi, tutte le citochine espresse sia dalle cellule HS5 che dalle cellule stromali ottenute dai pazienti affetti da LMC, sono state poi testate in vitro per verificare se e come andassero a modulare la risposta farmacologica delle cellule Ph+ ai TKI. Il saggio ELISA assay BioPlex è stato effettuato sul mezzo di 24 ore di coltura della linea stromale HS5 (HS5/SCM) e sui mezzi condizionati derivanti dalla coltura delle cellule stromali ottenute da 8 differenti pazienti affetti da CML (CML/SCM). Nel nostro saggio abbiamo identificato una serie di fattori altamente espressi in entrambe le linee cellulari, quali il fattore G-CSF, IL-6, IL-8 e VEGF, conosciuti avere un ruolo fondamentale nell'attivazione di STAT3 e quindi della via JAK/STAT3.

Recentemente utilizzando topi knock-out per STAT3 si è dimostrato il suo ruolo fondamentale nel dare origine alla malattia, ma non nel mantenimento della stessa. Nel contesto del microambiente midollare, STAT3 è attivato in maniera BCR-ABL indipendente dalle citochine presenti nel mezzo di coltura dello stroma midollare. Quindi l'attivazione della via JAK/STAT3 contribuisce alla sopravvivenza del clone staminale leucemico anche in presenza dei TKI. I nostri dati suggeriscono che la via JAK/STAT3 è un bersaglio terapeutico interessante nell'ottica di una terapia combinatoria con i TKI, e rappresenta una strategia vitale nell'eliminare o almeno ridurre la malattia minima residua dei pazienti affetti da LMC.

SUMMARY

The importance of tumor microenvironment for cancer progression is becoming widely recognized in recent years. The Bone Marrow (BM) is a dynamic microenvironment with high concentration of growth factors and cytokines necessary for haematopoiesis, making it a highly permissive zone for cancer haematopoietic staminal cell homing and survival. It is possible that the same factors that modulate hematopoiesis promote leukemogenesis, enhance blast survival and make them resistant to treatment within the BM microenvironment. In the era of molecular target therapy, whereas Imatinib has shown a cumulative best complete cytogenetic response rate of 82% and an estimated event free survival at 8 years of 85%, several in vitro data have confirmed that Ph+ CD34+ progenitor cells crammed in BM niches are resistant to TKI treatments. We attempted to define BM microenvironment markers that nurture and determine stem cell fate in leukemia associated-niches.
We treated Ph+ K562 cell lines and primary CD34+ BM cells derived from untreated CML patients with a dose range of TKIs (0-100μM) in the presence of a monolayer of human BM mesenchimal stromal cell line (HS-5) or HS5 conditioned media (HCM), to assess the role of BM niche in the regulation of TKI responsiveness. We demonstrated that BM stroma environment significantly protects K562 cell line from TKI-induced apoptosis. Indeed, we demonstrated that Half maximal inhibitory concentration (IC50) value (calculated on cell viability) of Imatinib, Nilotinib and Dasatinib significantly increased when leukemic cells are exposed to HS5 or HCM. Moreover, we prove that a significant TKI-resistance could be achieved also by Ph+ CD34+ primary CML cells exposed to HS5 or HCM.
Taken together, these findings indicate that BM-derived stroma cell line produces a strong effect on the regulation of TKI responsiveness in Ph+ CML cells by both a direct cell-to-cell contact and exposition to soluble factors. Moreover, the observed TKI resistance is associated to a BCR-ABL independent STAT-3 activation leading to a significant down-modulation of apoptosis when either Ph+ cell line or primary CD34+ progenitor cells derived from patients with CML are treated with TKIs in the presence of a direct mesenchymal stroma cell interaction or exposition to SCM. Finally, I proved that JAK inhibitor Ruxolitinib, that inhibits STAT3 phosphorylation (a marker of JAK activity), synergizes with TKIs in the induction of apoptosis in CML primary cells. Indeed, compared with single agent treatment, exposure of CML cells to the combination of TKI and JAK inhibitor Ruxolitinib significantly decreased viability of CML cells and increased their apoptosis in vitro. Taken together, our data show that the rational drug combination of TKI and Ruxolitinib may enhance the eradication of primary human Ph+ cells homed in BM stroma niche.

BONE MARROW MICROENVIRONMENT

The bone marrow provides a framework of microenvironmental domains or niches that support the function of immune cells and Haematopoietic Stem Cells (HSCs). Cellular niches are functional compartments within tissues that control cell numbers by providing signals that regulate self-renewal, differentiation and quiescence. Such signals can be transmitted both via direct cell-to-cell contact, or by growth factors cytokines and components of the extracellular matrix. The concept of the HSC niche was first proposed by Schofield in 1978 to describe the physiologically limited microenvironment in which stem cells reside. The niche hypothesis has been supported by a variety of coculture experiments in vitro **[1–2].** Normal hematopoiesis occurs in a unique Bone Marrow (BM) microenvironment that is of fundamental to the production and differentiation of normal hematopoietic progenitor cells. Such hematopoietic cell development is regulated by BM stromal cells (BMSC) through the production of cytokines and intracellular signals initiated by cellular adhesion **[3].**
BM resides within the protected confines of the bones. In adults, 4.6% of body weight is due to the BM that is distributed throughout the vertebrae, ribs, pelvis, skull and proximal ends of the long bones. The fundamental objective of the BM is to maintain the numbers of differentiated hematopoietic cells in the peripheral blood at a constant level throughout the life time of a human being. The BM does this by producing an estimated 500 billion cells per day **[4].** The structure of the BM consists of a rigid bone cortex enclosing a cavity containing the arterial vascular system, a complex sinusoidal system, hematopoietic cells and the stroma. Arterial vessels enter the BM and divide into arterioles and capillaries that span throughout the BM **[5].** The cellular component of the BM can be divided into Hematopoietic Cells and Mesenchymal-Derived Cells.
The mesenchymal-derived cells, along with the macrophages and the extracellular matrix (ECM), form the BM stroma.

The main constituents of the BM extracellular matrix (ECM) include fibronectin (FN), hyaluronan, collagen types I and IV, laminin, glycosaminoglycans heparin sulfate and chondroitin sulfate. In addition to providing the structural scaffold for cellular elements of the BM, ECM also exerts its effect on cell growth, differentiation, motility and viability. The ECM molecules function as a reservoir of many secreted growth factors, cytokines, matrix metalloproteinases and other processing enzymes whose availability is regulated by arrangement of the matrix, thus creating stromal niches within the BM **[6].**

Osteoblasts play a central role in skeletal development. Derived from pluripotent BMSC, they mature along a specific lineage to become highly specialized cells. Primary human osteoblasts produce growth factors, including granulocyte colony stimulating factor G-CSF, that are likely essential for the survival of CD34+ BM cells and support the growth of CD34+ human BM progenitor cells in vitro **[7].** Some recent studies have suggested that osteoblasts are critical regulators of hematopoietic stem cells (HSCs) microenvironment **[8-9].**

Adipocytes, which are the prevalent type of stromal cells in adult BM, also play an important role in the hematopoietic environment. In particular, the adipocyte differentiation of BMSC has been shown to positively correlate with the ability of the BM microenvironment to support the growth of lymphoid cells. **[10].** They produce the leptin and multiple cytokines, including stromal-derived factor-1 (SDF-1), M-CSF,

GM-CSF, SCF, thrombopoietin, IL-6, LIF, and transforming growth factor β (TFG-β) **[11].** Preadipocytes also strongly express fibronectin, an adhesive ECM protein that promotes leukemia development **[12].** These mechanisms may be important to the interactions between leukemic and stromal cells that occur in vivo, with a potential of promoting chemoresistance in leukemias **[13].** The hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) are held within the BM stroma not only through the adhesive interactions between VCAM-1, expressed by stromal cells, and integrin α4β1 (alpha 4 beta 1) expressed by the HSCs and HPCs, but also by the *chemotactic interaction* between the chemokine CXCL12 and its receptor CXCR4 expressed on the HSCs and HPCs. In steady-state conditions the majority of the HSCs reside in the BM with a few circulating in the peripheral blood. HSCs are localized in a non random manner at the bone–BM interface (osteoblastic niche) and around the blood vessels (vascular niche).

The Osteoblastic Niche

The majority of HSCs in the endosteum are found to be in direct contact with the osteoblasts. The HSCs home in on the endosteum with the help of a chemotactic Ca2+ sensing receptor that senses the Ca2+ gradient within the BM. The gradient is created by the release of Ca2+ in the BM due to the constitutive osteoclasts and osteoblasts-mediated bone remodeling.[54] Once reaching the endosteum, the HSCs are held in the endosteum by the expression of:

- Adhesive molecules like osteopontin,
- N-cadherin,
- Transmembrane c-KIT ligand stem cell factor (SCF)
- The polysaccharide hyaluronic acid.

This interaction is strengthened by the cytokines and chemokines that are produced by the osteoblasts, like:

- Chemokine CXCL12, also known as SDF-1
- Angiopoietin-1 (Ang1),
- Jagged-1, a ligand for Notch1 receptor also expressed by HSCs.

Osteopontin, a matrix glycoprotein expressed by the osteoblasts supports the adhesion of HSC to the osteoblastic niche and negatively regulates HSC proliferation, contributing to the maintenance of a quiescent state. N-cadherin, is expressed in HSCs or HPCs and they plays a critical role in the regulation of HSCs/HPCs engraftment. Osteoblasts in the bone marrow produce a significant amount of membrane-bound stem cell factor (SCF), which has the capacity to enhance the adhesion of HSCs to stromal cells. [56] SCF binds and activate c-KIT (a receptor tyrosine kinase), which is highly expressed by HSCs. Hyaluronan is synthesized by primitive HSCs, participates in their lodgment at the endosteum.

Secreted Factors

The bone-forming osteoblasts are crucial players for the homeostasis of the hematopoietic tissue with its high turn-over. Indeed, osteoblasts express several cell-signaling molecules such as BMP4, Jagged-1, and angiopoietin-1 (Ang1), both are important for HSCs self-renewal, survival, and maintenance. CXCL12 a member of the CXC subfamily of cytokines, also known as Stromal derived factor-1 (SDF-1) is produced by stromal cells and, acting through the G protein–coupled CXCR4, the sole receptor for SDF-1. SDF-1 functions as both a chemo-attractant and a modulator of cellular growth/ survival. Ang1 in osteoblasts interacts with the tyrosine kinase receptor Tie-2, a type of receptor tyrosine kinase that is expressed in HSCs, and the Tie-2/Ang1 interaction activates β1-integrin and N-cadherin. This enhanced adhesion contributes to the maintenance of the stem cell quiescence. Osteoblasts expressing the Notch ligand: Jagged1. Jagged1-mediated Notch1 signaling is dispensable for HSCs self-renewal and differentiation.

The Vascular Niche

The vascular niche promotes proliferation, differentiation of actively cycling, and short-term HSCs. The most purified HSCs, fractioned as CD150+ CD48− CD41− Lin−cells, were found in majority to be associated with the sinusoidal endothelium lining blood vessels, suggesting that endothelial cells create a cellular niche for HSCs.

Secreted Factors

The vascular niche has been shown to produce factors important for mobilization, homing, and engraftment of HSCs, such as CXCL12 (SDF-1, stromal cell derived factor-1) important for mobilization, homing, and engraftment for HSCs. SDF-1 and FGF-4 (fibroblast growth factor-4) induce upregulation of adhesion molecules, including VLA4/VCAM1, facilitating localization to the vascular niche. CXCL12/CXCR4 signaling plays important roles in HSCs trafficking and HSCs mobilization. HSCs were specifically located adjacent to cells expressing a high level of CXCL12, which surrounded the sinusoidal endothelial cells. They named these cells CXCL12-abundant reticular cells (CAR). HSCs have been shown to be associated with CAR cells in the sinusoidal region and are located between CAR and osteoblastic cells in the endosteal region, suggesting that those cells might be an important component of both the osteoblastic and the vascular niches in adult BM.

Membrane-Bound Factors

During the bone marrow transplantation, the ability of HSCs to “home” and engraft in the recipient’s bone marrow requires a cascade of events which includes specific molecular recognition. Adhesion molecules on the HSCs involved in the process of rolling are VLA4 (CD49d), LFA-1 (CD11a), and hyaluronan binding cellular adhesion molecule (HCAM/CD44) whereas the complementary binding partners on the BM

endothelial cells are VCAM-1, ICAM-1 (CD54), and E- and P-selectin (CD62E and CD62P).[62]

Difference between vascular and osteoblastic niches.

The major difference between both microenvironments is the oxygen level. Higher in the vascular niche than in the osteoblastic niche under hypoxia, HSCs would move to the vascular niche and resume then cell cycle in order to restore hematopoiesis. The osteoblastic niche localized at the inner surface of the bone cavity and with abundant osteoblasts, might serve as a reservoir for long-term HSC storage in a quiescent state. Whereas the vascular niche, which consists of sinusoidal endothelial cell lining blood vessel, provides an environment for short-term HSC proliferation and differentiation. Both niches act together to maintain hematopoietic homeostasis or restore it after damage.

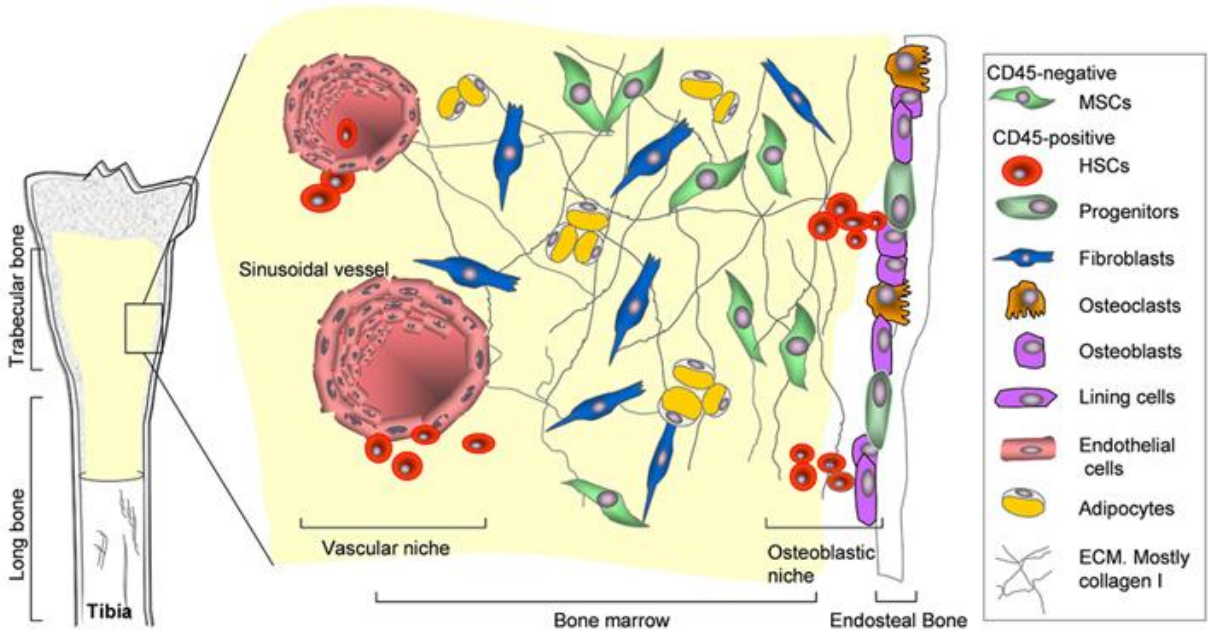

Figure 1. **Schematic representation of the molecular interactions between hematopoietic stem cells and their niches in the bone marrow**. In normal physiological conditions, the hematopoietic stem cells (HSCs) reside either at the endosteum, lodged with the osteoblasts (OBT) (osteoblastic niche) or at the sinusoidal vessels (SV) (vascular niche). The osteoblastic niche provides the microenvironment for HSC maintenance and quiescence and the vascular niche provides the microenvironment for HSC proliferation and differentiation. The oxygen and the calcium gradient might play a crucial role in maintenance of the different niches within the bone marrow.

Mesenchymal Stem Cells

The key component of the BM is Mesenchymal Stromal Cells (MSC). These plastic-adherent cells currently described as mesenchymal stem cells are termed multipotent mesenchymal stromal cells, while the term MSCs should be reserved for a subset of these cells that demonstrate stem cell activity by clearly stated criteria **[14].** MSCs are primitive cells originating from the mesodermal germ layer and were classically described to give rise to connective tissues, skeletal muscle cells, and cells of the vascular system. Friedenstein and colleagues (1974) first described MSC as fibroblast-like cells that could be isolated from BM via inherent adherence to plastic in culture. BM-derived MSC are primitive, undifferentiated cells that are capable of self-renewal and of giving rise to different cell lineages, including adipocytes, osteocytes, fibroblasts, chondrocytes, and myoblasts. Thus, MSCs are key components of the hematopoietic microenvironment. One of the main functions of MSCs is to provide, within the BM, a microenvironment that is conducive for hematopoiesis. It does so by not only differentiating into the various cellular components of the BM stroma, but also by providing a scaffold for cell to cell interactions and by secreting cytokines and growth factors. MSCs rely on the *transforming growth factor-β* (TGF-β) family of proteins in governing its development and differentiation. Even if TGF-β itself promotes the expansion of undifferentiated MSCs, other TGF-β family members in cooperation with specific transcriptional regulators promote the differentiation of MSCs. For example, transcription factor Runx2 drives MSCs to differentiate into osteoblasts and the transcription factor peroxisome proliferator-activated receptor (PPARγ) drives the differentiation of MSCs into adipocytes **[15-16]**. In addition, it has also been demonstrated that MSCs are capable of differentiating into cardiomyocytes, neurons, and astrocytes in vitro and in vivo **[17].** MSC constitutively express the mRNA for *interleukin (*IL)-6, IL-11, *leukemia inhibitory factor* (LIF), *macrophage colony-stimulating factor* (M-CSF), and *stem cell factor* (SCF) and can express *granulocyte colonystimulating factor* (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) upon IL-1α stimulation **[18].** MSCs are also considered the major source for the secretion of the homeostatic chemokine, CXCL12 which has a role in both homing and circulation of hematopoietic cells **[19].** MSCs play a fundamental role in lymphopoiesis by aiding the development of the naïve B cells in the BM and the development of T cells via the process of extra-thymic T cell lynphopoiesis **[20].** MSCs through the production of CXCL12, assist in the homing of CD8+ T cells into the BM and thus mediate the induction of adaptive immunity in response to antigen HSCs and in immune responses. This ability of MSC to produce cytokines coupled with the multiple mechanisms in the ECM that directly support MSC and their pleiotropic capacity are also conducive to the development of leukemic cells. Phenotypically MSCs express a number of markers, none of which are specific only to MSCs. It is generally agreed that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14, or CD11. They also do not express the costimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31, CD18, or CD56, but they can express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1 as well as the adhesion molecules CD106, CD166, intercellular adhesion molecule, and CD29 **[21-22].** Although there are no unique cell surface markers for the identification of MSCs, minimal criteria to define human MSC have been published. According to such criteria, MSC must be:1) plastic-adherent; 2) express CD105, CD90 and CD73; 3) lacking expression of

CD45, CD34 and CD14; 4) show in vitro differentiation capabilities into osteoblasts, adipocytes and chondroblasts **[22-23].**

Endothelial Cells and Angiogenesis

The microvasculature is an active component of the stroma and is responsible for supplying the appropriate oxygen and nutrients. It provides a route for homing and metastasis as well, and the BM endothelial cells are also involved in autocrine and paracrine interactions with leukemic cells **[13].** The synthesis of *Vascular endothelial growth factor (*VEGF) and other angiogenic factors such as *basic fibroblast growth factors* (bFGF) and hepatocyte growth factor (HGF) has been observed in leukemic cells. Interestingly, VEGF signaling in HSCs is involved in regulating cell survival by means of an internal autocrine loop mechanism that involves both *VEGF receptors* (VEGFR-1 and -2) **[25].** VEGF is an essential regulator of physiologic and pathologic angiogenesis. For example, VEGF secreted by leukemic cells activates receptors on both leukemic and endothelial cells. In chronic myeloid leukemia (CML), VEGF is expressed at high levels in the BM and peripheral blood; moreover, the CML-associated oncogene BCR-ABL induces VEGF- and *hypoxia inducible factor-1α* (HIF1α)-gene expression via *phosphoinositide 3-kinase (*PI3K)-mTOR– dependent pathway **[26].** It is therefore conceivable that the expression of HIF target genes, such as VEGF, plays an important role in leukemia progression, therapy response, and outcome **[27].** On the basis of in vivo and ex vivo studies, BM endothelial cells were found to express factors that promote haematopoiesis, such as *granulocyte colony stimulating factor* (G-CSF), *granulocyte–macrophage colony-stimulating factor* (GM-CSF), *macrophage colony-stimulating factor* (M-CSF), *stem cell factor* (SCF; also known as KIT ligand), *interleukin-6* (IL-6) and *FMS-related tyrosine kinase 3 ligand* (FLT3L; also known as FLK2 ligand). In addition, these cells were shown to express the adhesion molecules E-selectin, P-selectin, *vascular cell adhesion molecule 1* (VCAM1) and *intercellular adhesion molecule 1* (ICAM1)**[28].**

The BM vasculature is heterogeneous in its expression of molecules that are thought to facilitate cell homing, such as E-selectin and CXCL12. Such homing pathways can also be exploited by malignancies that can metastasize to the BM **[29-30-31].** In vitro, immortalized endothelial cells can support HSC function in culture through the expression of Notch ligands **[32]**. Similarly, the expression of a constitutively active mutant form of AKT in endothelial cells (using a cell-specific inducible transgenic mouse model) leads to an increase in HSC numbers **[33]**. These characteristics support the idea that endothelial cells can provide niche functions in vivo, although conclusive evidence that they do so is not yet in hand. Endothelial cells are surrounded by perivascular MSCs, which provide structural support and harbor populations of cells that can regenerate the BM stroma and interact directly with HSCs. Under steady-state conditions, most stem cells are in contact with BM stromal cells, including osteoblasts, and are maintained in G0 phase of cell cycle **[34].** Although a small fraction is in S or G2/M phase of the cell cycle. The equilibrium between these two compartments is dictated by the bioavailability of stem cell-active cytokines, which are bound to the extracellular matrix or tethered to the membrane of stromal cells. Stress, such as BM ablation by cytotoxic agents, switches on sequences of events where HSCs are recruited from their niches to reconstitute hematopoiesis. Local secretion of proteases may alter the stem cell-stromal cell interaction. The proteolytic cleavage of vascular cell adhesion molecule-1, expressed

by BM stromal cells, is triggered by the degranulation of neutrophils in the BM after G-CSF administration and may be an essential step contributing to the mobilization of hematopoietic progenitors. *Matrix metalloproteinases* (MMPs) promote the release of extracellular matrix-bound or cell-surface-bound cytokines such as VEGF, which then can regulate angiogenesis or osteoclast recruitment **[36-37-38].**

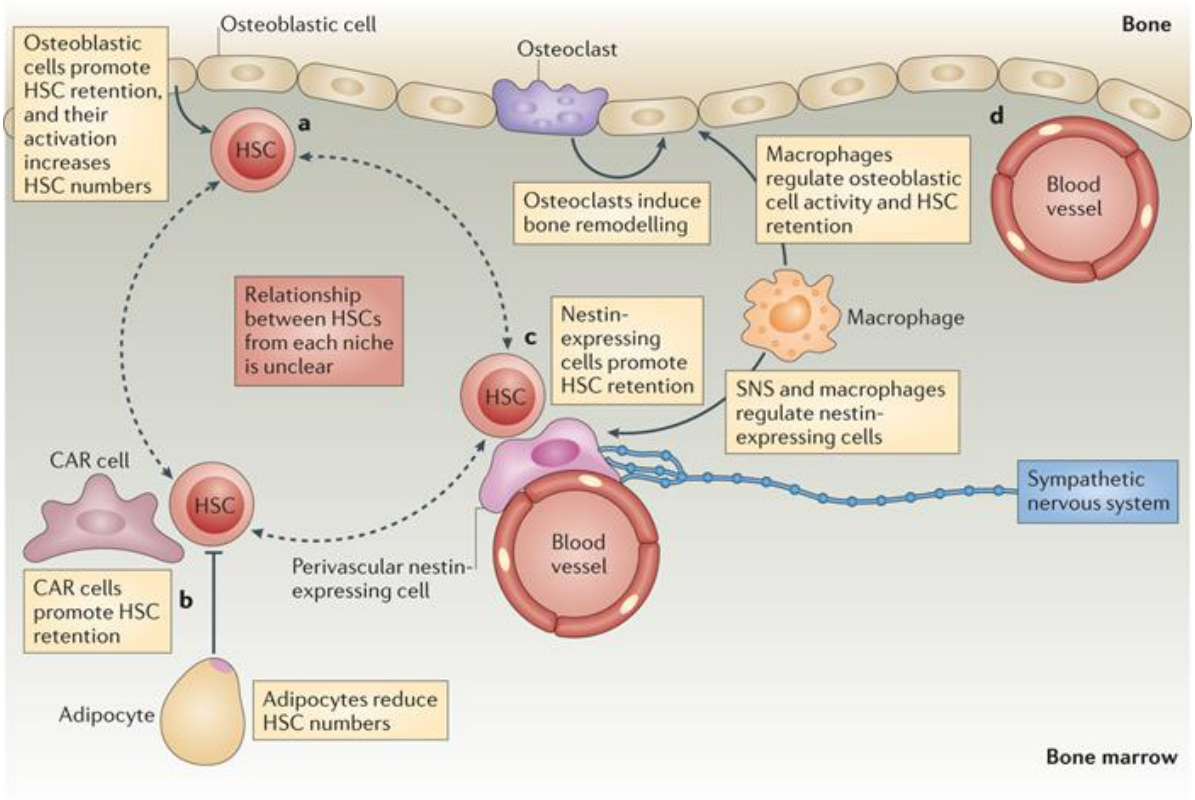

Figure 2. In the bone marrow, haematopoietic stem cells (HSCs) can be found near the endosteal surface (**a**); in association with CXCL12-abundant reticular (CAR) cells (**b**); and in the periphery of sinusoids and perivascular nestin-expressing cells (**c**). Each niche is thought to provide signals that support HSC behavior, although the relationship between HSCs that are present in different niches is still unclear (dotted arrows). Likewise, blood vessels in the bone marrow are often in close association with bone, although their interaction is still poorly understood (**d**). At the endosteal surface, osteoblastic cells express factors that participate in HSC retention; osteoclasts regulate osteoblastic cell function by inducing bone remodelling. In the bone marrow stroma, HSCs are associated with CAR cells, which express factors that promote HSC retention. Adipocytes negatively regulate HSCs in the steady state. In the perivascular area, HSCs are associated with nestin-expressing cells, which promote HSC retention and are regulated by macrophages and the sympathetic nervous system (SNS).

Hematopoiesis and vascularization occur concurrently during development. In fact, HSCs and endothelial cells are derived from the same progenitor cells (hemangioblasts) at the embryonic stage and are closely related to the ontogeny of hematopoiesis that occurs in the yolk sac, aorta-gonad-mesonephros, placenta, fetal liver, spleen, and adult BM. The BM vascular structure provides a barrier between the hematopoietic compartment and the peripheral circulation. Most primitive HSCs remain physiologically quiescent within the BM niche; however, a portion of HSCs leave this resting pool and initiate the process of mobilization **[37-38-39]**. There are some controversial reports suggesting that mobilized HSCs may be more quiescent than those that remain in the BM. Evidence to support this notion comes mainly from a study in which the CD34+ marker was used to identify HSCs and HPCs **[42].** Since the population of CD34+ cells included numerous progenitor cells BM endothelial cells have been proposed to play a role in HSC controlling within vascular niche. Primary CD34+ microvascular endothelial cells can restore hematopoiesis in mice when they receive lethal doses of irradiation **[43].** Selective activation of AKT in endothelial cells produced angiocrine factors mediated in the reconstitution, expansion, and maintenance of HSCs **[44].** Nonetheless, constitutively activation of AKT, a binding ligand of phosphoinositide 3 in the phosphoinositide 3-kinase pathway, impaired engraftment ability and generated leukemia in mice **[45].** Reticular cells located around the sinusoid endothelium could produce *stromal cell-derived factor 1* (SDF-1, also CXCL12) mediated in HSC niche. These cells have been named CXCL12 abundant reticular cells (CAR cells). The shuttle of HSCs between vascular and endosteal niches is a important phenomena involving CXCL12/CXCR4 signaling. This signaling is essential in maintaining the HSC pool, development of B cells and plasmacytoid dendritic cells **[46-47].**

Hierarchy of Human Hematopoiesis

The origin of all blood cells in hematopoietic system is believed to be derived from HSCs that contain self-renewal capacity and give rise to *multipotent progenitors* (MPPs) which lose self-renewal potential but remain fully able to differentiate into all multilineage cells. MPPs further give rise to oligopotent progenitors which are common lymphoid and myeloid progenitors (CLPs and CMPs, resp.).

All these oligopotent progenitors differentiate into their restricted lineage commitment: (1) CMPs advance to megakaryocyte/erythrocyte progenitors (MEPs), granulocyte/macrophage progenitors (GMPs), and dendritic cell (DC) progenitors, (2) CLPs give rise to T cell progenitors, B cell progenitors, NK cell progenitors and DC progenitors. Notably, DC progenitors (CD8α+ DC, CD8α− DC, and plasmacytoid DC) could be derived from both CMPs and CLPs **[48-49]**. Among the isolation and characterization of HSCs and progenitors, CD34 molecule is the first widely chosen for the study by several researchers. CD34 is comprised in the CD34 family of cell-surface transmembrane proteins together with podocalyxin and endoglycan **[50-51].** CD34 expression on blood cells is about 0.1–4.9% in human cord blood, bone marrow, and peripheral blood **[52-53]** .

The balance that controls between self-renewal and differentiation (or cell fate decision) of HSCs in the bone marrow is mediated by several factors. Most HSCs are in quiescent state (i.e., in G0/G1 phase of the cell cycle). However, when the hematopoietic cell disturbance occurs, hematopoiesis system will respond by **[54].** shutting down or turning on specific signals. Several pathways have been employed

in this regulation, including SDF-1 (CXCL12)/CXCR4, BMP, Mpl/Thrombopoietin (TPO), Tie2/Ang-1, Hedgehog and Notch, as well as Wingless (Wnt). SDF-1 belongs to α-chemokines that functions as chemoattractant for both committed and primitive hematopoietic progenitors and regulates embryonic development including organ homeostasis **[55].**

Bone morphogenic proteins (BMPs) are a group of growth factors that belongs to a TGF-β family member **[56].** BMPs are mainly produced by osteoclasts in HSC niche **[57].** BMP signaling impairment displayed an increase in the niche size, leading to the enhancement in the number of HSCs **[58].** c-Mpl and its ligand, *thrombopoietin* (TPO), are known to regulate megakaryopoiesis **[59].** c-Mpl receptor is expressed mainly on HSCs, with a lesser extent on megakaryocytic progenitors, megakaryocytes and platelets **[4].** *Angiopoietin-1* (Ang-1) is the ligand of Tie2, a receptor tyrosine kinase, which expresses predominantly on osteoblastic cells in endosteum **[60]** and in MSCs **[61].** Interaction of Tie2 with its ligand, Ang- 1, resulted in tightly adhesion of HSCs to the niche and become more quiescence. Hedgehog (Hh) is proposed as a negative regulator of the HSC quiescence **[62].** Hh ligand binds to the transmembrane receptor Patched (Ptc) and subsequently allows the signaling function of a second transmembrane protein, Smoothened (Smo), essentially for the Hh signal to be active, but in contrast it has been demonstrated that constitutive activation of the Hh signaling pathway in Ptc heterozygous (Ptc-1 +/−) mice resulted in induction of cell cycling and expansion of primitive BM hematopoietic cells **[63]**. Notch signaling plays a key role in several fundamental functions including proliferation, differentiation and cell fate decision **[63-64].** Four notch receptors (Notch 1–4) and five ligands (Jagged1-2 and Delta-like 1, 3, and 4) have been identified in mammals **[65].** Several studies support the role of Notch signaling mediating the regulation of HSC hematopoiesis and self-renewal maintenance **[66].**

CHRONIC MYELOGENOUS LEUKEMIA

Chronic myelogenous leukaemia (CML) results from the neoplastic transformation of a haematopoietic stem cell. CML leukemia stem cells (LSCs) retain the ability to regenerate multilineage hematopoiesis and generate a vast expansion of malignant myeloid cells, which retain differentiating capacity and displace residual normal hematopoiesis. CML cells also demonstrate altered trafficking resulting in increased numbers of circulating progenitors, extramedullary hematopoiesis, and massive splenomegaly.LSCs acquire additional genetic abnormalities over time, leading to disease progression from an initial chronic phase (CP) to advanced accelerated phase (AP) and blast crisis.

In CML, the course is marked by three distinct phases: 1) chronic phase with stable clinical situation and the absence or rarity of symptoms; 2) accelerated phase, which typically occurs after 3-5 years and in which there is a worsening of all the clinical and haematological and finally, 3) blast phase in which there is essentially a transformation in acute leukemia. The first step, as mentioned, is very variable, whose median duration is three years, which means that half of the patients undergoes blast crisis within the first three years after diagnosis. In the accelerated phase, which usually lasts few months, there is a loss of response to standard treatments. In the blast phase, both in the absence of therapy or in patients undergoing aggressive treatments, the survival is ranging from few months to few weeks, as it is difficult to induce a second chronic phase stability.

The hallmark genetic abnormally of CML is a t(9;22) (q34;q11) translocation, which was first discovered as an abnormal, small chromosome, named the 'Philadelphia chromosome' (Ph). At molecular level, the Ph translocation results in the juxtaposing of the 5' part of the BCR gene to the 3' part of the ABL gene, and, depending on chromosomal breakpoint locations, different parts of these two genes may be included in the oncogenic fusion gene. The ABL gene is a gene encoding a non receptor tyrosine kinase that is expressed in most tissue. It is generally accepted that acquisition of the BCR-ABL1 oncogene is the initiating event in the genesis of CML-CP, despite various lines of evidence suggesting that, at least in some cases, hematopoiesis may already be clonal before the acquisition of the Ph chromosome. It is believed that acquisition of the BCR-ABL1 gene occurs initially in a single HSC that gains a proliferative advantage and/or aberrant differentiation capacity over its normal counterparts, giving rise to the expanded myeloid compartment. In cells, the ABL protein is distributed in both the nucleus and cytoplasm of cells and shuttles between the two compartments. It transduces signals from cell-surface growth factor and adhesion receptors to regulate cytoskeleton structure. BCR is also signaling protein that contains multiple modular domains. The fusion of BCR sequences to ABL during the translocation associated with CML increases the tyrosine-kinase activity of ABL, and brings new regulatory domains/motifs to ABL, such as the growth factor receptor-bound protein 2 (GRB2) SH-2 biding site. ABL spans a 230 kb region at band q34 of chromosome 9 and consists of 11 exons, with two first alternative exons, i.e. exons Ia and Ib. In the vast majority of the Ph positive patients, breakpoints in the ABL gene appeared to be distributed over a rather large 300 kb fragment of chromosome 9 at band q34. BCR-ABL activates a number of downstream targets including RAS, PI3 kinase, NF-κB and *signal transducer and activator of transcription 5* (STAT5), resulting in cytokine-independent growth, resistance to apoptosis, and altered cellular adhesion. Classical CML usually shows,

at least in the initial chronic phase, a prominent, but often asymptomatic increase in peripheral blood of WBC, and in some cases of platelet count associated with the presence of immature granulocytic elements. This leukemia is almost invariably derived by the P210-encoding BCR/ ABL gene, and it is the prototype of a stem cell neoplasia, in which all the hemopoietic lineages derive from a staminal transformed cell and have the Ph chromosome. In the classical CML, however, only the myeloid and megakaryotytic compartments show a neoplastic expansion, and the granulocytic progenitors have a moderate degree of impairment of their differentiation and maturation capacity, whereas the erythroid, monocytic and B-and T-lymphoid lineages do not reveal any functional damage.

Expression of BCR-ABL in human CD34+ cells causes increased proliferation in response to growth factors, increased growth factor-independent survival, reduced adhesion to fibronectin and reduced chemotaxis to stroma-derived factor-1α. These properties of BCR-ABL-expressing human haematopoietic progenitor cells mimic those of CML progenitor cells isolated from patients. BCR-ABL activates diverse progrowth and prosurvival mechanisms, which confer resistance to apoptosis. These include increased phosphorylation and transactivation by STAT-5 (signal transducer and activator of transcription), which leads to increased expression of the antiapoptotic Bcl-xL and Pim-2 protein, and increased Ras/Raf/MEK/ERK1/2, AKT, and NFkB activity. BCR-ABL TK activity also leads to depletion of the cyclin-dependent kinase-2 inhibitor p27 and the BH3 domain-only–containing proapoptotic Bim protein.

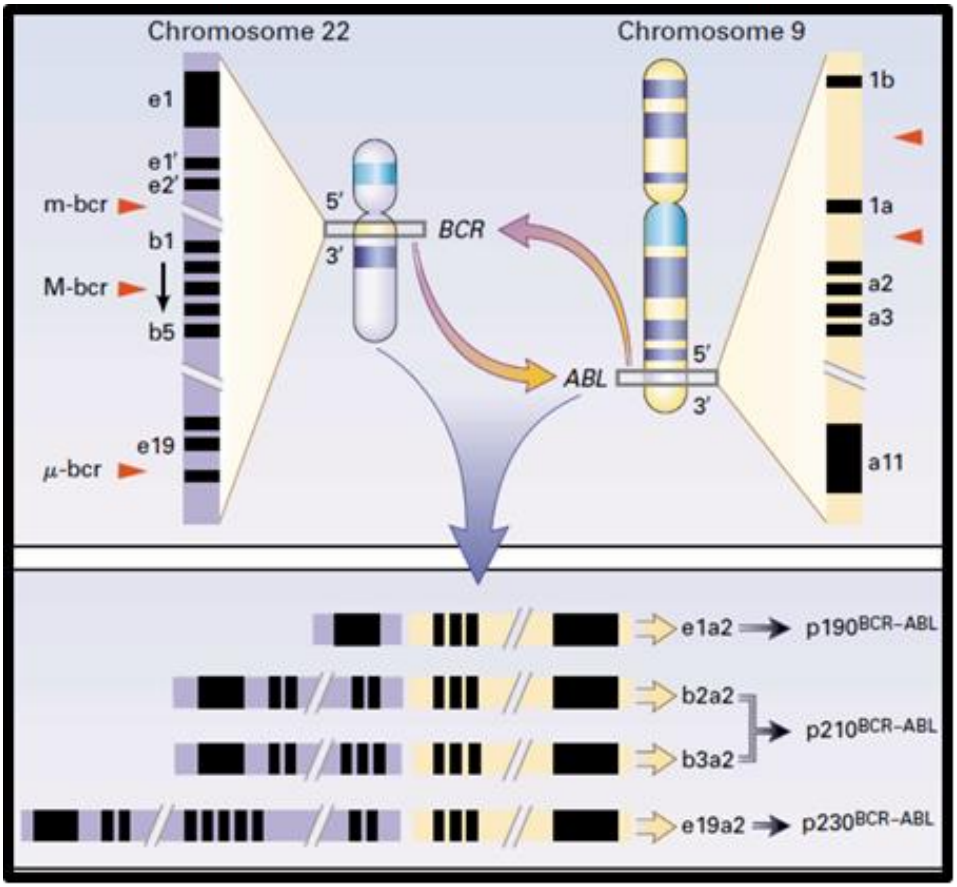

Figure 3: The Ph chromosome is result from translocation the 3 'end of the ABL gene on chromosome 9 with the 5' end of the BCR gene on chromosome 22. The breakpoints in ABL1 gene are distributed in an area of size greater than 300 Kb and are localized at the 5 'exon 2 in most cases. The BCR gene identified a number of break-point; different fragments of BCR will be merged with the sequences 3 'ABL1. This results in mRNA (e1a2, b2a2, b3a2 and e19a2) of different lengths, translated into different protein products (p190, p210 and p230).

CML Therapy

Tyrosine kinase inhibitors (TKIs) have transformed the natural history of CML by inducing cytogenetic and molecular responses in the majority of patients in chronic phase resulting in transient deep responses in many cases of advanced disease. [26] Currently, there are three commercially available TKIs for the treatment of CML; these include Imatinib, Dasatinib, and Nilotinib.

Imatinib was the first-choice treatment in CML with excellent results. It is a 2-phenylaminopyrimidine derivative that functions as a specific inhibitor of a number of tyrosine kinase enzymes. Imatinib mesylate is a tyrosine kinase inhibitor (TKI) that inhibits the BCR-ABL tyrosine kinase.It inhibits proliferation and induces apoptosis in BCR-ABL positive cell lines as well as fresh leukemic cells from Ph+ CML. Imatinib also inhibits the receptor tyrosine kinases for *platelet derived growth factor* (PDGF) and *stem cell factor* (SCF) called *c-kit*. Imatinib was identified in the late 1990s by Dr Brian J. Druker. Its development is an excellent example of rational drug design. Soon after identification of the BCR-ABL target, the search for an inhibitor began.

Dasatinib and *Nilotinib* are considered a "second-generation" BCR/ABL tyrosine kinase inhibitors, built on the success of imatinib. Like Imatinib mesylate, Nilotinib bind the inactive conformation of BCR-ABL, although Nilotinib binds with a higher affinity. In contrast, Dasatinib binds the active conformation of BCR-ABL kinase and is a less selective kinase inhibitor targeting Src kinase family members as well. They are more potent inhibitors compared with Imatinib mesylate and show activity against most of the well characterized BCR-ABL mutants. Indeed, after more than one decade of clinical trial appling Imatinib as front line therapy for patients with CML, it is well known that TKI is not able to eradicate leukemia, and primary or secondary drug resistance eventually occurs in the first two years of treatment. Thus, new drugs with ever-increasing specificity and anti-leukemia power are always under investigation by researchers.

New Alternative Drug Therapy in Leukemia

The mechanisms underlying persistence of LSC in Imatinib-treated CML patients are not well understood. Although reduced drug uptake or increased efflux together with high levels of BCR-ABL expression in primitive progenitors could theoretically contribute to Imatinib resistance, previous studies have found adequate drug levels and effective inhibition of BCR-ABL activity in CML progenitors following Imatinib treatment. Several studies show also that Imatinib effectively inhibits proliferation of CML primitive progenitors but only modestly increases progenitor cell apoptosis.

Growth factor (GF) or other microenvironmental signals may preserve viability of CML cells despite BCR-ABL kinase inhibition by Imatinib. Importantly Imatinib-induced apoptosis is restricted to dividing CML progenitors, whereas non-dividing CML progenitors are especially insensitive to Imatinib-induced apoptosis. The relative insensitivity of non-dividing CML progenitors may contribute to the persistence of BCR-ABL positive progenitors in patients achieving remission during Imatinib therapy. Similar results have been obtained with more potent BCR-ABL TKI including Dasatinib, Nilotinib and Bosutinib. These results suggest that BCR-ABL independent mechanisms contribute to survival of primitive CML cells after TKI

treatment, and indicate the need to identify additional strategies to eliminate CML LSC.

Since the discovery of the BCR-ABL kinase inhibitor Imatinib, great advances have been made in developing kinase inhibitors with exquisite selectivity and potency. Most of the drug discovery efforts on the identification of kinase inhibitors have been focused on ATP-mimetic small molecule inhibitors. Given that *janus kinase* (JAK2) is a tyrosine kinase, the discovery of activating mutations naturally resulted in a great deal of excitement and optimism in identifying and developing JAK2 inhibitors for the treatment of myeloproliferative neoplasms. JAK2V617F–mutated enzyme is often compared to the BCR-ABL oncoprotein from the standpoint of drug development. However, because no known vital function for endogenous ABL kinase has been identified, BCR-ABL inhibitors can be administered at doses that completely inhibit BCR-ABL and eliminate BCR-ABL–positive cells without concerns for mechanism related adverse effects. In contrast, it is important to recognize that because of the localization of the V617F mutation in a region outside the ATP-binding pocket of JAK2 enzyme, ATP-competitive inhibitors of JAK2 kinase are not likely to distinguish between wild-type and mutant JAK2 enzymes. Therefore, JAK2 inhibitors, by virtue of their near equipotent activity on wild-type JAK2, which is important for normal hematopoiesis, should have adverse myelosuppression as an expected side effect if administered at doses that aim to completely inhibit the mutant JAK2 enzyme. While they may prove to be effective at controlling hyper-proliferation of hematopoietic cells, they may not be able to eliminate mutant clones in a manner similar to BCR-ABL inhibitors. On the other hand, JAK inhibitors may have great therapeutic benefit by controlling the disease for patients with myeloproliferative neoplasms who suffer from debilitating signs (eg, splenomegaly) or constitutional symptoms that presumably result from high levels of circulating cytokines that signal through JAK enzymes.

Ruxolitinib (also known as INC424 or INCB18424) is an orally bioavailable, potent, and selective inhibitor of JAK1 and JAK2 that is approved for the treatment of intermediate and high-risk myelofibrosis. Ruxolitinib selectively inhibits the proliferation of *JAK2* V617F-driven Ba/F3 cells, and these effects are correlated with decreased levels of phosphorylated JAK2 and of STAT5 [77].

Histone deacetylase inhibitors (HDACi) are a class of agents that have shown promise as a therapy for several cancers [78]. HDACi can modulate gene expression through increased histone lysine acetylation. Anti-cancer effects may also be related to modulation of the acetylation status of non-histone proteins. In contrast to most other pro-apoptotic agents that preferentially target dividing cells, HDACi have been shown to induce apoptosis in non-proliferating cancer cell lines, which may have important implications for elimination of quiescent primitive LSC. Several clinical trials for patients affected by myeloproliferative neoplasms are based on *Panobinostat*, a new anti-cancer drugs within the family of deacetylase inhibitors (HDACi) with a marked anti-tumor activity across a broad range of cancer cell lines of hematologic malignancies. It has been demonstrated to be effective in several neoplastic disease, including cutaneous T cell lymphoma (CTCL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma (MM), Hodgkin lymphoma (HL), as well as solid tumors cell lines such as breast, colon, prostate and pancreas. Treatment with the hydroxamic acid analog Panobinostat-HDACi SAHA, LAQ824 (LAQ) or LBH589 (LBH), alone and in combination with TKI has been reported to induce apoptosis in CML cell lines and BC CML.

The Role of Bone Marrow in CML

The bone marrow microenvironment facilitates the survival, differentiation and proliferation of hematopoietic cells. These cells are supported by fibroblast-like bone marrow stromal cells, osteoblasts, and osteoclasts which secrete soluble factors and extracellular matrix proteins that mediate these functions. This rich environment serves as a safe haven not only for normal and malignant hematopoietic cells, but also for epithelial tumor cells that metastasize to bone, offering protection from chemotherapeutic agents by common mechanisms. Soluble factors produced in the bone marrow, such as stromal SDF-1 and IL-6, mediate homing, survival, and proliferation of tumor cells, and integrin-mediated adhesion sequesters tumor cells to this protective niche. Environment-mediated drug resistance includes a combination of soluble factors and adhesion, and can be subdivided into soluble factor mediated drug resistance and cell adhesion mediated drug resistance. Strategies to overcome BM stroma-mediated drug resistance should include not only disrupting the interaction between the CML cells and the BM stromal cells, but also, inhibit the signaling pathways mediated by cytokines and growth factors in the BM. Signals derived from the BM stroma can effectively reconstitute the downstream signaling pathway of BCR-ABL protein, such that CML cells can achieve BCR-ABL independent growth in the BM, making them resistant to BCR-ABL tyrosine kinase inhibitors.

AIM OF THE STUDY

BCR-ABL kinase inhibitors have provided proof of principal that targeted therapy holds great promise for the treatment of leukemia. However, despite the success of these agents in treating CML, the majority of patients continue to present Ph+ cells residing within the BM microenvironment. These clinical observations suggest that the BM microenvironment may provide survival signals that contribute to the treatment failure in eliminating minimal residual disease. Experimental evidence indicates that exposure of tumor cells to either BM derived soluble factors or the extracellular matrix can confer a multi-drug resistance phenotype. Strategies to overcome BM stroma-mediated drug resistance should include not only disrupting the interaction between the CML cells and the BM stromal cells, but also, inhibiting the signaling pathways mediated by cytokines and growth factors in the BM.

In this work, we evaluate whether the stromal microenvironment influence the drug resistance in CML, and whether we may suppress this resistance when in vitro leukemia cell line and CML progenitors are co-cultured in the presence of both stroma-derived supernatant or stromal cell line.

MATERIALS AND METHODS

Cell Cultures and tumor cells

K562 cell line

K562 is an erythroleukemia cell line derived from a chronic myeloid leukemia patient in blast crisis (obtained from the American Type Culture Collection). The cell population has been characterized as highly undifferentiated and of the granulocytic series. The morphology is round large, single cells in suspension. Cells carry the BCR-ABL1 e14-a2 (b3-a2) fusion gene. Human CML K562 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (L-Glu), and 1% penicillin/streptomycin (P/S), defined as regular media (RM), at 37°C in 5% CO_2 in a humidified incubator. K562 cells were kept at 0.5 x 10^6 cells/ml.

KT1 cell line

KT1 (CML) kindly provided by Dr Fujita (First Department of Internal Medicine, School of Medicine, Ehime University, Japan) is a leukemia cell line established from a patient in the blast crisis phase of CML. KT-1 has undifferentiated morphology that exhibited a high nuclear/cytoplasm ratio without cytoplasmic granules. KT-1 cells showed two Ph1 chromosomes and an absence of normal copies of chromosomes 9 and 22. The KT-1 cells are IFN-alpha or IFN-gamma sensitive. Human CML KT1 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (L-Glu), and 1% penicillin/streptomycin (P/S), defined as regular media (RM), at 37°C in 5% CO_2 in a humidified incubator. K562 cells were kept at 0.5 x 10^6 cells/ml.

BV173 cell line

BV173 (obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) is a B cell precursor leukemia cell line established from the peripheral blood of a 45-year-old man with chronic myeloid leukemia (CML) in blast crisis in 1980. The morphology of cell is round to elongate. Cells grow in suspension. Cells carry the t(9;22) leading to BCR-ABL1 e13-a2 (b2-a2) fusion gene. Human BV173 cells were cultured in RPMI supplemented with 20% FBS, 1% L-Glu and 1% P/S, defined as regular media (RM), at 37°C in 5% CO_2 in a humidified incubator. BV173 were maintained at 0.5 x 10^6 cells/ml.

HS-5 cell line

The human stromal cell line HS-5 (obtained from the American Type Culture Collection) is a fibroblastoid cell line immortalized by transduction with the human papilloma virus E6/E7 genes. Cells grow in adherent manner. it secretes detectable amounts of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), macrophage inhibitory protein 1-α, stem cell factor (SCF), leukemia-inhibitor factor, IL-11, IL-8, IL-6, and IL-1; and it is capable of sustaining proliferation of normal hematopoietic progenitors cells in serum-free media without additional growth

factors. Human HS-5 cell were cultured α- MEM supplemented with 10% FBS, 1% L-Glu, and 1% P/S at 37°C in 5% CO2 in a humidified incubator. HS-5 cultured 1,5 × 10^6 cells/mL to achieve 75% to 80% confluency. Subsequently, the medium was removed from the cells and was flirted with 0.22 µm filter for future use

Samples from CML Patients and Healthy Donors

Bone marrow samples and peripheral blood were collected from 20 newly diagnosed patients with CML-CP patients enrolled in our Institution and two healthy donors (HD), after signed informed consent was obtained in accordance with the Declaration of Helsinki and after approval by the Institutional Review Board (IRB) of University of Naples Federico II.

Isolation of CML Progenitor Cells CD34+

BM and PB samples were obtained from donors clinical protocol GIMEMA. Mononuclear cells were isolated using Ficoll separation. CD34+ cells were sorted with Miltenyi Biotec CD34 MicroBead Kit human. The CD34+ cells are magnetically labeled with CD34 MicroBeads. Then, the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD34+ cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD34+ cells. After removing the column from the magnetic field, the magnetically retained CD34+ cells can be eluted as the positively selected cell fraction. We resuspend cell pellet in 300 µL of buffer for up to 10^8 total cells. We add 100 µL of FcR Blocking Reagent for up to 10^8 total cells. We add 100 µL of CD34 MicroBeads for up to 10^8 total cells and Mix well and incubate for 30 minutes in the refrigerator (2−8 °C). After we re-suspended in 10ml of MACS BUFFER and centrifuged at 1000 rpm for 5 min and resuspended in 500µL in MACS BUFFER and proceed to magnetic separation. After they cultured media IMDM 10% FBS, 10% P/S. The CD34+ cells were cultured in AIM-V serum-free media (Invitrogen) supplemented with 2nM L-glutamine (GIBCO-BRL Invitrogen), 1% penicillin/streptomycin (Gibco-Invitrogen) at a starting density of $1x10^5$ cells/ml at 37°C, 5% CO_2, in a humidified incubator for 4 days. When specified, the following cytokines were included [high grow factor (GF) cocktail]:100 ng/ml SCF, 100 ng/ml FLT3 ligand, 20 ng/ml G-CSF, 20 ng/ml IL-3, and 20 ng/ml IL-6 (all from R&D Systems). Depending on the individual experiment, TKI were added at approximely a clinical relevant concentration (1µM Imatinib, 400nM Nilotinib and 2.5nM Dasatinib).

Generation of Mesenchymal Stroma Cell (MSC) and Mesenchymal Stroma Conditioned Media (SCM)

Mononuclear cells were isolated from BM aspirates by centrifugation on a Ficoll-Hypaque gradient. MSC cells were sorted with Miltenyi Biotec Anti MSCA-1 MicroBead kit human. Briefly, the MSC+cells are magnetically labeled with MSC MicroBeads. Then, the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled MSC cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of MSC cells. After removing the column from the magnetic field, the magnetically retained MSC cells can be eluted as the positively selected cell fraction.

Human MSCA-1+ cells were cultured in Mesenchymal Stem Cell Basal Medium (MSCBM) with Mesenchymal Cell Growth Supplement (MCGS), 2% (L-Glu), 1% Penicillin/Streptomycin and cultured at 37°C in 5% CO_2 in a humidified incubator. Conditioned Media (CM) was collected after at least two cell passages of two mesenchymaò stroma cell lines obtained from Healthy Donor (HD), named HD/SCM and eight stroma cell lines obtained each from a BM sample of a patient with CML, named CML/SCM, and stored at -80°C. After SCM collection, mesenchymal stroma cell lines have been immunophenotypically analyzed for the positive expression of CD73, CD90, and CD105 and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface antigens (human MSC Phenotyping Kit, Miltenyi).

The Trypan Blue Method: Assessment of Percentage of Viable and Non Viable Cells

Cells were stained with trypan blue (Sigma, St Louis, MO). The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. The numbers of non-viable cells were determined by counting the cells that showed trypan blue uptake using the Burker's chamber, and reported as percentage of untreated control cells.

Drugs and Reagents

Imatinib mesylate and Nilotinib, both supplied by Novartis Pharma, were dissolved in DMSO as a 10 mmol/L stock solution and stored in aliquots at −20°C. Dasatinib, supplied by Bristol-Myers Squibb, was treated similarly. Panobinostat and Roxolitinib .

Apoptosis Assay

Apoptosis was measured using FITC Annexin V Apoptosis Detection Kit II BD Pharmingen. Briefly, we wash cells twice with cold PBS and then resuspend them in 1X Binding Buffer at a concentration of 1 x 10^6 cells/ml. We transfer 100 µl of the solution (1 x 10^5 cells) to a 5 ml culture tube and add 5 µl of FITC Annexin V and 5 µl propidium iodide (PI). We gently vortex the cells and incubate for 15 min at RT (25°C) in the dark. After incubation we add 400 µl of 1X Binding Buffer to each tube. Analyze by flow cytometry within 1 h.
In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. FITC Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes.
Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V positive).

PI is an intercalating agent and a fluorescent molecule with a molecular mass of 668.4 Dalton that can be used to stain cells. Propidium iodide is used as a DNA stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis and microscopy to visualize the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells.
Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive.

Flow-Cytometric Assays

Apoptosis was measured using FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen). The percentage of apoptotic cells was determined by flow cytometry. Cell cycle analysis was detrmined by BRdU Flow Kits (BD Pharmigen), as specified by the manufactory.

Analysis of Proliferation

The immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and flow cytometric analysis provide to determine the frequency and nature of individual cells that have synthesized DNA. In this method, BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies. The levels of cell-associated BrdU are then measured by flow cytometry. Often, staining with a dye that binds to total DNA such as 7-aminoactinomycin D (7-AAD) is coupled with immunofluorescent BrdU staining. With this combination, two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their cell cycle position (ie, G0/1, S, or G2/M phase defined by 7-AAD staining intensities). The kit used is BRdU Flow Kits of Bd Pharmigen.

Evaluation of BCR-ABL Activity in K562 Cell Line

K562 cell have been treated with TKIs at the indicated concentration for 30’ (minute), than cells were transferred in ice and washed with cold PBS. To simultaneously asses the phosphorylation status of BCR-ABL target, we carried out intracytoplasmatic immunostaining with antibody against p-CrkL (Tyr207), p-STAT5 (Tyr694), p-ERK1/2 (Thr202/Tyr204) and p-STAT3 (Tyr705) (Cell Signaling Technology), following the manufacturer’s instructions. For each sample, a minimum of 100,000 cells were analyzed using a FACSCalibur (Becton Dickinson, San Diego, CA).

Colony-Forming Unit (CFU) Inhibition Assay of Leukemic and Normal Hematopoietic Progenitors

CD34+ cells are prepared by positive selection using Miltenyi Biotec CD34 MicroBead Kit human. After the count is made with 3% acetic acid. We prepare a 10X concentrated cell suspension of cells in IMDM with 2% FBS. Add 0.3 mL of cells to 3 mL of MethoCult. This 1:10 v/v ratio of cells:medium gives the correct viscosity to ensure optimal CFC growth and morphology. We vortex tube to mix contents thoroughly and then let stand for 2 - 5 minutes to allow bubbles to dissipate before dispensing and using a 3 mL syringe attached to a 16 gauge blunt-end needle, dispense 1.1 mL of the MethoCult mixture containing cells into a 35 mm dishes. Gently we tilt and rotate each dish to distribute methylcellulose evenly. Dishes are pre-screened to ensure low cell adherence. Cell adherence can inhibit CFC growth. Finally we add 3 - 4 mL of sterile water to an additional uncovered 35 mm dish. For duplicate assays, place all three dishes into a 100 mm culture dish. The cells are incubate at 37°C, in 5% CO2, with 95% humidity for 14 - 16 days.

High Grow Factor Cocktail

We used the same high grow factor cocktail reported by Susan M. Graham et al; Briefly, CD34+ cells were grown in Iscoves modified Dulbecco medium (Sigma) supplemented with a serum substitute (BIT; StemCell), supplemented or not with 100ng/mL recombinant human Flt3-ligand (Immunex Corporation, Seattle,WA) and Steel factor (Terry Fox Laboratory, Vancouver, BC, Canada), and with 20 ng/mL recombinant human interleukin-3 (IL-3) (Novartis, Basel,Switzerland), IL-6 (Cangene, Mississauga, ON, Canada), and granulocyte–colony-stimulating factor (G-CSF) (Chugai Pharma, United Kingdom; abbreviated as 5 growth factors [GFs], like reported by Susan M. Graham et al.)

Half Maximal Inhibitory Concentration (IC 50)

IC50 is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC50). It is commonly used as a measure of antagonist drug potency in pharmacological research.
To calculate the IC50 based on the level of viable cells after TKI treatment in the presence or absence of HS-5/CM, we analyzed our data by a specific software (MasterPlex ReaderFit), applying 4 Parameter Logistic (4-PL) and 5 Parameter Logistic (5-PL) model equations to calculate IC50 for dose response curves.

Q-RT-PCR

Single cell clones have been evaluated for the expression of P210 mRNA by quantitative real-time PCR (Q-RT-PCR) as previously described. Briefly, all amplification reaction was carried out in triplicate and the mean Ct value was used to interpolate standard curves and calculate the transcript copy number.

Bioplex ELISA Assay

The Bio-Plex multiplex system enables the detection and quantification of multiple analytes in a single sample volume. Utilizing xMAP technology licensed from Luminex, the Bio-Plex Multiplex system can multiplex up to 500 different assays simultaneously. We evaluated the secretory profiles between the HS5/SCM and CML/SCM from 8 mesenchymal stromal cell lines obtained from CML patient, in particular we tested them for 20 different analytes, reported to be involved in leukemia signaling pathways: IL-1a, IL-3, M-CSF, SCF, SDF1-a, TRAIL, HGF, PDGF-bb, IL1b, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, G-CSF, GM-CSF, MIP-1a, TNF-a, and VEGF; using the Bio-PLEX-200 System.

Statistical Analysis

All in vitro experiments were summarized as mean plus or minus one standard Deviation (SD). Student *t* test was used to determine the statistical significant differences by non parametric Mann-Whitney test, between values obtained in a population of leukemic cells treated with different experimental conditions, with *P* value less than .05 indicating a significant difference. The half maximal inhibitory concentration (IC50) was calculated based on the level of viable cells (Annexin-$V^{neg.ve}/PI^{neg.ve}$) residual after treatment with increasing doses of TKI ranging from 0 to 100μM in the presence or absence of HS-5/SCM. Data were analyzed by a specific software (MasterPlexReaderFit), applying 4 Parameter Logistic (4-PL) and 5 Parameter Logistic (5-PL) model equations to calculate the IC50 for dose response curves. Combination index (CI), defined as quantitative measure of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement [Z=(1-T/V), calculated from drug-treated samples with a measured response T (as a percentage of residual viable cells; Annexin-$V^{neg.ve}/PI^{neg.ve}$) relative to V, (a median level of viable cells of 10 vehicle-treated samples),[81] was calculated by CompuSyn software based on Chou's median-effect equation and its extension, combination index (CI) equation.
In particular, based on the CI value, we defined, as previously reported, a very strong synergism (CI<1), strong synergism (0.1-0.3), synergism (0.3-0.7), moderate synergism (0.7-0.85), slight synergism (0.85-0.9), nearly additive (0.90-1.10), slight antagonism (1.10-1.20), moderate antagonism (1.20-1.45), antagonism (1.45-3.3), strong antagonism (3.3-10) and very strong antagonism (>10).

RESULTS

Evaluation of the BM microenvironment activity on the modulation of TKI-induced apoptosis in BCR-ABL+ cell line.

To evaluate whether BM microenvironment has a potential role on modulation of first and second generation TKI activity on Ph+ cells, we treated K562, BV173 and KT1 cell lines with Imatinib, Nilotinib or Dasatinib at clinical relevant concentrations, in the presence of monolayer human stromal cell line HS-5.
Ph+ cell lines are incubated in direct contact of HS-5 or in the presence of supernatant derived from HS-5 culture; they are significantly protected from TKI-related cell apoptosis (Fig.4A-C) and mortality (Fig.5A-C).
Indeed, when K562 cell line (Figure 4A) are treated with Imatinib, Nilotinib or Dasatinib in the presence of a HS-5 monolayer, apoptosis is significantly reduced (18%±13%, 50%±6%, or 10%±10%, respectively), respect to Ph+ cell line treated in Regular Media (RM) (46%±12%, 84%±15%, or 53%±20%, respectively. $p<0.05$ in all the conditions. (Figure 4A). Similar results have been achieved in the context of BV173 and KT1 cell lines (Figure 5A and 5B). Moreover, we demonstrated that TKI-resistant is also related to soluble factors produced by HS-5 in the conditioned media (HS-5/SCM). Indeed, a significant level of protection is also achievable by treating Ph+ cells with TKIs in the presence of HS-5/SCM soluble factors (Figure 4A-C dark-gray barrel).
The apoptosis is also greatly reduced when K562 cell line is treated with Imatinib, Nilotinib or Dasatinib in the presence of HS-5/SCM (20%±9%, 29%±18%, or 17%±6%, respectively), respect to Ph+ cell line treated in RM.
To evaluate if the observed stroma-related TKI resistance is merely associated to HS-5 intrinsic characteristics, we generated stromal cell lines directly from two healthy BM donor identified as healthy donor/stroma cells (HD/SC) and 8 patients at diagnosis affected by CML identified as CML/ stroma cells (CML/SC) by the sorting MSCA1+ BM cells and after the second passage, we collected conditioned media from stroma cell culture.
We tested the effects of the derived conditioned media obtained from these stroma cell lines (HD/SCM and CML/SCM, respectively) on the regulation of TKI activities on Ph+ cell lines. As shown in Figure 4A-C, HD/SCM (white barrel) and CML/SCM (gray barrel) are equally able to significantly reduce TKI-related apoptosis in K562 (Figure 4A), KT1 (Figure 4B) or BV173 cell lines (Figure 4C). Indeed TKI-induced apoptosis is significantly inhibited in K562 cell line by CML/SCM exposition (22%±12%, 40%±16%, or 31%±16%, respectively) respect to control cell line treated in RM (46%±12%, 84%±15%, or 53%±20%, respectively. (Figure 4A). $p<0.05$ in all the conditions. Similarly, apoptosis was greatly reduced when KT1 and BV173 cell lines were treated with TKIs in the presence of HD/SCM or CML/SCM (Fig. 4B and 4C, respectively).
Thus, we demonstrated that BM mesenchymal stroma microenvironment, either represented by the selected clone HS-5 or by mesenchymal stroma cell lines derived from HD and CML patients, provides survival factor able to protect Ph+ cells from TKI-related apoptosis or cell death.

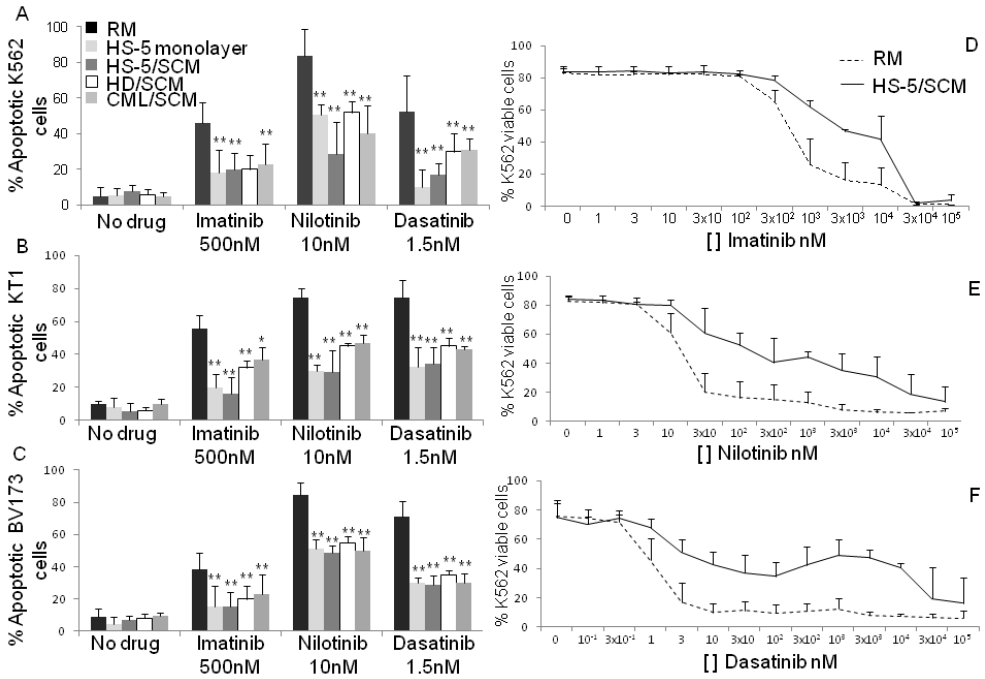

Figure 4. Analysis of Imatinib, Nilotinib and Dasatinib activity modulation by Mesenchymal Stroma cells exposition.

Ph+CML cell lines, K562, BV173 and KT1 were treated with the indicated concentrations of Imatinib, Nilotinib or Dasatinib in different culture conditions: in regular media (RM) (black barrel), in the presence of a HS-5 monolayer (light-gray barrel), in media supplemented with 50% of HS-5/SCM (dark-gray barrel), in media supplemented with 50% of HD/SCM (white barrel), or in media supplemented with 50% of patients derived CML/SCM (gray barrel). Apoptosis were monitored by Annexin V/PI staining after 24 hrs in K562 cell line (**A**), KT1 cell line (**B**) and BV173 cell line (**C**). Results represent the mean ± SD from 3 independent experiments. $p<0.01$ for all the condition. IC50 was calculated based on the level of viable cells (Annexin-$V^{neg.ve}$/$PI^{neg.ve}$) residual after treatment with increasing doses of Imatinib (**D**), Nilotinib (**E**) or Dasatinib (**F**) ranging from 0 to 100.000nM in the presence (continuous line) or absence (dot line) of HS-5/SCM.

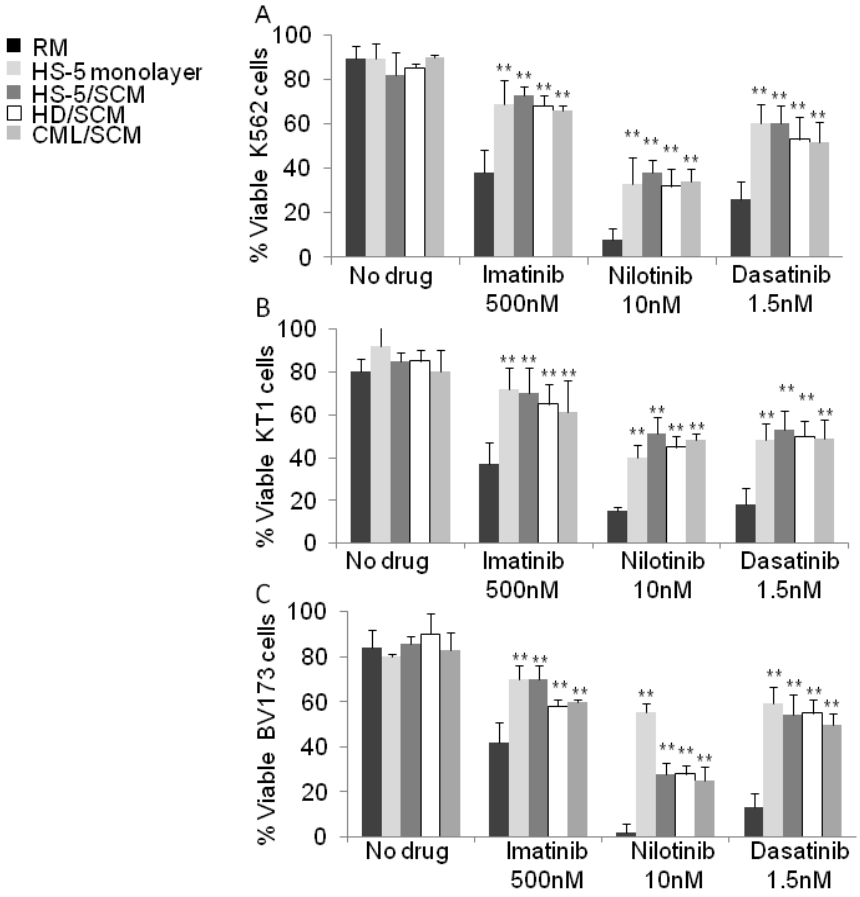

Figure 5. Cell viability in Ph+ cell lines treated with TKI upon Mesenchymal Stroma cells exposition.

Ph+CML cell lines, K562, BV173 and KT1 were treated with the indicated concentrations of Imatinib, Nilotinib or Dasatinib in different culture conditions: in regular media (RM) (black barrel), in the presence of a HS-5 monolayer (light-gray barrel), in media supplemented with 50% of HS-5/SCM (dark-gray barrel), in media supplemented with 50% of HD/SCM (white barrel), or in media supplemented with 50% of patients derived CML/SCM (gray barrel). Cell viability were monitored by Annexin V/PI staining after 72hrs, in K562 cell line (**A**), KT1 cell line (**B**) and BV173 cell line (**C**). Results represent the mean ± SD from 3 independent experiments. Significance values: * $p<0.05$; ** $p<0.01$.

Half Maximal Inhibitory Concentration (IC 50) of TKI

To quantify the effect of HS-5/SCM exposition on TKI activity, K562 cell line has been treated with an escalation dose of Imatinib, Nilotinib or Dasatinib (range 0.1-100,000 nM) in the presence or in the absence of HS-5/SCM. Cellular viability has been monitored after 72hrs. by Annexin-V/IP flow cytometric test and for cell viability estimating the number of PI negative cells.

We demonstrated that the IC50 of Imatinib, Nilotinib or Dasatinib is significantly increased when the Ph+ cell line K562 is cultured in the presence of HS-5/SCM (5309.29nM, 381.14nM, 2.31nM, respectively) vs the IC50 calculated on K562 viable cells residual after TKI treatment in RM (564.97nM, 14.26nM and 1.13nM, respectively).

In particular the IC50 of K562 cell line, based on cell viability at 72hrs after Imatinib treatment, increases about ten times from 564.97 nM in RM to 5309.29 nM in cell treated in presence of HS-5/SCM (Fig.6A), the IC50 increases about thirty times from 14.26 nM in RM to 381.14 nM in HS-5/SCM after Nilotinib treatment and the IC50 increases about two times from 1.13 nM in RM to 2.31 nM in HS-5/SCM, after Dasatinib treatment. Moreover, in average 47%, 40% and 51% of K562 cells exposed to HS-5/SCM were still viable after treatment with 3000nM Imatinib, 300nM Nilotinib or 3nM Dasatinib, respectively (11%, 15% and 17% of K562 cells treated with the same concentration of TKIs in the absence of HS-5/SCM (Fig.5A); $p<0.001$ in all the conditions). Similarly, we observed the same results when KT1 and BV173 cell lines were exposed to HS-5/SCM. (Fig.5B and 5C, respectively).

Finally, to evaluate whether the viable Ph+ cells residual after TKI treatments in the presence of HS-5/SCM are able to further expand or apoptosis is just delayed by the presence of soluble factors produced by stroma cell line, we cultured K562 cell line with TKIs at the calculated IC50 (Table 1) for 72 hrs in the presence of HS-5/SCM.

Cells were collected and re-plated in complete media for a total of 14 days without addition of TKIs. As shown in Fig 8A, residual K562 cells after TKIs treatment upon stroma exposition were able to significantly expand after drug withdrawal, respect to K562 cells exposed to a single dose of TKIs in the absence of HS-5/SCM.

However, HS-5/SCM withdrawal before TKI treatment is able to completely overcome the stroma-mediated TKI protection (Fig 8B). Moreover, serial dilutions of HS-5/SCM, ranging from 50% to 0.05%, show that soluble factors are associated to stroma-related TKI resistance in a dose dependent manner (Fig.8C).

	RM IC50 (nM)	HS5-SCM IC50 (nM)
Imatinib	564.97	5309.29
Nilotinib	14.26	381.14
Dasatinib	1.13	2.31

Table1. IC50 modulation in K562 cell line by stroma conditioned media.
Data were analyzed by specific software (MasterPlex ReaderFit), applying 4 Parameter Logistic (4-PL) and 5 Parameter Logistic (5-PL) model equations to calculate IC50 for each dose response curve.

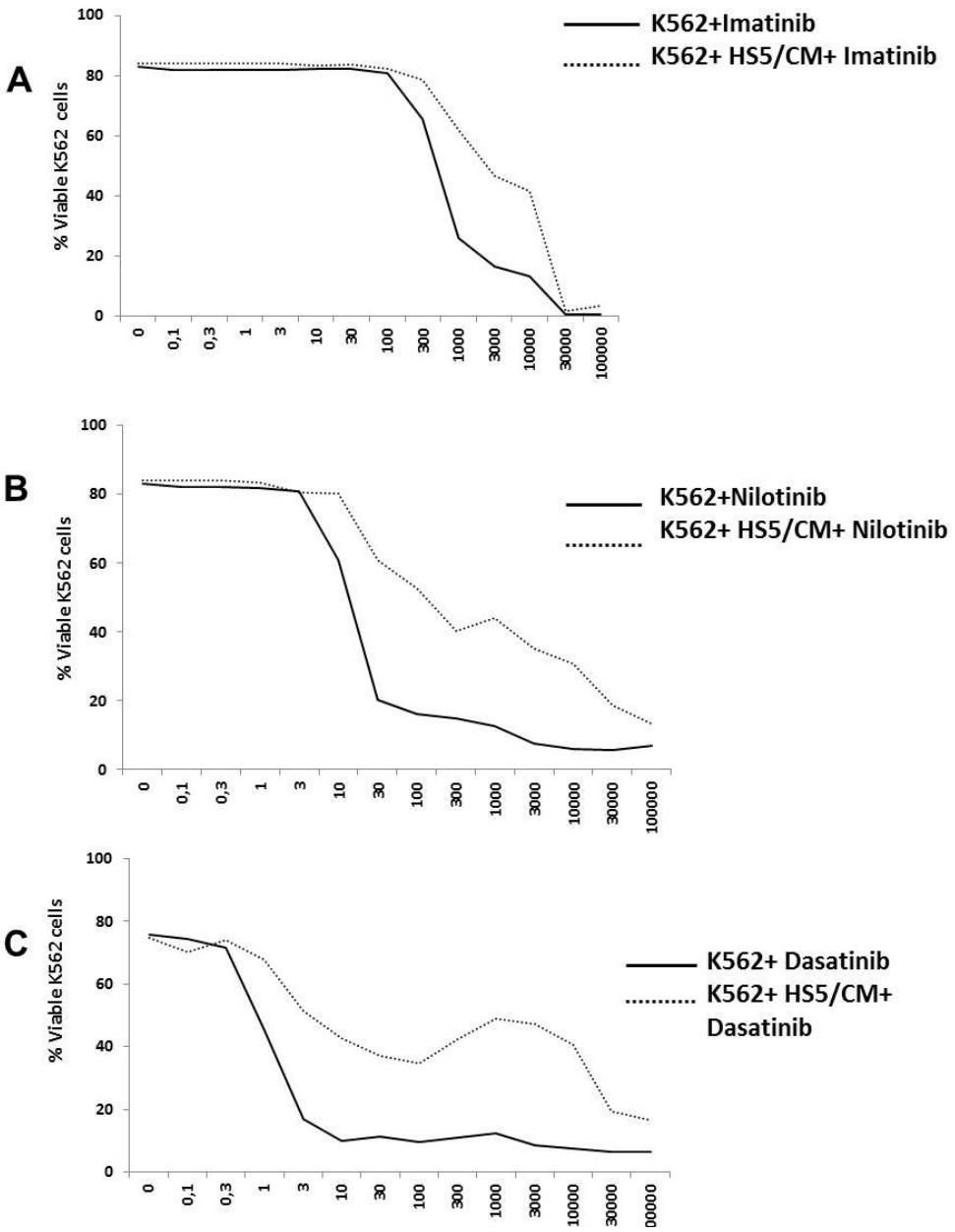

Figure 6: K562 cell line was evaluated for cell viability after 72hrs of treatment by Annexin-V/IP flow cytometric test and by estimating the number of PI negative cells. **A)** IC50 based on cell viability at 72hrs after Imatinib treatment : IC50 CNT= 564.97 nM; IC50 HS-5/CM=5309.29 nM; **B)** IC50 based on cell viability at 72hrs after Nilotinib treatment: IC50 CNT= 14.26 nM; IC50 HS-5/CM=381.14 nM **C)** IC50 based on cell viability at 72hrs after Dasatinib treatment: IC50 CNT= 1.13 nM; IC50 HS-5/CM=2.31 nM

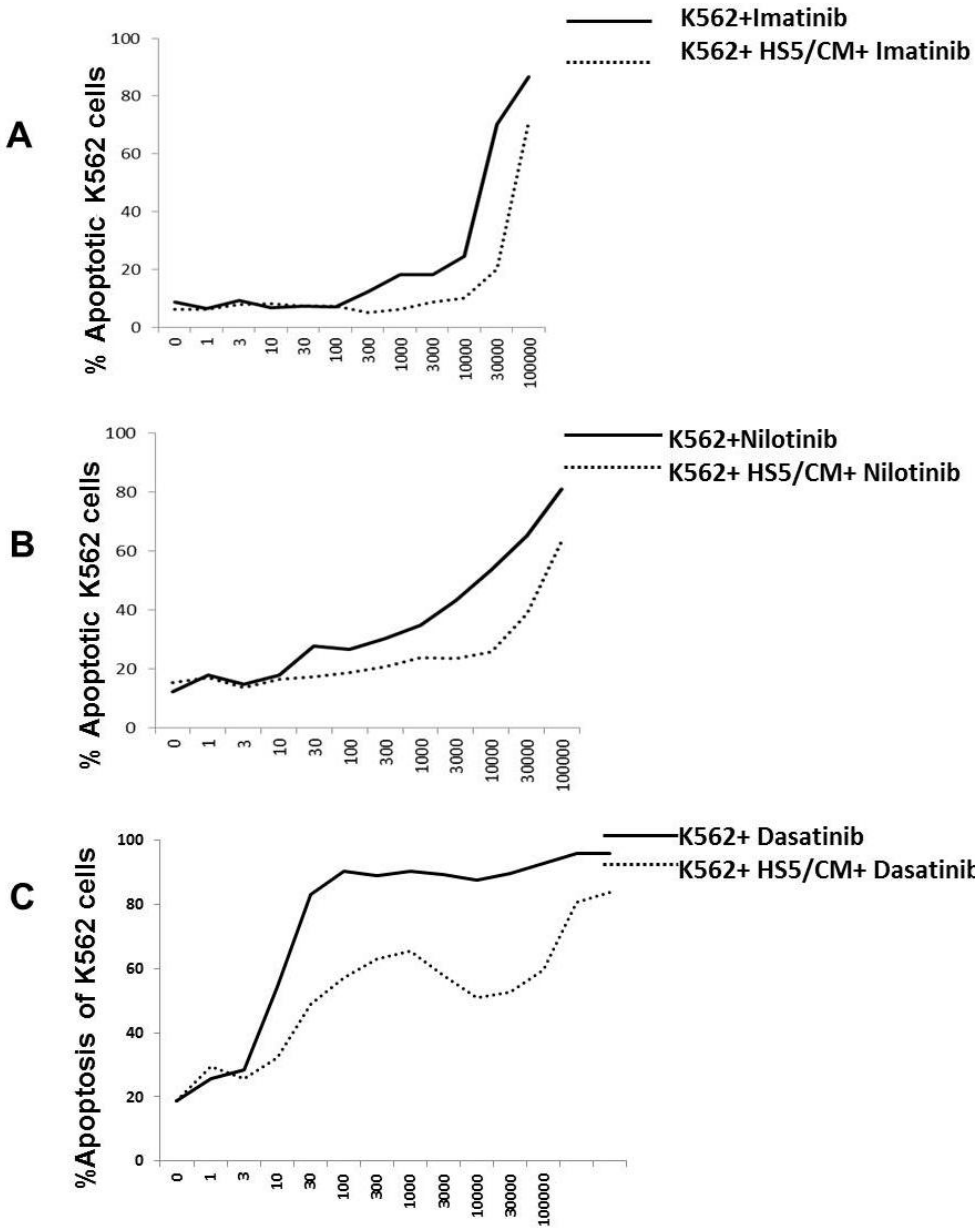

Figure 7: **K562 cell line was evaluated for apoptosis induction after 72hrs of treatment by Annexin-V/IP flow cytometric test. A)** IC50 based on cell viability at 72hrs after Imatinib treatment: IC50 CNT= 564.97 nM; IC50 HS-5/CM=5309.29 nM; **B)** IC50 based on cell viability at 72hrs after Nilotinib treatment: IC50 CNT= 14.26 nM; IC50 HS-5/CM=381.14 nM **C)** IC50 based on cell viability at 72hrs after Dasatinib treatment: IC50 CNT= 1.13 nM; IC50 HS-5/CM=2.31 nM.

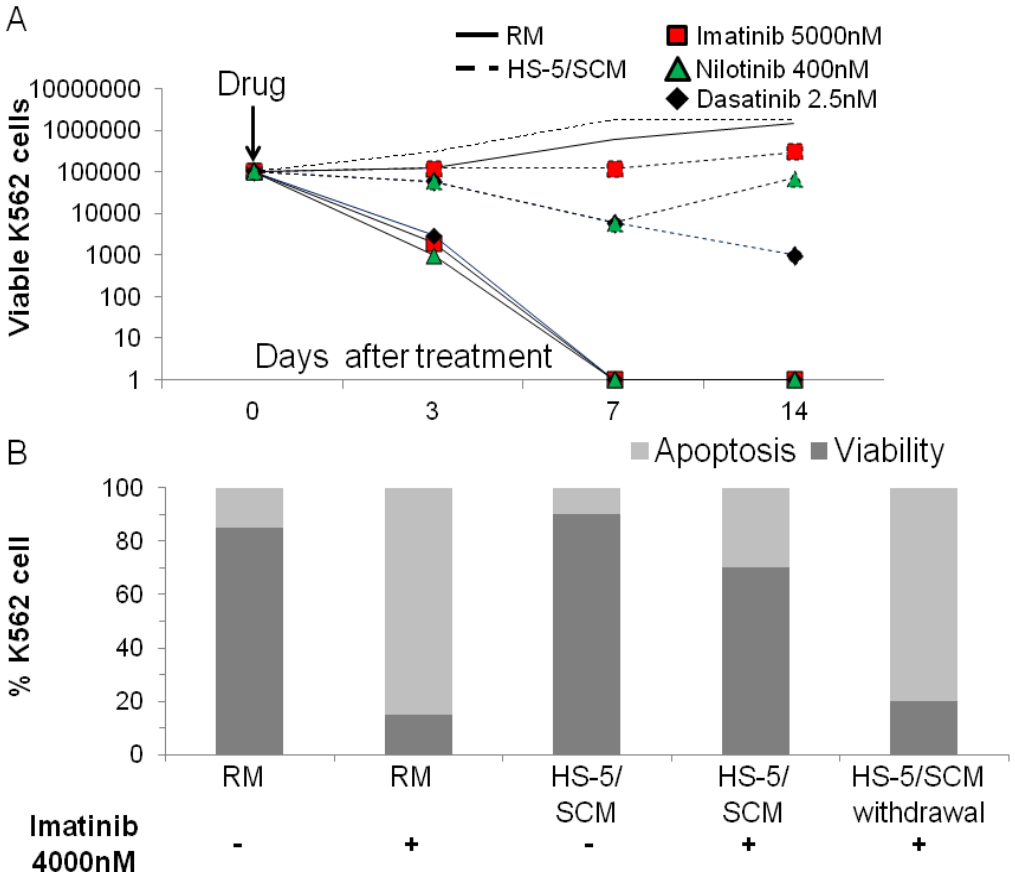

Figure 8A. Residual number of K562 cells after TKIs treatment upon stroma exposition were able to significantly expand after drug withdrawal. Cell proliferation has been monitored by trypan blu exclusion methode at the indicated time point. Long viability experiment: K562 cell line was treated with 5µM Imatinib (red square), 400nM Nilotinib (green triangle) and 2.5nM Dasatinib (black rhombus) at time 0 and day 3, day 7 and day 14 in RM (continuous lines) or HS-5/SCM (dotted lines).

Figure 8B. HS-5 media withdrawal before TKI treatment doesn't not protect from TKI induced apoptosis. K562 cell line was treated with 4000nM Imatinib at time 0 in RM or presence of HS-5/SCM. Cell viability (dark gray barrel) and apoptosis (light gray barrel) were monitored by Annexin-V/PI staining after 72hrs of treatment.

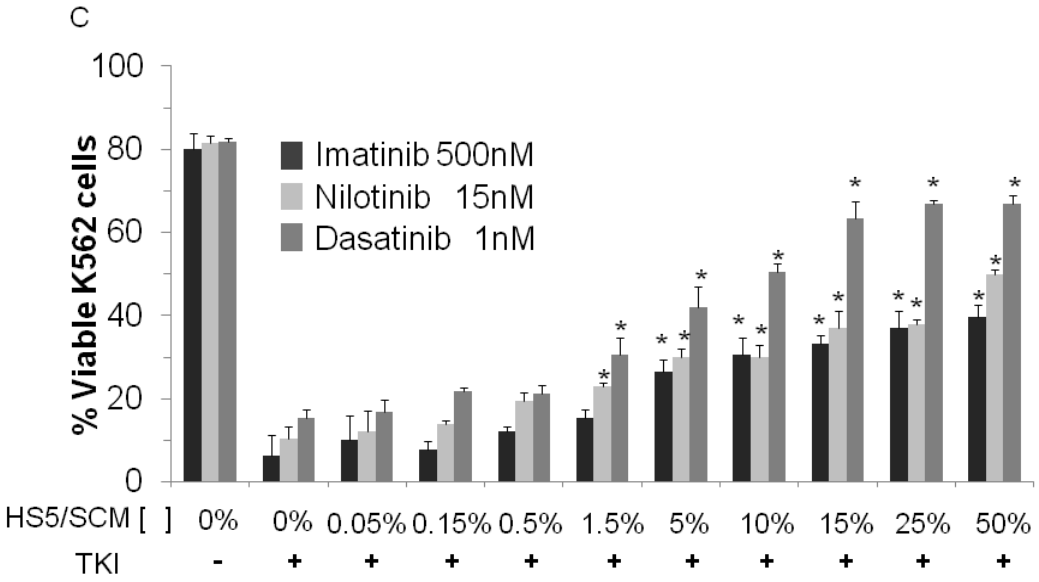

Figure 8C. **SCM protects K562 cell line from apoptosis in a dose dependent manner**. K562 cell line was evaluated for cell viability, by Annexin-V/PI staining after 72hrs of TKI treatment, in cell culture conditions where media was supplemented with increasing percentage of HS5/SCM. (N=3. * indicates $p<0.005$).

Analysis of Cell Proliferation in Ph+ Cells treated with TKI in the presence of Stromal Microenviroment.

It has been reported that Ph+ quiescent staminal cells are not sensible to TKI-induced apoptosis. Thus, to evaluate if stroma conditioned media protect Ph+ cells from TKI-induced apoptosis by reducing cell cycle induction, we treated K562 cells with Imatinib, Nilotinib or Dasatinib at the calculated IC50, either in the presence or absence of HS-5/CM. Thus, cell line was evaluated for cell proliferation after 24hrs of treatment by Bromo Uridine (BrdU) assay. Direct measurement of new DNA synthesis by using nucleoside analogs is a well established method. BrdU flow kit, based on the incorporation of BrdU into newly synthesized DNA strands of actively proliferating cells, is the method of choice compared to traditional 3H-thymidine incorporation assays, which are slow, labor intensive, and involve hazardous radioactive materials. When BrdU incorporation assays are combined with DNA dyes such as 7-aminoactinomycin D (7-AAD), it is possible to resolve cell cycle phases in a given cell population into G0/G1, S, and G2/M phases using flow cytometry.
As shown in Figure 8D, HS-5/CM did not significantly modify the percentage of K562 cell line in the G0-G1 cell cycle phase. Moreover, Imatinib, Nilotinib and Dasatinib treatment induced a significant increase in G0-G1 cell cycle phase, in a HS-5/CM independent manner. Indeed TKI treatment significantly increased the G0-G1 fraction of K562 cells and decreased proportion of S-phase cells compared with the untreated cells. Because most available chemotherapeutic agents show some degree of S-phase specificity, cells that are not actively dividing may prove resistant to such drugs. The quiescent leukemic cells are likely to survive to standard chemotherapy regimens, and it may explain the clinical observation that, unlike CML cannot be eradicated by TKI chemotherapy alone.

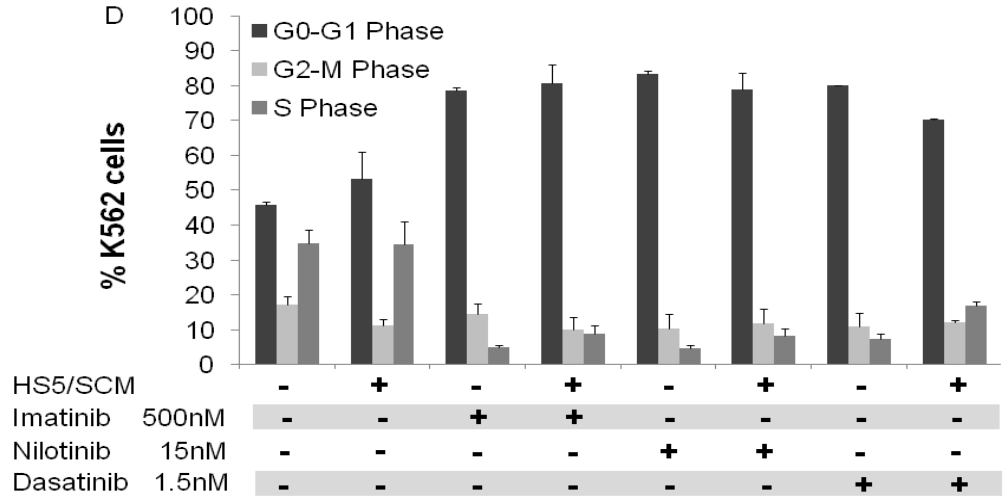

Figure 8D: TKI treatment significantly induce G0-G1 cell cycle arrest in Ph+ cell line independently by HS5/SCM. The K562 cell cycle state was evaluated after 24hrs of TKI treatment by BrdU assay. The graphic show the percentage of K562 cell line in the G_0-G_1 phase (black barrel); the percentage of K562 cell line in the G_2-M (gray-white barrel) and the percentage of K562 cell line in the S-phase (gray-iron barrel)**.**

CML derived BM mesenchymal cell lines produce several STAT3 activating cytokines

TKI treatment effect was not modified in the K562 cell line by the presence of HS-5/SCM, despite the effective inhibition of BCR-ABL kinase activity. Indeed, Imatinib (500nM), Nilotinib (15nM) or Dasatinib (1.5nM) treatment was able to reduce the phosphorylation level of the considered BCR/ABL targets as CkrL, STAT5 and ERK in Ph+ cells when cultured in either RM or in the presence of HS-5/SCM (Fig.9A-C respectively). In contrast, STAT3-Y705 was detected at the activated level in K562 cell line only when the cell line was exposed to HS-5/SCM, independently of TKI treatments (Fig. 9D), as previously reported. Constitutively active STAT3 induces the expression of anti-apoptotic genes including Bcl-2, Bcl-xl, Mcl-1, and also up-regulates the expression of inhibitors of apoptotic machinery like survivin and c-IAP2. Since, STAT3 has been demonstrated to be activated by a variety of cytokine receptors including G-CSF and members of the IL-3/IL-5/GM-CSF family. We also quantified by Bioplex ELISA assay the amount of several cytokines known to be related to survival signaling, in HS-5/SCM and CML/SCM derived from 8 BM mesenchymal stromal cell lines generated from BM samples of 8 patients affected by CP-CML. As shown in Figure 10, the majority of the cytokines are present at a significant higher level in HS-5/SCM than in CML/SCM (IL-1a, M-CSF, SCF, PDGF-bb, IL-1b, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, G-CSF, GM.CSF, MIP-1a, TNF-a and VEGF), whereas SDF-1a, TRAIL and HGF cytokines are present in HS-5/SCM and CML/SCM supernatants at similar levels. As already observed IL-3 was not found in any supernatant but not in one single tested CML/SCM at the concentration of 9pg/ml. Interesting we observed that both cell lines HS-5/SCM and CML/SCM produce high level of cytokine IL-6 which serves as a positive feedback loop to sustain CML development and acts at the level of leukemic MPPs (CML multipotent progenitors cells) to promote myeloid development at the expense of lymphoid differentiation. IL-6 is a major player in CML pathogenesis. Indeed BCR/ABL regulates IL-6 mRNA levels through a complex and indirect mechanism involving, at least, BCL6 and the LIN28/28b-Let-7 pathway, which are two known transcriptional regulators of the IL-6 gene. This paracrine mechanism appears exacerbated in CML myeloid blast crisis, most likely due to increased IL-6 production by blast cells. Pharmacological inhibition of BCR/ABL activity with TKIs in K562 cell line or in CML cells downregulate STAT5 activation and both LIN28 and LIN28b expression, that increase BCL-6 expression, a direct transcriptional repressor of the *IL-6* gene (Yu et al., 2005). Then exogenous IL-6 may activates the JAK-STAT pathway in CML CD34+ cells treated with TKIs. In our experiment we show that stroma cell lines producing high level of IL-6 may protect leukemic cell fate by TKI effect.

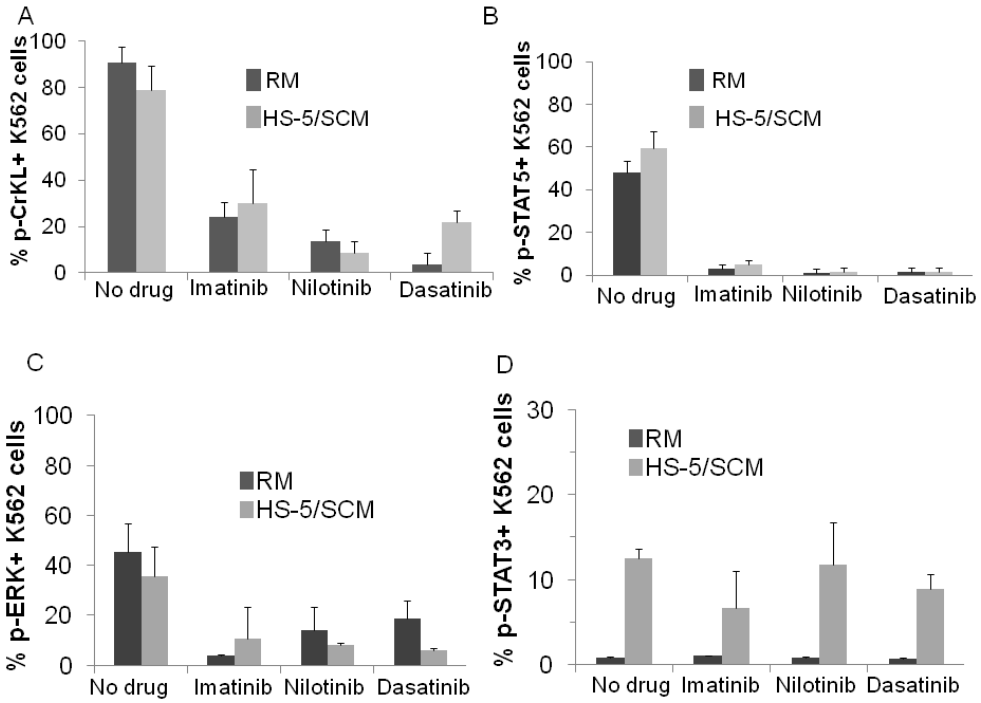

Figure 9. **HS-5/CM phosphorylates STAT3 and confers BCR/ABL indipendent resistance against TKI treatment**. K562 cells were treated with Imatinib (500nM), Nilotinib (15nM) or Dasatinib (1.2 nM) for 2.5 hours, and the extracts from these cells were subjected to immunoblot analysis for phosphorylated (p) or total forms of proteins (not showed) associated with the BCR/ABL signaling pathway. The immunoblot analyses were showed for p-CrKL (**A**), pSTAT5 (Tyr694) (**B**), p-ERK (**C**) and pSTAT3 (Tyr705) (**D**).

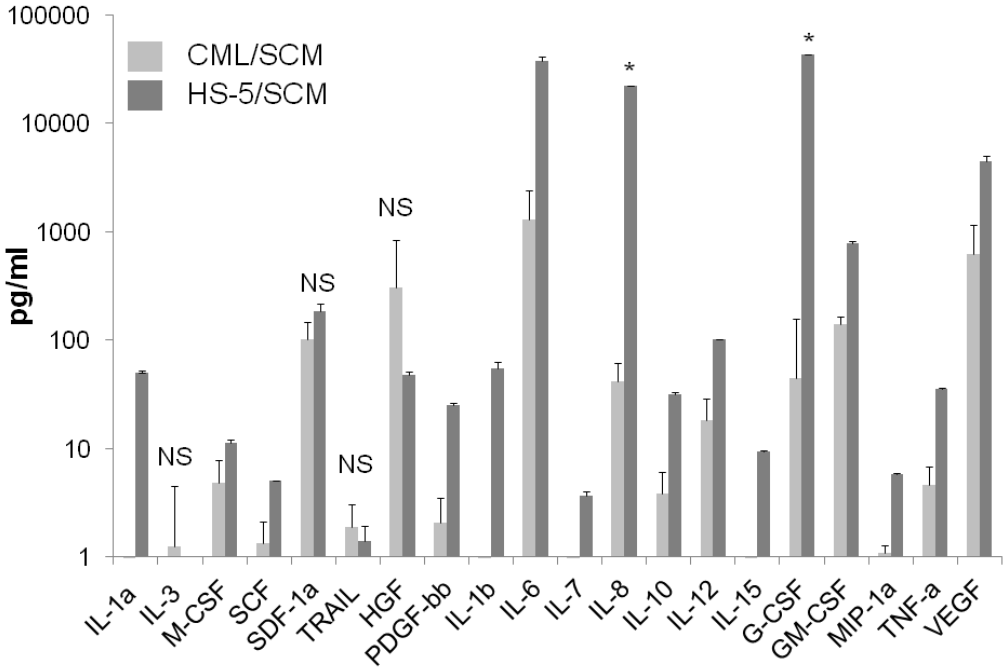

Figure 10. Bioplex ELISA assay. Comparation of secretory profiles between the HS5/SCM and CML/SCM. The human bone marrow stromal cell line HS-5 or the mesenchymal stromal cell derived from 8 BM CML patients were seeded in 75-cm^2 flasks and grown to 70%–80% confluency. Thereafter, culture media were exchanged for in α-MEM supplemented with 20% FBS, 1% L-Glutamine, and 1% penicillin/streptomycin. After 24 hours, the conditioned medium was carefully harvested, centrifuged for 10 minutes at 5,000*g to remove debris, 0.22 µm filtered and stored at* -80°C before the Bioplex ELISA analysis.

Ph+ Cell fate after TKI treatment in the Presence of Stroma-Derived Soluble Factor

In our study, we attempted to evaluate whether the viable cells residual after TKI treatment in the presence of stroma derived media, were already death-committed or instead, they were capable of additional cell divisions. Thus, we treated K562 cells with Imatinib, Nilotinib or Dasatinib at the calculated IC50, either in the presence or absence of HS-5/CM. After 72hrs, cells have been re-plated in new fresh media without addition of TKI. Cell proliferation and cell viability have been monitored by Trypan blu exclusion every 3 days for two consecutive weeks. On day 3, cell proliferation was significantly reduced by Imatinib, Nilotinib and Dasatinib treatment in all control conditions, where K562 cells were cultured in the absence of stroma derived soluble factors. Moreover, at a TKI concentration clinically achievable, cell viability and proliferation of K562 cell line continue to decrease overtime, showing that Ph+ cell line may be completely eliminated. In contrast, HS-5/CM is able to reduce TKI activity as previously demonstrated, and viable K562 cells were also able to further expand even if exposed to high concentration of Imatinib, Nilotinib and Dasatinib.

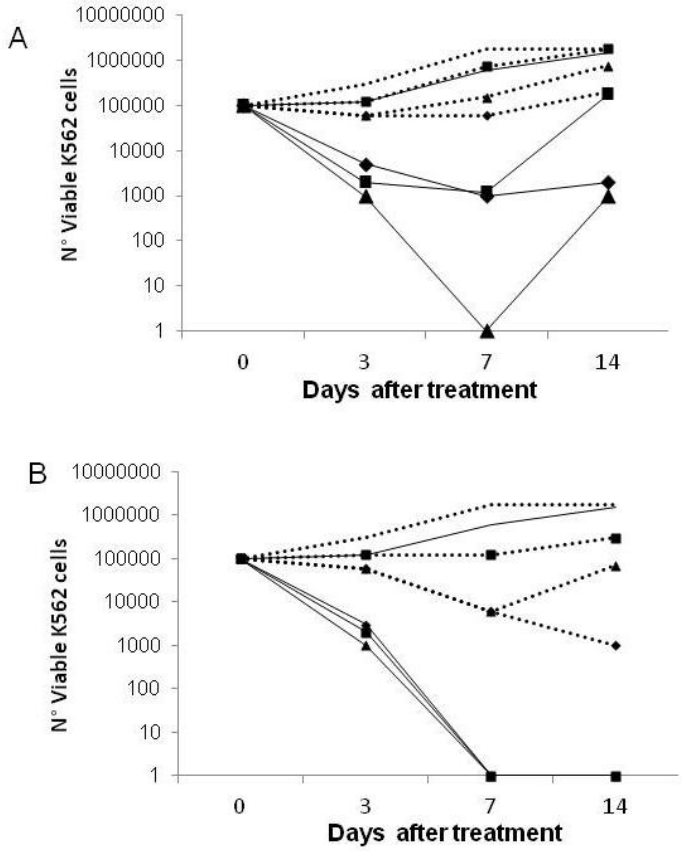

Figure 11. **Cell proliferation has been monitored by Trypan blu exclusion at the indicated time point. A)** Long viability experiment: K562 cell line was treated with 1µM Imatinib (square), 30nM Nilotinib (rhombus) and 2nM Dasatinib (triangle) at time 0 in the absence (continuous lines) or presence of HS-5/CM (dashed lines). **B)** Long viability experiment: K562 cell line was treated with 4µM Imatinib (square), 300nM Nilotinib (rhombus) and 10nM Dasatinib (triangle) at time 0 in the absence (continuous lines) or presence of HS-5/CM media (dashed lines).

CD34+ CML Progenitor Cells are Protected from TKI Toxicity by Stroma Conditioned Media and Cytokines

We evaluate if the observed stroma derived TKI resistance may have some relevance also in a patient related setting. In particular, we selected haematopoietic progenitor cells from BM and peripheral blood samples of 10 untreated patients affected by CML, by sorting with a magnetic system CD34+ cells.
The CD34+ cells were treated with clinical relevant concentration of TKIs, and drug-related cell death and apoptosis have been monitored after 72 and 24hrs, respectively. As shown in Fig. 12A-B, and as already demonstrated by several other independent groups, immature CML CD34+ cells, grown in the absence of grow factors, are inherently resistant to TKI treatment since 20±11%, 19±11% and 19±9% of CD34+ cells are still viable after treatment with Imatinib, Nilotinib or Dasatinib, respectively.
Moreover, drug-related cell death and apoptosis are significantly reduced when CML CD34+ cells are treated with Imatinib, Nilotinib or Dasatinib in the presence of either high growth factor cocktail or HS5/SCM (Figure 12A-B).
To confirm that the viable residual cells were not irreversible un-functional after TKI treatment, primary progenitor CML CD34+ cells residual after TKI treatment in the presence or absence of stroma derived grow factors, were washed and plated in a methylcellulose-based medium that is widely used to detect and quantify hematopoietic progenitor cells in the *colony-forming unit* (CFU) assay.
Imatinib, Nilotinib and Dasatinib treatments significantly reduce CFU growth of progenitor CD34+ from CML patients. Nevertheless, a significant increase in CFU grow was observed when progenitor CD34+ from CML patients were treated with TKIs in the presence of high growth factor cocktail or HS5/SCM. These data indicate that not only stroma SCM protects from apoptosis or cell death but also results in a higher percentage of cells capable of dividing and repopulating. Moreover, after 14 days of colony grow, hematopoietic colonies were single plucked from methylcellulose and analyzed by Q-RTPCR for the presence of BCR-ABL mRNA, revealing that a large proportion of CD34+ cells remain BCR-ABL positive, after TKI treatment, independently from the in vitro applied culture conditions.

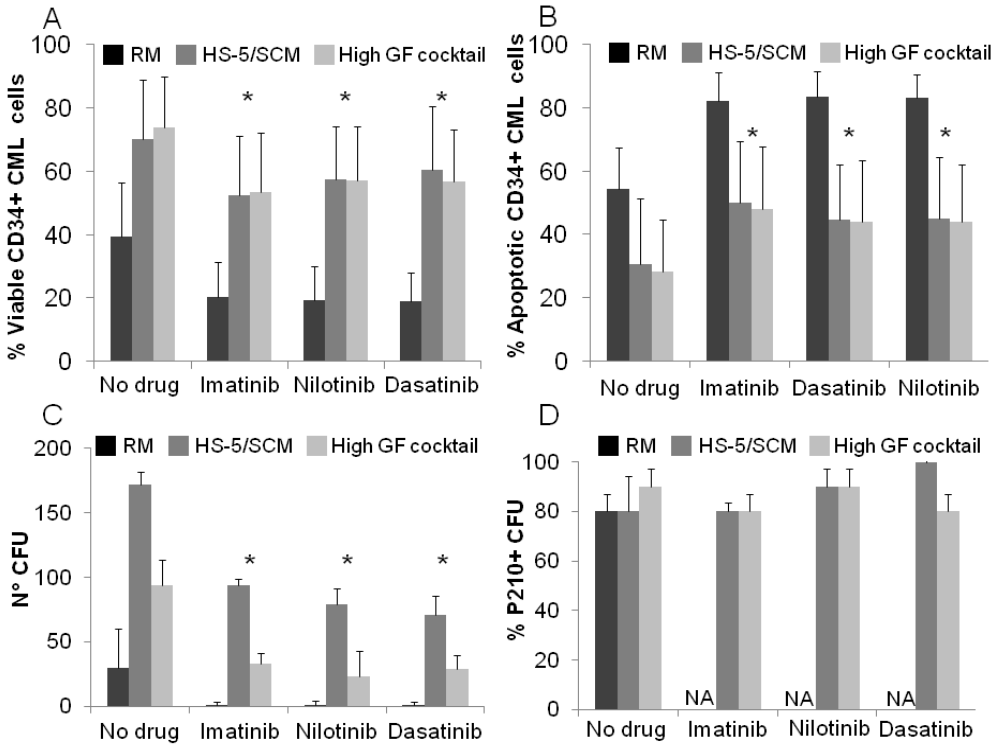

Figure 12. CD34+ CML progenitor cells are protected from TKI toxicity by stroma conditioned media. CD34+ CML progenitor cells (**A**) cells were treated respectively in regular media (RM) (black barrel), or with 50% HS-5/SCM (gray-iron barrel), or high grow factor (GF) cocktail (gray-white barrel) with clinical relevant concentrations of Imatinib (500nM), Nilotinib (15nM) or Dasatinib (1.2 nM) for 72 hours. The Figure 12A show that the residual percentage of viable cells after TKI treatment, assessed by flow cytometry, was significantly higher in CD34+ cell treated in presence of HS5/SCM or high GF cocktail. (**B**) The induction of overall apoptosis (early + late) was significantly reduced in presence of HS5/SCM or high GF cocktail. The Figure (**C**) show that granulocyte-macrophage colony-forming units and erythrocyte colony-forming units (CFU) originate from CD34+CML progenitor cells was significantly reduced but persistent, after TKI exposition in HS-5/SCM or high GF cocktail. The Figure (**D**) show that a percentage of CFU BCR-ABL positive, after TKI treatment, is independently from the in vitro applied culture conditions. Results are expressed as mean ± SD from 10 independent experiments. The symbol [*] is for p value ≤0.05.

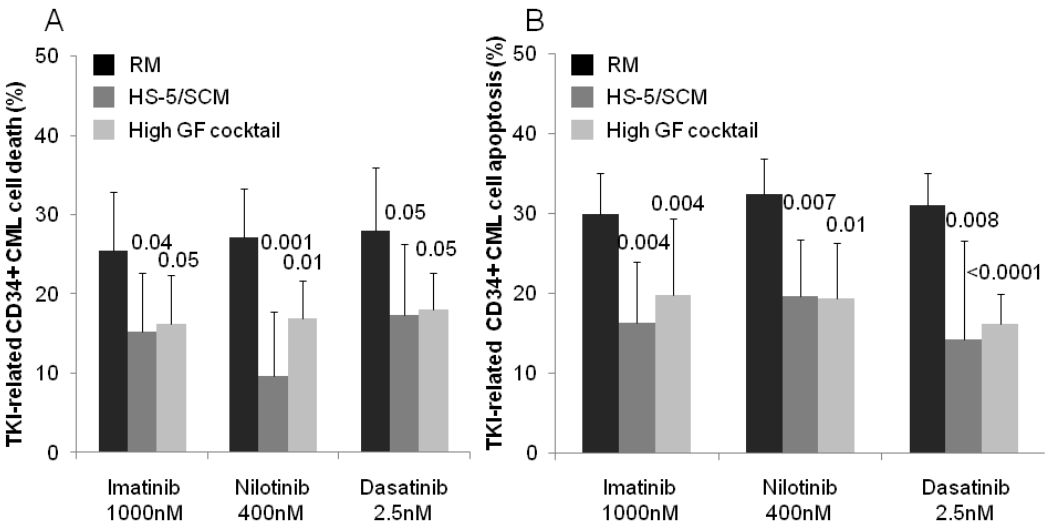

Figure 13: SCM or stroma derived cytokines protects CML CD34+ from TKI-specific cell death and apoptosis.
TKI-specific cell death (**A**) and apoptosis (**B**) were calculated by subtracting the percentage of Annexin-V+/PI+ CML CD34+ cells from un-treated samples to the percentage of Annexin-V+/PI+ CML CD34+ cells relative to the specified samples treated with TKIs in the presence of RM (black barrel), in media supplemented with 50% of HS-5/SCM (dark gray barrel) or in media supplemented with high grow factor (GF) cocktail (light-gray barrel).

Synergistic Effect of JAK2 Inhibitors and TKI to overcome Stroma Related Drug Resistance

We attempted to evaluate if could be possible to reduce the negative effects of stroma microenvironment on TKI activity. We suppose that one mechanism could be to reduce cell signaling induced by the interaction of a putative soluble factor with its specific receptor. In particular, it has been established that cytokines induce JAK/STAT pathways through the activation of a specific receptor. Thus, we exploit the possibility to down regulate JAK signaling induced by stroma soluble factors, by treating K562 cell line with two different JAK2 inhibitors Panobinostat (Pan) or Ruxolitinib (Ruxo). In particular, we set-up the following experiments: K562 cells were treated with TKI and/or JAK2 inhibitors either in the presence or absence of HS-5/CM. Apoptosis induction has been monitored after 24hrs by Annexin-V flow cytometric test. Panobinostat and Ruxolitinib are not able to reduce cell viability (Figure 14 A-B) or induce apoptosis (Figure 14 C-D) in Ph+ cells by their self, as expected. Indeed, JAK2 inhibitors do not down-regulate the activity of the oncogene BCR-ABL. In contrast, the co-treatment of TKI and JAk2 inhibitors are able to significantly reduce cell viability and induce apoptosis in Ph+ cells treated in the presence of HS-5/CM. This experiment prove a strong synergistic effect of TKI and JAK2 inhibitors to overcome stroma derived TKI resistance.
It has been recently proved by a carefully conducted study that BM stroma conditioned media activates Jak2-Tyk2-Stat3 pathway in CML cells and that knocking down JAK signaling by the JAK inhibitor Ruxolitinib (INCB018424), CML cells are sensitized to a subsequent Nilotinib treatment. We next sought to determine a potential synergistic effect between TKIs and JAK inhibitor Ruxolitinib, by applying a dose matrix cell-based assay. In particular, drugs were serially diluted and all possible pairs of diluted drug combinations were tested on three independent experiments. In particular, K562 cell line was exposed to increasing dose of Imatinib or Nilotinib in combination with Ruxolitinib. Cell survival was assessed by AnnexinV/PI staining at 72 hours after treatment.
Ruxolitinib was not effective on K562 cells, demonstrating a modest effect on overall viability relative to untreated controls (Fig. 15). However, the combination of Imatinib or Nilotinib and Ruxolitinib demonstrated greater toxicity in K562 cells than treatment with either TKI alone or Ruxolitinib alone. Next, we tested the effects of drug synergy in the presence of HS-5/SCM, with similar results. In particular, combination indices (CIs) to determine synergy for the drug combinations (ie, CI<1) were calculated according to Chou-Talalay method, which indicated that the combinations were synergistic either in the absence or in the presence of HS-5/SCM. Nevertheless, a very strong synergism has been achieved in K562 cell line treated with Nilotinib in combination with Ruxolitinib in the presence of HS-5/SCM. Moreover, we looked after to evaluate the relevance of these findings, also in the modulation of the survival of progenitor CD34+ cells from patients with CML. Thus, we co-treated BM CD34+ cells with TKIs and Ruxolitinib for 72hrs in the presence or absence of stroma conditioned media (HS5/SCM). As shown in Fig. 15A, Ruxolitinib significantly synergize with either Imatinib or Nilotinib to reduce cell viability in CD34+ cells derived from CML patients, treated in the presence of either HS-5/SCM or high growth factor cocktail. In particular, although Ruxolitinib exert a significant cellular effect as single agent in CD34+ cells from both CML patients, or healthy donors treated in the presence of stroma conditioned media or high growth factor cocktail , the observed increase in cell death is significantly lower than the one observed by applying the proposed drug

combination. Moreover, the synergistic effect of TKI and Ruxolitinib on the modulation of cell viability seems to be selective for CD34+ cells derived from patients with CML, since we observed a modest cell death when we treated CD34+ cells from healthy donors (CD34+HD cells), irrespective of the concomitant TKI treatment. Indeed, Imatinib, Nilotinib and Ruxolitinib each demonstrated a moderate ability to impair the formation of CML colonies (CFU) in the presence of HS5/SCM (83%, 50% and 61% colonies, respectively, relative to untreated controls). However, Imatinib or Nilotinib in combination with Ruxolitinib demonstrated a substantial improvement over either Imatinib alone ($p<0.0001$) or Nilotinib alone ($p<0.0001$), significantly reducing the formation of CML colonies to 6% and 2%, respectively, relative to untreated controls. Importantly, Imatinib, Nilotinib, and Ruxolitinib, alone or in combination did not significantly impair formation of normal erythroid and myeloid colonies.

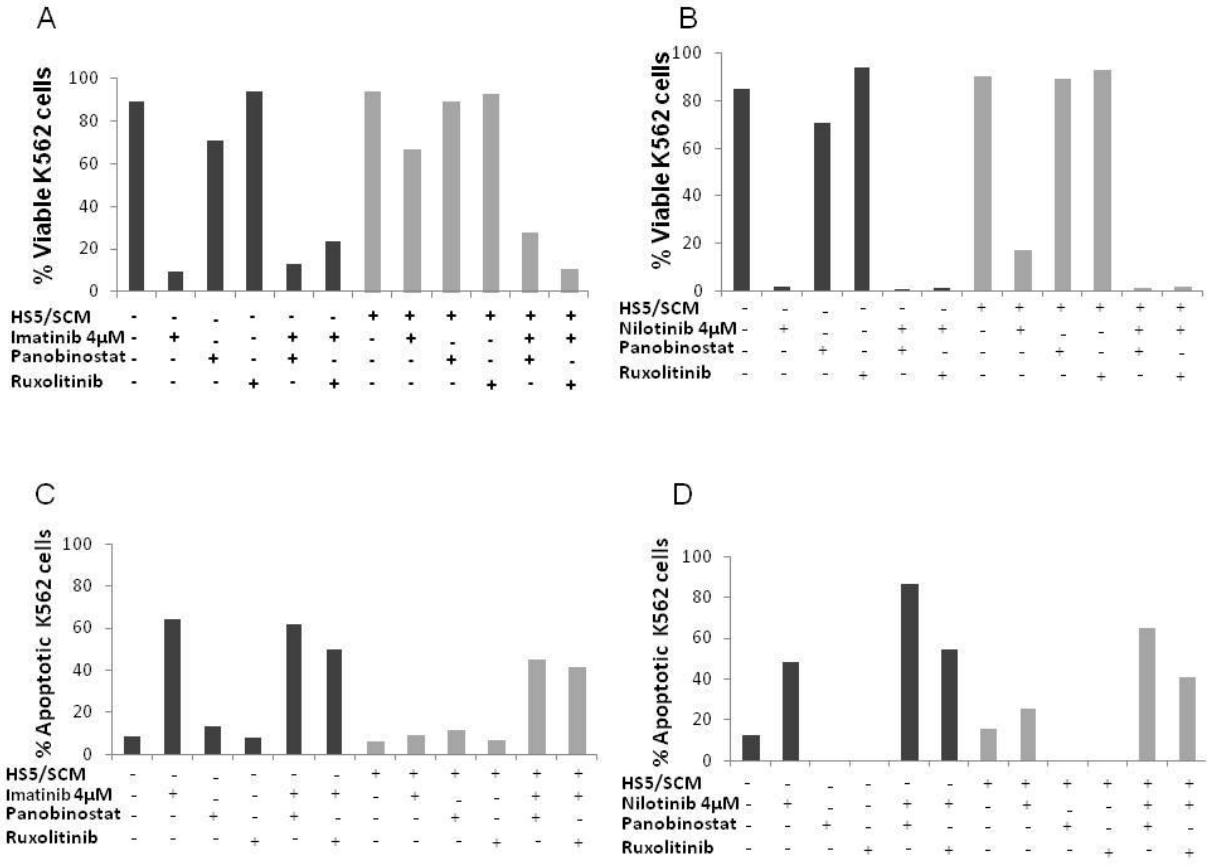

Figure 14: **A)** Apoptosis induction has been monitored after 24hrs by Annexin-V flow cytometric test. Viable of K562 cells treated with 4µM Imatinib and/or JAK2 inhibitors either in the absence of HS-5/CM (Black) or in presence of HS-5/CM (Grey). **B)** Viable of K562 cells treated with 4µM Nilotinib and/or JAK2 inhibitors either in the absence of HS-5/CM (Black) or in presence of HS-5/CM (Grey). **C)** Apoptosis of K562 cells treated with 4µM Imatinib and/or JAK2 inhibitors either in the absence of HS-5/CM (Black) or in presence of HS-5/CM (Grey). **D)** Apoptosis of K562 cells treated with 4µM Imatinib and/or JAK2 inhibitors either in the absence of HS-5/CM (Black) or in presence of HS-5/CM (Grey).

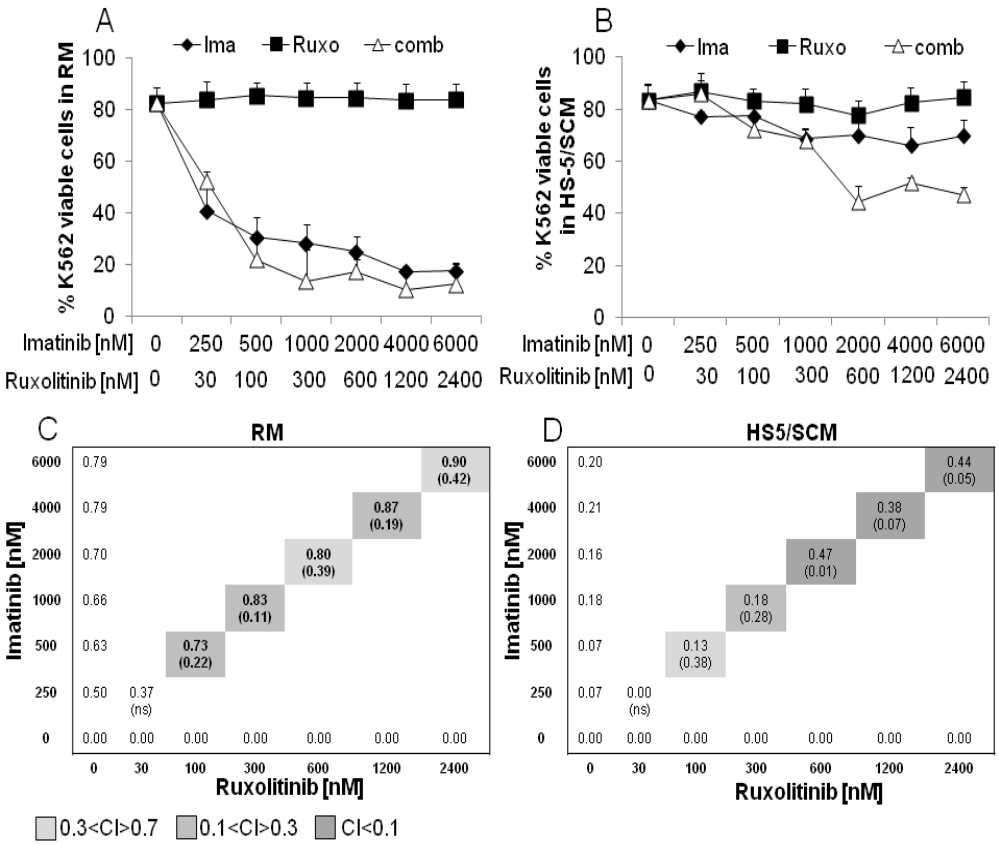

Figure 15: Very strong synergism of Imatinib and JAK2 inhibitor Ruxolitinib on overcoming stroma derived TKI resistance. K562 cell line was exposed to increasing dose of Imatinib (Ima) in combination with increasing doses of Ruxolitinib (Ruxo). Cell viability was assessed by AnnexinV/PI staining at 72 hours after treatment in RM (**A**) or in media supplemented with 50% of HS-5/SCM (**B**). Dose matrix summarizes the cell based cytotoxic effect (Z) relative to the drug combination (comb) of Imatinib and Ruxolitinib in K562 cells treated in RM (**C**) or in media supplemented with 50% of HS-5/SCM (**D**). Cell based cytotoxic effect (Z) for each combination dose is reported into the tables C and D; and CI value for each combination dose are reported in parenthesis. In particular, based on the CI value, we defined, as previously reported, a very strong synergism (dark gray), strong synergism (gray), synergism (light gray), from moderate synergism to no synergism (white).

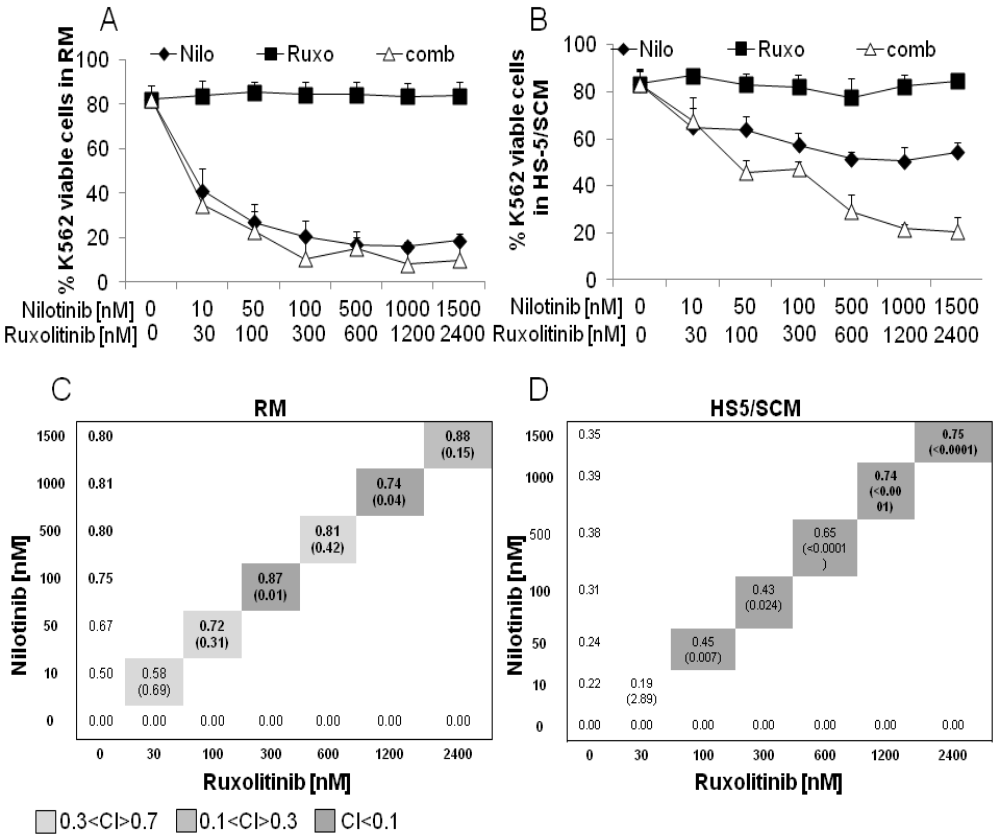

Figure 16: Very strong synergism of Nilotinib and JAK2 inhibitor Ruxolitinib on overcoming stroma derived TKI resistance. K562 cell line was exposed to increasing dose of Nilotinib (Nilo) in combination (comb) with increasing doses of Ruxolitinib (Ruxo). Cell viability was assessed by AnnexinV/PI staining at 72 hours after treatment in RM (**A**) or in media supplemented with 50% of HS-5/SCM (**B**). Dose matrix summarizes the cell based cytotoxic effect (Z) relative to the drug combination of Nilotinib and Ruxolitinib in K562 cells treated in RM (**C**) or in media supplemented with 50% of HS-5/SCM (**D**). Cell based cytotoxic effect (Z) for each combination dose is reported into the tables. CI value for each combination dose are reported in parenthesis. In particular, based on the CI value, we defined, as previously reported, a very strong synergism (dark gray), strong synergism (gray), synergism (light gray), from moderate synergism to no synergism (white).

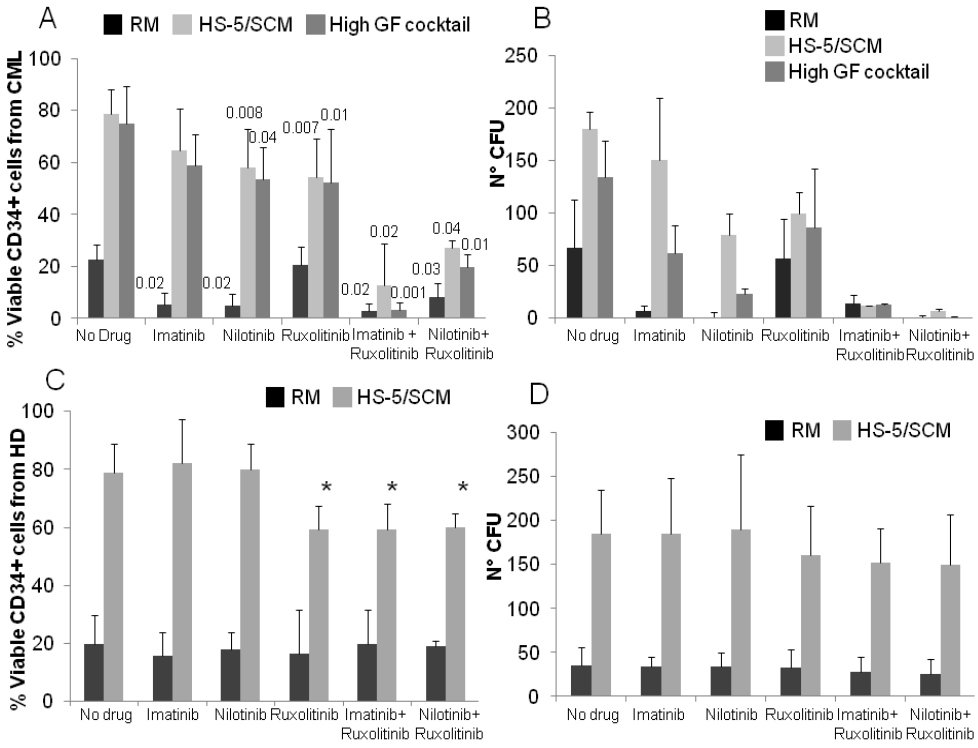

Figure 17. Ruxolitinib synergize with TKI in the elimination of CFU from patients with CML.

CD34+cells derived from either BM of five patients with CML-CP or PB of two immobilized HDs, were treated respectively in regular media (RM) (black barrel), or with 50% HS-5/SCM (gray-white barrel), or high grow factor (GF) cocktail (gray-iron barrel) with clinical relevant concentrations of Imatinib (500nM), Nilotinib (15nM), Ruxolitinib (300nM) or drug combination for 72 hours. The Figure (**A**) show that the residual percentage of viable CD34+ CML progenitor cells after TKI treatment or/and Ruxolitinib, assessed by flow cytometry, was higher in CD34+ CML progenitor cell treated in presence of HS5/SCM or high GF cocktail than RM, but significantly lower in cells treated with the drug combination (TKI+ JAK2 inhibitor). Interesting the CFU originate from CD34+CML progenitor cells (**B**) were significantly reduced, but still persistents, after Nilotinib or Ruxolitinib exposition in HS-5/SCM or high GF cocktail. (**B**) Data from a clonogenic assay clearly evidenced a significant down-regulation of CFU grow when CD34+ CML progenitor cells were treated with the drug combination (TKI+JAK2 inhibitor).

The Figure (**C**) show that the residual percentage of viable CD34+HD cells exposed to HS-5/SCM and treated with Ruxolitinib or drug combination was slightly but significantly reduced. (The symbol [*] is for p value ≤0.05). (**D**) Data from a clonogenic assay clearly evidenced that CFU originate from CD34+HD cells still grow when treated with clinical concentrations of TKI or/and Ruxolitinib in RM and in HS5/SCM.

Effects of TKI and JAK2 Inhibitors treatment on CML Progenitor Apoptosis

To evaluate the synergistic effect of TKI and JAK2 inhibitors on CD34+ progenitor cells derived from patients with CML, we treated CML CD34+ cells with 1µM Imatinib, 2.5nM Dasatinib and 400nM Nilotinib in the following three different media condition: 1) serum free media, 2) serum free media supplemented with haemaopoietic pro-proliferating cytokines (FLT3, SCF, IL3, GCSF and IL6), 3)HS-5/CM. After 72hrs, CD34+ cells were analyzed for viability and apoptosis level by Annexin-V/PI assay. As shown in Figure 18, the down-regulation of JAK2 signaling by the cotreatment with Panobinistat or Ruxolitinib results to be significantly synergic with TKI for the reduction of cell viability and induction of apoptosis also if applied on CD34+ progenitor cells derived from patients affected by CML. Moreover, we evaluate if the CD34+ progenitor cells residual after treatment have clonogenic activity. Indeed, it is well known that clonogenic activity is strongly increased in leukemia derived progenitor cells.
Thus, after the described cultures, CD34+ cells were plated in a semi-solid media to allow the grow of colony forming unit. In particular, we used methylcellulose-based medium, that is widely used to detect and quantify hematopoietic progenitor cells in the *colony-forming unit* (CFU) assay. This approach is suitable for identifying and enumerating CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. After 14 days, we counted CFU obtained in all conditions.
Clonogenic activity of CD34+ cells are only modestly reduced after Imatinib treatment, whereas the co-treatment of TKI and JAK2 inhibitors is able to significantly affect colony growth.

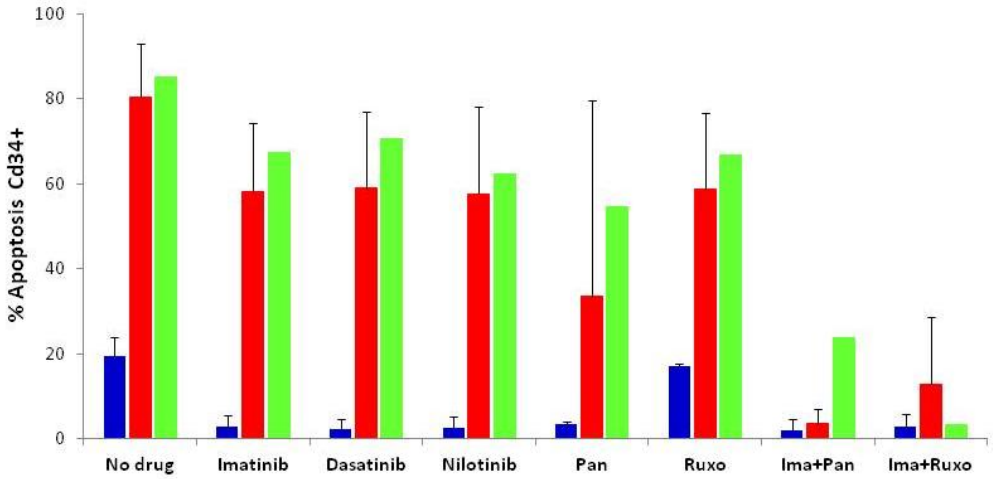

Figure 18: CML CD34+ cells were analyzed for viability and apoptosis level by Annexin-V/PI assay. CML CD34+ were treated with 1µM Imatinib, 2.5nM Dasatinib and 400nM Nilotinib in the following three different media condition: serum free media (Blu), serum free media supplemented with haematopoietic pro-proliferating cytokines (FLT3, SCF, IL3, GCSF and IL6) (Red), HS-5/CM (Green).

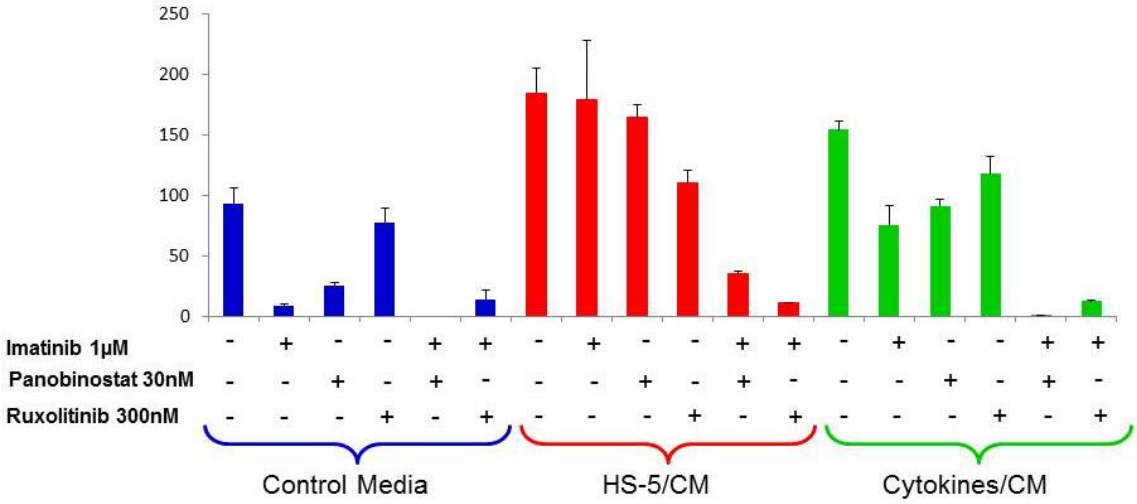

Figure 19: Count of CFU growth after 14 days of grow in a semi-solid media. CML CD34+ cells treated with 1µM Imatinib, 2.5nM Dasatinib and 400nM Nilotinib in the following three different media condition: serum free media (Blu), serum free media supplemented with haemaopoietic pro-proliferating cytokines (FLT3, SCF, IL3, GCSF and IL6) (Red), HS-5/CM (Green). After 72hrs of treatment, CD34+ cells were plated in a semi-solid media.

DISCUSSION

The emergence of drug resistance continues to limit the success in finding a cure for hematologic malignancies. CML represents a disease that is initially driven by the well-established oncogenic event resulting in the expression of the BCR-ABL fusion oncogene. This uniform transforming event common to CML results in an ideal disease model to delineate the emergence of drug resistance. Clinical data arising from studies targeting BCR-ABL inhibitors indicate that, although BCR-ABL inhibitors are very effective in reducing leukemia burden, targeted therapy using a single agent does not eliminate minimal residual disease.

In this work we have shown that resistance to BCR-ABL tyrosin kinase inhibitors is related to soluble factors produced by both BM stroma immortalized cell line HS-5 and CML patient-derived BM stroma cell line. Indeed, we proved that stroma-derived microenvironment drug protection is achievable not only by leukemia cell exposition to a direct stroma cell contact during TKI treatment, but also by setting up cell culture condition implying soluble factors produced by HS-5 or CML patient-derived BM stroma cell line. Thus, co-culturing Ph+ K562 cell line with the conditioned media derived from HS-5 cell line, CML cells survive to the action of drugs by observing a significant increase of viability compared to controls. We also observed the same evidence when Ph+ K562 cell line was treated with TKI in the presence of conditioned media derived from CML patient BM stroma cell line, that we established in our laboratory. Moreover, we demonstrated that the higher cell viability, noticed in K562 cells treated with TKI in the presence of stroma conditioned media respect to control cell culture condition, is not related to cytokine cell cycle regulation but to a significant reduction in apoptosis induction. Indeed, BrdU analysis show a relevant accumulation in G0-G1 cell cycle phase of K562 cells treated with TKI, but we detected no significant differences in cell cycle phases between K562 cells treated with TKI in presence or absence of conditioned media derived from the stroma cell line HS-5. To understand whether TKI treatment is able to completely eliminate leukemic cells, we conducted a long-term experiment, in which K562 cell viability and proliferation were monitored overtime for 14 consecutive day after TKI treatment in presence or absence of conditioned media derived from the stroma cell line HS-5. We observed that K562 cells treated with TKI in the absence of HS-5/CM cease to proliferate and are not more able to encounter cell divisions. In contrast, if K562 cells are treated with TKI in the presence of HS-5/CM, after a steady-state of three days from the TKI culture addition, cells start to proliferate at the same level of the untreated cells. This evidence, strongly suggests that TKIs are not able to completely eradicate leukemic cells when stroma derived factor are present in the microenviroment.

Our study may have same clinical relevance, since we also demonstrated in vitro that IC50s of the three studied TKI (Imatinib, Nilotinib and Dasatinib) are strongly modulated by HS-5/CM. Indeed, we show that we can reduce the Ph+ cell viability below the 50% only implying high TKI concentration that are not reasonable achieved in vivo in patients. These evidences also confirm what it is coming out from several clinical trials applying TKI: target molecular therapy, including Imatinib, induces remission in patients with CML but does not eliminate leukemia stem cells, which remain a potential source of relapse. Thus, we investigated the activity of two different JAK2 inhibitors, Panobinostat and Ruxolitinib, to be synergic with TKI in the elimination of CML stem cells. In particular, the first inhibitor Panobinostat is a pan-

HDAC inhibitor. Histone acetyltransferases and histone deacetylases (HDACs) play a central role in modifying the acetylation status of the lysine residues of histone and non-histone proteins. Treatment with pan-HDAC inhibitor, such as Panobinostat, induces hyperacetylation of histones and non-histone proteins, including heat shock protein 90 (Hsp90). Hyperacetylation of Hsp90 inhibits its chaperone function and promotes the proteasomal degradation of Hsp90 client proteins, including FLT-3, AKT, c-RAF, and JAK2, in leukemia cells **[81-82].** Concomitantly, HDAC inhibitor treatment has been shown to cause cell cycle growth arrest and apoptosis of leukemia cells. In a phase 1 clinical trial, it has been observed that treatment with Panobinostat reduced leukemia blast cell numbers in the blood of 7 of 11 patients. The second inhibitor Ruxolitinib is a potent and selective JAK1 and JAK2-inhibitor (IC_{50} of 3.3 and 2.8 nmol/L, respectively, in “naked” kinase assays in cell-free in vitro systems). It demonstrates modest selectivity against Tyk2 (~6-fold) and ≥ 130-fold selectivity against JAK3. Treatment with Ruxolitinib is associated with a dramatic decrease in circulating levels of proinflammatory cytokines, IL-6, and *tumor necrosis factor* (TNF)-α, which have been implicated in the pathogenesis of MPNs **[83].**

We demonstrated that either co-treatment with HDACi or JAK2 inhibitor combined with TKI effectively induced apoptosis in both K562 cell line and CD34+ progenitor quiescent CML cells resistant to elimination by Imatinib, Nilotinib and Dasatinib alone, due to BM stroma microenviroment signaling. Thus, HDACi treatment represents an effective strategy to target LSC in CML patients receiving tyrosine kinase inhibitors. It was very interesting to discover that the synergistic effect of TKI and HDACi reduces drug resistance.

Finally, we attempted to evaluate if the synergic combination of TKI and JAK2 inhibitors is able to significantly reduce leukemia clonogenic activity in an in vitro assay. Thus, we selected CD34+ progenitor cells from BM samples of patients with CML. After 72hrs of treatment with TKI and JAK2 inhibitors, residual cells were plated in methylcellulose media supplemented with cytokine allowing grow of CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. CFUs were encountered after 14 days and we demonstrated that Imatinib treatment is able to significantly reduce colony grow only in the absence of conditioned media from HS-5 or stroma derive cytokines with anti-apoptotic and pro-proliferating activity. Moreover, the co-treatment of Ph+ CD34+ cells with Imatinib and Panobinostat or Ruxolitinib strongly decrease CFU counting in a stroma-independent manner.

This data strongly suggest that stroma related drug resistance has a relevant role in the regulation of TKI responsiveness in patient with CML and that the drug concentration needed to eliminate in vitro Ph+ cells in the presence of stroma microenviroment is not achievable in vivo clinical trial. Thus, we propose a novel drug combination able to be effective in the leukemia eradication: the combined down regulation of the BCR/ABL oncogene plus the down-regulation of the signaling induced by soluble factors present in the stroma BM microenvironment through JAK/STAT pathway may be likely relevant in vivo for the treatment of patients and the reduction of relapses. Moreover, since we demonstrated that CML cells are protected from TKI activity by soluble factors either derived from immortalized HS-5 cell line or stroma CML cell lines (data not shown), we performed a quantitative evaluation of cytokines present in this two cellular settings. The majority of the investigated cytokines, such as M-CSF, SCF, PDGF-bb, IL-6, IL-8, IL-10, IL-12, G-CSF, GM-CSF, TNF-α and VEGF but not the chemotactic factor SDF-1, whereas present in significantly higher levels in HS-5/SCM, were also found in CML/SCM. In particular, previous studies showed that stroma-mediated drug resistance is due to

the increase in the phosphorylation level of Stat3 (Tyr705), thus indicating that the stromal protection passes through the activation of this clinically relevant pathway, which is known to sustain cell viability in vivo, and to trigger the activation of Jak2–Tyk2 signaling. Moreover, it was observed that turning off the JAK pathway using a JAK inhibitor sensitizes CML cells to Nilotinib treatment in the context of the BM microenvironment. Thus, we investigated whether Ruxolitinib differentially synergizes with Imatinib, Nilotinib and Dasatinib to overcome stroma related TKI resistance in CML cell line or CD34+ progenitor cells from CML patients. Our data indicate that Ruxolitinib effectively strongly synergizes with Imatinib, and achieves a very strong synergism with second generation TKIs, either Dasatinib or Nilotinib, being able to induce apoptosis in both K562 cell line and CD34+ progenitor CML cells resistant to the elimination by the single TKI agent, in the presence of BM stroma signaling. Indeed, co-treatment of Ph+ CD34+ cells with Ruxolitinib and Imatinib, Nilotinib or Dasatinib significantly decreased CFU outgrowth, providing evidence that it is able to overcome stromal protection. In contrast with Traer et al., we prove that the JAK inhibitor Ruxolitinib, instead the TG101209, used by Traer et al., allows to achieve a therapeutic window of drug combination that spear progenitor cells from healthy donors or CML patients in MR4. From a clinical point of view, although second generation TKIs achieve a deeper and prolonged cytogenetic and molecular response rate than Imatinib in patients with CML-CP, the use of TKIs as a single agent seems unable to eradicate the disease, despite the increase in MR4.0 rate. Our data strongly support the hypothesis that a drug combination of Ruxolitinib and second generation TKIs (either Nilotinib or Dasatinib) may be more effective in eradicating leukemia rather than the single agent TKI, with minimal toxic effect on normal hematopoietic cells. However, the in vivo side effects related to the proposed drug combination, need to be determined by a specific clinical trial. The combined deep down-regulation of BCR/ABL oncogene plus the down-regulation of the signaling induced by the soluble factors present in the stroma BM microenvironment through the JAK/STAT pathway may be likely relevant in vivo for the treatment of CML patients and may significantly increase the rate of MR4.0, which is the only condition that may prelude the decision of treatment interruption.

We are planning to carry out a secretoma assay. In particular, we will isolate acrylamide gel bands derived from running a concentrated preparation of stroma conditioned media. Thus, bands will be hydrolyzed and analyzed by mass spectrometry. All identified factors will be functionally analyzed.

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