Unraveling the Role of Lyn Kinase in the Extracellular Vesicle-Based Crosstalk Between Primary Chronic Lymphocytic Leukemia Cells and Stromal Cells

Inaugural-Dissertation

zur Erlangung des Doktorgrades der

Mathematisch−Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Thaís Dolzany de Oliveira

aus Nova Iguaçu

Köln

2022

Berichterstatter: Prof. Dr. Ines Neundorf

Prof. Dr. Stefan Höning

Prof. Dr. Ana J. Garcia-Saéz

Tag der mündlichen Prüfung: 23.06.2022

Acknowledgments

The work presented in this thesis was performed at the Cluster of Excellence Cellular Stress Responses in Aging-Associated Diseases (CECAD) at the University of Cologne in association with the Department of Internal Medicine I of the University Hospital of Cologne. I would like to express my gratitude to everyone that has helped and encouraged me during the work of this thesis.

I especially would like to thank PD Dr. Hinrich P. Hansen, my supervisor, thank you for your fantastic support all these years with thesis supervision, and friendship, and for being always available and enthusiastic about work and life.

I also express further gratitude to all members of the Hallek group, in special Prof Dr. Michael Hallek and Dr. Phuong Hien Nguyen for adding value to this work with great discussions, questions, and ideas; Alexander vom Stein for providing the knockout cells for this thesis and all support with experiments and discussions; Liudmila Lobastova and Dr. Maximillian Koch that has been always so helpful, and Dr. Rocio Rebollido-Rios for helping with proteomic data. Many thanks to everyone in the lab (Krause group, Pallasch group, Frenzel group, and Rebollido-Rios group) that were very supportive and added great input to my work.

I would like to thank Dr. Marcus Lettau (University Medical Center Schleswig-Holstein) for helping with the ImageStream experiments and analysis.

I would like to thank the CECAD Imaging Facility and CECAD Proteomics Facility, especially Dr. Christian Jüngst, Peter Zentis, and Dr. Astrid Schauss. Also, thank the CLL Biobank and the patients. I gratefully acknowledge the German Research Foundation (DFG) and the University Hospital of Cologne for grants supporting this work.

“Knowledge has a beginning but no end”

Geeta Iyengar

Abstract

In Chronic Lymphocytic Leukemia (CLL), the tumor cells receive decisive survival support from non-malignant cells within the bone marrow. This support is stimulating tumor growth, progression and helps leukemic cells to evade chemotherapy. Within this microenvironment, the crosstalk between stromal and tumor cells plays an important role. The cell types interact directly and through the paracrine release of intercellular messengers, such as growth factors, and released plasma membrane-enclosed extracellular vesicles (EVs). In particular, the communication by EVs is not fully understood. It has already been shown that members of the Src family of kinases (SFKs) can influence the EV secretion and the SFK member Lyn is aberrantly expressed and highly active CLL-affected tissue, not only on malignant CLL cells but also in macrophages and stromal cells. This thesis aimed to study the mechanism of the Src kinase Lyn in the EV-based communication between the malignant B-CLL and stromal cells. Comparing Lyn proficient and deficient stromal cell lines revealed that the lack of Lyn raised the cell adhesion and podosome formation but decreased the number of filopodia per cell. Moreover, Imaging Flow cytometry and nanoparticle tracking analysis revealed that the EV release and EV uptake are significantly reduced in Lyn deficient stromal cells as compared to wild-type (WT) counterparts, resulting in a 36% reduction of the EV release and a 16% reduction in EV uptake, indicating that Lyn influences both, the cellular release and uptake of EVs. In addition, the same amount (4 µg/ml) of EVs from Lyn proficient stromal cells induced significantly higher support on primary CLL cells as compared to EVs from Lyn deficient counterparts. These data suggest that Lyn not only influences the EV release but also the molecular composition of the EVs. Proteomic comparison of the Lyn proficient and the deficient stromal cell line HS5 highlighted 72 significantly differentially expressed proteins. Among them, CD248 was prominently decreased in Lyn-deficient HS5 cells and a knockdown of CD248 in HS5 cells resulted in a diminished B-CLL cells survival feeding capacity compared to WT cells. In conclusion, the presented data provide initial preclinical evidence, that the tyrosine kinase Lyn crucially influences the EV-based communication between primary B-CLL and supporting bystander cells by raising the EV release and their concentration of functional molecules, such as CD248.

Abbreviations

ADC	Antibody-Drug Conjugates
BCR	B-Cell Receptor
BMSC	Bone Marrow Stromal Cells
CAF	Cancer-Associated Fibroblast
Cas9	CRISPR associated protein 9
CFSE	Carboxyfluorescein Succinimidyl Ester
CLL	Chronic Lymphocytic Leukemia
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXCR4	C-X-C motif receptor 4
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EV	Extracellular Vesicles
FA	Focal Adhesions
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
H	Hour
ISEV	International Society of Extracellular Vesicles
KO	Knockout
MEF	Murine Embryonic Fibroblast
MHC	Major Histocompatibility Complexes
MS/MS	Mass spectrometry/mass spectrometry
MVB	Multivesicular Bodies
NTA	Nanoparticle Tracking Analysis
PBS	Phosphate-Buffered Saline
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
SFKs	Src Family of Kinases
sgRNA	Single Guide Ribonucleic Acid
TEM	Transmission Electron Microscopy
TME	Tumor Microenvironment
TNT	Tunneling Nanotube
TSG101	Tumor Susceptibility Gene 101 Protein
WASP	Wiskott Aldrich Syndrome Protein
WT	Wild-Type
µg	Microgram
µL	Microliter

List of Illustrations

Figure 1. Schematic representation of extracellular vesicle biogenesis.. 11
Figure 2. Illustration of filopodia, lamellipodia, and podosome. 15
Figure 3. Domain structure of Src family kinases.. 25
Figure 4. Illustration of the study aims. 27
Figure 5. HS5 cell proliferation 37
Figure 6. Cell morphology comparison. 37
Figure 7. EV release of HS5 cells upon adherent and suspension conditions. 38
Figure 8. EV release counting. 39
Figure 9. TEM images of EVs. 40
Figure 10. EV release upon Dasatinib treatment.. 41
Figure 11. EV dose-dependent uptake 42
Figure 12. HS5 cells EV uptake... 43
Figure 13. B-CLL cellular EV uptake. 44
Figure 14. B-CLL cell EV survival support is dose-dependent. 44
Figure 15. EVs and protrusions of HS5 and CLL cells. 45
Figure 16. B-CLL cell and EVs co-culture. 46
Figure 17. BCL2 activation on B-CLL cells upon HS5 EVs treatment. 47
Figure 18. Volcano plot of EVs protein. 48
Figure 19. B-CLL cell and HS5 CD248 KO cells co-culture. 49

Table of Contents

Extracellular Vesicles 9
- Extracellular vesicles biogenesis 11
- Extracellular vesicle secretion and release 12

Cellular Protrusions 14
- Filopodia 15
- Lamellipodia 16
- Podosomes 18
- Tunneling Nanotubes 19
- Extracellular vesicles transported by protrusions 20

Tumor microenvironment in chronic lymphocytic leukemia 21
- Stromal cells tumor support 22
- Extracellular vesicles in the chronic lymphocytic leukemia tumor microenvironment 24

Scr family kinases 25
- Lyn kinase 26

Study Aims 27

Materials 28
- Reagents 28
- Tools 29
- Softwares 29
- Biological samples and cell culture 30

Methods 31
- Extracellular vesicles isolation and characterization 31
- Extracellular vesicles uptake analysis 31
- Extracellular vesicles release analysis 32
- Primary cells and extracellular vesicle co-culture 32
- Primary cells and stromal cells co-culture 32
- Protrusions analysis with confocal microscopy 33
- Generation of HS5 CD248 knockout cells 33
- Western Blot 34
- Statical analysis 34

Results 36
- HS5 WT and Lyn KO cell characterization 36
- Extracellular vesicles release 38
- Extracellular vesicles release upon Dasatinib treatment 40

Uptake of Extracellular vesicles 41
Extracellular vesicles influence on primary B-CLL cells 44
Proteomics Analysis 48
Discussion 50
Conclusions 55
References 56
Erklärung zur Dissertation 63

Introduction

Extracellular Vesicles

Extracellular vesicles (EVs) are a collective name for all membrane-enclosed particles secreted by cells into the extracellular environment. Depending on their diameter, EVs are often divided into three subtypes. Apoptotic bodies have the largest diameter (1 – 5 µm) (Jimenez et al., 2019). Smaller particles are referred to as microvesicles with a diameter ranging between 200 nm and 1000 nm. The smallest particles are exosomes (50 nm – 100 nm) (Szatanek et al., 2017. Apoptotic bodies are likewise considered EVs (Jimenez et al., 2019) as well as microvesicles and exosomes. Due to the breadth of their effects and ramifications, EV research has primarily focused on exosomes and microvesicles (Zarà et al., 2019). Microvesicles are formed when the plasma membrane spontaneously buds and cracks, while exosomes are formed when the plasma membrane and a multivesicular body fuse.

Even though this category was previously unknown, the larger particles and smaller vesicles that pass through the plasma membrane originate from multivesicular formations. Therefore, the International Society of Extracellular Vesicles (ISEV) rejects the use of exosome and microvesicle designations for particles with uncertain biogenesis, preferring to refer to them as EVs or small EVs, medium EVs, or huge EVs (Stahl and Raposo, 2019). When examining the chemicals delivered by an EV, both the membrane and the interior payload must be inspected (Zarà et al., 2019). Although the membranes of EVs are mainly composed of proteins and lipids, each EV has a unique protein and lipid composition based on its origin and function. Additionally, the nature of the EV membrane influences the EV's destiny and absorption by recipient cells. The

components of the EV lumen, which are composed of proteins and other nucleic acids, are substantially more diverse (Stahl and Raposo, 2019).

When observed under scanning electron microscopy, EVs are composed of lipid bilayers that primarily include sphingolipids, cholesterol, and ceramide components and have a round or cup-shaped morphology. EVs are characterized by a typical protein payload including integrins, tetraspanins (CD9, CD63, CD81), the cytoplasmic heat shock protein HSP70, as well as GAPDH, Tsg101, and Alix (Keerthikumar et al., 2016). These molecules are typically used as EV detection markers. Furthermore, the surface of EVs may contain major histocompatibility complexes (MHC) such as MHC-I and MHC-II, as well as adhesion molecules. These molecules define the characteristic composition of EV populations.

Along with mRNA and miRNA, EVs include non-coding RNAs, circular RNAs, and double stranded DNA fragments (Jimenez et al., 2019). However, investigations into EV secretion and its contents have revealed that assembling components into a formed particle is a deliberate process rather than a random arrangement of accessible molecules in the secreting cell. A similar process regulates the adoption of EVs. Numerous studies on the binding and internalization of EVs by recipient cells have been conducted, and a range of different components, including tetraspanins, integrins, lipids, and lectins, have been identified to be involved (Bebelman et al., 2018; Jimenez et al., 2019). Membrane fusion with the host cell or other endocytic routes may bring EVs into touch with the cells from whence they are derived. A comprehensive explanation of the EV biogenesis, release, and targeting processes was just published (Szatanek et al., 2017).

Extracellular vesicles biogenesis

The biogenesis of EVs and its release are controlled by endocytosis proteins and lipids. This process starts with the budding from the early endosome membrane of the multivesicular bodies (MVBs). MVBs after the maturation process entering in the lysosomal degradation pathway. The MVBs enter the endosomal recycling system, where MVBs can fuse with the cell membrane to release the exosomes in the extracellular space (Colombo et al., 2014).

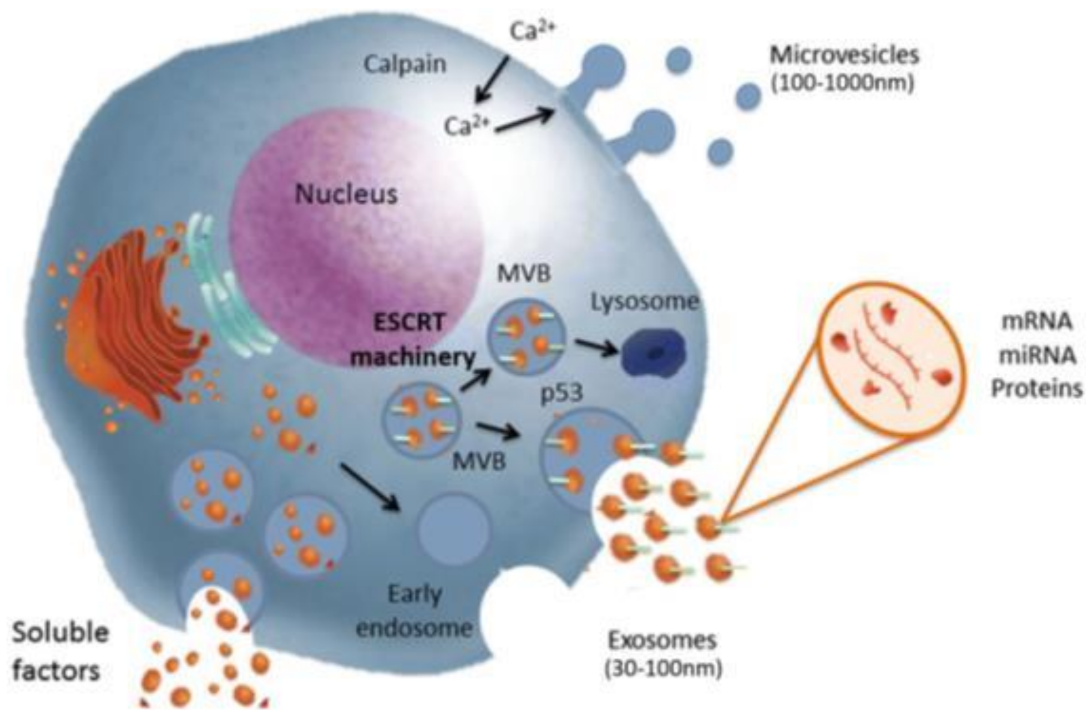


Figure 1. Schematic representation of extracellular vesicles biogenesis. Microvesicles bud directly from the plasma membrane, whereas exosomes originate from intraluminal vesicles that are generated by inward budding of the limiting membrane of a subgroup of late endosomes called multivesicular bodies (MVBs). MVBs can be directed towards the cell periphery and, after fusion with the plasma membrane, release their content (miRNA, microRNA, and proteins) into the extracellular space (adapted from Abreu et al. 2016).

The process used to produce the EV affects whether it is classified as a microvesicle or an exosome (Chivero et al., 2021). Exosome and microvesicle biogenesis may share properties based

on the source of the cells. Exosome and microvesicle biogenesis are mutually exclusive in terms of processes of biogenesis and molecular components at the endosomal membrane and plasma membrane, respectively. The processes behind the biogenesis of EVs have just recently begun to be unraveled. It looks as if EV release necessitates changes to the tumor microenvironment.

Changes in ionic homeostasis have been linked to EV biogenesis. When intracellular Ca^{2+} levels increase, phospholipid asymmetries in the plasma membrane collapse, cytoskeletal anchors in the plasma membrane disintegrate, and EVs are discharged (Bebelman et al., 2018).

EV shedding, invasion and metastasis may be enhanced in the tumor microenvironment by acidity. Cancer cells produce EVs in response to the destruction of the extracellular matrix (ECM) and the release of bioactive peptides, such as those produced from elastin (Chivero et al., 2021). Several mechanisms have been implicated in EV biogenesis, including the ESCRT-dependent mechanism, the involvement of asymmetric lipid required for the formation and release of budding, the tetraspanin-dependent system needed for exosome cargo selection, and the syndecan and syntenin pathways for budding (Bebelman et al., 2018). Rab GTPases and SNARE have a role in the trafficking of the EVs, exosome release, and plasma membrane fusion. Cytoskeletal components are required for EV budding and release. A reorganization of the actomyosin cytoskeleton will facilitate the release of microvesicles and, maybe, exosomes at the plasma membrane (Verderio et al., 2018).

Extracellular vesicle secretion and release

Disruption of the cell membrane or interaction with lysosomal MVBs may result in the release of exosomes. Their passage through cells and towards the cell membrane is regulated by many

proteins and depends on interactions with the cytoskeleton's actin and microtubules (Mir and Goettsch, 2020). Although the Rab protein GTPase family is critical in this situation, it seems to have a cell-specific function. For example, Rab27a and Rab27b have been implicated in the release of exosomes from HeLa cells, with Rab27b driving MVB motility toward the cell membrane and Rab27a promoting MVB fusion (Catalano and O'Driscoll, 2020). On the other hand, Rab27 isoforms are rare. Rab11 is expressed in K562 cells (bone marrow chronic myelogenous leukemia cells) and Rab35 is expressed in Oli-neu cells to illustrate that Rab proteins are used to regulate exosome release in distinct cell types (Juan and Fürthauer, 2018).

Additionally, proteins belonging to the SNARE (Soluble NSF Attachment Protein Receptor) family play a role in orchestrating the final exosome release after membrane fusion. This is accomplished by generating a SNARE complex composed of three-fourths of proteins with coiled-coil helices. Regulating exosome release is feasible via altering the connection between SNARE proteins and their phosphorylation partners (Catalano and O'Driscoll, 2020). Numerous *in vitro* research, including cell lines and a limited number of preclinical *in vivo* studies, indicate that a range of drugs may prevent or significantly decrease exosome and/or microvesicle formation and release. Only a few examples are imatinib (NSC23766), dimethyl amiloride, glibenclamide, pantethine, and imipramine (GW4869), bisyndoylmaleimide I (U0126), manumycin A, calpeptin (Y27632), and sulfisoxazole (Chivero et al., 2021). Extensive research is now required to evaluate the impacts of these drugs — both alone and in combination – in a far broader range of cell lines and preclinical *in vivo* models (Catalano and O'Driscoll, 2020).

Cellular Protrusions

Tumor invasion and metastases are facilitated by cell migration. It is also required for embryogenic morphogenesis, immunological surveillance, tissue repair, and regeneration. Filopodia, lamellipodia, podosomes, and invadopodia are all terms that refer to protruding structures formed by migrating and invading cells (Yang et al., 2020). These structures are generated via spatially and temporally regulated actin polymerization at the leading edge of migrating cells. Cellular protrusions have been shown to communicate between cells. These protrusions are seen in cytotonemes, tunneling nanotubes (TNTs), and microtubule-based nanotubes (MT nanotubes) (Yang et al., 2018).

These protrusions may be identified by their width and length and the cytoskeletal components that contribute to their formation, most notably actin filaments or microtubules (Figure 2). Rather than simply 'broadcasting' signals from a source cell to many cells, cellular protrusions enable selective signal transmission from a source cell to target cells across a wide range of distances (Miao et al., 2019). For instance, protrusions might occur between neighboring cells immediately near or between far adjacent cells. In both cases, specificity precludes other cells from interacting (Miao et al., 2019).

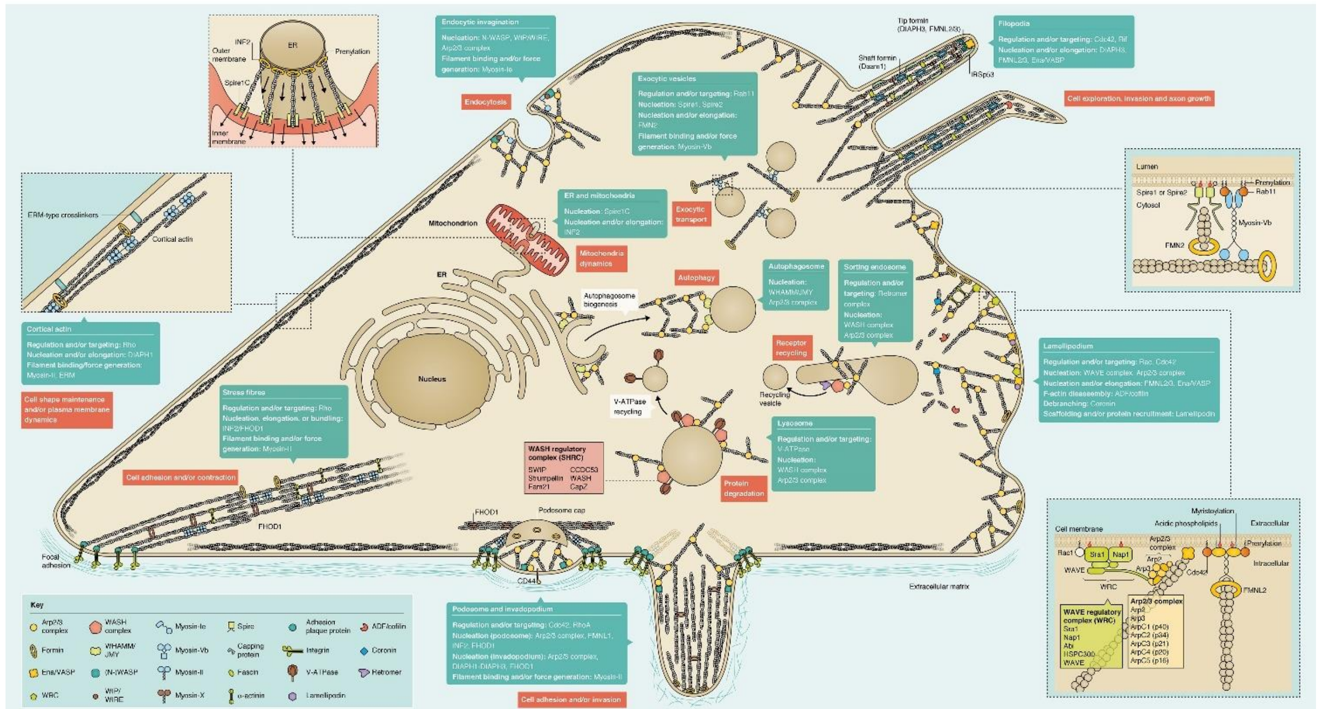


Figure 2. Illustration of filopodia, lamellipodia, and podosome. Filopodia may arise from lamellipodia or other areas of the cell and contain actin filaments organized as bundles. Lamellipodia is rather flattened cell expansions formed by actin filament polymerization. A podosome is a focal point of cells attached to the extracellular matrix through integrins, but it can also release metalloproteinases for extracellular matrix degradation. It contains a central branched scaffold of actin filaments and a ring of non-branched actin filaments (Klemesn, et al., 2017).

Filopodia

Filopodia are dynamic, finger-like cell surface protrusions present in most cell types, including microspikes within lamellipodia (Mattila and Lappalainen, 2008; Small et al., 2002). They play roles in neuronal growth cone navigation, epithelial morphogenesis, and promoting 3D migration and cancer cell invasion (Millard and Martin, 2008; Viveiros et al., 2011; Jacquemet et al., 2015). Canonical filopodia, typically a few micrometres wide and up to 10 µm long, consist of actin filaments compacted by cross-linkers like fascin and Daam1 (Mellor, 2010; Hoffmann et al., 2014; Jaiswal et al., 2013). Despite the accumulation of proteins such as VASP and myosin-X at

their distal tips, the precise molecular mechanisms governing filopodial actin assembly remain unclear (Mattila and Lappalainen, 2008). Filopodia grow by actin incorporation at their tips and shrink through rearward filament pulling or cofilin-mediated disassembly (Mallavarapu and Mitchison, 1999; Breitsprecher et al., 2011). Two main models explain filopodia formation: the 'convergent elongation' model, where actin filaments arise from the Arp2/3 complex-nucleated network, and the 'de novo nucleation' model, which involves formins like mDia2 and FMNL proteins (Korobova and Svitkina, 2008; Svitkina et al., 2003; Block et al., 2008; Gauvin et al., 2015). Filopodia formation can occur independently of the Arp2/3 complex, as seen in various cell types (Gomez et al., 2007; Nicholson-Dykstra and Higgs, 2008). Further research is needed to definitively identify the nucleators required for filopodia formation in mammals (Young et al., 2015).

Lamellipodia

Lamellipodia are prominent cellular protrusions composed of intricate networks of actin filaments, which are typically formed by various migrating cells on flat and rigid substrates (Small et al., 2002). When lamellipodia are elevated and retracted onto the cell surface, they are referred to as membrane ruffles, and are thought to contain actin assembly machinery similar to that involved in micropinocytosis and phagocytosis. Notably, some bacterial pathogens can induce structures resembling ruffles, facilitating their entry into non-phagocytic cells, which allows them to spread within tissues and evade the immune system.

Lamellipodia are characterized by branched actin networks that rely on the activity of the Arp2/3 complex (Suraneni et al., 2012; Svitkina & Borisy, 1999; Wu et al., 2012), which is activated

downstream of the Rac subfamily of small GTPases (Ridley et al., 1992; Steffen et al., 2013). The activation of the Arp2/3 complex and subsequent formation of lamellipodia is driven by Rac's direct interaction with the WAVE regulatory complex (WRC) via its Sra-1 (also known as CYFIP1) subunit or its isogene PIR121 (CYFIP2) (Innocenti et al., 2004; Leithner et al., 2016; Steffen et al., 2004), facilitating lamellipodia formation (Steffen et al., 2014; Stradal & Scita, 2006).

Other critical components in the formation of lamellipodia include the heterodimeric capping protein, which accumulates near the lamellipodial edges (Iwasa & Mullins, 2007; Lai et al., 2008; Mejillano et al., 2004; Svitkina et al., 2003), and members of the ADF/cofilin family of actin disassembly factors that sustain cellular actin filament turnover, essential for protrusion (Hotulainen et al., 2005; Kanellos & Frame, 2016). The maintenance of actin filament turnover is vital for all actin structures, regardless of the nucleation process employed, albeit to varying extents.

Additional regulators of lamellipodia include Arpin, an inhibitor of the Arp2/3 complex that plays a role in guiding Arp2/3-dependent protrusion and migration (Dang et al., 2013), and lamellipodin, an interactor of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of actin polymerases, which may also link Rac with the WRC (Krause & Gautreau, 2014; Law et al., 2013). Furthermore, actin turnover in lamellipodia is modulated by cortactin, a well-known lamellipodial marker (Lai et al., 2009), and coronin family proteins, which are believed to regulate the destabilization of Arp2/3-dependent branches (Cai et al., 2008). Finally, the generation of lamellipodial actin is enhanced downstream of Cdc42 by the formins FMNL2 and FMNL3 (Block et al., 2012).

Podosomes

Podosomes are actin-rich, sticky structures seen on the ventral surface of monocytic myeloid cells, activated endothelium cells, and cancer cells treated with Src. These structures are not confined to the cell periphery but rather migrate in a polarized fashion toward the lamellipodium-labellum junction (Miao et al., 2019). A succession of distinct processes forms a podosome. As sticky structures, podosomes include many of the same proteins found in focal adhesions (FAs), including talin, vinculin, paxillin, and Src family members (Graf and Frey, 2017). Tks5 (tyrosine kinase substrate containing five SH3 domains) is an example of this since it is found in podosomes and invadopodia but not in FAs or other actin-based structures. Because WASP (Wiskott Aldrich Syndrome protein) is present only in podosomes, it is far more selective. Tks5 and WASP, in combination with actin, cortactin, and the Arp2/3 complex, may therefore be used as markers for differentiating these protrusions (Dufrançais et al., 2021).

Although podosomes were first found as sticky structures in Rous sarcoma virus-transformed fibroblasts in the 1980s, they were later revealed to be naturally present in average, highly mobile myelomonocytic cells (Graf and Frey, 2017). According to the current understanding, podosomes are structures that arise in both normal and sick cells. Additionally, podosomes have been found in various cell types in response to cell stress or matrix receptor activation. They are tiny protrusions measuring around 0.4 μm in length and 1 μm in diameter. These cellular activities are identified by the production of unique F-actin-rich protrusions on the inner cell membrane of migratory cells at areas of cell-ECM contact (Miao et al., 2019). Columnar arrays of actin filaments surround a small tubular invagination of plasmalemma that runs almost parallel to the

substratum. Cells may pass through these surfaces due to their high adhesion levels. It is hypothesized that podosomes in expected cells aid cancer cell invasion in pathologic situations (Yang et al., 2020).

Tunneling Nanotubes

Tunneling nanotubes (TNT) are dynamic connections between cells that provide an entirely new intercellular communication mode. TNT are synthesized by various cell types, including cells, epithelial cells, and almost all immune cells. TNT promotes communication between myeloid cells (macrophages, dendritic cells, and osteoclasts), which is necessary for their proliferation and immunological activity (macrophages, dendritic cells, and osteoclasts) (Ljubojevic et al., 2021). The role of TNT in intercellular signaling, chemical exchanges, and organelle and pathogen activation is becoming increasingly apparent (Ljubojevic et al., 2021).

Notably, TNT enable myeloid cells to communicate with a range of different cell types and local or distant cells, resulting in a rich array of cellular connections. TNT also contribute to disease transmission by acting as a corridor between cells. Time-lapse microscopic examination of the cells showed two different pathways for creating TNT (Graf and Frey, 2017). Adjacent cells might initially split but stay connected by a thin membrane thread stretched after cell separation. Alternatively, cells would first expand and extend its filopodia until they encounter another cell, which would convert to TNT (Dufrançais et al., 2021).

While the former is more prevalent in lymphoid cells, the latter occurs in DC, where TNT are formed mainly by filopodia conversion. While macrophages may synthesize TNT through either

mechanism, the murine macrophage cell line synthesizes TNT primarily via actin-driven protrusions, also known as TNT precursors. It is critical to remember that these two processes are not mutually exclusive and may occur simultaneously in any two cells (Ljubojevic et al., 2021). Latrunculin or cytochalasin D cannot inhibit TNT synthesis in the absence of F-actin. It cannot open the conduit or switch from closed to open-ended TNT. The generation of open-ended TNT is thought to include a mechanism analogous to virus-to-cell membrane fusion or cell-to-cell fusion, which results in the formation of multinucleated giant cells (MGC) (Yang et al., 2020).

Extracellular vesicles transported by protrusions

The formation of invaginated membrane ruffles and their subsequent ejection into the intracellular space is referred as micropinocytosis. Protrusion of ruffled extensions of the plasma membrane enables molecules or EVs to be caught and internalized after fusing with the plasma membrane or the protrusions. Macropinocytosis is Na^+/H^+ exchanger-dependent and requires cholesterol to recruit activated Rac1 GTPase to invagination sites for actin cytoskeleton remodeling (Dufrançais et al., 2021). Researchers significantly reduced oligodendrocyte-derived EV absorption in microglia cells by blocking the Na^+/H^+ exchanger and rac1, highlighting the importance of this pathway for EV internalization. Exosomes are minuscule extracellular vesicles (EVs) generated by the endosomal route via multivesicular bodies (MVBs) (Bhat et al., 2020).

Additionally, vesicles, like retroviruses, may bud off the plasma membrane, resulting in EVs with sizes ranging from 200 to 500 nm (Dufrançais et al., 2021). These ejected vesicles are called microvesicles or exosomes. Smaller vesicles have been seen to bud from the plasma membrane

and may be retrieved in association with exosomes. Another method of release is through EV formation at the terminals of microvillar-like protrusions (Ljubojevic et al., 2021), which may be amplified by increased cellular hyaluronan content. Even larger EVs, dubbed gigantic endosomes, may bleb off the cell membrane in cancer cells. Additionally, when cells die, they generate membrane-bound apoptotic bodies that resemble other types of EVs but contain a greater quantity of genomic DNA (Yang et al., 2020).

TNTs between astrocytes and glioma cells allow oncogenic material to be transferred and alter glioma cell proliferation (Zhang and Zhang, 2015). Surprisingly, there is a dose-dependent positive correlation between TNT formation and EV release in glioblastoma cells and cocaine (Carone et al., 2015). This suggests that TNT and EVs work together to promote intercellular communication and glial-neuronal plasticity and that they may play a role in the processes associated with cocaine addiction. TNTs have recently been reported to be capable of transporting micro-sized particles produced by cancer cells in response to radiotherapy (Ware et al., 2015). TNTs may be used by cancer cells to develop resistance to therapies by transferring P-glycoprotein and mitochondria (Pasquier et al., 2012; Pasquier et al., 2013).

Tumor microenvironment in chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a type of cancer that affects mature B lymphocytes, which survive and expand in large part because of their interactions with the surrounding tissue (von Heydebrand et al., 2021). Numerous cell types, including monocyte-derived nurse-like cells, mesenchymal stromal cells, T cells, and natural killer cells, interact with CLL cells through a complex network of adhesion molecules, chemokine receptors, tumor necrosis factor family

members, and other soluble factors (van Attekum et al., 2017). Autonomous mechanisms or antigens activate the B cell receptor (BCR) and its downstream signaling cascade in secondary lymphatic tissues, hence playing a critical CLL pathogenetic role. Small molecule inhibitors of BCR signaling such as ibrutinib, which inhibits Bruton's tyrosine kinase (BTK), and idelalisib, which inhibits phosphoinositide-3-kinase delta (PI3K), have proven the most successful new therapy for this illness (Svanberg et al., 2021).

The interactions between CLL cells and the tissues in which they reside are critical to the disease's pathogenesis (Fierle, 2020). CLL cells multiply at a daily rate of 1–2% of the total clone, as determined by deuterated water labeling, at diverse tissue sites known as pseudofollicles between the peripheral blood and secondary lymphoid organs. The complex interaction between chemokines generated by stromal cells inside the tissues, which attract and hold CLL cells through chemokine receptors, adhesion molecules on the leukemia cells, and suitable tissue ligands, enables homing to tissues (Gonder et al., 2020). Numerous cellular components of the CLL microenvironment and signaling pathways involved in CLL survival, homing, and proliferation have been identified throughout the years, offering a paradigm for addressing the CLL microenvironment (Svanberg et al., 2021).

Stromal cells tumor support

B lymphocyte growth in CLL (B-CLL) is highly dependent on a complex and dynamic tumor microenvironment that is specifically primed by diverse, bidirectional interactions to promote leukemic homing, proliferation, and progression. Extracellular vesicles (EVs) might functionally

substitute for tumor-supporter cell contact. EVs are small membrane-enclosed particles that are released by practically all cell types and contain traits (characteristic proteins, nucleic acids) of the releasing cells (Coccuti et al., 2009; Théry et al., 2009).

The cells in the bone marrow niche, act as feeder cells for normal hematopoietic progenitor cells. Secondary lymphatic tissues in individuals with CLL often include mesenchymal stromal cells, which act as survival and migration signals for CLL cells (van Attekum et al., 2017). When CLL cells encounter bone marrow stromal cells (BMSCs), they may promote differentiation in the normal structure of bone marrow. Stromal cells continuously produce chemokines, which impact CLL cell movement and tissue localization and provide additional signals that promote CLL survival and resistance to treatment. While BMSCs suppress C-X-C motif receptor 4 expressions (CXCR4), they promote the development of aggressive disease indicators such as ZAP70 and CD38 in CLL cells (Svanberg et al., 2021).

When BMSCs reduce CD20 expression on the surface of CLL cells, anti-CD20 antibody treatment seems to be unsuccessful (von Heydebrand et al., 2021). Additionally, stromal cells increase glutathione synthesis and glycolysis in CLL cells through NOTCH-mediated c-MYC activation, facilitating the survival and resistance of the cells to treatment. The interaction of stromal cells with CLL cells in the bone marrow increases PKCII expression and activation of the NF-κB pathway in stromal cells (Gonder et al., 2020). This benefits the CLL cells and stimulates the stromal cells simultaneously. Additionally, CLL cells may create microvesicles containing active signaling proteins that might activate the Akt pathway in BMSCs, highlighting the critical importance of bidirectional contact between CLL cells and stromal cells (Fierle, 2020).

Extracellular vesicles in the chronic lymphocytic leukemia tumor microenvironment

Through the intercommunication of autocrine and paracrine mechanisms, EVs regulate many cellular functions, including cell proliferation, survival, and transformation (*Zhang et al., 2019*). Since their discovery, EVs have been shown to carry information between cells in the body. Surface-attached ligands and receptors on EVs makes it possible to connect selectively cells that express the required/corresponding ligands or receptors (van Attekum et al., 2017). EVs and their payload may be carried by several methods to destination cells. EVs may dock with the plasma membrane of a target cell, and linked EVs can integrate directly into the recipient cell plasma membrane. Phagocytosis, macropinocytosis, or endocytosis mediated by a lipid raft, clathrin, or caveolin may all be used to endocytose linked EVs (Gonder et al., 2020).

Following endocytosis, EVs may be guided to lysosomes and removed. Additionally, EVs may fuse with the membrane of an endocytic compartment, allowing the contents of the EV to be released into the cytoplasm of the recipient cells. EVs may affect the features and phenotypes of recipient cells in various ways. These include de novo translation, post-translational modification of target transcripts, and activation of several signaling cascades. It has been shown that EVs aid in embryonic development and growth and immunological avoidance in pregnant females. Bidirectional communication between the embryo and the uterine endometrium is required for the complete implantation of the embryo, and EVs have been shown to influence angiogenesis, tissue remodeling, and fetal development (Fierle, 2020).

Scr family kinases

The domain organization of members of the src family of kinases is conserved, with a myristoylated *N*-terminal segment followed by SH3, SH2, linker, and tyrosine kinase domains, and a short *C*-terminal tail (Figure 3). Src kinase structural dissection reveals canonical mechanisms of phosphotyrosine recognition by the SH2 domain and proline-motif recognition by the SH3 domain (Parsons & Parsons, 2004).

The capability of oncogenic forms of SFKs to induce cell transformation suggested an early role for Src and its family members in cell growth regulation. Bromann et al. provide an overview of how SFKs interact with and contribute to RTK signaling. These authors provide an overview of how RTKs activate SFKs and how SFKs activate RTKs. Furthermore, SFKs promote growth factor receptor signaling in a variety of ways, including direct participation in DNA synthesis pathways, controlling the turnover of cell surface receptors, modulating actin cytoskeleton rearrangements, and promoting cell motility and survival (Bromann et al., 2004; Parsons & Parsons, 2004).

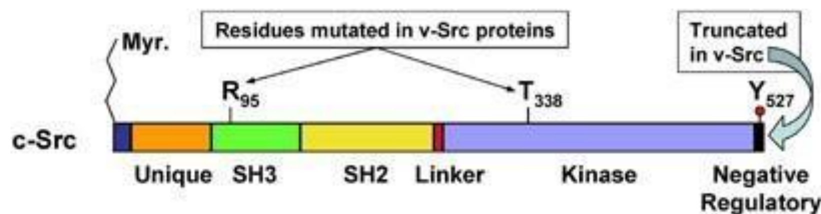


Figure 3. Domain structure of Src family kinases. Members of the family exhibit a conserved domain organization, which includes a myristoylated *N*-terminal segment, followed by SH3, SH2, linker, and tyrosine kinase domains, and a short *C*-terminal tail (Parsons & Parsons, 2004).

Lyn kinase

Lyn kinase, a member of the SFK has been reported as an important molecule for CLL progression (Nguyen et al., 2016). Lyn deficiency in a CLL mouse model resulted in a significant decrease in the CLL burden, the lack of Lyn impairs the BCR signaling pathway in leukemic cells, including BTK phosphorylation, but does not impair leukemic cell proliferation (Svanberg et al., 2021).

Additionally, in another study, primary human B-CLL cells were cocultured *in vitro* with Lyn-deficient mouse embryonic fibroblasts cell line (MEF) or Lyn ko human HS5 cells and compared with wt counterparts.

All Lyn deficient fibroblasts had a significantly lower feeding capacity for B-CLL cells than their wt counterparts, indicating the functional importance of Lyn in leukemia-associated fibroblasts. In addition, the transcriptomic, proteomic, and phosphoproteomic alterations associated with Lyn knockout (KO) in HS5 cells showed an extensive change in gene and protein expression patterns that appeared to be primarily regulated at the transcriptional level. The differentially expressed genes were frequently ECM, cytoskeleton, or cytokine-related (vom Stein et al., 2019). In this context, this thesis aimed to study the mechanism of the Src kinase Lyn in the EV-based communication between the malignant B-CLL and stromal cells.

Study Aims

This thesis aimed to study the mechanism of the Src kinase Lyn in the EV-based communication between the malignant B-CLL and stromal cells. Using the Lyn-proficient and deficient stromal cell lines (HS5) the following topics were investigated: Cellular protrusions, EV release, EV uptake, and HS5 EVs survival support capacity on primary B-CLL cells.

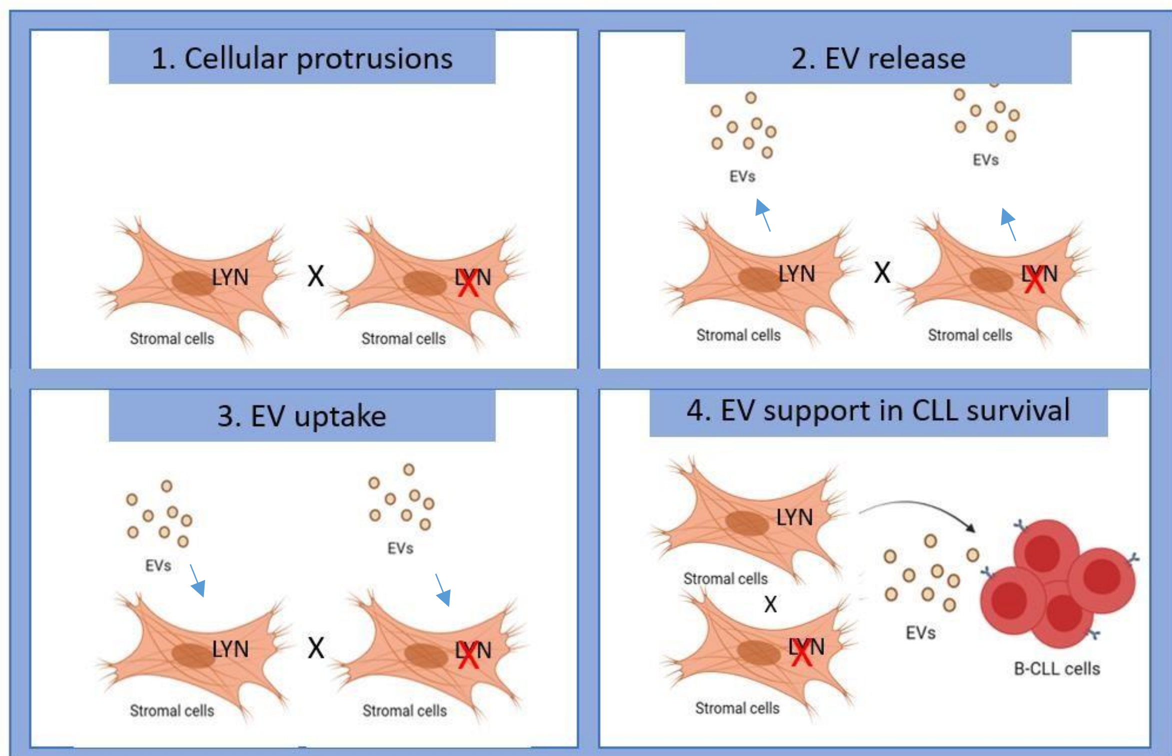


Figure 4. Illustration of the study aims. Lyn-proficient and Lyn-deficient stromal cells were used to study the role of the tyrosine kinase Lyn in the EV-based support of primary CLL cells. The following aspects were investigated in the present study: 1. cellular protrusions, 2. EV release, 3. EV uptake, and 4. EV support in CLL survival Illustration created with BioRender.com

Materials

Reagents

NAME	SUPPLIER
7AAD	Thermo Scientific
Annexin V	Thermo Scientific
Anti-Adherence Rinsing Solution	STEMCELL TECHNOLOGIES
Casy clean	OMNI Life Science
Casy ton	OMNI Life Science
Cellmask deep red	Thermo Scientific
Celltracker™ Dii (553/570 nm)	Thermo Scientific
Dimethyl sulfoxide (DMSO)	Thermo Scientific
Ethanol	Carl Roth
Fetal bovine serum	GIBCO
Flow cytometry running buffer	MACSQuant
Gene Knockout kit v2 – CD248	Synthego
Lipofectamine CRISPRMAX	Thermo Scientific
Nucblue live cell stain (Hoechst 33342)	Thermo Scientific
Penicillin	Thermo Scientific
Phosphate buffer saline	GIBCO
Plasmid CD63-eGFP	Kindly provided by AG Pogge, Marburg
Polybead carboxylate 4.5 micron	Polyciences
Roti® histofix 4%	Carl Roth
RIPA lysis buffer	Thermo Scientific
RPMI-1640 medium	GIBCO
Streptomycin	Thermo Scientific
Triton X-100 Octoxinol 9	Sigma-Aldrich
Trypan blue	Thermo Scientific

sgRNA sgRNA

	SUPPLIER
GUCGCCCCCCAGCUCGCGGC	Synthego
AAGAGAGCGUAGCAGCUGCU	Synthego
CGGCCGCCCAGGCCAGCAAC	Synthego

Devices

NAME	PRODUCER
Cell couter Casy Model TT 150 µm	Innovatis
Centrifuge 5415 R	Eppendorf AG
Centrifuge 5810 R	Eppendorf AG
Freezer -150°C Ultra-Low Temperature	Panasonic
Freezer -20°C Comfort NoFrost	Liebherr
Freezer -80°C MDF-U51868	Sanyo
Gallios Flow Cytometer	Beckman coulter
Heating block Thermomixer Comfort	Eppendorf AG
Heracell 150	Thermo Scientific
Laminar flow hood Mars Safe Class 2	SCANLAF
Leica TCS SP8 gSTED 3X	Leica
MACSQuant X	MACSQuant
Microscope TELAVAL 31	Zeiss
NanoDrop 1000 Spectrophotometer	Thermo Scientific
Nanoparticle Tracking Analysis (NTA)	Malvern Panalytical
Optima MAX-XP Ultracentrifuge	Beckman Coulter
Optima XPN-80 Ultracentrifuge	Beckman Coulter
Pipette Research plus	Eppendorf AG

Tools

NAME	PRODUCER
0.22µm Sterile Filter	Sartorius
96-well plate	Thermo Fisher
CASYcup	OMNI Life Science
Cell culture flask	Thermo Fisher
Falcon tubes	Thermo Fisher
Microscopy slides 76 x 26mm	Thermo Fisher
Polycarbonate Bottle Assembly 38x102 mm	Beckman Coulter GmbH
Polypropylene Eppendorf tubes 1.5ml	Beckman Coulter GmbH
qEVoriginal / 35nm Legacy	IZON Science

Softwares

NAME
FIJI
LAS X Leica

FlowJo™ v10.8
Nanosight 4.0
BioRender
GraphPad Prism v.8.0
R Studio

Biological samples and cell culture

B cells from CLL patients were obtained from CLL-Biobank of University Hospital of Cologne, after written informed consent. Stromal cells (HS5 and MEF WT and Lyn KO) were generated and kindly provided by Alexander vom Stein from our group using the CRISPR-Cas9 technique. WI38 cell line were kindly provided by AG Krönke, Cologne.

Methods

Extracellular vesicles isolation and characterization

To obtain extracellular vesicles, HS5 WT and HS5 Lyn KO cells were cultured in 80% confluency with serum deprivation for 16 hours to avoid contaminations by fetal bovine serum (FBS) derived vesicles. Serum deprivation over 16 h did not induce senescence and morphology modifications of HS5 cells. Cell-free supernatants were obtained by 2 successive centrifugations at 300 xg for 10 min, next ultracentrifuge at 10,000 xg to remove apoptotic bodies, and then the supernatant was centrifuged at 100,000 xg for 90 min at 4°C (Ultracentrifuge Beckman Coulter MAX-XP). Finally, the EV pellet was collected resuspended in 500 µl of PBS, and purified using a size exclusion column (qEVoriginal 35 nm, IZON Science). The EVs were collected in fractions 4-6. The amount and size of HS5 EV were determined using nano tracking analysis technology (Nanosight NS300, Malvern Panalytical), and the protein content was measured with UV-Vis Spectrophotometer (NanoDrop® ND-1000, Thermo Scientific) and BCA protein assay.

Extracellular vesicles uptake analysis

HS5 WT and HS5 Lyn KO derived EV were stained with DID (red) or CFSE (green) for 20 min at 37 °C. Next, washed twice with PBS by ultracentrifugation at 100.000 xg for 90 min at 4 °C. EV were incubated with the CLL B-cells for 24 h. After the incubation period, the cells were washed with PBS and analyzed by Flow cytometry. For ImageStream uptake analysis,

the cells were fixed with paraformaldehyde 1% for 20 min on ice, washed, and resuspended with FACS buffer.

Extracellular vesicles release analysis

The cells ($2x10^6$/mL) were incubated in a 6-well-plate with RPMI-1640 medium. After 24 h, the cells were washed 3 times with PBS and RPMI-1640 medium serum-free was added and incubated for 3 hours. The supernatant was collected, and the EV isolation method proceeds as mentioned above and analyzed by nano tracking analysis technology, 1 single measured was composed of 5 videos with 60 seconds each. (Nanosight NS300, Malvern Panalytical, UK).

Primary cells and extracellular vesicle co-culture

Primary B-CLL cells ($0.3x10^6$ cells/well) were cultured with or without 4 µg/mL of EVs in 96-well plates in 10% FBS RPMI-1640. Cell viability was determined by CellTiter-Glo 2.0 (Promega), according to the manufacturer´s protocol.

Primary cells and stromal cells co-culture

Primary B-CLL cells ($1.5x10^6$ cells/well) were cultured with or without HS5 WT or Lyn KO cells ($1x10^4$ cells/well) in 24-well plates in 10% FBS RPMI-1640. Cell viability was determined by CellTiter-Glo 2.0 (Promega), according to the manufacturer´s protocol.

Protrusions analysis with confocal microscopy

HS5 cells were previously transfected with CD63-eGFP plasmid for EV tracking. HS5, CLL cells, or co-culture, were seeded (1x106 cells/ml) in a 6-well-plate with a coverslip for 24 h. Next, the cells were fixed with paraformaldehyde 4% and permeabilized with triton x-100 0,01% for 15 min, followed by washing, nuclei, and actin stain steps. The images were acquired using SP8 Leica confocal microscopy with 63x magnification and analyzed using Fiji software. The Fiji plugin Poji was used to measure podosomes (Herzog et al., 2020) and FiloQuant to measure the filopodia (Jacquemet et al., 2015).

Generation of HS5 CD248 knockout cells

HS5 CD248 KO cells were generated using the Gene Knockout kit v2 – CD248 (Synthego) according to the producer´s protocol. Briefly, HS5 cells ($5x10^4$ cells/mL) were seeded and incubate in incubated in a 2x 24-well-plate with RPMI-1640 medium serum-free for 24 h. Next, a ribonucleoprotein (RNP) complex solution (1.3:1 sgRNA to Cas9 ratio) or control (Cas 9 only) were mixed with a transfected solution (Lipofectamine CRISPRMAX transfection reagent and Opti-MEM reduced serum medium) and incubate for 10 minutes at room temperature. Then, the cells were treated with this RNP-transfection solution or control and incubated at humidified 37 °C, 5% of CO_2 for 3 days with medium replacement after 24 h. After 3 days, the cells were split and grown into single cells clones and maintained for several weeks for further selection, which was later validated with western blot.

Western Blot

Lysates were made in RIPA lysis buffer (0.607 g Tris, 0.876 g NaCl, 0.1 g SDS, 0.5 g sodium deoxycholate, 1 ml Triton X-100 in 49 ml H_20). A protein inhibitor cocktail mix (Sigma Aldrich, cat# P2714) was added to prevent protein degradation. Before gel loading, lysate concentrations were established via Bradford assay (Sigma). 10 µg of HS5 lysates were loaded per condition and separated by SDS-PAGE on 12% acrylamide gels under reducing conditions. Proteins were transferred to a PVDF membrane (Merck Millipore). Membranes were blocked with bovine serum albumin (5% w/v;
1 h; room temperature) in Tris-buffered saline containing 0.1% Tween-20 (TBST), after which membranes were incubated with the desired primary antibodies at 4 °C for overnight incubation, for example: β-actin (Santa Cruz). After washing with TBST and subsequent milk block (5% w/v; 1 h), membranes were incubated for 2 h at room temperature with anti-rabbit IRDye 800CW, or anti-mouse IRDye 800CW (both LI-COR, 1:10,000 and 1:10,000 respectively). Images were collected using a scanner imaging system (Odyssey CLx LI-COR).

Statical analysis

The t-test (non-parametric) was used to analyze the statistical significance of experimental results, p-value (p) <0.05 was considered statistically significant. Using the following legend: nonsignificant (ns) = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$. Data were analyzed and graphics were constructed using GraphPad Prism v.8.4 (GraphPad Software, San Diego, CA, USA).

Results

HS5 WT and Lyn KO cell characterization

The Scr kinase Lyn plays an important role in the tumor-supporting survival signaling within the tumor microenvironment of the CLL (Nguyen et al., 2016). It is expressed in both, the tumor, and the bystander cells. The mechanism of how Lyn facilitates this support is not entirely understood. In addition to its central role in triggering downstream kinases, it is also participated in the generation of protrusions by indirectly stimulating actin nucleation. Protrusions, such as filopodia or podosomes reach out to sense the environment, they guide EVs to the target or are themselves a source of EVs by pinching the protrusion. To unravel the role of Lyn in EV-based communication we used the human stromal cell line HS5. A Lyn-deficient clone, generated by CRISPR-Cas9 supported primary CLL cells significantly less than the wild-type counterpart. We initially compared the viability of the cell types by measuring the metabolic activity in a kinetic study. As shown in Figure 5, both cell types showed no significant difference in cell growth, indicating that the lack of Lyn did not significantly influence the viability/proliferation of the sister cell lines, but significantly higher cell adhesion to culture plates is shown on KO cells as compared to the WT cells.

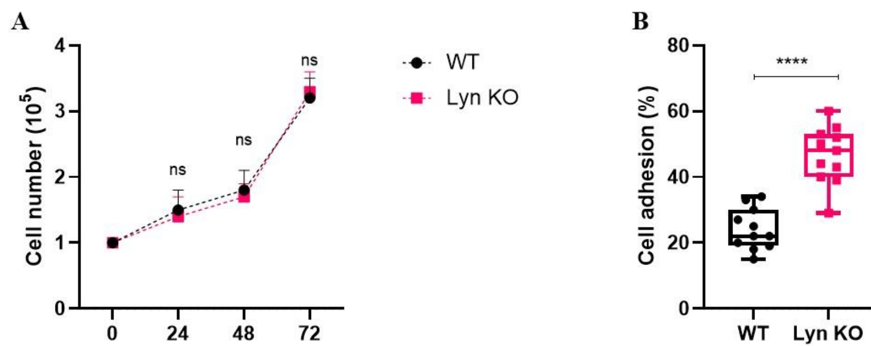


Figure 5. HS5 WT and Lyn KO cell characterization. (**A**) Cell proliferation rate between WT and Lyn KO HS cells was compared by measuring the cell count after indicated time points. The graph shows no difference in the growth rate between the cell types (p-value ns). (**B**) The percentage of adherent cell was calculated after 24 h of incubation in 11 different experiments.

To observe the effect of Lyn kinase-on HS5 cells protrusions, the podosome and filopodia count as well as the filopodia length were analyzed. Lyn-deficient HS5 cells showed a decrease in filopodia count but a higher length in comparison to WT cells. Surprisingly, the podosome count significantly increased in cells with a Lyn defect (Figure 6). The results might explain the better adhesion of cell with defective Lyn.

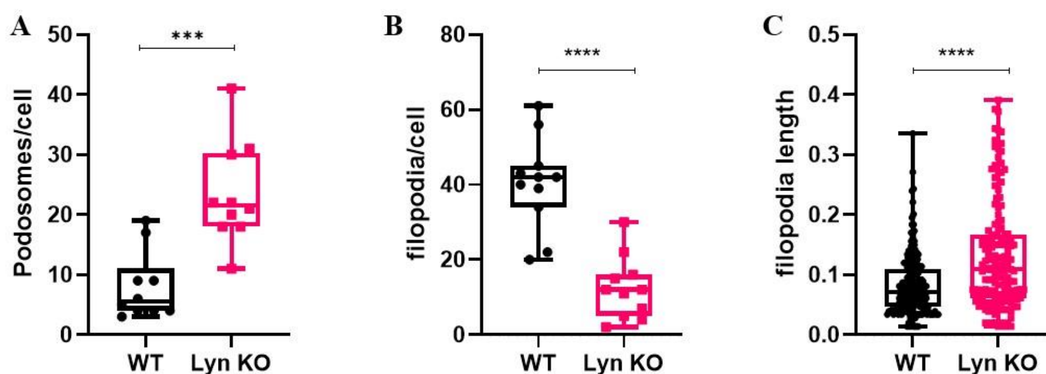


Figure 6. Cell morphology comparison. (**A** and **B**) Filopodia and podosome count was determined in Lyn KO (red) and WT HS5 cells (black). The results show the means of 11 independent measurements. (**C**) The filopodia length was also determined in both cell types. The results show the mean length of 100 – 300 determinations.

Extracellular vesicles release

HS5 cells EV release was tested in normal conditions (adherent) and in suspension to investigate the role of cell adhesion in the EV release. As the HS5 cells normally tend to attach to the cell culture plates, the plates were pretreated with Anti-Adherence Rinsing Solution (STEMCELL TECHNOLOGIES). Next, the plates were washed with PBS, and then cells were seeded and incubated with a serum-free medium for 24 h. Cell viability remained comparable with the control (Figure 7, right side diagrams), but EV release was about 60% higher in the suspension cell culture than in the adherent control group (Figure 7, left side).

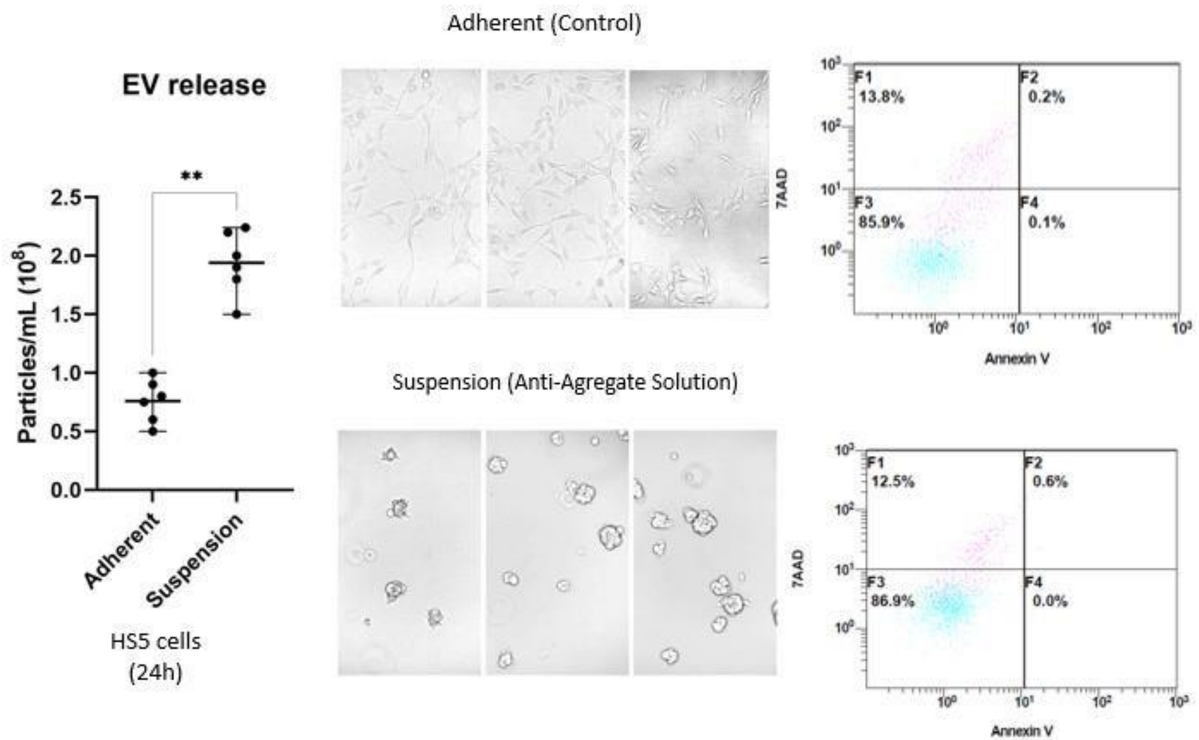


Figure 7. EV release of HS5 cells upon adherent or suspension conditions. Suspension cells show about 50% higher EV release in comparison to the same cell line in adherent conditions.

Next, the Lyn kinase influence on the EV release in HS5 cells was investigated (Figure 8A). Comparing the Lyn WT and Lyn KO HS5 cells, we observed that the EV release is 66% reduced in the Lyn KO cells. To confirm the effect caused by Lyn deficiency, mouse endothelial fibroblasts (MEF) were tested as well. Also here, Lyn KO cells released about 70% less EVs than the WT counterparts (Figure 8B). This strong reduction in Lyn KO cells, suggests that the Lyn kinase pathway influences EV release.

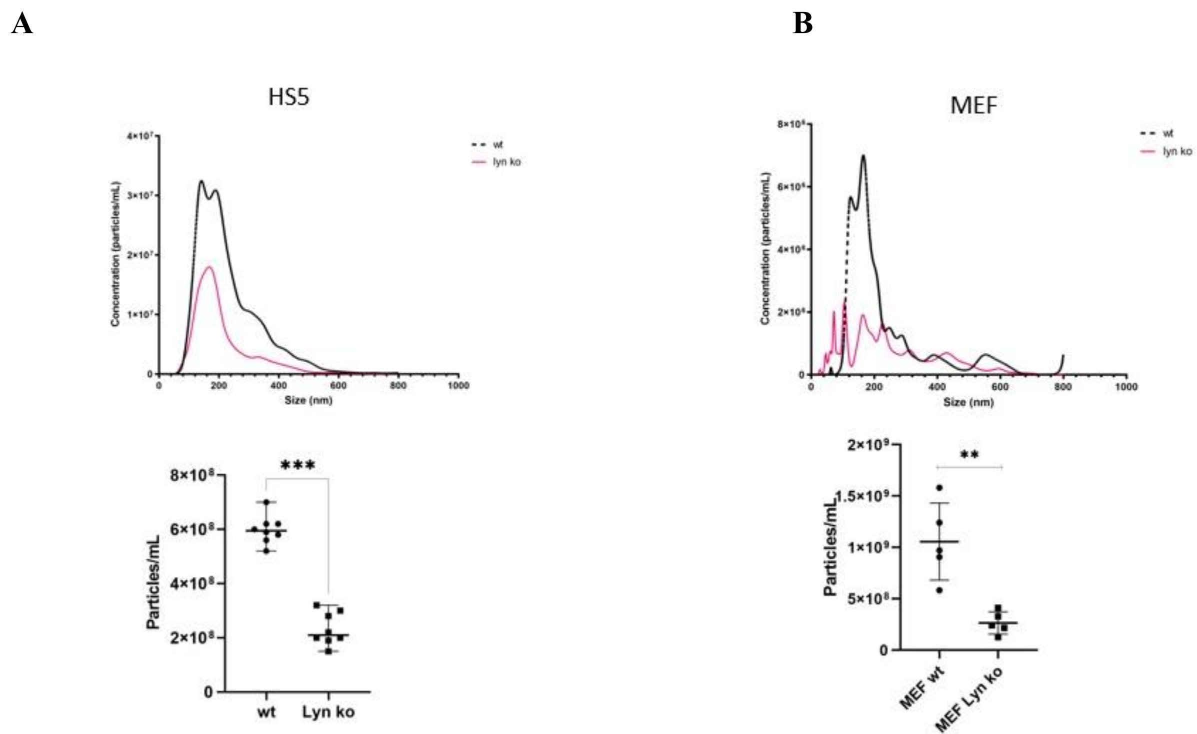


Figure 8. EV release counting. Nanoparticle tracking analysis of (**A**) EVs from HS5 cells, and (**B**) MEF cells (WT and Lyn KO). The KO cells present a strongly reduced EV release in comparison to the WT counterparts.

To confirm the presence of EVs in the preparations, we used TEM to visualize the vesicles. Membranes were stained with uranyl acetate. They then appear dark in TEM. The middle part of

the vesicle also shows a dark stain because the membranes collapse due to dehydration, resulting in the classical cup-shaped shown in Figure 9. The EV diameter ranges between approximately 80 and 200 nm, the typical diameter of EVs.

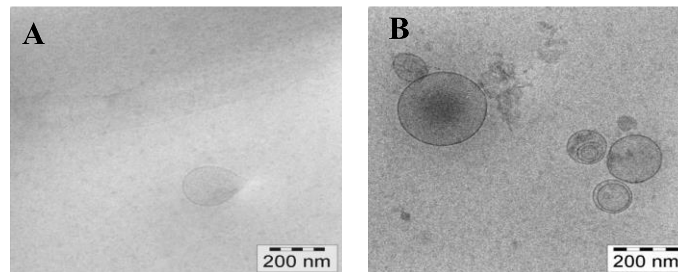


Figure 9. TEM images of EVs. (A) HS5 WT EVs. (B) HS5 Lyn ko EVs (Reduced to 10 pixel)

Extracellular vesicles release upon Dasatinib treatment

To further understand that Lyn kinase can be involved with the EV release. We investigated the HS5 and WI38 (human lung fibroblast cell line) cells upon Dasatinib treatment, a Src kinase inhibitor, including Lyn, in different concentrations (1, 3, 10, 30, and 100 nM) for 24 h serum-free, then the EV were collected by ultracentrifugation and size exclusion chromatography and analyzed by NTA.

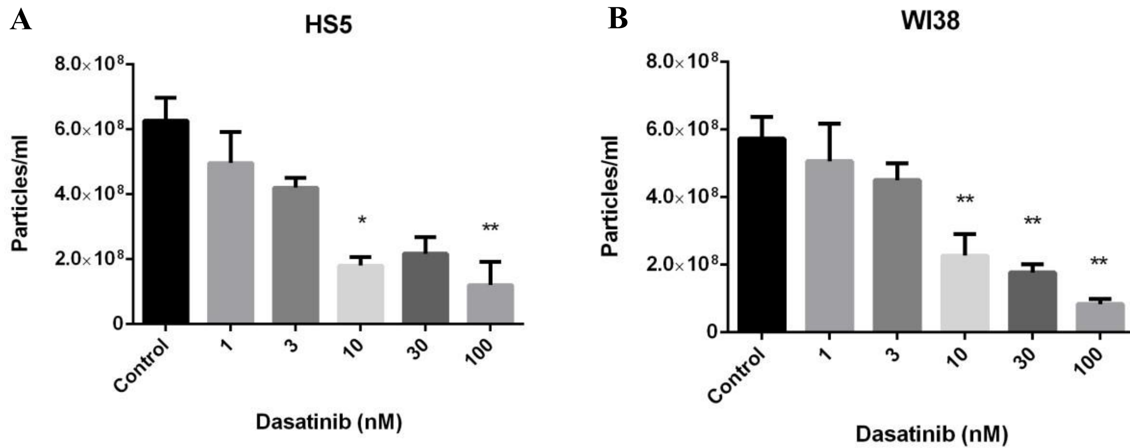


Figure 10. EV release upon dasatinib treatment. (**A**) HS5 and (**B**)WI38 cells were treated with dasatinib in different concentrations (1, 3, 10, 30, and 100 nM) for 24 h serum-free, then the EVs were collected by ultracentrifugation and counted by NTA. HS5 cells treated show a reduction in EV release with 10 and 100 nM and WI38 cells with 10, 30, and 100nM in comparison to the non-treated cells (control).

As shown in Figure 10, treated HS5 cells show a reduction in the EV release already with a low concentration (1 nM) of Dasatinib when compared with the non-treated control. The reduction of the EV release was more prominent with higher concentrations of Dasatinib. A similar reduction was also determined with WI38 cells, another stromal cell line, demonstrating that similar to a KO of Lyn kinase, the pharmacological inhibition of tyrosine kinases also inhibits the release of EVs.

Uptake of Extracellular vesicles

Next, we investigated the influence of the EV concentration on the EV uptake in HS5 cells (**Figure** 11). HS5 Lyn WT and KO were incubated with several concentrations (1, 2, 5, 10 mg/mL) of EVs isolated from Mec-1 (CLL) cells. EVs were stained with CFSE dye (green) to observe the fluorescence and analyzed by flow cytometry.

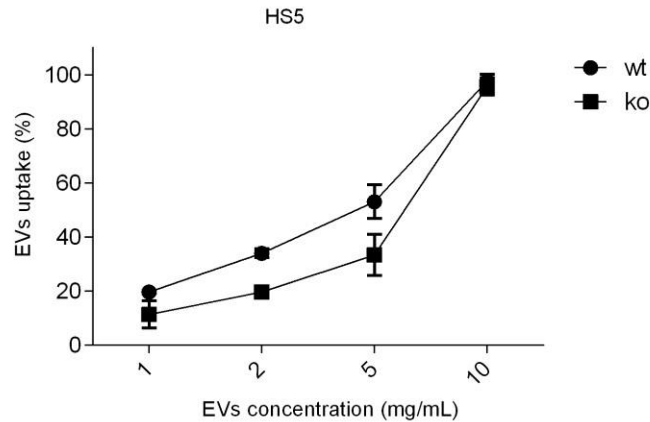
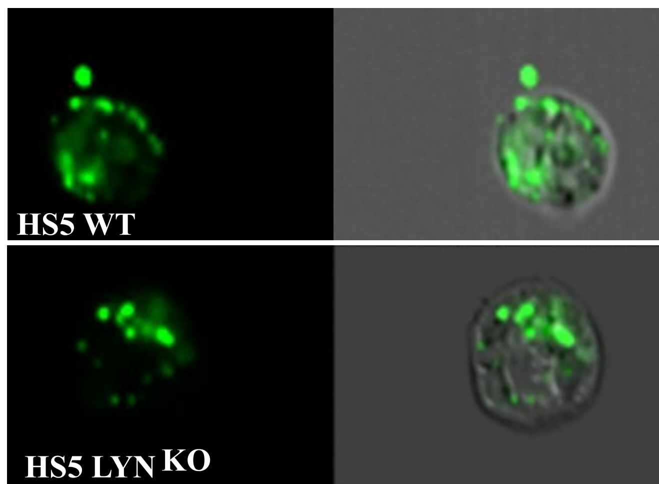


Figure 11. EV dose-dependent uptake. HS5 cells WT and Lyn KO were incubated with several concentrations (1, 2, 5, 10 mg/mL) of EV isolated from the Mec-1 (CLL) cell line. EV cellular uptake was analyzed by flow cytometry. HS5 Lyn KO demonstrates less uptake in the 2mg/mL and 5mg/mL concentrations compared to HS5 wt cells.

We observed that the EV uptake is dose-dependent in both cells (WT and KO) and with low EV concentration (1 mg/mL) and high EV concentration of 10mg/mL there is no difference in uptake of WT and KO cells, however, there is a difference with 2 and 5 mg/mL, which was confirmed with ImageStream analysis (Figure 12), with this technique is possible to differentiate the EVs bind on the surface from the EV that is inside the cells.

A



B

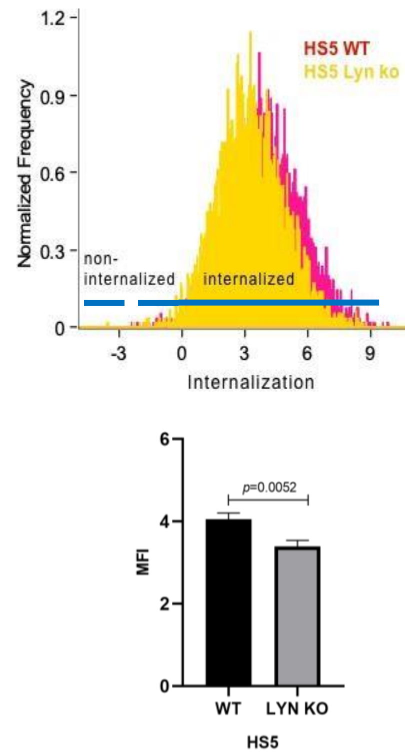
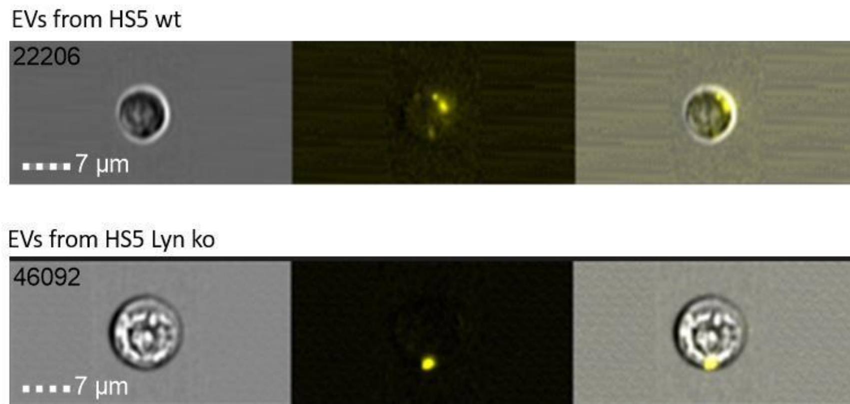


Figure 12. HS5 cells EV uptake. HS5 cells WT and Lyn KO were incubated with 2 mg/mL of EV isolated from the Mec-1 (CLL) cell line. The EVs were stained with CFSE dye (green) to detect EVs. (**A**) Sample pictures of HS5 cells WT and Lyn KO with EV from ImageStream analysis. (**B**) Graphics showing the median internalization rate of 3 individual measurements (10,000 images each). HS5 Lyn KO demonstrates 16% less uptake compared to HS5 WT cells.

Next, using the same technique (ImageStream) the B-CLL EV cellular uptake was also tested. In this case, the EVs were collected from HS5 cells (WT and Lyn KO) and incubated with primary B-CLL cells (3 patients). We observed cellular uptake in both cases, without difference (Figure 13).

A **B**



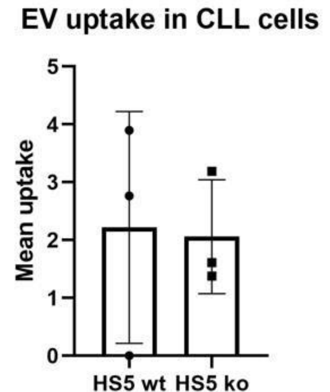


Figure 13. B-CLL cellular EV uptake. Primary B-CLL cells (3 patients) were incubated with 2 mg/mL of EV isolated from the HS5 cells (WT or Lyn KO), and the EVs were stained with CFSE dye (green) to observe the fluorescence EVs. (**A**) Sample picture of HS5 cells WT and Lyn KO with EV from ImageStream analysis. (**B**) Graphics showing the median internalization rate of 3 individual measurements (10,000 images each). The tested cells do not show a difference in the uptake of the EVs.

Extracellular vesicles influence on primary B-CLL cells

To test whether the functional effects of EVs are dose-dependent, B-CLL cells were incubated with an increasing amount of EVs derived from HS5 WT cells (µg/mL) for 24 h. The Figure 14 shows higher B-CLL cell survival with increasing EV concentration, therefore EVs effects are dose-dependent in this case. Further experiments were performed using 4 µg/mL of EV protein concentration, which showed a promising response.

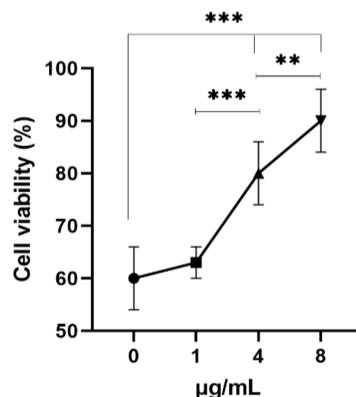


Figure 14. B-CLL cell EV survival support is dose-dependent.

To test whether EV functionally bind or are taken up by the target cell, we evaluated a series of microscopic images. Therefore, HS5 cells were transfected with CD63-eGFP for EV tracking since CD63 is considered as an EV marker. These cells were co-cultured with primary CLL cells and confocal microscopy was used to follow the location of EVs in the target cells. As shown in Figure 15, EVs were predominantly observed on protrusions of HS5 and B-CLL cells (Figure 15).

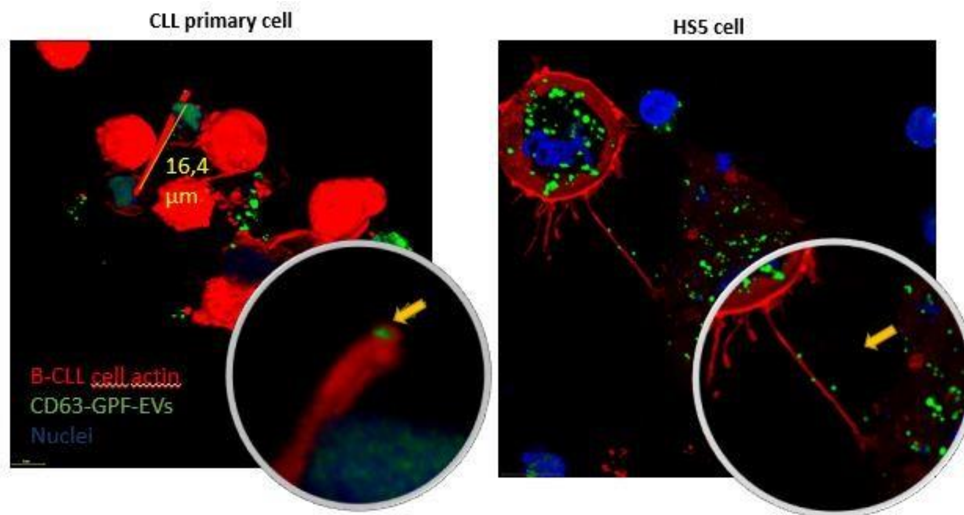


Figure 15. EVs were found on protrusions of HS5 and CLL cells.

EVs are considered as an important mechanism of bidirectional crosstalk between CLL tumor cells and non-malignant cells within the tumor microenvironment. They play a critical role in activating tumor survival and proliferation. Stromal cells release EV that carries molecules that can attract CLL B cells in the tumor microenvironment and protect them from spontaneous apoptosis (Crompot et al., 2017). Therefore, we isolated EV from HS5 WT and KO cells and performed coculture to analyze their influence on the survival of primary CLL cells. To be closer to the physiological condition we applied lower concentrations of EV (between 2 and 5 mg/mL) and

observed significant effects. We found that coculture with EV protected CLL cells from spontaneous apoptosis at all time points. However, the same amount of EV from WT cells protected effect is higher compared to the EV KO, suggesting a different composition of the EVs from the tested cells (Figure 16).

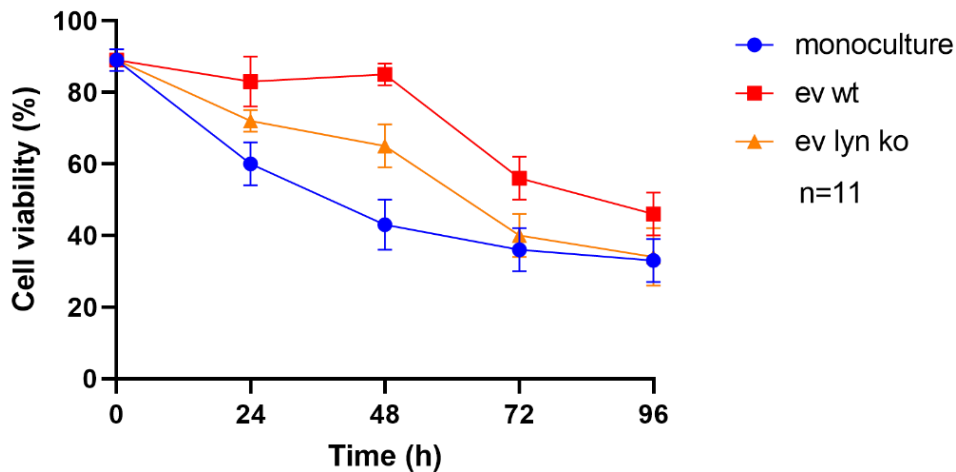
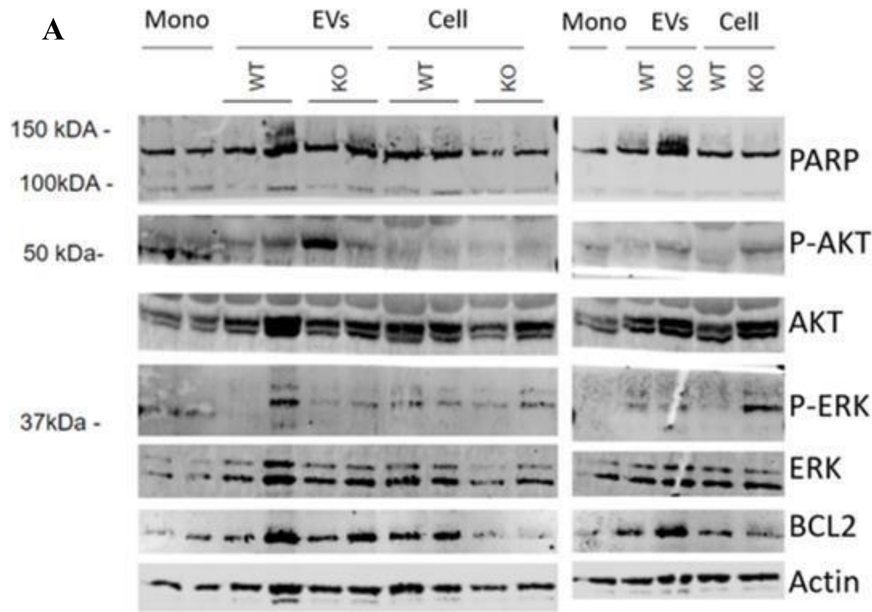
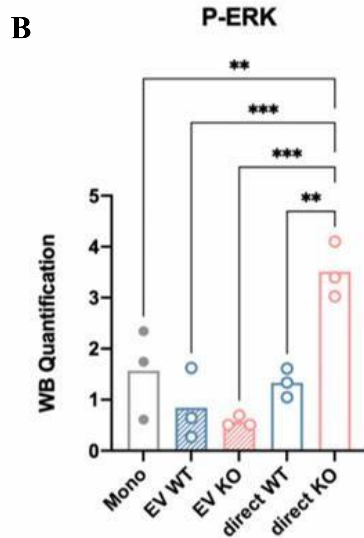


Figure 16. Co-culture of B-CLL cell and EVs from stromal cells. We tested the B-CLL cell viability in co-culture with EVs from HS5 (WT and Lyn KO). Both EVs were able to support B-CLL survival, but the EVs from Lyn KO cells shows reduced support in comparison to the WT counterparts.

Lysates from B-CLL cells (3 patients) after 48 h of co-culture with HS5 cells or EVs from HS5 cells (WT and Lyn KO) were tested by western blot with several markers (Figure 17A). P-ERK was a higher indirect co-culture between B-CLL cells and HS5 Lyn ko cells in comparison with the other groups (Figure 17B). In addition, although not significant in total, one patient has shown higher BCL2 signaling with the EV groups in comparison with direct contact and monoculture (Figure 17C). Therefore, more patients should be tested to observe whether BCL2 signaling is influenced by EV-mediated contact. The other markers tested remain unaltered.





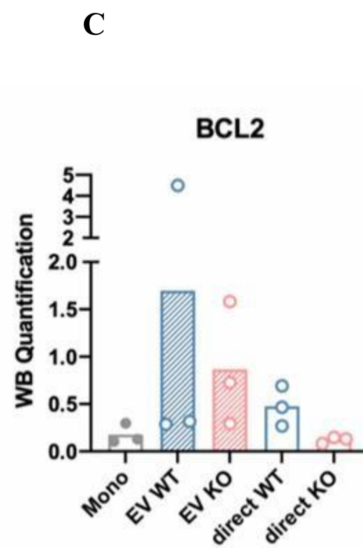


Figure 17. BCL2 activation on B-CLL cells upon HS5 EVs treatment. (**A**) Western blot, (**B**) P-ERK western blot quantification, and (**C**) BCL2 western blot quantification.

Proteomics Analysis

To investigate the difference in composition, EVs from WT and KO cells were submitted to label-free (MS/MS) proteomic analysis. From a total of 1200 proteins, 72 were differentially expressed. EVs from Lyn KO cells present about 60% of downregulation in comparison with the EVs WT, and the major group belongs to extracellular matrix cell composition (Figure 18).

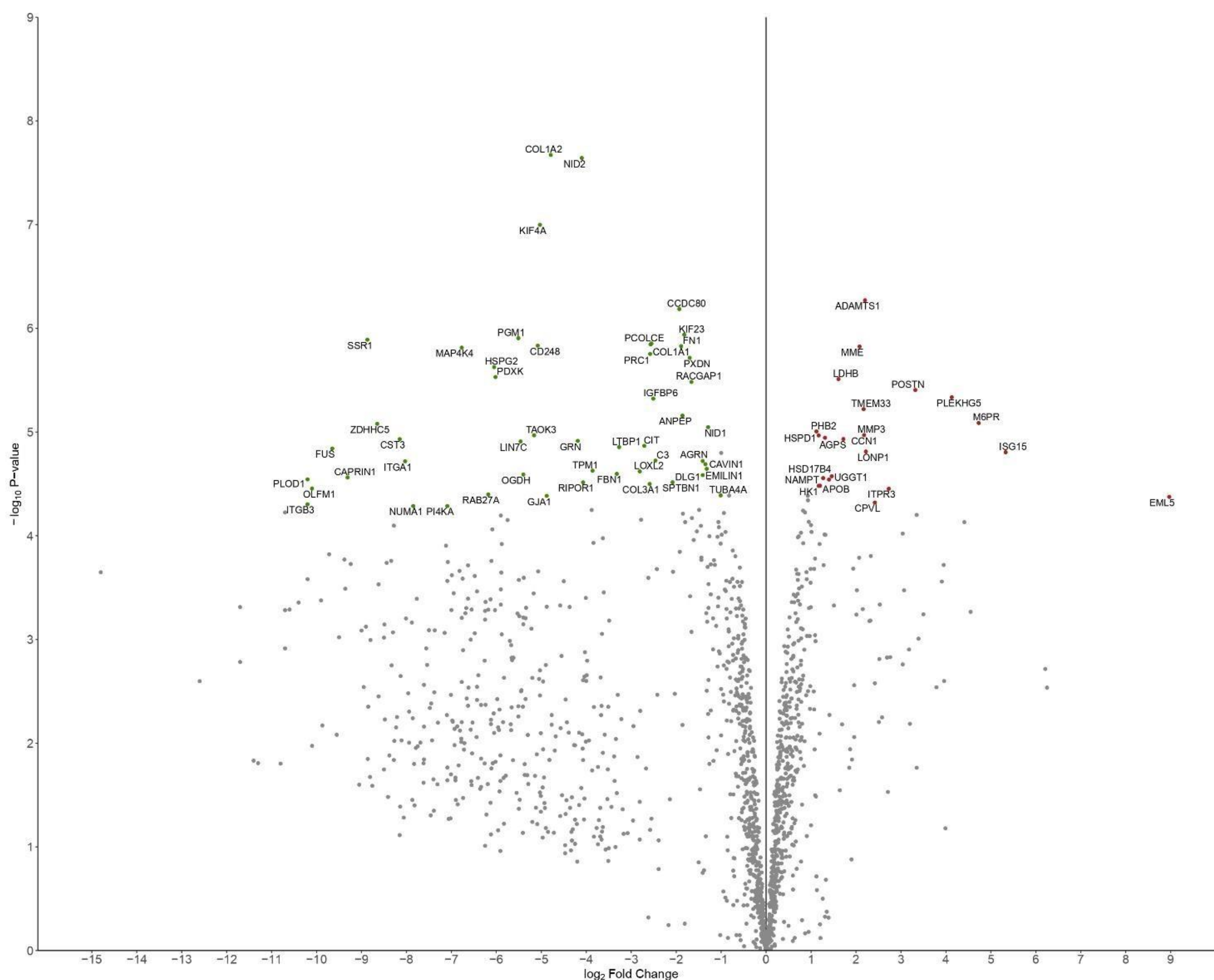


Figure 18. Volcano plot of EVs protein. Proteins downregulated (green) and upregulated (red) in comparison with the WT counterpart.

From the significant downregulate proteins, the transmembrane protein CD248 was selected to be further investigated, since it is a potential biomarker due to its higher specificity and role in cancer progression and survival (Becker, et al., 2020). Therefore, we generated HS5 cells with a CD248 KO and performed a coculture with the primary B-CLL cells (11 patients), as shown in Figure 19.

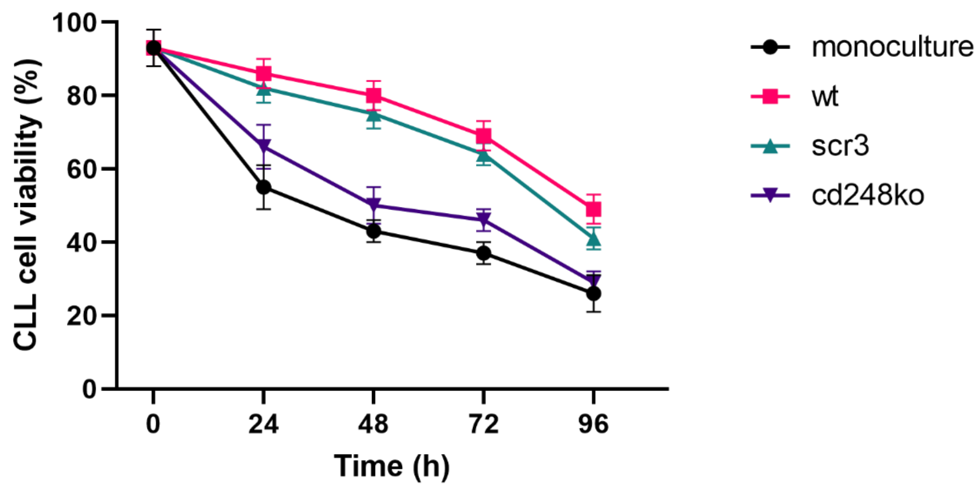


Figure 19. B-CLL cell and HS5 CD248 KO cells co-culture. We tested the B-CLL cell viability in co-culture with HS5 cells (WT, scramble control, and CD248 KO). Both cells were able to support B-CLL survival, but the KO cells show reduced support in comparison to the WT counterparts.

The cell viability was analyzed using CellTiter-Glo based on ATP luminescent measuring. We observed that the CD248 KO cells shows less survival support in comparison with the WT and scramble control, but still shows some support when compared to the monoculture 72 h, but not in the others time points tested.

Discussion

Interactions between B-CLL cells and stromal cells are important in the physiopathology of CLL and EVs which are composed of signaling cargo such as protein, lipids, and RNA, are required for cell communication. Because so little is known about their role in CLL microenvironment interactions and the previously known importance of Lyn in leukemia-associated fibroblasts, this study raises the question of the role of this kinase in this interaction. Therefore, here we investigated the role of tyrosine kinase Lyn in the extracellular vesicle-based crosstalk between primary chronic lymphocytic leukemia cells and stromal cells.

Comparing Lyn proficient and deficient stromal cell lines revealed that the lack of Lyn raised the cell adhesion and podosome formation but decreased the number of filopodia per cell. There is a morphologic change in the cytoskeleton composition as previously shown, and podosomes are enriched in the Lyn ko cells which may explain the higher cell adhesion when compared to the WT

cells.

Moreover, nanoparticle tracking analysis revealed that the EV release was significantly reduced in Lyn deficient stromal cells as compared to wild-type (WT) counterparts, resulting in a 36% reduction of the EV release. Additionally, HS5 cells EV release was tested in adherent and in suspension to investigate the role of protrusions role in adhesion in the EV release. The EV release was higher in the suspension cells than in the control group. The attached cells present more contact with each other than in suspension, therefore more EVs are necessary to reach another

cell for cell-cell communication, rising the importance of the guidance EV transport that could be mediated by the cell protrusions.

Furthermore, Imaging Flow cytometry (ImageStream) analysis revealed that the EV uptake is significantly reduced in Lyn deficient stromal cells as compared to wild-type (WT) counterparts, resulting in a 16% reduction in EV uptake, indicating that Lyn influences both, the cellular release and uptake of EVs. The uptake of exosomes is not fully established but endocytosis is the most reported method, predominantly under the mediation of clathrin (Tian et al. 2014a). Phagocytosis (Feng et al. 2010), direct fusion with the plasma membrane (Parolini et al. 2009), or binding through exosomal adhesion molecules (Pan et al. 1985) are all possible alternatives but it's not clear whether this uptake is cell-specific (Feng et al. 2010).

To keep EVs as close to their native state as possible, we did not use any activators to boost EV production, and serum deprivation was used on the stromal cell cultures to avoid fetal bovine serum vesicle contamination. For EV concentrations, we tested 1, 4, and 8 µg/mL and we saw significant effects in 4 and 8 µg/mL, showing a dose-dependent effect.

Because of this EV dose-dependent effect was important to investigate whether the transport of EVs can be guided. Currently, it is believed that these EVs passively reach the target cell through diffusion, bind there through specific molecular interactions, trigger a signal, and/or transmit specific molecules. However, previous work with a Hodgkin lymphoma model showed that only high EV concentrations of tumor cells stimulate tumor support in bystander cells. In both, Hodgkin lymphoma and CLL, the tumor cells receive the survival stimulus in a tissue-based tumor microenvironment *(Cook et al., 2022).* However, in tissue, the EV concentration decreases with the distance from the donor cell. Therefore, we raised the question of how cells optimize EV-

based intercellular communication. A previous study was shown that in a semi-solid 3D model, EVs were transported to and taken up by the target cell with the help of long, actin-based cell protrusions. Such protrusions lead EVs in both directions and high concentrations to the target cell (Hansen, 2015; Hansen et al., 2014).

We performed confocal microscopy with HS5 cells transfected with CD63-eGFP for EV tracking, since CD63 is considered an EV marker, and actin staining phalloidin. These cells were cocultured with primary CLL cells and employing confocal microscopy EVs were observed surfing on protrusions from one cell to another in HS5 and primary B-CLL cells. Similarly, this kind of transport was also observed by Polak and collaborators with primary B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells and primary mesenchymal stromal cells (Polak et al., 2015). This suggests that TNT signaling is critical for the survival of patient-derived BCP-ALL cells.

HS5 cells were transfected with CD63-eGFP for EV tracking since CD63 is considered an EV marker. These cells were co-cultured with primary CLL cells and employing the confocal microscopy EVs were observed on protrusions of HS5 and B-CLL cells (Figure 13).

In addition, the same amount (4 μg/ml) of EVs from Lyn proficient stromal cells induced significantly higher support on primary CLL cells as compared to EVs from Lyn deficient counterparts. These data suggest that Lyn not only influences the EV release but also the molecular composition of the EVs.

Proteomic comparison of the Lyn proficient and the deficient stromal cell line HS5 highlighted 72 significantly differentially expressed proteins. The differentially expressed proteins were

frequently ECM and cytoskeleton, similar to the proteomic profile identified from their origin cells (vom Stein et al., 2019).

Among them, CD248 was prominently decreased in Lyn-deficient HS5 cells. CD248 is a very specific and sensitive indicator of tumor vascularity. Anti-CD248 imaging may be used to track cancer responses and identify individuals who may benefit from CD248-targeted therapy (Fierle, 2020). Concentrations of CD248 are increased in cancer blood vessels. CD248 expression is increased in a variety of solid malignancies, including colorectal cancer, glioma, and breast cancer. In metastatic melanoma, CD248 is expressed preferentially in subsets of small and medium-sized tumor vasculature (von Heydebrand et al., 2021).

Therefore, we generated a HS5 CD248 knockout cells and performed co-culture with primary B-CLL cells and it resulted in a diminished survival potential compared to HS5 WT cells support. However, a more detailed mechanism is still missing concerning the survival support capability of such molecules, including immunoregulatory functions.

It has already been shown that tumor suppressor properties of TGFβ, observed in early-stage cancer, are likely mediated in part via suppression of CD248, the latter which is mediated via canonical Smad-dependent pathways (Babu et al., 2014). Upregulation of CD248 could be an early detection marker of tumor growth and metastasis, and it could be useful in monitoring TGF-based therapies. Understanding how CD248 is regulated has clinical relevance, as evidenced by ongoing Phase 1 and 2 clinical trials in which the anti-CD248 antibody, MORAb-004, is being tested for efficacy in solid tumors and lymphomas (www.clinicaltrials.gov). Determining the molecular mechanism(s) by which TGF loses its ability to suppress CD248 will be critical in

developing additional therapeutic interventions to prevent and/or reduce CD248-dependent tumor cell proliferation and metastasis (Babu et al., 2014).

In conclusion, the presented study provides initial evidence, that the tyrosine kinase Lyn crucially influences the EV-based communication between primary B-CLL and supporting bystander cells by raising the EV release and their concentration of functional molecules, such as CD248.

Conclusions

EVs are currently the subject of intense research to better understand their role in intercellular communication, the dissemination of bioactive cargo, and their role in the progression of various diseases. Improved understanding of EVs in tumor-stromal crosstalk could aid in the identification of new selective targets for cancer therapeutics.

Lyn kinase deficient in stromal cells, strongly impairs the survival support for B-CLL cells mediated by EVs. In addition, Lyn deficiency leads to changes in cellular protrusions, amount of EVs release and cellular uptake and alters the proteomic EV composition.

Proteomic comparison of EVs of Lyn proficient and deficient stromal cell line HS5 highlighted CD248 downregulated in Lyn-deficient HS5 EVs. Co-culture experiment showed that the lack of CD248 impairs the survival support for B-CLL cells by HS5 cells.

For the future, other molecules highlighted by the proteomics study might be also tested to better understanding of the EV mediated support mechanism in CLL. Collectively, these data demonstrate that Lyn kinase plays an important role in the EV-mediated communication between B-CLL and stromal cells. Moreover, the different protein composition between EVs Lyn proficient and deficient might be important to discovering functional molecules, such as CD248.

References

Abreu, S. C., Weiss, D. J., & Rocco, P. R. (2016). Extracellular vesicles derived from mesenchymal stromal cells: a therapeutic option in respiratory diseases? *Stem cell research & therapy*, *7*(1), 1-10.

Aldinucci, D., Celegato, M., & Casagrande, N. (2016). Microenvironmental interactions in classical Hodgkin lymphoma and their role in promoting tumor growth, immune escape, and drug resistance. *Cancer letters*, *380*(1), 243-252.

and Largeot, A., 2020. Method for the analysis of the tumor microenvironment by mass cytometry: application to chronic lymphocytic leukemia. *Frontiers in Immunology*, *11*.

Bebelman, M.P., Smit, M.J., Pegtel, D.M. and Baglio, S.R., 2018. Biogenesis and function of extracellular vesicles in cancer. *Pharmacology & therapeutics*, *188*, pp.1-11.

Becker, L.M., and O'Connell, J.T., 2020. Endosialin/CD248: A Potential Therapeutic Target Across Multiple Tumor Types. Molecular Cancer Research, 18(10), pp.1468-1478.

Bhat, S., Ljubojevic, N., Zhu, S., Fukuda, M., Echard, A. and Zurzolo, C., 2020. Rab35 and its effectors promote the formation of tunneling nanotubes in neuronal cells. *Scientific reports*, *10*(1), pp.1-14.

Block, J., Breitsprecher, D., Kühn, S., Winterhoff, M., Kage, F., Geffers, R., Duwe, P., Rohn, J.L., Baum, B., Brakebusch, C., and Geyer, M., 2008. FMNL2 drives actin-based protrusion and migration downstream of Cdc42. Current Biology, 22(12), pp.1005-1012.

Breitsprecher, D., Kiesewetter, A.K., Linkner, J., and Faix, J., 2011. The mechanism of the Arp2/3 complex function and its link to filopodia formation. Molecular Biology of the Cell, 22(20), pp.3980-3991.

Catalano, M. and O'Driscoll, L., 2020. Inhibiting extracellular vesicles formation and release: a review of EV inhibitors. *Journal of extracellular vesicles*, *9*(1), p.1703244.

Cell-Derived Evs to Immune Cells of the Tumor Microenvironment. J Clin Exp Pathol, 226

Chivero, E.T., Dagur, R.S., Peeples, E.S., Sil, S., Liao, K., Ma, R., Chen, L., Gurumurthy, C.B., Buch, S., and Hu, G., 2021. Biogenesis, physiological functions and potential applications of extracellular vesicles in substance use disorders. *Cellular and Molecular Life Sciences*, pp.1-

Cocucci, E., Racchetti, G., & Meldolesi, J. (2009). Shedding microvesicles: artifacts no more. Trends in cell biology, 19(2), 43-51.

Cook, M.R., Dunleavy, K. Targeting the Tumor Microenvironment in Lymphomas: Emerging Biological Insights and Therapeutic Strategies. *Curr Oncol Rep* **24**, 1121–1131 (2022).

Crompot, E., Van Damme, M., Pieters, K., Vermeersch, M., Perez-Morga, D., Mineur, P., ... & Stamatopoulos, B. (2017). Extracellular vesicles of bone marrow stromal cells rescue chronic lymphocytic leukemia B cells from apoptosis, enhance their migration and induce gene expression modifications. *Haematologica*, *102*(9), 1594.

Dang, I., Gorelik, R., Sousa-Blin, C., Derivery, E., Guérin, C.,

Dufrançais, O., Mascarau, R., Poincloux, R., Maridonneau-Parini, I., Raynaud-Messina, B. and Vérollet, C., 2021. Cellular and molecular actors of myeloid cell fusion: podosomes and tunneling nanotubes call the tune. *Cellular and Molecular Life Sciences*, *78*(17), pp.60876104.

Fierle, J.K., 2020. *Novel Antibody-based Therapeutic Approaches for Redirecting T Cell Effector Functions Towards Tumors Expressing CD248 (TEM1)* (Doctoral dissertation, éditeur non identifié).

Gauvin, T.J., Young, L.E., and Higgs, H.N., 2015. The formins FMNL2 and FMNL3 specifically associate with and modify the Arp2/3 complex. Journal of Biological Chemistry, 290(50), pp.29662-29672.

Gomez, T.M., and Letourneau, P.C., 2014. Actin dynamics in growth cone motility and navigation. Journal of Neurochemistry, 129(2), pp.246-258.

Gonder, S., Botana, I.F., Wierz, M., Pagano, G., Gargiulo, E., Cosma, A., Moussay, E., Paggetti, J.

Graf, I.R. and Frey, E., 2017. Generic transport mechanisms for molecular traffic in cellular protrusions. *Physical review letters*, *118*(12), p.128101.

Hansen, H. P., Engels, H. M., Dams, M., Paes Leme, A. F., Pauletti, B. A., Simhadri, V. L., Dürkop, H., Reiners, K.S., Barnert, S., Engert, A., Schubert, R., Quondamatteo, F., Hallek, M. & Pogge von Strandmann, E. (2014). Protrusion-guided extracellular vesicles mediate CD30 transsignaling in the microenvironment of Hodgkin's lymphoma. The Journal of pathology, 232(4), 405-414.

Hansen, H.P., Reiners K.S., & Pogge von Strandmann E. (2015) Targeting Functionality of Cancer

Hoffmann, A.K., Nosaka, Y., and King, M.M., 2014. A journey to the center of filopodia: radiating actin filaments reveal a common theme in cellular protrusions. Molecular Biology of the Cell, 25(11), pp.1690-1701.

Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Areces, L.B., Steffen, A., Stradal, T.E., Di Fiore, P.P., and Scita, G., 2004. Abi1 regulates the activity of N-WASP and WAVE in distinct actin-based processes. Nature Cell Biology, 6(3), pp.299-307.

Iwasa, J.H., and Mullins, R.D., 2007. Spatial and temporal relationships between actin-filament nucleation, capping, and disassembly. Current Biology, 17(5), pp.395-406.

Jacquemet, G., Paatero, I., Carisey, A.F., Padzik, A., Orange, J.S., and Ivaska, J., 2015. Filopodome mapping reveals molecular principles of organization and function of filopodia. Developmental Cell, 34(4), pp.384-397.

Jaiswal, R., Breitsprecher, D., Collins, A., Corrêa, I.R., Xu, M.Q., Goode, B.L., 2013. The formin Daam1 and fascin directly collaborate to promote filopodia formation. Current Biology, 23(14), pp.1373-1379.

Jimenez, L., Yu, H., McKenzie, A.J., Franklin, J.L., Patton, J.G., Liu, Q., and Weaver, A.M., 2019. Quantitative proteomic analysis of small and large extracellular vesicles (EVs) reveals enrichment of adhesion proteins in small EVs. *Journal of proteome research*, *18*(3), pp.947959.

Juan, T. and Fürthauer, M., 2018, February. Biogenesis and function of ESCRT-dependent extracellular vesicles. In *Seminars in cell & developmental biology* (Vol. 74, pp. 66-77). Academic Press.

Klemens Rottner,Jan Faix,Sven Bogdan,Stefan Linder,Eugen Kerkhoff, Actin assembly mechanisms at a glance, *J Cell Sci*, 2017.

Korobova, F., and Svitkina, T., 2008. Arp2/3 Complex contributes to the emergence of filopodia by participating in their initiation and elongation. Journal of Cell Biology, 180(6), pp.1233-1249.

Lai, F.P., Szczodrak, M., Block, J., Faix, J., Breitsprecher, D., Rottner, K., and Sixt, M., 2008. Arp2/3 complex-independent lamellipodium formation in fibroblasts. Journal of Cell Science, 121(22), pp.4075-4084.

Leithner, A., Eichner, A., Müller, J., Reversat, A., Brown, M., Schwarz, J., Merrin, J., de Gorter, D.J., Schur, F., Bayerl, J., and Rottner, K., 2016. WAVE regulatory complex associates

with YAP/TAZ to regulate cancer cell migration and invasion. Nature Communications, 7, p.10918.

Ljubojevic, N., Henderson, J.M. and Zurzolo, C., 2021. The ways of actin: why tunneling nanotubes are unique cell protrusions. *Trends in Cell Biology*, *31*(2), pp.130-142.

Mallavarapu, A., and Mitchison, T., 1999. Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. Nature Cell Biology, 1(3), pp.136-143.

Mattila, P.K., and Lappalainen, P., 2008. Filopodia: molecular architecture and cellular functions. *Nature Reviews Molecular Cell Biology*, 9(6), pp.446-454.

Mejillano, M.R., Kojima, S.I., Applewhite, D.A., Gertler, F.B., Svitkina, T.M., and Borisy, G.G., 2004. Lamellipodial actin assembly complex kinetics revealed by real-time imaging. Nature Cell Biology, 6(4), pp.364-373.

Mellor, H., 2010. The role of formins in filopodia formation. Biochemical Society Transactions, 38(5), pp.1829-1832.

Miao, Y., Bhattacharya, S., Banerjee, T., Abubaker-Sharif, B., Long, Y., Inoue, T., Iglesias, P.A., and Devreotes, P.N., 2019. Wave patterns organize cellular protrusions and control cortical dynamics. *Molecular systems biology*, *15*(3), p.e8585.

Millard, T.H., and Martin, P., 2008. Dynamic analysis of filopodial interactions during the zippering phase of Drosophila dorsal closure. Developmental Biology, 319(2), pp.468-481.

Mir, B. and Goettsch, C., 2020. Extracellular vesicles as delivery vehicles of specific cellular cargo. *Cells*, *9*(7), p.1601.

Nicholson-Dykstra, S.M., and Higgs, H.N., 2008. Arp2/3 complex-independent assembly of lamellipodia and filopodia in fibroblasts. Journal of Cell Science, 121(1), pp.166-177.

Parsons, S. J., & Parsons, J. T. (2004). Src family kinases, key regulators of signal transduction. Oncogene, 23(48), 7906-7909.

Polak, R., de Rooij, B., Pieters, R., & den Boer, M. (2015). B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment. *Blood*, 126(21), 2404–2414.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A., 1992. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. Cell, 70(3), pp.401-410.

Small, J.V., Stradal, T., Vignal, E., and Rottner, K., 2002. The lamellipodium: where motility begins. Trends in Cell Biology, 12(3), pp.112-120.

Stahl, P.D. and Raposo, G., 2019. Extracellular vesicles: exosomes and microvesicles, integrators of homeostasis. *Physiology*, *34*(3), pp.169-177.

Steffen, A., Faix, J., Resch, G.P., Linkner, J., Wehland, J., Small, J.V., and Rottner, K., 2004. Targeting of the WAVE complex to lamellipodia depends on a complex containing Abi and Sra-1/CYFIP1. Current Biology, 14(7), pp.627-632.

Steffen, A., Ladwein, M., Dimchev, G.A., Hein, A., Schwenkmezger, L., Arens, S., Ladwein, K.I., Holleboom, J.M., Schur, F., Victor Small, J., and Rottner, K., 2013. Rac1 and Cdc42 are necessary for lamellipodia formation and cell migration. Journal of Cell Science, 126(15), pp.3542-3552.

Steffen, A., Rottner, K., and Bohl, B.P., 2014. The role of RAC1 in the formation of lamellipodia. The Journal of Biological Chemistry, 289(19), pp.13132-13141.

Stradal, T.E., and Scita, G., 2006. Protein complexes regulating Arp2/3-mediated actin assembly. Current Opinion in Cell Biology, 18(1), pp.4-10.

Suraneni, P., Rubinstein, B., Unruh, J.R., Durnin, M., Hanein, D., and Li, R., 2012. The Arp2/3 complex is required for lamellipodia formation, migration, and invasion. Journal of Cell Biology, 197(2), pp.239-251.

Suresh Babu, S., Valdez, Y., Xu, A., O'Byrne, A.M., Calvo, F., Lei, V., & Conway, E.M. (2014). TGFβ-mediated suppression of CD248 in non-cancer cells via canonical Smad-dependent signaling pathways is uncoupled in cancer cells. BMC Cancer, 14, 113Svanberg, R., Janum, S., Patten, P.E., Ramsay, A.G. and Niemann, C.U., 2021. Targeting the tumor microenvironment in chronic lymphocytic leukemia. *Haematologica*.

Svitkina, T.M., and Borisy, G.G., 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization of actin filaments in lamellipodia. Journal of Cell Biology, 145(5), pp.1009-1026.

Szatanek, R., Baj-Krzyworzeka, M., Zimoch, J., Lekka, M., Siedlar, M. and Baran, J., 2017. The methods of choice for extracellular vesicles (EVs) characterization. *International journal of molecular sciences*, *18*(6), p.1153.

Ten Hacken, E., & Burger, J. A. (2016). Microenvironment interactions and B-cell receptor signaling in Chronic Lymphocytic Leukemia: Implications for disease pathogenesis and treatment. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1863(3), 401413.

Théry, C., Ostrowski, M., & Segura, E. (2009). Membrane vesicles as conveyors of immune responses. Nature reviews immunology, 9(8), 581-593.

van Attekum, M.H., Eldering, E. and Kater, A.P., 2017. Chronic lymphocytic leukemia cells are active participants in microenvironmental cross-talk. *Haematologica*, *102*(9), p.1469.

Verderio, C., Gabrielli, M. and Giussani, P., 2018. Role of sphingolipids in the biogenesis and biological activity of extracellular vesicles. *Journal of lipid research*, *59*(8), pp.1325-1340.

Viveiros, M.M., Hirao, Y., and Eppig, J.J., 2011. Evidence that the instability of microfilaments underlies the spontaneous breaking of the mouse oocyte meiotic spindle. Developmental Biology, 363(2), pp.1-15.

Vom Stein, A. F., Koch, M., Reinartz, S., Lucas, A., Rebollido-Rios, R., Oellerich, T., ... & Hallek, M. (2019). Lyn Kinase Contributes to the Reprogramming of Fibroblasts Promoting Chronic Lymphocytic Leukemia Progression. Blood, 134, 4283.

von Heydebrand, F., Fuchs, M., Kunz, M., Voelkl, S., Kremer, A.N., Oostendorp, R.A., Wilke, J., Leitges, M., Egle, A., Mackensen, A. and Lutzny-Geier, G., 2021. Protein kinase C-βdependent changes in bone marrow stromal cells' glucose metabolism in chronic lymphocytic leukemia. *STEM CELLS*.

Wu, C., Asokan, S.B., Berginski, M.E., Haynes, E.M., Sharpless, N.E., Griffith, J.D., Gomez, S.M., and Bear, J.E., 2012. Arp2/3 is critical for lamellipodia and stroke recovery. Nature Medicine, 18(9), pp.1287-1290.

Yang, J.M., Bhattacharya, S., West-Foyle, H., Hung, C.F., Wu, T.C., Iglesias, P.A. and Huang, C.H., 2018. Integrating chemical and mechanical signals through dynamic coupling between cellular protrusions and pulsed ERK activation. *Nature communications*, *9*(1), pp.1-13.

Yang, W., Wu, P.F., Ma, J.X., Liao, M.J., Xu, L.S. and Yi, L., 2020. TRPV4 activates the Cdc42/Nwasp pathway to promote glioblastoma invasion by altering cellular protrusions. *Scientific reports*, *10*(1), pp.1-15.

Young, L.E., Latario, C.J., Higgs, H.N., and Smith, M.A., 2015. Mammalian actin filament nucleators regulate cytoskeletal dynamics in cells lacking functional Arp2/3 complex. Molecular Biology of the Cell, 26(2), pp.231-242.

Zarà, M., Guidetti, G.F., Camera, M., Canobbio, I., Amadio, P., Torti, M., Tremoli, E. and Barbieri, S.S., 2019. Biology and role of extracellular vesicles (EVs) in the pathogenesis of thrombosis. *International journal of molecular sciences*, *20*(11), p.2840.

Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J., and Mi, S., 2019. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics, Proteomics & Bioinformatics, 17(5), pp.473-484.