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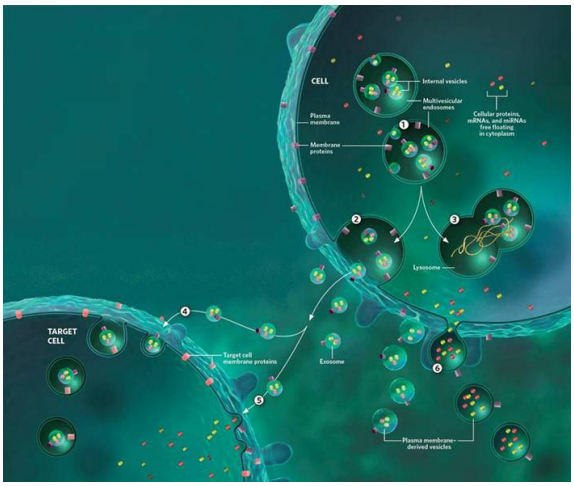
Role of exosomes released by Chronic Myelogenous Leukemia in the modulation of tumor microenvironment



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Introduction

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder of the hematopoietic stem cell (HSC). CML makes up approximately 15% to 20% of all cases of leukemia, with an incidence of 1 to 1.5 cases per 100,000 population per year (1). Similar to other myeloproliferative disorders, CML is usually triphasic, having a chronic, an accelerated, and a blast phase (2). In the initial chronic phase, myeloid progenitors and mature cells accumulate in the blood and extramedullary tissues; in half of the patients the disease progresses directly from the chronic to the blastic phase characterized by a maturation arrest in the myeloid or lymphoid lineage (3). The main functional changes that occur with progression of CML are marked changes in proliferation, differentation, apoptosis and adhesion (4). CML is characterized by deregulated proliferation of cells of the myeloid lineage, in which leukemic blasts display a distinctive shortened chromosome, the Philadelphia (ph) chromosome generated from a reciprocal t(9:22) (q34:q11) translocation (5)(Fig. 1).

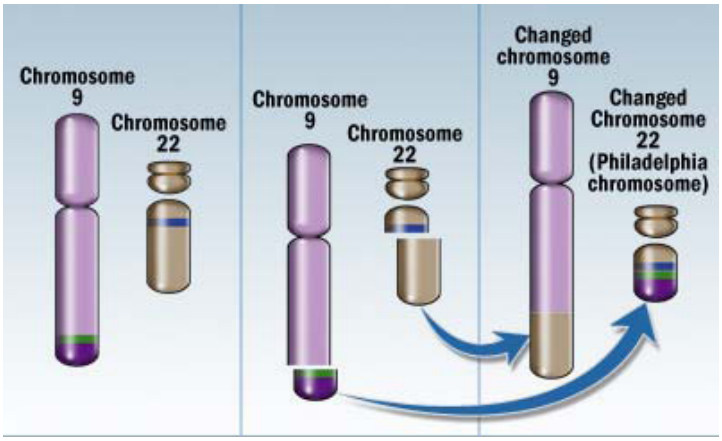

Fig. 1: Reciprocal t(9:22) translocation in Chronic myeloid leukemia generate Philadelphia chromosome

This translocation results in the head-to-tail fusion of the breakpoint cluster region (BCR) gene on chromosome 22 with the ABL proto-oncogene on chromosome 9 (6). The encoded chimeric bcr-abl oncoprotein exhibits constitutively active tyrosine kinase activity as a result of oligomerization of the coiled coil region of $p210^{BCR/ABL}$ (7) and deletion of the inhibitory SH3 domain of ABL (8). This results in the activation of a number of downstream signaling cascades (9). The targets for bcr–abl include members of the Ras, phosphotidylinositol-3 kinase (PI3K)/Akt, and Jak/Stat signaling pathways, which regulate cell proliferation and apoptosis. Bcr–abl abrogates cell dependence on external growth factors by upregulating interleukin-3 production and alters the cell adhesion properties by modulating expression and activation of focal adhesion kinase and associated proteins. (10,11). The features of chronic phase CML, expansion and premature circulation of the malignant myeloid population can therefore be explained by activation of mutagenic pathways, antiapoptotic pathways and abnormal cytoskeletal function (Fig. 2).

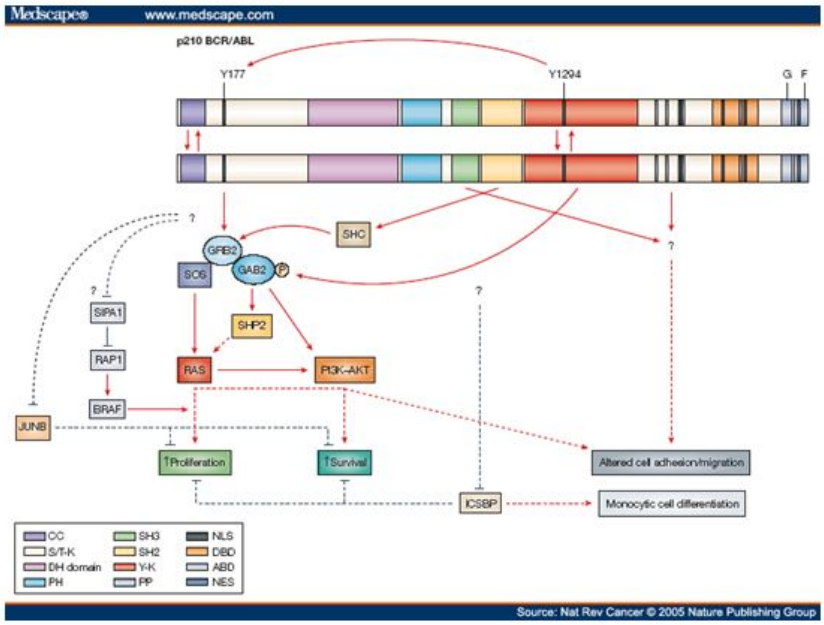

Fig. 2: Schematic representation of some signal transduction pathways activated by BCR-ABL

These same characteristics, increased mutagenicity and decreased susceptibility to apoptosis ay also be responsible for disease progression (12). Moreover, leukemic progenitors can acquire secondary aberrations that make them able to overcome cell cycle checkpoints and acquire an advantage in growth compared to normal cells and make them resistant to drug therapies.

Therapy for Chronic Myelogenous Leukemia

Since the description of BCR–ABL oncogene, a myriad of treatment options have been explored in CML, including arsenic trioxide, spleenic irradiation, busulphan, hydroxycarbamide. These compounds could only control the proliferation of white blood cells and enlargment of the spleen, but didn't avoid the disease progression to accelerated phase and blast crisis. Most relevant results were obtained with the introduction of therapy with recombinant Interferon-α (rIFN-α), which compared to conventional chemotherapy, is more effective, especially in patients with early chronic phase (13,14). The introduction of rIFN-α induced cytogenetic remission and increased survival (15). The exact mechanisms of action of rIFN-α have not been identified, but it is widely believed that they involve immune response to tumor cells, abnormal expression of adhesion molecules, and induction of tumor cell death (16). In spite of the effectiveness of rIFN-α treatment, however, there are limitations, from poor patient compliance to development of anti–rIFN-α antibody. The only curative approach is stem cell transplantation, however, as the average age of onset is >50 yrs of age, this factor, combined with the inability to identify suitably matched donors in all cases, limits this option to a minority of patients (17). The most exciting breakthrough in the treatment of CML has been the development of Imatinib Mesylate (IM) a selective kinase inhibitor, able to inhibit the activity of constitutive tyrosine kinase activity of

Bcr-Abl protein (18). Imatinib acts as a competitive inhibitor of ATP, it binds to the ATP binding site located on ABL and block the action of the tyrosine kinase in an irreversible way. The inhibition of kinase activity of bcr-abl inhibit neoplastic clone proliferation and has a significant effect on haematological and cytogenetics level. However, some patients with an advanced disease develop resistance to Imatinib due to secondary point mutations in the bcr-abl tyrosine kinase domain or due to BCR-ABL gene amplification (19). It has been proposed that the interaction between leukemic cells and the bone marrow microenvironment may play an important role in CML pathogenesis (20). A better understanding of the mechanisms involved in the disease progression may provide information to develop new treatment strategies.

Chronic Myelogenous Leukemia and Angiogenesis

Angiogenesis is the formation of new blood vessels from an existiting vasculature. It involves degradation of extracellular matrix proteins and activation, proliferation and migration of endothelial cells and pericytes in a multistep process (21). Angiogenesis is mediated by a balance of various positive [eg vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), interleukin-8 (IL-8), transforming growth factors (TGF-α, TGF-β), matrix metalloproteinases (MMPs)] and negative [eg platelet factor-4 (PF-4) tissue inhibitors of metalloproteinases (TIMPs)] angiogenic molecules released by tumor cells (22,23). Tumor angiogenesis is linked to a switch in this balance, and mainly depends on the release by neoplastic cells of growth factors specific for endothelial cells and able to stimulate the growth of the host's blood vessels (24). This complex pathomechanism may comprise a direct stimulation of endothelial cells (paracrine loop) resulting in proliferation, sprouting and release of growth factors and on the other hand, an

autocrine stimulation by inhibition of apoptosis and promotion of growth (Fig. 3), finally leading to an expansion of the neoplastic cell clone (25,26).

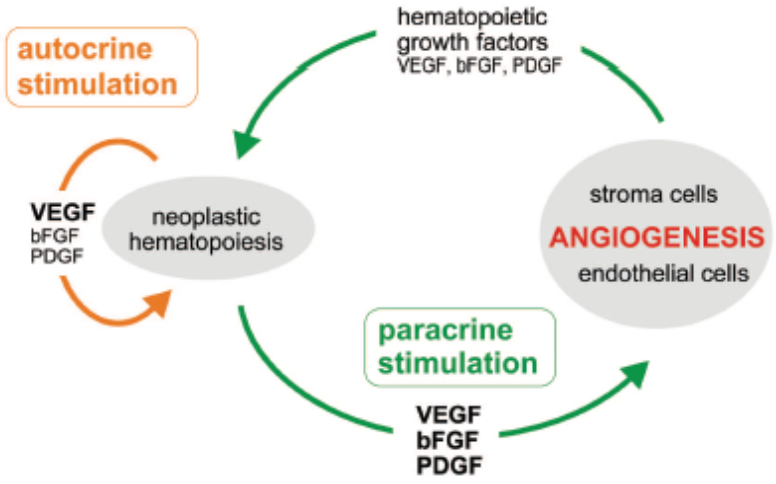

Fig. 3: Dynamics of angiogenesis in hematological neoplasias: angiogenic factors secreted by neoplastic cells promote cell growth and inhibit apoptosis (autocrine stimulation). On the other side, these angiogenic mediators can also stimulate endothelial cell proliferation and enhance the production and release of hematopoietic growth factors (paracrine stimulation).

Recently, there are evidence that implicates angiogenesis in the growth and progression of CML (27). Lundberg LG et al., have found increased plasma levels of VEGF as well as other angiogenic factors such as FGF-2 and HGF and increased marrow vascularity in patients with CML (28). Bcr-Abl has also been involved in VEGF-mediated angiogenesis in CML and evidence indicates that the formation of new vessels plays an important role in the development and progression of CML. It has been proposed that the interaction between leukemic cells and the bone marrow microenvironment may play an important role in CML pathogenesis (20). An important aspect in the study of CML is the cross-talk between leukemic cells and bone marrow microenvironment. This microenvironment may facilitate tumor progression by the pertubation of CXCR4-SDF1

axis. Kalinkovich et al, have shown that microvesicles isolated from patients with leukaemia contain CXCR4, and these vesicles CXCR4+ are more abundant in leukemic patients compared to healthy individuals (29). CXCR4-SDF1 leads the increase of cytokines as IL8. IL8 stimulates the expression of VEGF, SDF-1 and IL6 in endothelial cells resulting in promoting angiogenesis. Many studies have shown over-expression of IL8 by tumor cells, often induced in response to chemotherapeutic interventions or environmental stresses such as hypoxia. The increased synthesis and secretion of IL8 from tumor cells activate many signalling pathways through the binding of IL8 to two cell-surface G-protein-coupled receptors, CXCR1 and CXCR2 (30,31) in cancer cells, endothelial cells and neutrophils/tumor associated macrophages. Activation of IL8 signalling pathways, through Akt, PKC and MAPK, have been detected in multiple forms of cancer and its role in modulating cell survival, angiogenesis and cell migration have estabilished this kinases as an important therapeutic target in cancer (32). As a consequence of the activation of these signalling pathways, IL8 may affect different cell types within tumor microenvironment. For example, activation of IL8 receptors on endothelial cells is known to promote an angiogenic response, inducing proliferation, survival and migration of vascular endothelial cells (33). IL8 secretion from cancer cells can enhance the proliferation and survival of cancer cells throug autocrine signalling pathway. In addition, tumor-derived IL8 will activate endothelial cells in the tumor vasculature to promote angiogenesis and induce a chemotactic infiltration of neutrophils into tumor site. It is reported also that cell adhesion molecules play an important role in angiogenesis. Adhesion is in part mediated by β1 integrins (VLA4 and VLA5) and CD44 expressed on leukemia cells, and the fibronectin and vascular cell adhesion molecule-1 (VCAM1), expressed on the stromal layer (34). During new capillary development, coordinated signals from both integrins and growth factor receptors

regulate the survival, proliferation and invasion of endothelial cells.

Exosomes Formation

Exosomes are small, 40-100 nm, membrane vesicles of endocytic origin that are secreted by a variety of cell types like B-cells (35), T-cells (37), mast cells (37), DCs (38), platelets (39), neurons (40) and epithelial cells (41) into the extracellular space. They were first described as microvesicles containing 5′-nucleotidase activity secreted by neoplastic cell lines (42). Few years later another group reported secretion of small vesicles of endocytic origin by cultured reticulocytes, and these small vesicles contained the transferrin receptor. Using electron microscopy (EM) they observed these vesicles in late endosomes, and the fusion of these late endosomes with the cell membrane resulted in the release of the vesicles extracellularly (43,44). In addition to cultured cells, exosomes have today further been isolated from a number of body fluids such as plasma (45), urine (46), synovial fluid (47), malignant effusions (48), epididymal fluid (49) and from seminal plasma, in which the vesicles are derived from prostate cells and called prostasomes (50). Exosomes correspond to the intraluminal vesicles (ILVs) of late endosomal compartments called multivesicular bodies (MVBs). These intraluminal vesicles are formed by inward budding of the limiting endosomal membrane and contain cytosol from the cell. MVBs are involved in transporting proteins for degradation in lysosomes. Alternatively, the MVBs can fuse with the plasma membrane leading to the release of the intraluminal vesicles extracellularly which are then called exosomes (35),(44). Proteins and lipids are sorted at the limiting membrane of endosomes during the formation of the intraluminal vesicles and as a consequence the released exosomes will contain molecules reflecting their origin from late endosomes (51). The mechanisms leading to exosome release are unknown. However, the

transmembrane protein TSAP6 has been suggested to be involved in regulating exosome production (52). Furthermore, Rab11, a member of the small GTPase family, together with calcium were shown to be important for the docking and fusion of MVBs with the plasma membrane (53, 54, 55) (fig. 4).

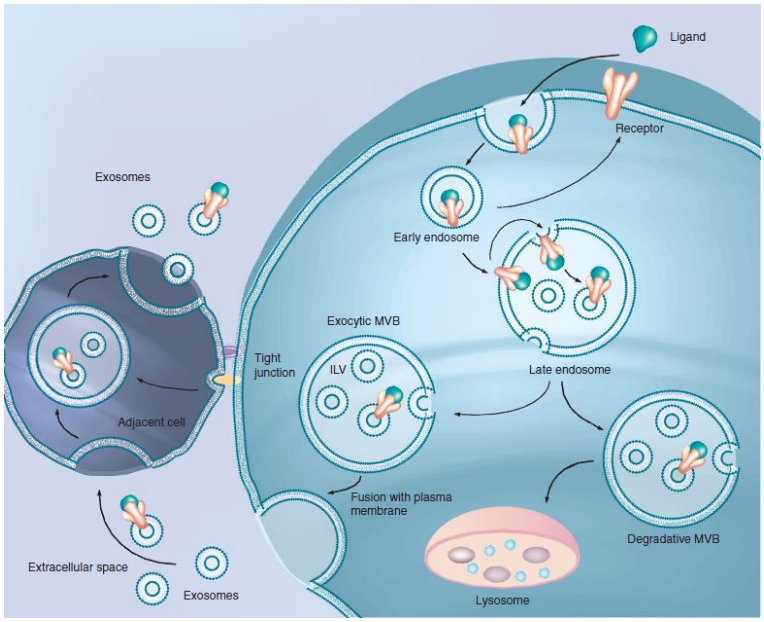

Fig. 4: Mechanism of exosomes release

A machinery responsible for sorting proteins in intraluminal vesicles has recently been identified and called ESCRT (Endosomal Sorting Complex Required for Transport) (56). The ESCRT-0, -I and -II complexes recognize and sequester ubiquitinated proteins in the endosomal membrane, whereas the ESCRT-III complex seems to be responsible for membrane budding (57). The ESCRT proteins are required for the targeting of membrane for lysosomal degradation while the function of the ESCRT machinary in the formation of ILVs that are further secreted as exosomes is not clear. Proteomic analysis of purified exosomes from different sources show an enrichment of ESCRT components and ubiquitinated proteins (58, 59). The expression of the Nedd4 family interacting-protein1, that is associated with elevated levels of protein ubiquitination in exosomes (60), and Alix, a protein associated with ESCRT machinary, is required for the sorting

of the transferrin receptor into exosomes (61). This leads to the hypothesis that ESCRT could be required for exosomal cargo. Lipid rafts has also been suggested to be involved in protein sorting into intraluminal vesicles (62) and typical raft components has been identified on exosomes such as glycolipids, Src tyrosine kinases and cholesterol (62,63). How MVBs discriminate between proteins that are destined for exosomal secretion or lysosomal degradation remains to be determined. In APC, MHC class II are accumulated in MVBs which are called MIICs. These MIICs are the major site for peptide loading, and subsequently exosomes from APC bear peptide-loaded MHC on their surface.

Exosome composition

Physical properties and purification of exosomes

The common procedure to purify exosomes from cell culture supernatants involves a series of centrifugations to remove cells and cell debris. Filtration of the cell-culture supernatant through 0.22-μm filters, followed by direct high-speed ultracentrifugation (64), reduces the contamination of exosome preparations with larger vesicles that are shed from the plasma membrane. In addition, because exosomes are present in serum it is crucial to avoid contamination with bovine exosomes from the fetal calf serum (FCS) that is used to culture the exosome-producing cells (65). For this reason, serum for culture medium can be depleted from serum exosomes by overnight high speed ultracentrifugation (65,36). To be sure that the isolated vesicles are exosomes must be used other criteria to identify exosomes. Exosomes float on sucrose gradients, and their density ranges from 1.13 g ml^{-1} to 1.19 g ml^{-1} (66). Contaminating material, such as protein aggregates or nucleosomal fragments that are released by apoptotic cells, are separated from exosomes by flotation on sucrose gradient (64). Electron microscopy is

also used to recognized exosomes by revealing the characteristic “cup-shaped” morphology, a flattened sphere limited by a lipid by-layer (67). These characteristics are consistent with the observed size and morphology of internal vesicles in multivesicular endosomes (68).

Molecular composition of exosomes

The molecular composition of exosomes depending on the cell from which they are secreted and their endosomal origin. Immuno-Electron Microscopy localization studies, Western blot analysis and peptide mass mapping of exosomal proteins from Dendritic Cells (DCs) (64,65), B lymphocytes (68,69), intestinal epithelial cells (41), and other cell types revealed the presence of common as well as cell-type-specific proteins. For example, exosomes from DCs, mast cells and B cells are enriched in MHC class I and MHC class II and express co-stimulatory molecules like CD54 (also called ICAM-1), CD80 and CD86 (66,69), exosomes from T cells bear CD3 (36) and exosomes from cytotoxic T cell contain perforin and granzymes (70), exosomes from reticulocytes contain the transferrin receptor (71). In addition to cell-specific molecules, exosomes also contain common components (Fig. 5)

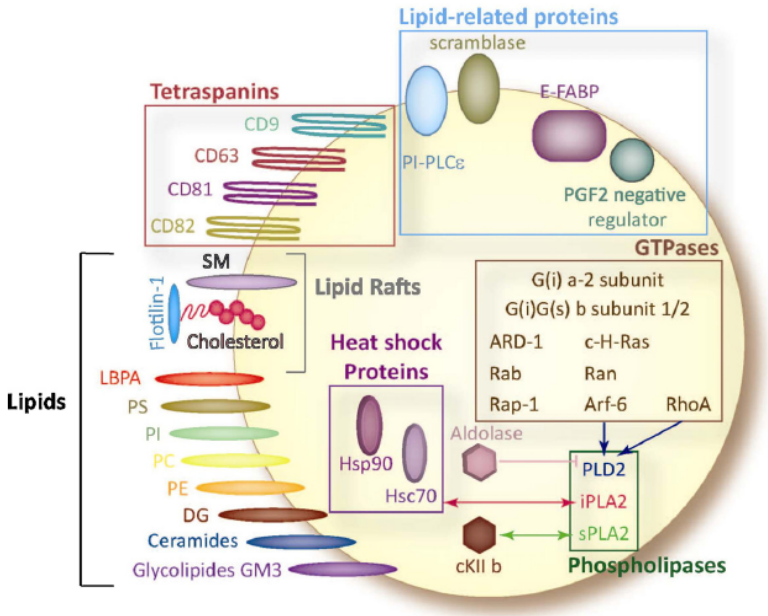

Fig. 5: Common molecular components of exosomes.

They are enriched in a family of proteins called tetraspanin which are cell-surface proteins that span the membrane four times (69). Tetraspanin proteins are found on the surface of many cell types but also in endosomal compartments. Tetraspanins form complexes by interacting between themselves as well as with a large variety of transmembrane and cytosolic proteins (72). These tetraspanin complexes are located in microdomains, termed TEM domains (tetraspanin-enriched membrane domains) (73). TEM domains are different from rafts and clathrin-coated pits, but share with these structures a specific capacity to facilitate vesicular fusion and/or fission (74,75). Tetraspanins act as 'molecular facilitators', which modulate, stabilize or prevent activities of associated molecules (76). They promote spreading, migration and cable formation by adjusting integrin compartmentalization, internalization, recycling and signalling (77). By regulating protein traffic, tetraspanins become involved in cell adhesion (78), and by modulating biosynthesis of associated molecules, such as MMPs (matrixmetalloproteinases), they may influence invasiveness (79). The main functions are cellular motility, invasion and fusion (80). Examples of tetraspanin proteins found on exosomes are CD9, CD63 and CD81. Exosomes have also been demonstrated to contain heat shock proteins (Hsps) like Hsp70, Hsc70, Hsc73 and Hsp90 (81,65). Heat shock proteins are a family of proteins which act as chaperones to facilitate the folding of protein intracellularly. Hsps can also be secreted and have extracellular functions such as immuno-regulatory activities. Hsps can be both constitutively expressed and be induced by cellular stress. Heat stressed cells have been shown to increase the expression of Hsps on their released exosomes (81). These ubiquitous proteins are involved in antigen presentation, as they can bind antigenic peptides and participate in loading peptides onto MHC molecules (82). MHC class I molecules are also present in exosomes from most cell types. Moreover, exosomes contain cytoskeleton proteins like

actin, tubulin and moesin, ESCRT proteins like Tsg101 and alix and proteins involved in intracellular membrane fusions and transports and fusion like Rab proteins and annexins (64,83). They also include molecules that are involved in signal transduction (such as protein kinases, 14-3-3 and heterotrimeric G proteins) and various metabolic enzymes (such as peroxidases, pyruvate and lipid kinases, and enolase-1). Furthermore, exosomes express CD55 and CD59 which have been shown to protect them from complement lysis (84) suggesting them to be stable in vivo. Exosomes also contain proteins that are involved in specific cell functions. Exosomes from antigen-presenting cells (APCs) have been analysed in most detail. MHC class II molecules are very abundant in exosomes from all cells that express MHC class II. Exosomes from DCs also contain CD86, which is an important co-stimulatory molecule for T cells. T-cell receptors are also specifically enriched on T-cell-derived exosomes. Exosomes contain a series of cell-specific transmembrane proteins including α- and β-chains of integrins (such as αM on DCs, β2 on DCs and T cells, and α4β1 on reticulocytes), immunoglobulin-family members (such as intercellularadhesion molecule 1 (ICAM1)/CD54 on B cells, A33 antigen on enterocytes and P-selectin on platelets) or cell-surface peptidases (such as dipeptidylpeptidase IV/CD26 on enterocytes and aminopeptidase N/CD13on mastocytes).

Milk-fat-globule EGF-factor VIII(MFGE8)/lactadherin (85), a milk-fat-globule protein that is expressed by DCs and some tumour-cell lines, was found very abundant in exosomes that are produced by these cells (65). These proteins probably ‘address’ exosomes to target cells.

Exosome function

Exosomes have been suggested to participate in different physiological and/or

pathological processes within the extracellular space and in biological fluids (e.g. urine, serum,) (86,87,88). Exosomes functionality seems to be determined by cell-type specific proteins that reflect the specialized function of their original cells. When exosomes were initially discovered from reticulocytes, they were shown to function as a way of removing unnecessary proteins such as the transferrin receptor during the maturation process of reticulocytes into erythrocytes (89). Another function of exosomes that was later described is as cell free messengers, which can be released from one cell and have an effect on another one. This communication with other cells may occur either in the microenvironment, or over a distance (90). Since exosomes have been found in blood plasma (45), they may be transported between organs via the systemic circulation. How this interaction occurs between exosomes and cells is not fully known. However, several mechanisms describing the interactions of exosomes and cells have been hypothesized. Exosomes can bind to cells through receptor-ligand interactions, similar to cell to cell communication, mediating for example antigen presentation (35,91). Clayton et al. showed that B cell exosomes express functional integrins, which are capable of mediating adhesion to extracellular matrix components and activated fibroblasts. This adhesion was strong and resulted in an increase in intracellular calcium concentration (92). Alternatively, exosomes can attach to or fuse with the target cell membrane, thus delivering exosomal surface proteins and perhaps cytoplasm to the recipient cell (93). MHCII positive exosomes have been shown to be attached to follicular DCs. These cells do not express MHCII themselves, and the exosomes provide them with new properties (93). Finally, exosomes may be internalized by the recipient cells due to mechanisms such as endocytosis. Immature DCs have been shown to internalize and process exosomes for antigen presentation to CD4+ T cells (94). Later this was also shown for the integrin α4β1 which was down

regulated from the red blood cell surface and instead found on the surface of released exosomes, and in addition this made the exosomes able to bind to fibrinonectin (95). Through their ability to bind target cells, they are likely to modulate selected cellular activities such as vascular homeostasis, and participate in the signaling events contributing to antigen presentation to T cells (35) and the development of tolerance (96).

Exosomes in Cancer

Cancer cells begin to mould their stromal environment starting at early phases of the neoplastic process mainly by pathways involving cell-to-cell contact and the release of soluble factors, as TNFα, TGFβ, VEGF, that are able to influence the recruitment of different cell types (e.g. myeloid cells, hematopoietic progenitors cells). Recently, an alternative novel mechanism involves the active release by tumor cells of exosomes. Tumor exosomes, seem to have dual functions with completely opposite effects, either they can activate or suppress immune response against cancer.

Protumorigenic role of tumor-derived exosomes

Tumor-derived exosomes have been implicated in facilitating tumor invasion and metastasis. By stimulating angiogenesis, modulating factors released by stromal cells, and remodelling extracellular matrix, tumor-derived exosomes have been found to contribute to the establishment of a premetastatic niche, generating a suitable microenvironment in distant metastatic sites (97). Exosomes can increase extracellular matrix degradation and augment tumor invasion into the stroma (98,99). It was suggested that CD44 is required for the assembly of a soluble matrix that, in cooperation with exosomes, promotes leukocyte, stroma, and endothelial cell activation

in the (pre)metastatic organ. Tumor-derived exosomes may also transport apoptosis inhibitory proteins, induced under stress conditions, to promote tumor survival. For example, survivin, a member of the inhibitor of apoptosis protein family, can be absorbed by cancer cells from extracellular media and inhibit their apoptosis following genotoxic stress as well as increase their replicative and metastatic ability (100). Another pronounced effect of tumor-derived exosomes is their ability to modulate the function of stromal cells such as fibroblasts. It was recently shown that exosomes shed by prostate, colorectal and breast cancer cells lines contain TGF-β on their surface in association with betaglycan and can trigger SMAD-dependent signaling. Exosomal delivery of TGF-β is capable of driving the differentiation of fibroblasts into myofibroblasts, whose enrichment in solid tumor represents an altered stroma that supports tumor growth, vascularization, and metastasis (101). These observations suggest a protumorigenic role of tumor-exosomal TGF-β in addition to their immunosuppressive functions. However, it was also noted that TGF-β is not universally present on exosomes derived from all cancer cells. Tumor-derived exosomes were shown to directly suppress the activity of effector T cells. Exosomes from melanoma and colorectal cancer cells express death ligand such as FasL and TRAIL, both of which can trigger the apoptotic death of activated T cells (102,103). Furthermore, ovarian tumor derived exosomes are able to down-modulate CD3-ζ chain expression and impair TCR signaling (103,104), suggesting that tumor-derived exosomes can also downregulate T cell function in addition to direct killing. In addition, NKG2D dependent cytotoxicity of NK cells and CD8+ T cells was inhibited by NKG2D ligand-containing exosomes derived from human breast cancer and mesothelioma cell lines (105,106). Similarly, murine mammary carcinoma exosomes were shown to promote tumor growth *in vivo* by suppressing NK cell function (107). These observations

suggest that tumor-derived exosomes can negatively regulate immune system, in particular T cells and NK cells. In addition, tumor-derived exosomes can also support the function of regulatory T (Treg) cells. Mesothelioma and prostate-derived exosomes were found to selectively impair the IL-2 response to cytotoxic effector cells while supporting Treg cell activities through a TGF-*β*-dependent mechanism (108). Exosomes from sera of patients with head and neck cancer were also reported to induce, expand, and upregulate the suppressor functions of human Treg cells as well as enhance their resistance to apoptosis via a TGF-*β*- and IL-10-dependent mechanism (109) (Fig. 6)

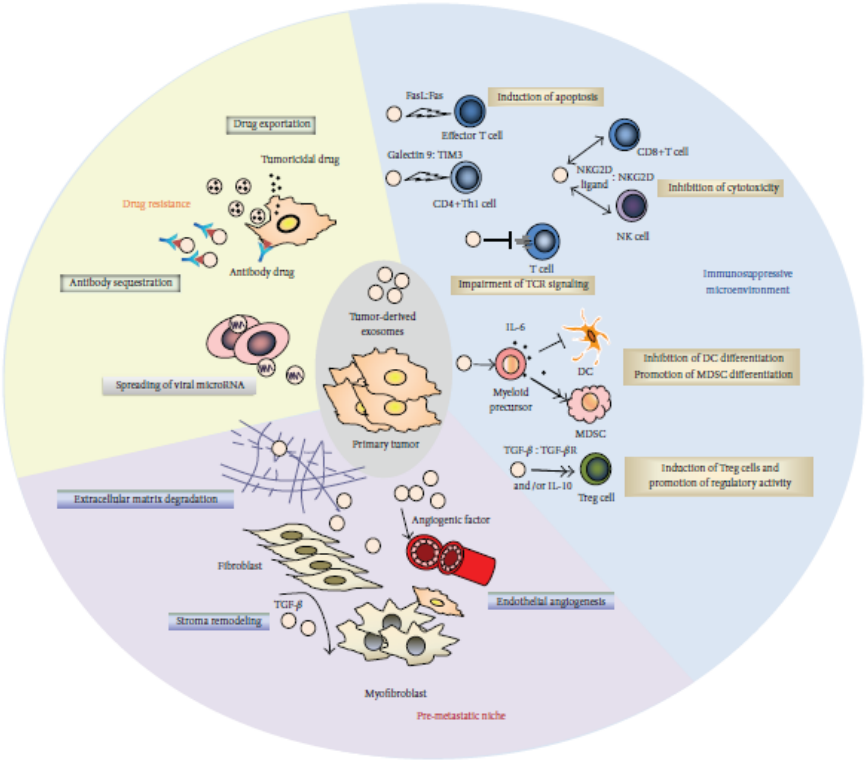

Fig. 6: The protumorigenic role of tumor-derived exosomes

Another mechanism that have a role in cell-cell communication within the tumor microenvironment is the intercellular exchange of proteins and genetic materials via exosomes (110). In particular, transport of mRNAs and microRNAs, from tumor cells to neighboring cells could have significant effects on tumorigenesis. It has been suggested

that the ability of exosomes to deliver nucleic acids to distal cells also makes them ideal candidates for gene therapy (111).

Antitumorigenic role of tumor-derived exosomes

Immune responses can be triggered by tumor-derived exosomes that contain tumor-specific antigens and peptide-MHC complexes. Antigens that are commonly present in tumour-derived exosomes include melan-A (112), carcinoembryonic antigen (CEA) (113), and mesothelin (108). Immunization of mice with Dendritic cells (DCs) pulsed with cancer cell–derived exosomes demonstrate that it is possible to induce protective antitumour immune responses using cancer-derived exosomes as a source of antigens (114). Similarly, in an *ex vivo* human model system, exosomes taken from malignant effusions proved an effective source of tumour antigens for cross-presentation to $CD8^+$ cytotoxic T cells by DCs (112). Expression of heat shock proteins (such as Hsp70) at the exosome surface is important, not only as a cofactor for efficient receptor-mediated uptake, but also for "danger" signals that trigger DC maturation and that subsequently enhance immunologic activation. Thus, exposing cancer cells to stress may render their exosomes significantly more immunogenic (115). DC have an active role in processing and cross-presenting exosomally delivered antigens, but this immune function depends from cancer exosomes phenotype and the influence of the microenvironment. Exosomes are able to activate also monocytes to secrete pro-inflammatory cytokines (116) and activate B cells through CD40L, thereby bypassing the need for CD4+ T cell help (117). Recently, ligands for NK cell-activating receptors on exosomes derived from immature human DCs were shown to promote NK cell activation *in vitro.* This occured through UL16 binding protein 1, MHC class I polypeptide-related sequence A (MICA) and MICB (which are ligands for NKG2D) on exosomes secreted by DCs from healthy

volunteers or patients with melanoma (118). The ability of these exosomes to activate NK cells was apparently due to the simultaneous presence of the IL-15 receptor α-chain (Il-15Rα) on the DC-derived exosomes, which could bind to exogenous Il-15 and thereby promote NK cell proliferation (119).

In addition to the potential immunostimularoty effects, a proapoptotic function of tumor-derived exosomes directly on tumor cells was also reported. Exosomes from human pancreatic tumor cells were reported to increase Bax and decrease Bcl-2 expression, inducing tumor cells toward apoptotic pathway (120).

Exosomes and Angiogenesis

It has been showed that exosomes are able to alter microenvironment through their protein content. Tumor derived exosomes can contribute to establish an oncogenic niche via delivery of protein, mRNA and miRNA that support angiogenesis, cell proliferation and cell survival (Fig. 7).

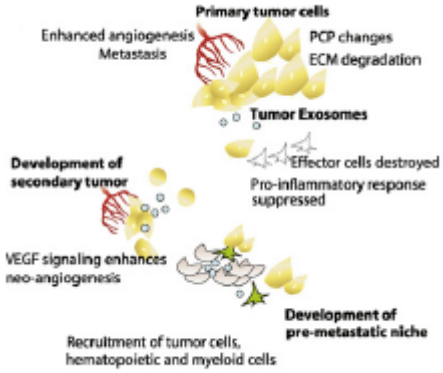

Fig. 7: Exosomes alter microenvironment and recipient cells. Exosomes help to establish a metastatic niche to aid tumor growth and tumor metastatis.

Tumor derived exosomes contain several cytokines and growth factors such as tumor necrosis factor TNF-α, IL-1β, and TGF-β as well as functional receptors like TNFR1 and TfR2 and through these molecules enhance recruitment of hematopoietic and

endothelial precursor cells to enhance neoangiogenesis in the tumor (121). Proteomic analysis of mesothelioma cell-derived exosomes, detected the presence of angiogenic factors that can increase vascular development in tumor microenvironment (122). Hood et al, showed that melanoma-derived exosomes stimulated endothelial signalling important for tissue matrix remodelling and endothelial angiogenesis (123). Recent studies showed that pancreatic tumour-derived exosomes increase matrix metalloproteinase (MMP) secretion and VEGF expression in target cells through the expression of proangiogenic molecules, such as members of the Tetraspanin family, thus promoting neo-angiogenesis even at tumour distant sites (124). Tetraspanins, which are constitutively enriched in exosomes, have been found to contribute to exosome-mediated angiogenesis. It was reported that exosomes derived from a pancreatic tumor line over-expressing D6.1A, strongly induced endothelial cell branching *in vitro* and angiogenesis *in vivo* in a rat model (124). Tumor derived D6.1A stimulates the secretion of matrix metalloproteinase and urokinase-type plasminogen activator, enhances the expression of vascular endothelial growth factor expression in fibroblasts, and upregulates the expression of endothelial growth factor receptor. Exosomal Tspan8 (D6.1A) was found to contribute to the selective recruitment of proteins and mRNA into exosomes, including CD106 and CD49d, both of which were implicated in the binding and internalization of exosomes by endothelial cells. Induction of several angiogenesis-related genes, including von Willebrand factor, Tspan8, chemokines CXCL5 (C-X-C motif chemokine 5), MIF (macrophage migration inhibitory factor), chemokine receptor CCR1, together with enhanced endothelial cell proliferation, migration sprouting and maturation of endothelial cell progenitors, were seen upon exosome internalisation (125). Circulating exosomes obtained from plasma of glioma patients were positive for the mutant/variant mRNA of epidermal growth factor receptor (EGFRvIII), which

defines a clinical subtype of glioma. Interestingly, these exosomes display proangiogenic properties, indicating that glioma derived exosomes play a role in initiating angiogenesis (126). Skog et al, showed that these exosomes mediate transfer of mRNA. Transfer of mRNA can modify the translational profile of these cells and promote acquisition of the angiogenic phenotype. Nevertheless, those particles also contain growth factors and proteases and have been shown to stimulate tubule formation in endothelial cells (126). Recently, it has been shown that Delta-like ligand 4, a transmembrane ligand for the Notch family of receptors, is incorporated in exosomes. Notch signaling is a cell-cell signaling pathway that have a role in several biologic processes, such as cell fate determination and differentiation (127), and also it is implicated in vascular development and angiogenesis (128). Delta-like 4 (Dll4) expression is particularly critical for angiogenesis (129), and its expression is restricted to the endothelium of developing vessels and regulate the vessel sprouting and branching (128). Exosomes that have Dll4 can transfer this protein from one cell type to another and incorporate it into the plasma membrane *in vitro* and *in vivo*. Dll4-containing exosomes increases capillary-like structure formation *in vitro* and *in vivo* by a mechanism that implicates the transfer of Delta-like-4 into the endothelium. This suggests that Delta like/Notch pathway does not require direct cell–cell contact to expand its signaling potential on angiogenesis (130). Taraboletti et al, have shown that matrix metalloproteinases contained in exosomes released from endothelial cells are functionally active and lead to endothelial cell invasion and capillary-like formation (131). Owing to the multifunctional protein repertoire transported by tumour exosomes, it could be hypothesized that they may also contribute to the process of stromal remodelling, thus widening the role of these microvesicles in tumour progression and metastasis formation. Exosomes are critical for some pathological disease processes,

then find a strategy of targeting the formation and/or release of exosomes in specific cell types, would represent a novel route of therapeutic intervention.

Exosomes as biomarkers

Exosomes have been shown in physiological and pathological fluids such as bronchoalveolar lavage (132), human plasma (45), malignant and pleural effusions (48,133), urine (46), breast milk (134), human saliva (135), synovial fluid (136) and malignant ovarian ascites. The presence of these bioactive vesicles in plasma and malignant effusions of patients with cancer (48) suggests that exosomes may lead to potential diagnostic biomarkers of disease conditions and play an important role in cancer diagnosis. Most cancers, such as ovarian cancer, are diagnosed at advanced stage and prospects for significant improvement in survival reside in early diagnosis then it is necessary to find non-invasive cancer biomarkers to detect cancer in its early stages. Currently, effective screening protocols are not available, because the classical biomarkers, for example CA125 for ovarian cancer, is thought to be robust only in following the response or progression of the disease, but not as a diagnostic or prognostic marker (137). Thus, it is important to identify additional diagnostic and prognostic markers for this disease. The increased levels of plasma-exosome in patients with advanced disease [e.g., mean 2.85 mg/mL exosomes for lung cancer adenocarcinoma patients compared with 0.77 mg/mL exosomes in the blood of normal volunteers (138)] and the discovery of cancer miRNA profiles in circulating plasma-derived exosome, giving exosomes an attractive biomarker candidate. Because exosomes transport molecules involved in cancer progression they can be used as markers, for example in exosomes may be present cancer-associated antigens not available as soluble molecules within biological fluids, such as the oncofetal

glycoprotein-5T4; which is over expressed by epithelial cancers but not shed from the cell surface (139). In a recent study, prostate cancer biomarkers, PCA-3 and TMPRSS2; ERG, were detected in exosomes isolated from the urine of prostate cancer patients (140), and they have been proposed as a possible source of multiple biomarkers of renal disease (46,141) and urological cancer. Exosomes are also an attractive biomarker candidate for bladder cancer. The differentially expressed proteins include psoriasin, kertain-14, galectin-7, epidermal fatty acid binding protein (E-FABP), migration inhibitor factor-related protein (MRP8) and stratifin, which may be useful markers for the diagnosis of bladder cancer (46).

In the past years, the importance of miRNA in cancer cells has been recognized. MicroRNAs, small (22–25 nucleotides in length) noncoding RNAs, suppress the translation of target mRNAs by binding to their 3′ untranslated region (142,143). Post-transcriptional silencing of target genes by microRNA can occur either by cleavage of homologous mRNA or by specific inhibition of protein synthesis. A correct control of miRNA expression is essential for cellular processes, such as proliferation, differentation, development and cell death. Valadi et al. reported the presence of mRNA and miRNA in exosomes from mouse and human mast cell lines. Exosomes are able to transport biologically mRNA and miRNA to neighboring cells that confer new functions to the recipient cells (111). These studies highlight the potential of exosomal microRNA profiles for use as diagnostic biomarkers of disease through a non invasive blood test. The expressions of individual microRNAs and specific microRNA signatures have now been linked to the diagnosis and prognosis of many human cancers. A possible obstacle in body fluid based exosome analysis is the presence of contaminating exosomes secreted by normal cells (e.g., normal ovarian cells as compared to ovarian cancer cells). To discriminate two population of exosomes can be used specific purification

strategy, as a magnetic bead immune capture technique was employed to isolate circulating epithelial cell adhesion molecule-positive exosomes from the plasma of ovarian cancer and lung cancer (Fig.).

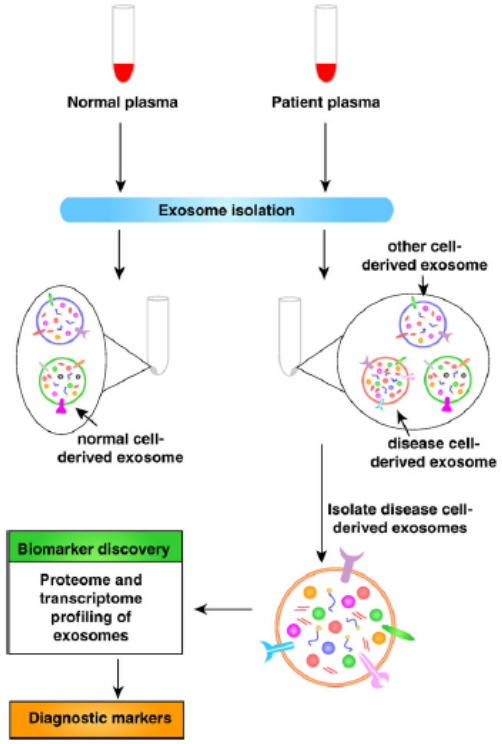

Fig. 8: Circulating exosomes can be a rich source for identifying potential biomarkers. Patient plasma contains exosomes that are released by disease cells (e.g., colorectal cancer cells), normal counterpart (e.g., normal colon cells) and other normal cells (e.g., liver). Exosomal tissue signatures can be used to isolate disease cell-derived exosomes for proteomic and transcriptomic profiling.

Currently, a major goal is to identify disease biomarkers in biological fluids, in a non-invasive manner, that can be measured relatively inexpensively for early diagnosis of disease and treatment success.

Exosomes in Immunotherapy

The first antitumor effects of exosomes was demonstrated by Zitvogel et al, they showed that exosomes from DCs pulsed with tumor peptides could prime a specific cytotoxic T lymphocyte (CTLs) *in vivo* and suppress growth of established tumors when injected into mice (38,144). Particularly in combination with TLR 3 and 9 ligands, exosomes efficiently induced anti-tumor responses in mice (145). These promising

results in mice led to the development of techniques for the clinical testing of tumor antigen loaded DC exosomes in human advanced cancer patients (66).

When dendritic cells are pulsed with cancer antigens or tumor peptides (38) dendritic cell-derived exosomes (DEX) has been shown to elicit stronger immune responses toward cancer cells (146) with up-regulation of specific antibody release and cytokine production (147). Furthermore, it has also been reported that DEX trigger NK cells in the immune response toward cancer cells via NKG2D-dependent NK cell activation and IL-15Rα-dependent cell proliferation (119).

Some clinical trials have been performed in humans with advanced staged melanoma and non small cell lung cancer. Patients were treated with tumor peptide loaded DC exosomes and the outcome was promising. The treatment was well tolerated and some patients showed long term stability of the disease (148,149) (Fig. 9).

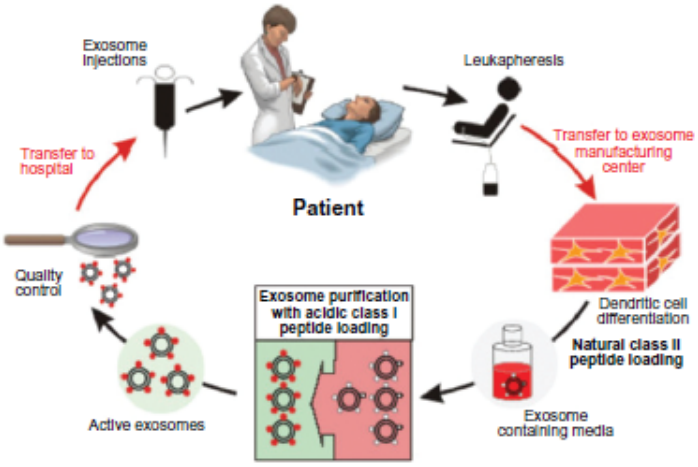

Fig. 9: Clinical grade exosomes in immunotherapy. The process of how DEX can be derived, purified, and utilized in cancer treatment. Creative Commons. Reproduced with permission from Escudier B, Dorval T, Chaput N, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. *J Transl Med*. 2005;3(1):10.

Although it has been established from the results in clinical trials that exosomes can be safely administered, it is necessary to increase their potency to elicit appropriate immune A phase II clinical trial is planned to follow-up the initial positive results (150).

responses to kill cancer cells. Different strategies have been applied to increase the immunogenicity of exosomes, for example the addition of adjuvant such as double stranded RNA or CpG (150). Another strategy that has been tested is the modulation of the exosome composition. Exosomes from mature DC have been shown to be more immunogenic than those from immature DC (151). Recent evidence suggests that a new strategy is represented by artificially coating and engineering exosomes (152) with tumor antigens to make it more recognizable to the immune system. It has been experimentally demonstrated that using nanotechnology, exosomes can be engineered to bear an optimal number of MHC I and ligands that would activate T cells, and their *in vivo* activity can be traced by encapsulating superparamagnetic iron oxide nanoparticles (153). Exosomes can also carry cytokines, DNA, RNA, adjuvants, labels, costimulatory signals, and gene therapy vectors, which makes it ideal to develop new immunotherapy strategies.

Immune system and tumor

The immune system is essential in tumor progression modulation and elimination of cancer cells. Immune cells, from both the innate and adaptive immune systems, are able to recognize, attack, and kill tumor cells in various ways. They collaborate and stimulate each other (e.g. with IFN-γ) to induce a potent immune response against the tumor cells. However, cancer still occurs. Tumor cells are able to fight back or hide from the immune system. The interaction between tumor and immune system consist of three phases and is known as cancer immunoediting (154). During the first phase (the elimination or cancer immunosurveillance phase) the immune system is able to protect the host from a developing tumor. However, some transformed cells may escape the immunological pressure, thereby entering the second phase (the equilibrium phase). During this period of immune-mediated latency, the tumor persists and acquires new mutations. This may allow the tumor to enter the third phase (tumor escape) during which established tumors become clinically manifest (155).

The innate immune system provides the first line of immediate defense and includes natural killer (NK) cells, γδ T cells, monocytes, macrophages, neutrophils and dendritic cells. NK cells are able to discriminate between self and non-self. The first activating signal for NK cells is a loss of MHC class I molecules on the surface of cancer cells (156). MHC class I molecules act as an inhibitory signal for NK cells, thus making MHC class I lacking tumor cells a target for NK cells. NK cells use their large repertoire of inhibitory and activating receptors to detect the transformed-self. Many tumor cells express activating NK ligands (like MICA and MICB) recognized by NKG2D and are thus efficiently eliminated by NK cells and γδ T cells (157). Tumors express antigens that are recognized as foreign by the immune system of the tumor-bearing host. The immunogenicity of tumors implies that tumor cells express antigens that are recognized

as foreign by the adaptive immune system. These tumor antigens, presented on the surface of antigen presenting cell (APCs), are recognized by T lymphocytes cells and leading to a tumor specific reaction. To mount an adaptive immune response, two signals are mandatory. The first signal is provided by the presentation of an antigen in the groove of an MHC molecule to the T cell receptor (TCR).The second signal is provided by the ligation of co-stimulatory molecules (the most important being CD28 on T cells interacting with CD80 and CD86 on the APCs (158). These two signals activate the adaptive system to recognize and kill tumor cells. After successful recognition of the tumor cell by innate and adaptive effector cells, cytotoxic T lymphocytes, γδ T cells and NK cells both utilise a pore-forming protein, perforin, and a battery of serine proteases (granzymes) that activate caspases, leading to apoptosis. The apoptotic program can also be activated after binding of TRAIL or Fas-ligand, expressed on the cell surface of the effector cell, to one of its cognate receptors. These lymphocytes are able to kill tumor cells via cytokines, such as IFNγ, TNFα and TNFβ. Tumor cells have developed numerous strategies to change their phenotype and mislead the immune system. Tumor cells can change their cell surface phenotype to prevent recognition and binding by immune effector cells. The most wellknown example of this strategy is the downregulation of MHC class I molecules (or molecules involved in antigen processing by MHC class I molecules) resulting in defective binding to the TCR of cytotoxic CD8+ T cells (159,160). However, downregulation of MHC class I renders cells susceptible for NK cell mediated killing. Consequently, tumor cells have also developed mechanisms to escape from NK surveillance: for example upregulation of the NK inhibitory HLA-G3 (161). Other strategies are the down-modulation of co-stimulatory molecules or upregulation of pro-apoptotic molecules like FAS-ligand on their cell surface to induce apoptosis of effector cells (162). Another strategy is to

prevent the pro-apoptotic signals after effective recognition and binding of immune cells. An example of this evasion of immune mediated killing is to downregulate proapoptotic death receptors or to upregulate anti-apoptotic or decoy receptors (for example TRAIL-R3). Over-expression of anti-apoptotic proteins, like XIAP (X-linked inhibitor of apoptosis) (163,164) or molecules that block the granzyme B/perforin pathway like (pI-9)(165) are other examples. The third way, is to produce immune suppressive factors that inhibit T cell function and lead to a local state of tolerance and anergy. Examples of this strategy are the production of transforming growth factor beta (TGF-β)(166), or indoleamine 2,3-dioxygenase (IDO)(167) or IL-10.

Recently, mounting evidence is indeed pointing to exosomes as major participants in immune evasion with several mechanisms. Among the earliest reports there is a description of melanoma-derived exosomes that were lethal to T cells (102). These cancer cells naturally express Fas ligand, and may expel by the multivesicular endosomal route at least a proportion of this molecule in exosomes. FasL–bearing exosomes, upon encountering activated (Fas-positive) T cells, can essentially crosslink T cell Fas and trigger apoptotic death (102). Other influences of exosomally expressed members of the tumour necrosis factor superfamily may include downmodulation by ovarian cancer exosomes of the CD3-ζ chain. This molecule is an integral component of the TCR complex, which is essential for competent signalling after TCR–MHC–peptide interactions (168). Melanoma exosomes expressing TNF-α may also affect the CD3–TCR complex in a reactive oxygen species–mediated manner (169). Thus, cancer exosomes can exert drastic effects on T-cell functions and may constitute an important mechanism by which tumours eliminate activated T cells that may recognize and kill them (102). Other death-independent effects of cancer exosomes on the immune system have been reported. Liu *et al.*, for example, pretreated mice with breast cancer

exosomes before implanting tumours and documented accelerated tumour growth (170). This accelerated growth was result of the negative influence of cancer exosomes on NK cell functions, inhibiting NK cell proliferation and impairing cytotoxic functions. Other studies showed that exosomes from cervical cancer harbor NKG2D ligand such as MICA*008 or ULBP3 proteins, which suppress Natural Killer cell toxicity (171).

Thus, tumor exosomes bearing NKG2D ligands can act on effector cells to down regulate NKG2D, impairing part of the immune response (106). In addition to NKG2D ligands, it was shown that tumor exosomes carry and express growth factors such as TGFβ1. Membrane-bound TGFβ1 on these vesicles displays the same function as NKG2D ligands i.e. the down regulation of surface NKG2D expression by NK cells and CD8+T cells following a direct interaction between exosomes and cells. Tumor-derived exosomes promote other immune escape mechanisms by triggering myeloid suppressive cells proliferation (MDSC). These cells are found in large number in lymphoid organs, blood and tumor tissues in cancer patients and are immature myeloid cells. These cells express myeloid marker stimulatory molecules (CD14 and CD11b) and are devoid of co-stimulatory molecules (HLA DR, CD80, CD86). They spontaneously secret TGFβ and have suppressive activity on activated T lymphocytes since they are able to inhibit T cells proliferation and cytolytic functions (172). It was shown that melanoma and colorectal carcinoma derived exosomes altered the monocyte differentiation into dendritic cells, leading to the generation of myeloid suppressive cells (172). Furthermore, it was demonstrated that MDSC-mediated promotion of tumor progression was dependent on TGF-β present on exosomes, but also depended upon the lipidic mediator prostaglandin E2 (PGE2) transported by tumor exosomes (173) (Fig. 10). These pleiotropic effects lead to hypothesize that interfering with exosome release by tumour cells may perhaps represent a novel strategy for simultaneously recovering

multiple immune functions in cancer patients, then the knowledge on the immune evasion mechanism exosomes-mediated could help for developing a successful, multi-level and multi-target anti-cancer therapy.

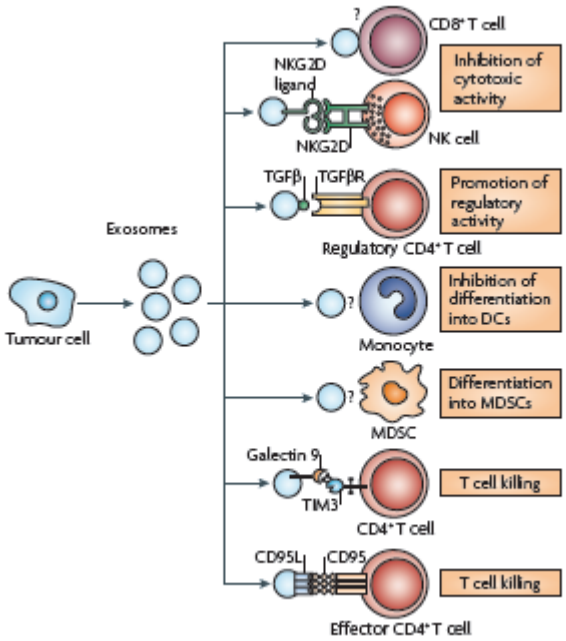

Fig. 10: Inhibitory functions of exosomes derived from tumor cells. Inhibition of T cell activation, inhibition of NK cell and CD8+ T cell cytotoxicity, promotion of regulatory T cell activity, inhibition of monocyte differentiation into DCs and promotion of monocyte differentiation into myeloid-derived suppressor cells (MDSCs), T cell killing through ligation of CD95 by CD95 ligand (CD95L)

γδ T cells

γδ T cells are innate effector cells able to act as firs-line defense against tumors and infections, but also to enhance antigen-specific immune responses mediated by conventional T cells and antibodies. They account for 5–10% of CD3+ peripheral blood T cells but constitute a dominant T-cell fraction at other anatomical sites such as the intestinal epithelia. γδ T cells differ from conventional αβ T cells in several aspects. αβ T cells are “conventional” T cells with a T cell receptor (TCR) composed of the higly variable α and β chains. They comprise two subpopulations CD4+ and CD8+ T cells and their activation depends on the interaction with professional APCs, such as dendritic

cells. γδ T cells are “unconventional” T cells that express a TCR composed of the δ and γ chains. Most γδ T cells lack CD4 or CD8 antigens and hence display a "double-negative" phenotype, although a sizeable fraction expresses CD8 (174). The absence of CD4 or CD8 expression on the majority of circulating γδ T cells is well in line with the lack of MHC restriction in antigen recognition of this T-cell subset. γδT cells often show tissue specific localization of oligoclonal subpopulations sharing the same TCR chains (175). For instance, murine skin γδ T cells, so-called dendritic epidermal T cells (DETCs), are largely Vγ5/Vδ1+ while in the human peripheral blood, the majority of γδ T cells express a TCR composed of Vγ9 and the Vδ2 chains (Vγ9/Vδ2 T cells). Similar to αβ T cells, Vγ9Vδ2 T lymphocytes comprise distinct populations distinguishable on the basis of surface markers, effector functions and trafficking properties. Naive (T_{naive}, CD45RA+CD27+) and central memory (T_{CM}, CD45RA–CD27+) cells home to secondary lymphoid organs and lack immediate effector functions, whereas the so-called effector-memory (T_{EM}, CD45RA–CD27–) and terminally differentiated (T_{EMRA}, CD45RA+CD27–) cells home to sites of inflammation where they display immediate effector functions such as cytokine production and cytotoxicity (176,177). Vγ9/Vδ2 T cells recognize through their TCR a set of non-peptidic small molecular compounds in an MHC-independent manner. This interaction is sufficient for their activation and does not require interaction with professional APC. γδ TCR enables them to recognize families of unprocessed nonpeptide compounds of low molecular weight (100-600 Da) with conserved patterns, comprising natural phosphoesters derived from mycobacteria and other pathogens, referred to as phosphoantigens, and to a lesser extent several ubiquitous metabolites such as alkylamines from plant extracts, xylosyl-or ribosyl-1-phosphate, 2,3-diphosphoglycerate and several synthetic aminobisphosphonates (NBPs)(178,179,180,181). The most potent Vγ9/Vδ2 T cell ligand, (*E*)-4-hydroxy-3-

methyl-but-2-enyl pyrophosphate (HMB-PP) is an intermediate metabolite of the microbial non-mevalonate pathway (MEP) of isoprenoid biosynthesis (182,183). Through this particular specificity for microbial molecules, Vγ9/Vδ2 T cells display an anti-microbial immune response (184). After their activation, Vγ9/Vδ2 T cells expand rapidly and secrete different proinflammatory cytokines. These cytokines mediate the activity of other immune cells to eliminate the microbe, such as interferon-γ (IFN-γ) increasing the microbial degradation in macrophages lysosomes. Activated Vγ9/Vδ2 T cells also recognize infected cells and induce their cell death through the secretion of apoptosis-inducing factors such as perforin, granzyme and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Fas-ligand expressed on Vγ9/Vδ2 T cell membrane can also directly interact with its receptor Fas on target cells and induce apoptosis. γδ T cells also recognize metabolites of the mevalonate pathway of the isoprenoid biosynthesis, present in all eukaryotic cells. Isopentenylpyrophosphate (IPP) is one prototype of these phosphorylated nonpeptidic ligands. Overproduction of these metabolites may allow γδ T cells to target cells with deranged metabolic activity of the mevalonate pathway (185). Notably, an increased expression of hydroxy-methylglutaryl-CoA reductase (HMGR), the rate limiting enzyme of the mevalonate pathway, has been reported in hematological malignancies and mammary carcinoma cells (186). Accumulated IPP in tumour cells is suggested to bind the Vγ9/Vδ2 TCR, thus acting as an activation signal, although the mode of recognition of this ligand remains unclear. When activated they release pro-inflammatory chemokines (e.g. MIP-1, RANTES) and T-helper cell type 1 (Th1) cytokines (e.g. IFNγ and TNFα), and proliferate in the presence of IL-2. Some hemopoietic tumor cell lines, such as the Daudi Burkitt's lymphoma and the RPMI 8226 myeloma line are spontaneously recognized and lysed by Vγ9/Vδ2 T cells in vitro (187,188). The molecular basis of

such a spontaneous tumor cell recognition has not been fully elucidated yet. One possibility is that tumor cells express induced self ligands like stress-inducible MHC class I-related MICA/MICB molecules, heat shock proteins (HSP), and/or other ligands that are recognized through the Vγ9/Vδ2 TCR or through coreceptors like the NK receptor member D of the lectin-like receptor family (NKG2D)(189). Most tumour cells express ligands for the NKG2D receptor such as the MHC class I chain-related genes (MIC) A/B, and proteins of the UL16-binding protein (ULBP) family (190). These molecules bind to the NKG2D receptor expressed on Vγ9/Vδ2 T cells, acting also as an activation signal (Fig. 11).

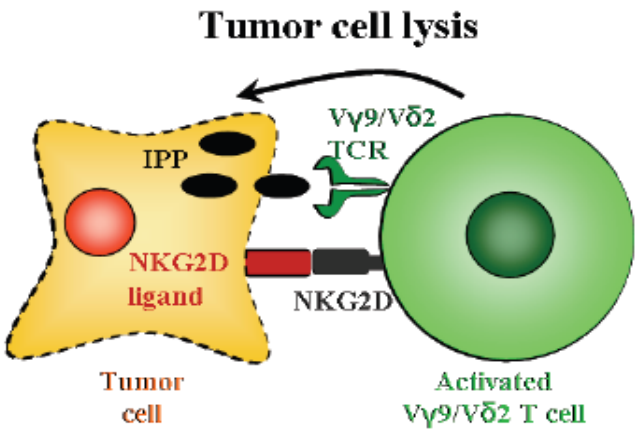

Fig. 11: Tumour cell killing capacity of activated Vγ9/Vδ2 T cells. Vγ9/Vδ2 T cells recognize via their TCR isoprenoid metabolites such as IPP that may accumulate in tumour cells. They also recognize via NKG2D stress-induced ligands that are often expressed on tumour cells. Stimulation of either or both receptors activates Vγ9/Vδ2 T cells and leads to tumour cell lysis.

After recognition of these molecules, γδ T-cells become activated to produce cytokines and eliminate a target cell. Killing is performed via the perforin / granzyme pathway and death-receptor-dependent pathways (180). Upon activation of a cytotoxic lymphocyte, granules containing perforin and granzymes are released in the synaptic cleft between the killer and target cell. Perforin makes pores in endosomes and granzymes enter in the cytoplasm of the target cells and activate apoptosis-pathway

(180). There are different death-receptor-dependent pathways involved in the killing of tumor cells (181). The most important death ligands are FasL, TRAIL and TNF. These death domain-containing adaptor molecules start the proteolytic caspase cascade by activating caspase-8 and -3 in the tumor cell, leading to apoptosis. Recently, it has been shown that activated Vγ9/Vδ2 T cells are a novel type of professional APC (191) similarly to DCs, macrophages and B cells. APCs present MHC molecules linked to a endogenous or exogenous peptide on the cell surface and interact with the αβ TCR of CD8+ T cells and CD4+ T cells. Activated Vγ9/Vδ2 T cells have been shown to present all these functions, these cells efficiently processed and displayed antigens and provide costimulatory signals sufficient for strong induction of naïve αβ T cell proliferation and differentiation (192). Thus, Vγ9/Vδ2 T cells have the ability to bridge innate and adaptive immune responses.

Vγ9/Vδ2 T cells are important contributors to innate and adaptive immune responses against tumor cells and pathogens, then it is possible to develop a novel strategy for treatment of solid and haematological cancer utilizing the mode of action of γδ T cells.

Bisphosphonates and γδ T cells

Bisphosphonates (BPs) are synthetic drugs that prevent bone resorption and are used for the treatment of Paget's disease, tumour-associated bone diseases and osteoporosis. Based on their chemical structure, BPs are traditionally divided into two pharmacological classes with distinct molecular mechanisms of action: nitrogen-containing (N-) and non-nitrogen containing (non-N) drugs. Bisphosphonates that lack a nitrogen atom are metabolized into non-hydrolyzable proapoptotic ATP analogues that accumulate in the cytosol of osteoclasts (193). The resulting accumulation of non-hydrolysable analogues of ATP induces osteoclast apoptosis and inhibits bone resorption

(194). By contrast, N-BPs interfere with a specific enzyme in the mevalonate pathway, farnesyl pyrophosphate synthase (FPPS), thereby depleting the osteoclasts of isoprenoid lipids (195,196). More specifically, FPPS inhibition by N-BPs blocks the covalent attachment of isoprenyl chains to small GTPases (*e.g.*, Ras, Rac, Rho), which is crucial for their intracellular localization and functions in osteoclasts. In addition to the effects on the function of small GTPases, the disruption of the mevalonate pathway by N-BPs results in the accumulation of isopentenyl pyrophosphate (IPP), which is then converted to a cytotoxic adenosine triphosphate analogue (ApppI) that can directly induce osteoclast apoptosis (197,198). Thus, N-BPs may exert their pharmacological effects on osteoclasts through the formation of ApppI or via the inhibition of protein prenylation, particularly of small GTPases. Moreover, N-BPs have been tested for a wide range of solid and haematopoietic cancers. *In vitro* studies show that N-BPs have cytostatic activity against tumor cells, induce apoptosis and also inhibit cell adhesion. Zoledronic acid (ZA) is a newer N-BP with a tertiary amino group included within a ring structure. It can be considered the most potent and widely used intravenous BP that prevent the delayed onset of skeletal related event (SRE) in patients with bone metastases from any type of tumour and also for the treatment of hypercalcemia of malignancy (199,200). ZA is approved for the treatment of patients with bone metastasis from breast cancer, hormone refractory prostate cancer, as well as other solid tumors and multiple myeloma (201). A very interesting property of N-BPs in antitumor immune responses is the ability to activate Vγ9Vδ2 T cells. Through inhibition of FPPS of the mevalonate pathway, N-BPs trigger the intracellular accumulation of IPP and exerting strong activation of Vγ9Vδ2 T cells. Furthermore, zoledronate induces functional changes in Vγ9Vδ2 T cell subsets (202). *In vivo*, it promotes the differentiation of Vγ9Vδ2 T cells toward CD45RA-CD27- γδ T cells, which produce interferon-γ and exert cytotoxicity,

while decreasing CD45RA+ CD27+ naive and CD45RA-CD27+ memory γδ T cells (202). This effect is specific to Vγ9Vδ2 T cells. Neither human γδ T cells expressing the Vγ9Vδ1 TCR, nor human αβ T cells, monocytes, NK or B cells are responsive to N-BPs (202,203).

Previous studies have demonstrated that zoledronate sensitizes chemotherapy-resistant tumor target cells (prostate,breast and lung cancer) to Vγ9Vδ2 T cell cytotoxicity, rendering these cancer cell lines highly susceptible to Vγ9Vδ2 T cell-mediated killing (204). Recently, interest has emerged on the use of zoledronate in CML, because this drug synergistically augments the anti-Ph+ leukaemia activity of imatinib both in vitro and in vivo (205,206), and inhibits proliferation and induces apoptosis of imatinib-resistant CML cells (207). D'Asaro et al. showed that pre-treatment of sensitive and resistant CML cells with zoledronate alone or in combination with imatinib significantly increased killing by Vγ9Vδ2 T lymphocytes (208). They showed also that Vγ9Vδ2 T cells kill cells freshly isolated from patients with CML, at time of diagnosis and before therapy, but exclusively when cells were pretreated with zoledronate alone or in combination with imatinib (208).

The unique ability of bisposphonates to render tumour cells susceptible to Vγ9Vδ2 T cell attack makes these drugs particularly interesting candidates for use in γδ T cell therapy.

AIMS

The overall aims of this project were to assess the release of exosomes by LAMA84 CML cell lines and to elucidate their role in angiogenesis. To evaluate this, I tested if the addition of those exosomes to human vascular endothelial cells (HUVEC) affects several step of in vitro angiogenesis including motility, cytokine production, cell adhesion and cell signalling, as well as stimulation of angiogenesis in a nude mouse assay. To confirm the data obtained with exosomes derived from LAMA84 cells, I used exosomes isolated from blood of CML patients to understand better how CML cells can induce an angiogenic phenotype. Another field of investigation was the study of the effects of exosomes on γδ T cells. These lymphocytes exhibit a potent MHC-unrestricted lytic activity against several tumor cells and are able to kill CML cells pretreated with zoledronic acid. Zoledronic acid is a syntethic aminobiphosphonate that is able to activate γδ T cells, augments the anti-Ph^+ leukemia activity of imatinib both *in vitro* and *in vivo* and inhibits proliferation and induces apoptosis of imatinib-resistant CML cells. I started to analyze if exosomes from CML cell lines have an activatory or inhibitory effects on these lymphocytes and if zoledronic acid affect the release or the effects of exosomes. The understanding of the mechanism of communication between leukemic cells and the microenvironment and the mechanism by which tumor cells can escape immune system could promote the development of new therapies to overcome drug resistance observed sometimes in the treatment of CML.

The more specific aims were to investigate:

- Detect and characterize microvesicles released from CML cell lines (western blot and enzymatic assays)
- Evaluate the effects of exosomes on endothelial cells (motility, wound healing, adhesion assays)

- Evaluate the effects of exosomes on *in vivo* angiogenesis assay
- Evaluate the effects of exosomes on γδ T cells functions (cytokines release, NKG2D, CD69 and CD25 expression)
- Evaluate the effect of zoledronic acid on the release and effects of exosomes

Materials and Methods

Cell culture

LAMA84 and K562 chronic myelogenous cells, were cultured in RPMI 1640 (Euroclone UK) supplemented with 10% FBS (Euroclone UK), 2mM L-glutamine (Euroclone UK), 100 U/ml penicillin and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC, Lonza, Clonetics, Verviers, Belgium) were grown in endothelial growth medium (EGM) supplemented with Hydrocortisone, hFGF-B, 2 ml; VEGF, 0.5 ml; R3-IGF-1, 0.5 ml; Ascorbic Acid, 0.5 ml; Heparin, 0.5 ml; FBS, 10 ml; hEGF, 0.5 ml; GA-1000, 0.5 ml. LAMA84 and K562 cells were maintained at concentration of $0{,}3x10^6$/ml to grown, and were expanded at $1x10^6$/ml to recover exosomes. For treatment with Zoledronic acid (gift from Novartis Pharma, Milan Italy), 5 μM/ml of the drug was added when K562 cells were seeded for exosomes at concentration of at $1x10^6$/ml. HUVEC cells were cultured in Petri dish, were harvested using trypsin-PBS 1:1 and seeded for the experiment. Cultures are placed in a humidified 95% air and 5% CO2 atmosphere at 37° C.

PBMC isolation

Human blood samples were obtained from healthy donors, after written informed consent was obtained, in accordance with the Declaration of Helsinki guidelines and University of Palermo Ethics committee. Human peripheral blood mononuclear cells (PBMC) were isolated using the Ficoll-Paque (GE Helthcare-Bio Science, Uppsala, Sweden) separation technique. Whole blood was diluted in RPMI, layered over Ficoll-Paque and centrifuged at 1400 × *g* for 20 min. The mononuclear cells (interphase layer) were collected, washed twice in complete medium (RPMI 1640 medium, 10% FBS, 1%

penicillin-streptomycin) and finally resuspended in complete medium. PBMC were cultured at concentration of $1x10^6$/ml to recover exosomes.

γδ T cell isolation

For γδ T cells isolation, whole blood were taken from healthy donors at Cardiff University, according to the local ethical guidelines on experimentation with human samples. Blood was layered on Lymphoprep (Axis-Shield), and centrifuged at 1700 rpm for 20 min. PBMC were recovered and washed twice with MACS buffer (5mM EDTA; 2%FBS; PBS 1X) and centrifuged at 1500 rpm for 10 min and 1000 rpm for 10 min. Pellet was resuspended in 1ml of MACS buffer and was added a blocking IgG antibody (1:100) for 10 min on ice. The cells were staining with Vγ9 PE-Cy5 (1:400) for 15 min on ice. Cells were washed in MACS buffer at 1300 rpm for 8 min and resuspended in 400 μl MACS buffer plus anti-PE microbeads (Milteny) for 15 min in the fridge. Cells were resuspended in 2 ml of MACS buffer and placed onto LS column. After 3 washes with MACS buffer, γδ T cells were recovered a 15ml falcon tube. γδ T cells was counted and seeded for all experiment at 50000 cells/well at 37 °C in 5% CO2. Cultures were incubated in 96 well plate overnight in the presence of 10 nM HMB-PP to activate Vγ9/Vδ2 T cells, and 20 U/mL interleukin-2 (IL-2) (Proleukin, Chiron).

Exosome isolation

Exosomes produced by LAMA84, K562 treated with ZA and K562 cells during a 24 hr culture period, were isolated from conditioned culture medium supplemented with 10% FBS (previously ultracentrifuged) by differential centrifugation. Conditioned medium (CM) was recovered after 24h. CM were centrifuged at 300 g x 5', 3000 g x 10', 10000 g x 20'. CM was filtered (0,22μm filter) and was ultracentrifuged in "Ultra Clear-Queck

Seal” tubes at 100000 g x1h30’. Pellet was washed and then resuspended in PBS.

30%sucrose/D2O cushion

To further verify the identity of vesicles as exosomes, vesicles were isolated on a 30% sucrose/D2O cushion (density of 1.13–1.19 g/mL), as described by Lamparski et al (66) Exosomes from differential centrifugation method were resuspended in 25 ml of PBS. 4ml of 30%sucrose/D2O was loaded on the bottom of the ultracentrifuge tube and diluited exosomes were layered above the sucrose cushion, gently without disturbing the interface. Samples were ultracentrifuged at 100000xg, 4°C for 75 min in SW 28 rotor. The cushion, which now contains exosomes, was recovered with a 5ml syringe from the side of the tube. The cushion was transferred to a fresh ultracentrifuge tube and were added 40 ml of PBS. Samples were ultracentrifuge at 100000xg 70 min in 70 Ti rotor. Pellet was resuspended in PBS. Exosome protein content was determined by the Bradford method. On average, the amount of exosomes obtained was 100 μg/40 ml from LAMA84 and K562 conditioned medium and 50 μg/40 ml from K562 treated with ZA.

Scanning Electron Microscopy

Exosomes were next examined by scanning electron microscopy analysis. They were fixed with 2% glutaraldehyde in PBS for 10 min, attached onto stubs, coated with gold in a sputterer (Sputter Coater 150A, Edwards, UK) and observed using a field emission scanning electron microscope (FEGESEM QUANTA 200 FEI) at working voltage 30 kV.

Patients

Blood samples were obtained from two newly diagnosed CML patients. Informed consent was obtained from patients, according to the Declaration of Helsinki and with hospital Ethics Committee approval. Whole blood samples were treated with red blood cell lysing buffer (Sigma, St. Louis, MO) for 2 min at room temperature, then centrifuged at 350g for 7 min to recover and discard lysed red cells. The interphase layer containing CML cells was collected, resuspended in PBS and lysated for controls. Exosomes released in fresh patient's plasma were prepared with the same protocol of CML cell lines.

Western blot and immunoprecipitation assay

CML cells and exosomes were lysed in lysis buffer (300 mM NaCl, 50 mM Tris HCl pH 7.6, 0.1% Triton, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 4 mM EDTA, 2 mM sodium orthovanadate, 10mMNaPPI, 100mMNaF) on ice for 1h. The cell lysates were clarified at high speed centrifugation for 15 min and an aliquot of the supernatant was assayed to determine protein concentration by the Bradford method.

Total cell or exosome lysates were subjected to SDS-PAGE electrophoresis and immunoblot. Antibodies used in the experiments were: HSC70, CD63 and VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), actin, MAPK and phosphoMAPK (Cell Signaling Technology, Beverly, MA). Five million HUVEC were incubated with 50 μg/ml of LAMA 84 exosomes for 6 hr or with 10 ng/ml TNF a for 2 hr (positive control) or with low serum medium for 6 hr (negative control) and processed for immunoprecipitation experiments using precleared lysates. Samples were resolved in 8% SDSPAGE followed by immunoblotting with anti-VCAM1. Aliquots of the

precleared cell lysates were resolved independently by 8% SDS-PAGE and examined for actin quantity as a surrogate of IP input (named as starting material or St) .

Acetylcholinesterase assay

A total of 10 µg of exosomes in 100 µl of PBS and 10 µg of total cell lysate were resuspended in a solution of 1.25 mM acetylthiocoline and 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid) in a final volume of 1 ml. The incubation was carried out in cuvettes at 37 °C, and the change in absorbance at 412 nm was followed at different time points (from 0 to 180 min).

Flow cytometry for VCAM1

Expression of HUVEC cell surface VCAM-1 was determined by flow cytometry analysis. HUVEC were treated with or without 50 µg/ml of LAMA 84-exosomes in low serum medium (EGM:RPMI, 1:9). 500,000 cells were washed in PBS and incubated with 0.5 lg VCAM-1-FITC (Santa Cruz Biotechnology**,** Santa Cruz, CA) for 15 min at 4°C. Viable cells were gated by forward and side scatter and the analysis was performed on 100,000 acquired events for each sample. Samples were analyzed on a FACS Calibur with the use of the CellQuest software (BD Biosciences).

Flow cytometry of VCAM-1 expression on the surface of latex bead-coupled exosomes

Exosomes are too small for FACScan analysis, and therefore were bound to surfactant-free white aldehyde/sulfate latex beads, 4 µm diameter (Interfacial; Dynamics, Portland, OR). Beads were incubated with 30 µg of exosomes for 15 min and then for 2h at room temperature in a final volume of 1 ml PBS. Glycine (100 mM) in PBS was added to the

beads at room temperature to saturate the remaining binding sites. Beads were then washed in PBS/0,5% BSA and resuspended in 0,5 ml of PBS/0,5% BSA . 10 µl of coated beads were incubated with 50 µl VCAM-1 antibody diluted in PBS/0,5% BSA for 30 min, washed twice with PBS/0,5% BSA and analyzed with a FACScan and CellQuest software (241).

Flow cytometry for NKG2D, CD69, CD25, IFNγ and TNFα

γδ T cells after overnight incubation with 1,5,10 µg/ml of exosomes from K562 and K562 zol were stimulated with 10 µg/ml Brefeldin A (Sigma) for 4 hours. The plate was centrifuged at 1300 rpm for 2 min and washed with PBS. Cell pellet was stained with Live/Dead aqua (1:10) (Invitrogen) at room temperature for 15 min. Cells were washed with PBS, spinned down at 1300 rpm for 2 min and resuspend with IgG blocking antibody (1:100) in 50 µl of FACS buffer (PBS containing 2% bovine serum albumin and 0.02% sodium azide). Cells were incubated for 15 min in dark on ice and washed on FACS. Cells were staining with surface antigens: CD69 FITC (Pharmigen); CD25 PE-Cy7(BD biosciences); CD3 APCH7 (BD biosciences); Vγ9 PE-Cy5 (Coulter); NKG2D PE (Pharmigen). At the same time was prepared also a control Isotype for surface antigens with: IgG1 FITC; Vγ9 PE-Cy5; CD3 APCH7; IgG1 PE-Cy7; IgG1 PE. Cells were staining for 20 min on ice and washed twice in PBS. Were added 50 µl of Fixation buffer to fix surface staining, for 15 min room temperature in dark. To eliminate all fixation buffer, cells were washed twice in PBS and washed with 50 µl of Permeabilization buffer. At this point cells were stained for intracellular cytokines with IFNγ Pacific Blue (eBiosciences) and TNFα APC (BD Biosciences), and were stained also with control Isotype: IgG1 Pacific blue and IgG1 APC. Cells were incubated 15

min room temperature in dark and washed with FACS buffer. Stained cells were analysed by eight-colour flow cytometry on a FACS CANTO II (Becton Dickinson) using FlowJo software (Tree Star).

Immunofluorescence and cell cytoskeleton analysis

HUVEC monolayers were grown to confluence on coverslips coated with type I collagen (Calbiochem, Darmstadt, Germany) and were treated with increasing doses of CML exosomes or low serum medium for 6 hr to study VE cadherin, β-catenin protein distribution and expression level. After incubation with exosomes or BSA control, cells were fixed in 3.7% paraformaldehyde for 10 min followed by permeabilization with 0.1% Triton X-100 for 3 min. Antibodies used in the experiments were: VE cadherin and b-catenin (1:100; Santa Cruz Biotechnology, Santa Cruz CA). Incubation with antibodies was performed in PBS with 1% BSA overnight at 4° C. Cells were stained with Texas Red-conjugated secondary antimouse antibodies (1:100; Molecular Probe, Eugene, OR) and analyzed by confocal microscopy (Olympus 1x70 with Melles Griot laser system). Analyses of the actin cytoskeleton were performed as described (ref20) The semi-quantitative analysis of fluorescence intensity was performed using IMAGE-J software (http://rsbweb.nih.gov/ij/).(242)

RNA extraction and Real-Time PCR

HUVEC cells were grown to confluence in 6-well plates and incubated for different times with the indicated stimuli. IL-8, VCAM-1, ICAM-1 transcript levels were measured by reverse transcription (RT) and TaqMan real-time quantitative polymerase chain reaction (RT-PCR) and analyzed as described (243) The following primers were

used: IL-8, HS00174103_m1; VCAM-1, HS 00174239 m1; ICAM-1, HS 00277001_m1 and, GAPDH, Hs99999905 m1 (Applied Biosystems, Foster City, CA). GAPDH was used as the internal control.

Motility assays

Migration assays were performed following two standard protocols, Transwell chemotaxis chambers (NeuroProbe, Cabin John, MD) and wound repair assay. LAMA84 cells (2 x 10^6/ml) were suspended in serum-free RPMI 1640 medium supplemented with 0.1% BSA in transwell chemotaxis above 8 μm pore filters and exposed to chemoattractants with increased amount of exosomes (10-20-50 μg/ml), 10 ng/ml of recombinant IL8 (Sigma, St. Louis, MO), or neutralizing antibodies anti IL8 (5 μg/ml) (R&D system, Minneapolis**,** MN) as indicated. Filters were removed after 6 hr, fixed in ethanol and stained with Diff-Quick (Medion Diagnostics GmbH, Du¨dingen, Switzerland). Each test group was tested in three independent experiments; the number of migrating cells in five high-power fields per well were counted at 400x magnification. For the wound healing assay, a wound was created by manually scraping the confluent endothelial cell monolayer with a p1000 pipette tip. After washing with PBS, cells were incubated for 3 hr with medium containing exosomes or control medium without exosomes. Images of cell-free spaces were taken with a digital camera at the indicated times and measured manually with the IMAGE-J software (http://rsbweb.nih.gov/ij/) (242). The data are reported as the percentage of the distance migrated relative to the control cultures for each experiment.

Adhesion assay

For Adhesion assays HUVEC monolayer was incubated for 6 hr with 10, 20, 50 μg/ml of LAMA84 exosomes, 50 μg/ml of LAMA84 exosomes plus anti-actin antibody (5 μg/ml), 10 ng/ml of recombinant IL8, 50 μg/ml of CML patients exosomes, EGM as positive control, 50 μg/ml of exosomes plus neutralizing antibody anti-IL8 (5 μg/ml), 50 μg/ml of PBMC exosomes and low serum medium as negative control. After treatment, cells were washed with PBS and $0.5x10^6$ CML cells were added for 1 hr at 37°C. Adherent cells were stained with hematoxylin/eosin, each test group was assayed in triplicate; five high power (400X) fields were counted for each condition.

ELISA

HUVEC conditioned medium (CM) was collected from cells stimulated for 6 hr with 20 and 50 μg/ml of PBMC exosomes, 10,20,50 μg/ml of LAMA84 exosomes. CM aliquots were centrifuged to remove cellular debris and IL-8 protein concentrations were quantified using an ELISA kit (R&D Systems, Minneapolis), according to the manufacturer's protocol. IL8 was also measured directly in LAMA84 exosomes (10,20,50 μg/ml)

HUVEC tube formation on Matrigel

Matrigel was used to test the effects of exosomes on in vitro vascular tube formation as described (ref22). HUVEC were plated on Matrigel at concentration of 70000 cells/well in endothelial basal medium containing 0.2% of FBS, and 50 μg/ml of LAMA84 exosomes, 50 μg/ml of LAMA84 exosomes plus anti-actin antibody (5 μg/ml), 10 ng/ml

of recombinant IL8, 50 μg/ml of CML patients exosomes, EGM as positive control, 50 μg/ml of exosomes plus neutralizing antibody anti-IL8 (5 μg/ml), 50 μg/ml of PBMC exosomes and low serum medium as negative control were added to wells. Cells were incubated for 6 hr and then evaluated by phase-contrast microscopy and photographed.

Matrigel plug assay

All animal experiments were conducted in full compliance with University of Palermo and Italian Legislation for Animal Care. Four week old BALB/c nude mice (Charles River Laboratorie International, Wilmington, MA) were injected subcutaneously with 400 μl Matrigel (BD Biosciences Pharmingen, San Diego, CA) containing 100 μg LAMA84-derived exosomes with or without 10 lg/ml non specific anti-actin antibody or anti-IL-8 neutralizing antibody or PBS (negative control). The degree of vascularization was evaluated by determination of hemoglobin content using the Drabkin method (Drabkin's reagent kit, Sigma Saint Louis, Missouri) (244)

Statistics

Data were expressed as means ± SEMs of the indicated number of experiments. Statistical analysis was performed by using a unpaired Student's t test. Differences were considered to be significant when p values were <0.05.

Results and Discussion

Characterization of the vesicles released from LAMA84, K562 and K562 treated with zoledronic acid CML cell lines

I examined the ability of LAMA84, K562 and K562 treated with 5 μM zoledronic acid (ZA) cells to release exosomes into the culture medium during a 24 hr period. Before the recover of the medium I performed an acridina-orange/BrEt to check the vitality of the cells. The percentage of apoptotic cells were less than 3% (Fig. 12), this result allow to exclude the presence of apoptotic bodies in the pellet after the ultra centrifugation.

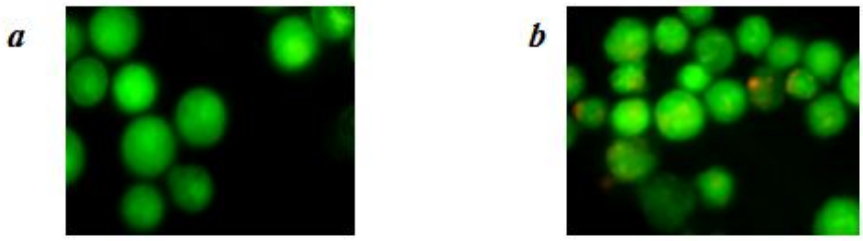

Fig. 12: Cultured LAMA84 (a) and K562 (b) CML cells (10^6/ml) for 24h revealed a small percentage of apoptotic cells (<3%)

Vesicles secreted by LAMA84 CML cells were also purified on a sucrose gradient and analysed by scanning electron microscope (Fig. 13a) and Western blotting using antibodies specific for HSC 70 and CD63. These proteins were detected in cell lysates and found more expressed in exosome fractions (Fig. 13b). Acetylcholinesterase activity, a characteristic enzyme localized in exosomes, was found associated with the exosome fraction while negligible amounts were found in conditioned medium deprived of exosomes (Fig. 13c).

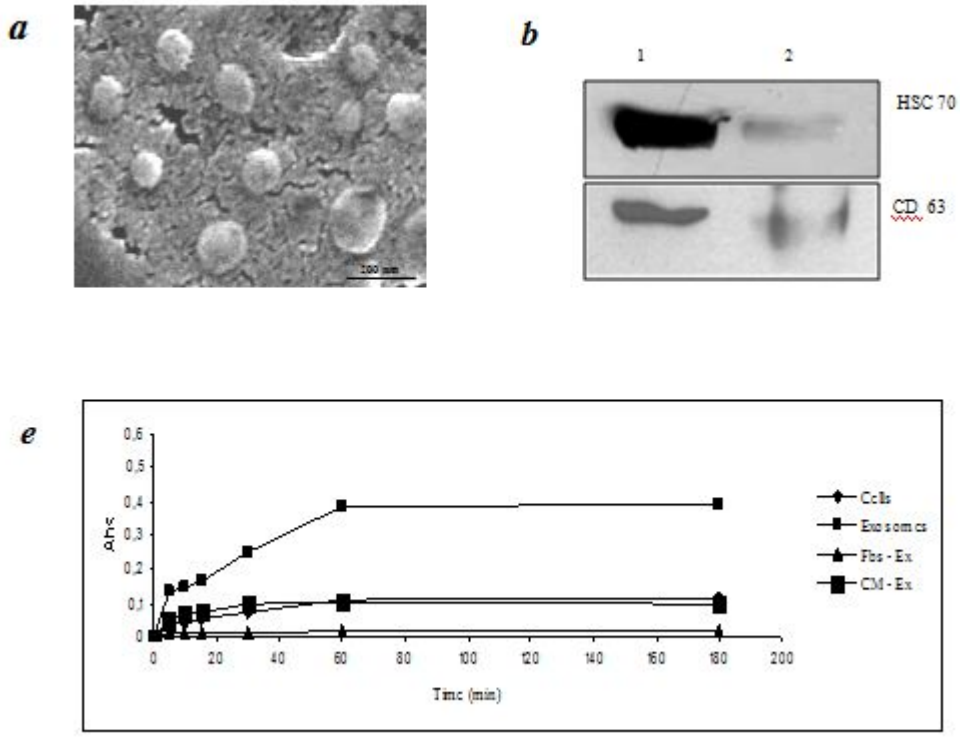

Fig. 13: (a) Exosomes released by LAMA84 cells observed by scanning electron microscopy. (b) Detection of Hsc 70 and CD63 in 30 µg of exosomes purified after ultracentrifugation on 30% sucrose/D2O gradient (lane 1) and 30 µg of cell lysate (lane 2). (c) Acetylcholinesterase assay. The activity of acetylcholinesterase, an exosome-specific protein marker, was determined in exosomes (10 µg) (▪), total cell lysates (10 µg) (♦), exosome-deprived Fbs (▲) and exosome-deprived conditioned medium (CM) (■) as negative control

Exosomes purified from whole blood of 2 patients with chronic myelogenous leukemia displayed same properties of vesicles isolated from LAMA84 cells (fig.14).

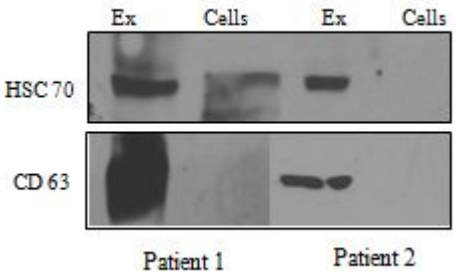

Fig. 14: Characterization of exosomes from CML patients. Exosomes are enriched in HSC70 and CD63

I also characterized exosomes from K562 and K562 treated with zoledronic acid for HSC70, CD63 and Acetylcholinesterase enzyme (fig.15).

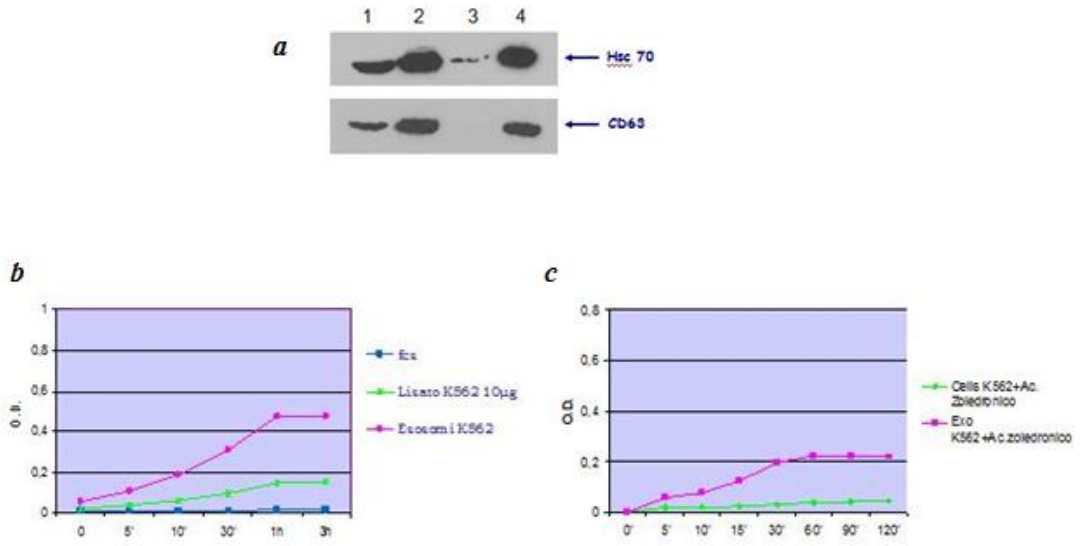

Fig. 15: (a) Detection of Hsc 70 and CD63 in 30 ug of exosomes from K562 and K562zol purified after ultracentrifugation on 30% sucrose/D2O gradient (lane 2 and lane 4) and 30 µg of cell lysate (lane 1 and lane 3). (b,c) Acetylcholinesterase assay. The activity of acetylcholinesterase, an exosome-specific protein marker, was determined in exosomes (10 µg) (pink lane), total cell lysates (10 µg) (green lane), exosome-deprived Fbs (blue lane) as negative control.

It is important to underline that the treatment with 5 µM of ZA affect the release of exosomes from K562 cells. On average, I obtained 100 µg of exosomes/40 ml of K562 conditioned medium, while after the treatment with ZA the amount recovered is 50 µg of exosomes/40 ml.

Scanning electron microscope, Hsc70, CD63 and acetylcholinesterase confirmed that the vesicles released from LAMA 84, K562, K562 treated with zoledronic acid CML cell lines were exosomes.

Exosomes treatment of HUVECs induces cell-cell adhesion molecules

To evaluate if exosomes shed by LAMA84 are able to affect cell-cell adhesion molecules mRNA expression, I treated HUVECs with different amount of exosomes for different times (6h, 12h, 24h).

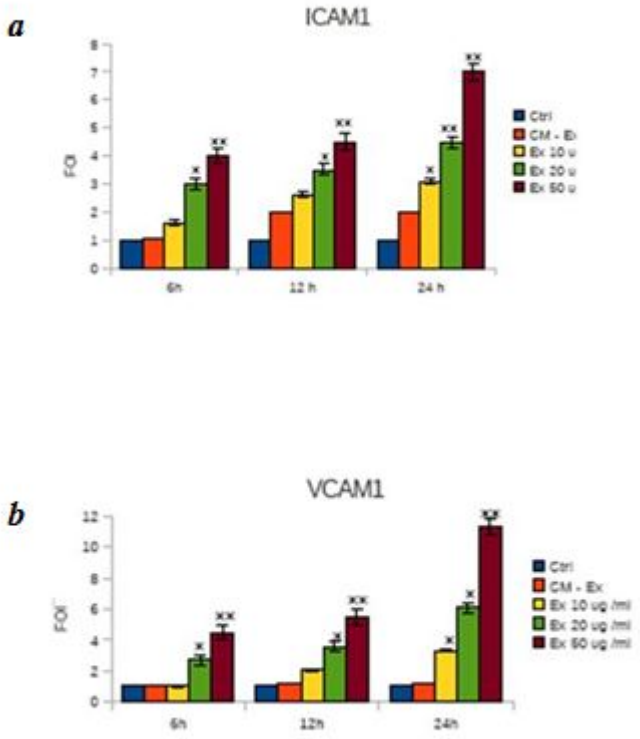

Fig. 16: ICAM1 (a) and VCAM1 (b) mRNA expression increased in a time- and dose-dependent (10, 20, 50 lg/ml) manner after addition of exosomes to endothelial cell monolayer. Exosome-deprived conditioned medium (CM-Ex) and low-serum medium were used as negative controls.

As shown in figure 16 a and b, addition of increasing doses of exosomes to endothelial monolayer caused a dose- and time-dependent increase in VCAM-1 (13 fold increase) and ICAM-1 (6 fold increase) mRNA expression ($p \leq 0.01$).

Figure 17a shows that incubation of HUVEC with LAMA84 exosomes or TNFα, used as positive control, induced an increase of VCAM1 protein levels. FACS analysis confirmed that incubation of HUVEC with LAMA84 exosomes resulted in the detection of VCAM-1 on the surface of HUVEC (Fig. 17b).

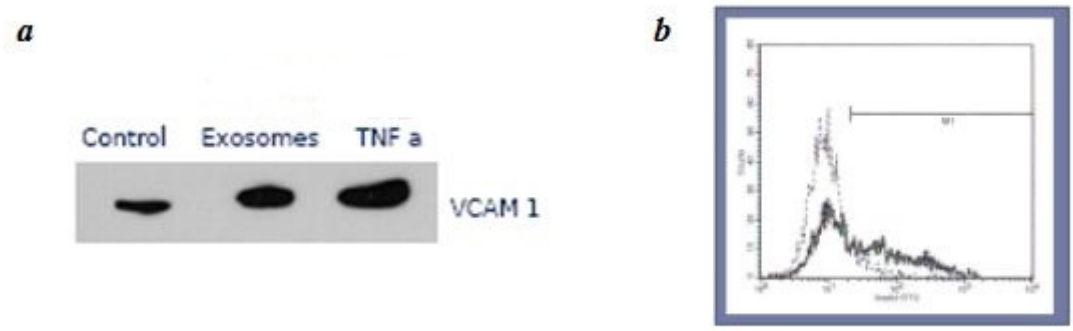

Fig. 17: a) Immunoprecipitation assay with anti-VCAM1 antibody. HUVEC were incubated for 6 hr with low-serum medium (lane 1); 50 lg/ml LAMA84 exosomes (lane 2) or 10 ng/ml TNFα (lane 3); results indicate an increased amount of VCAM-1 in exosome-treated cells. b) Representative overlay histogram showing an increase of surface expression of VCAM 1 on HUVEC treated with 50 lg/ml of LAMA84 exosomes (solid line) compared untreated HUVEC, as control (dot line)

Immunoprecipitation and western blotting assays showed that VCAM-1 was undetectable in LAMA84 exosomes and flow cytometry analysis of latex bead-coupled exosomes confirmed the absence of VCAM-1 on membrane particles (fig 18).

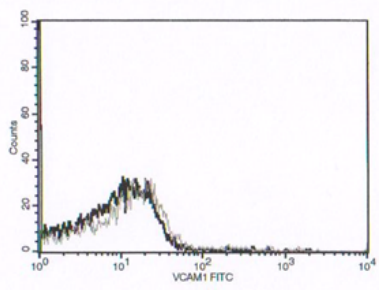

Fig. 18: Representative overlay histogram showing 10 µl of latex beads coupled with 30 µg of LAMA84 exosomes (black line) or BSA (grey line) tested for surface expression of VCAM-1. No shift in fluorescence was observed when exosomes were stained with VCAM-1 antibody.

Cell–cell interaction mediated through cell-adhesion molecules occurs after endothelial activation in angiogenesis. In leukaemia, the adhesion molecules have been thought to play an important role in various processes, including release of blast cells from bone marrow to the circulation, homing of blast cells to various organs and interaction of

blast cells with each other (209). These data showed that the amount of ICAM1 and VCAM1 was significantly higher in cells treated with increasing doses of LAMA84 exosomes. To support this statement VCAM1 was detected in exosomes-treated and untreated HUVEC cells. FACS analysis shows an increase in surface expression of VCAM1 in HUVEC cells after treatment with exosomes compared to untreated HUVEC cells. VCAM1 was tested also on exosomes directly, and the absence of surface expression confirm that this protein was produced by HUVEC and not transferred by adding LAMA84 exosomes. Then exosomes can be have a role on the interaction between CML cells and endothelium. Infact, it is described that leukemic cells interact with endothelium through adhesion molecules and cell adhesion is necessary to trigger the survival signals on leukemic cells. The increased expression of cell-cell adhesion molecules on endothelial cells may be associated with an augmented dissemination of leukemia blast cells to extramedullary sites (210), because ICAM1 and VCAM1 mediated the adhesion of myeloblasts to activated endothelium (211).

I also tested also the expression of IL8, IL6, VEGF and TGFβ genes that are involved in tumor growth and angiogenesis. HUVECs treated with exosomes from LAMA84 induced a dose dependent increase in IL8 mRNA (fig.19).

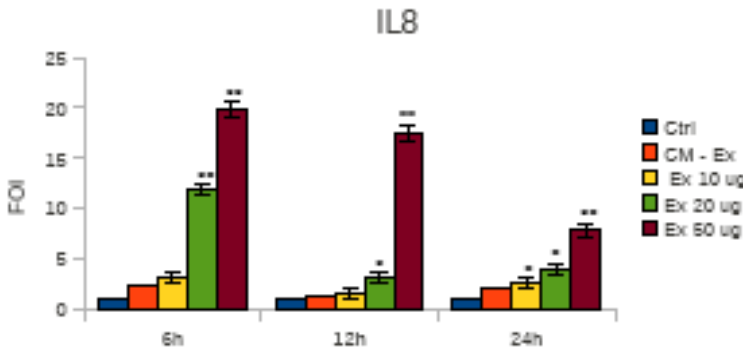

Fig. 19: IL8 mRNA expression increased in a dose-dependent (10, 20, 50 lg/ml) manner after addition of exosomes to endothelial cell monolayer. Exosome-deprived conditioned medium (CM-Ex) and low-serum medium were used as negative controls.

Increased IL8 mRNA production was statistically significant and reached approximately a 20-fold induction after 6 hr of stimulation of the endothelial monolayer with 50 μg/ml of vesicles. No effect was observed on IL-6, VEGF or TGF-β (data not shown). The use of IL-8 neutralizing antibody inhibited exosome-stimulated increase of ICAM-1, VCAM-1 adhesion molecules and IL8 as shown in figure 20.

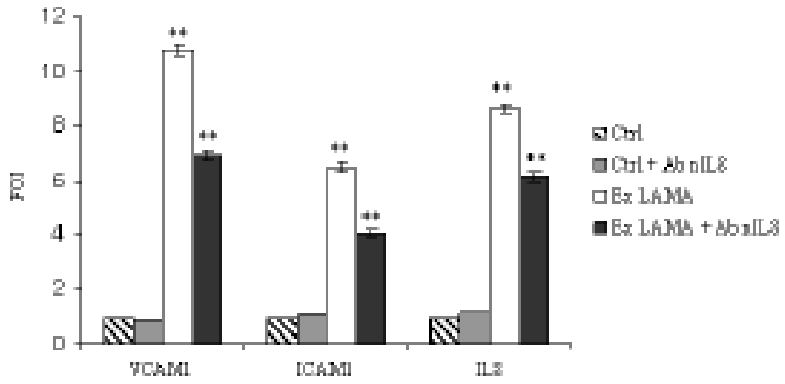

Fig. 20: VCAM1, ICAM1 and IL8 mRNA expression in HUVEC treated for 12 h with low serummedium (Ctrl), Ctrl plus 10 lg/ml of a neutralizing anti-IL8 antibody, 50 lg/ml exosomes or 50 lg/ml exosomes plus 10 lg/ml of a neutralizing anti-IL8 antibody.

I evaluated also the release of IL8 into HUVEC conditioned medium and the data confirmed that there is an increase in the synthesis of IL8 in endothelial cells after treatment with exosomes. A small amount of IL8 was found in LAMA84 exosomes. No significant induction of IL-8 release was observed when HUVEC were stimulated with exosomes purified from PBMC (fig. 21).

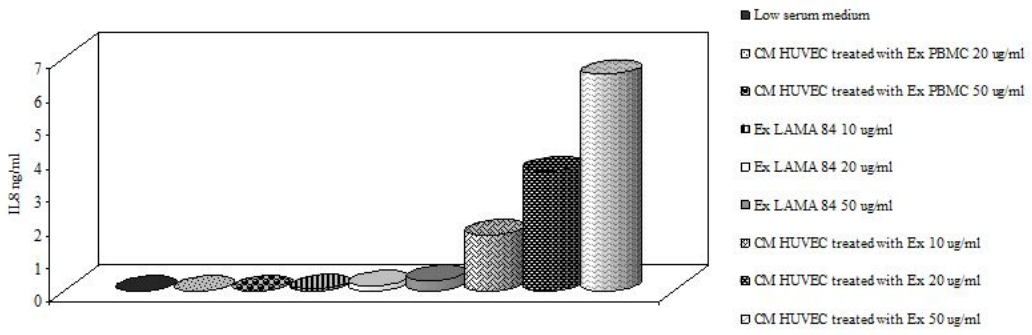

Fig. 31: Elisa Assay. Treatment with exosomes induce an increase in the release of IL8 by HUVEC.

I demonstrated increased mRNA and protein expression of interleukin-8 in exosomes-stimulated HUVEC cells. IL-8, a member of the CXC family of chemokines, is best known for its leukocyte chemotactic properties and associated role in inflammatory and infectious diseases (212). IL8 has other biological functions in addition to its well-recognized role in regulating inflammatory responses (213), it has been shown that IL8 is a potent proangiogenic factor (214). It was demonstrated that IL8 has the ability to exert a strong effect on tumor microenvironment. For example, secretion of IL-8 from cancer cells can enhance the proliferation and survival of cancer cells through autocrine signaling pathways. In addition, tumor-derived IL-8 will activate endothelial cells in the tumor vasculature to promote angiogenesis and induce a chemotactic infiltration of neutrophils into the tumor site (215). It was demonstrated that IL8 plasma protein levels and IL8 mRNA expression by leukemic blasts were associated with a worse outcome in ALL (216) and CLL (217) patients, and it was described that IL8 plasma concentration are significantly increased in patients with CML (218). These data shows that IL8 were able to enhance the ability of endothelial cell to support adhesion of CML cells, and exert its pro-adhesion effect by stimulating VCAM-1 and ICAM-1 expression on HUVEC. Involvement of IL8 in exosome-mediated increase of ICAM1 and VCAM1 was also demonstrated by using recombinant IL8 and IL-8 neutralizing antibodies.

Exosomes stimulate binding of CML cells to HUVEC monolayer

A hallmark feature of leukemia progression is the adhesion of cancer cells to endothelial cells for extramedullary infiltration. Then, I tested the ability of leukemia cells to adhere to an endothelial monolayer to investigate functional effects of the observed increase of ICAM-1 and VCAM-1 expression in exosome-treated HUVECs. Figure 22a shows a dose-dependent increase in leukemia cell adhesion to HUVEC after 6 h treatments. Figure 22b shows the increase in adhesion of LAMA84 cells (arrows) to HUVEC

monolayer after a 6 hr treatment with 50 μg/ml of exosomes. Figure 22c shows that addition of recombinant IL-8 to endothelial cells causes an increase of CML cells adhesion to HUVEC monolayer similar to that produced by LAMA84 or CML patient exosomes. The addition of exosomes from CML patients to endothelial monolayer is similar to that produced by LAMA84 exosomes, while the treatment with exosomes from PBMC or with exosomes plus IL8 neutralizing antibody didn't increase the adhesion of leukemic cells to endothelial cells.

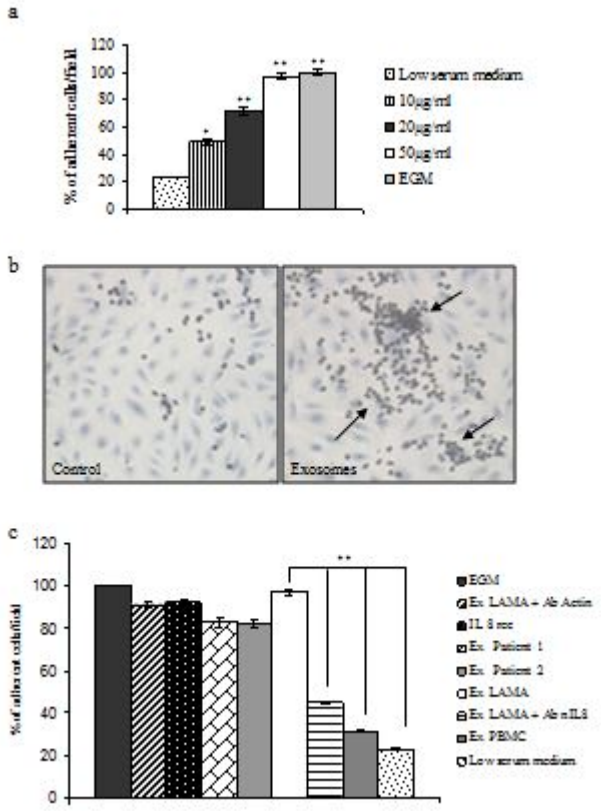

Fig. 22: a) Adhesion of LAMA84 cells to endothelial cell monolayer treated for 6 hr with different amount of LAMA84 exosomes or with EGM, used as positive control; b) Adhesion of LAMA84 cells to endothelial cell monolayer treated for 6 hr with LAMA84 exosomes observed at contrast phase microscopy; c) Adhesion of LAMA84 cells to HUVEC treated with 50 μg/ml of LAMA84 exosomes, 50 μg/ml of exosomes plus antibodies anti actin (5 μg/ml), 10 ng/ml of recombinant IL8, 50 μg/ml of CML patients exosomes, EGM (as positive control), 50 μg/ml of exosomes plus neutralizing antibodies anti IL8 (5 μg/ml), 50 μg/ml of PBMCexosomes in low serum medium and low serum medium (as negative control). Values are the mean 6 SD of 5 fields in three independent experiments CTRL: control. *p≤ 0.05; **p≤0.01.

These data confirmed that CML cells exhibited increased adhesion to HUVEC monolayer when LAMA84 exosomes were added. Interestingly, the same effects was observed with exosomes from CML patients. The effect of IL8 neutralizing antibody, that didn't increase the adhesion of leukemia cells to HUVEC monolayer, suggest that this chemokine may provide a supportive effects for CML cells in this angiogenic step.

CML exosomes promote migration of endothelial cells

In the angiogenic process one important step is the increase of the motility of the endothelial cells. Confluent, scrape-wounded endothelial cell monolayers were incubated with various concentrations of CML vesicles, and the percentage of closure was observed after 3 hr. Figure 23a shows that endothelial cell migration was significantly increased in exosome-treated cultures but not in the control medium.

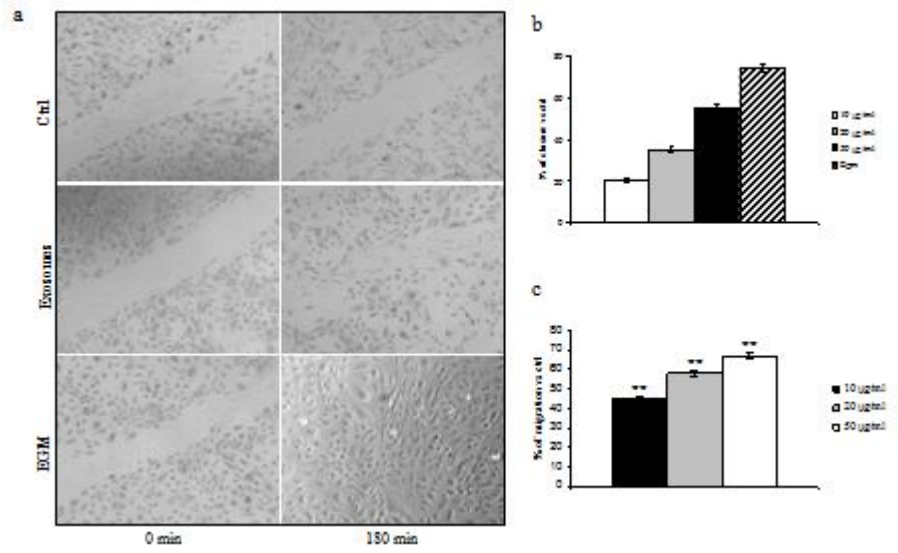

Fig. 23: (a) Confluent, scrape-wounded endothelial cell monolayer incubated with low serummedium (negative control), 50 µg/ml of LAMA84 exosomes, and EGM medium (positive control), for 3 hr. b) Percentage of closure of the wounded area measured after addition of different amount of exosomes; c)Effects of exosomes on endothelial cell migration as measured by Boyden chamber assay. Addition of exosomes (10, 20, 50 µg/ml) for 6 hr to the bottom wells of the chamber induced a dose-dependent increase of HUVEC migration. Values are the mean 6 SD of 3 fields in three independent experiments *p ≤ 0.05; **p≤ 0.01.

As positive control, EGM-treated cells migrated into the denuded area, almost completely covering the exposed surface after 3 hr. Measurement of wounded area evidenced, compared to control, a 55% percentage of closure when endothelial cells were treated with the dose of 50 μg/ml of exosomes (Fig.23b). I further analyzed the effects of exosomes on cell migration by Boyden chamber assay. Figure 15c shows that addition of a range of concentrations of vesicles (10–50 μg) to the bottom wells of the chamber caused, after 6 hr, a dose-dependent increase of CML cell migration. A similar, statistically significant, effect in the stimulation of endothelial cell migration was obtained when recombinant IL-8 or CML patients exosomes were added as chemoattractant in the Boyden assay (Fig.23c); on the contrary the presence of anti IL-8 neutralizing antibodies or PBMC exosomes in the bottom wells of boyden chamber didn't increase the motility of leukemia cells (Fig.24).

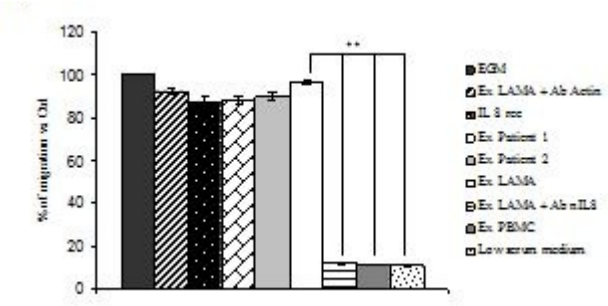

Fig. 24: 50 μg/ml of LAMA84 exosomes, 50 μg/ml of LAMA84 exosomes plus antibodies anti actin (5 μg/ml), 10 ng/ml of recombinant IL8, 50 μg/ml of CML patients exosomes, EGM (as positive control), 50 μg/ml of LAMA84 exosomes plus neutralizing antibodies anti IL8 (5μg/ml), 50 μg/ml of PBMC-exosomes in low serum medium and low serum medium (as negative control) were added as chemoattractants to the bottom wells.

Exosomes released from leukemia cells in close proximity of endothelial cells may also contribute in the exacerbation of endothelium activation and increase migration of endothelial cells during angiogenesis.

This data demonstrate that the chemotactic effects on endothelial cells were potentiated by LAMA84 and CML patients exosomes in a dose-dependent manner both in Boyden chamber and in a wound healing assay. It was described that chemokines have been implicated in the migration and tissue infiltration of leukaemic cells (219), and here it has been demonstrated that the addition of IL-8 neutralizing antibodies to CML exosomes in both assays inhibited the process of cells motility thus reinforcing the role of IL-8 in exosomes-induced cells migration.

Exosome treatment alters VE-cadherin and β-catenin localization

To investigate if alteration of cell junctional components could be responsible for the increase in cell motility, I evaluated the effects of exosomes on expression of VE-cadherin and β-catenin. VE-cadherin is an endothelial-specific adhesion molecules which under normal condition, is located at adherens junctions and is essential for the mainteinance and control of endothelial cell contacts (220,221), and the association with β-catenin is required for junction stabilization. The extracellular domain of VE-cadherin mediates initial cell adhesion,whereas the cytosolic tail is required for interaction with the cytoskeleton and junctional strength, mediated through β-catenin (222). The functional role of VE-cadherin changes during early phases of angiogenesis. Endothelial cells treated with exosomes from LAMA84 cell lines show a delocalization of this molecule. VE-cadherin staining decreased in intensity and became patchy at the membrane concomitant with the appearance of a granular cytoplasmic staining in HUVEC treated with 50 µg/ml of exosomes, compared to control cells that had continuous peripheral VE-cadherin staining (Fig. 25b). I evaluated also the effect of exosomes on β-catenin, a protein that interact with VE-cadherin and is involved in signal transduction. HUVEC treated with exosomes show a reduction of membrane

immunostaining compared to control cells after 6h. The treatment with exosomes caused a translocation from the plasma membrane to the cytoplasm and nucleus (fig.25 c and d). Furthermore, staining of actin filaments with rhodamine-conjugated phalloidin confirmed the alteration of endothelial integrity when exosomes are added to HUVEC monolayer (fig.25a).

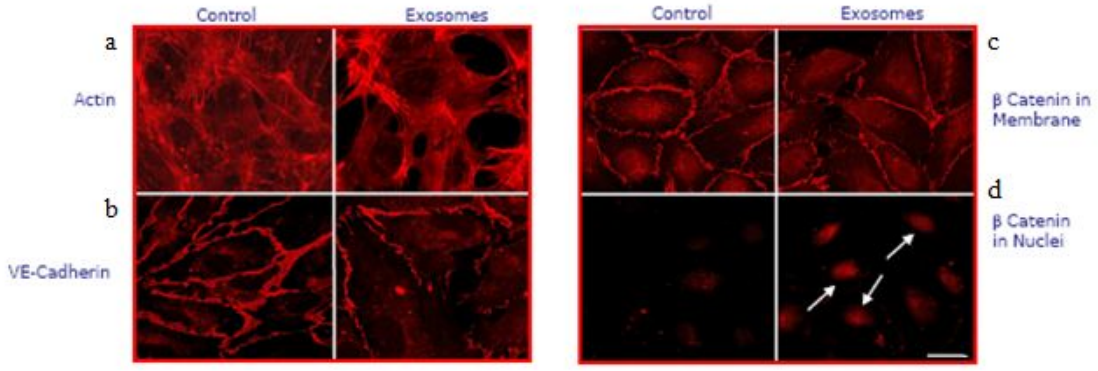

Fig. 25: (a) modification of cytoskeletal structures as observed with actin localization in HUVEC monolayer treated with 50 µg/ml of exosomes compared to control cells b) Analysis at confocal microscopy of VE Cadherin localization in HUVEC cells treated with LAMA84 exosomes revealed a decrease of immunostaining compared to untreated cells (control). (c) Decrease of immunostaining for β catenin in cell membranes was revealed after 6 hr incubation of HUVEC with 50 µg/ml of LAMA84 exosomes compared to control cells (d) figure shows the translocation of b catenin in the cytoplasm and nucleus compared to control

Treatment of HUVECs with LAMA84 exosomes induced a cytoskeletal reorganization with a concomitant translocation of VE-cadherin and β-catenin from cell surface to cytoplasm and nuclei. Overall, these results indicate that addition of exosomes to endothelial cells reduced intercellular adhesion, as a biological consequence of loss of zonulae adherens components, VE-cadherin and β-catenin. These loosen endothelial cell-to-cell contacts, is indicative of weakened cell-cell adhesion mechanism and may be responsible for increased motility (223).

In vitro Angiogenesis Assay

To evaluate the effects of exosomes on *in vitro* models of angiogenesis, I performed an angiogenesis assay on Matrigel; as shown in figure 26, a treatment for 6h with exosomes from LAMA84 induce in HUVECs an endothelial network formation in a

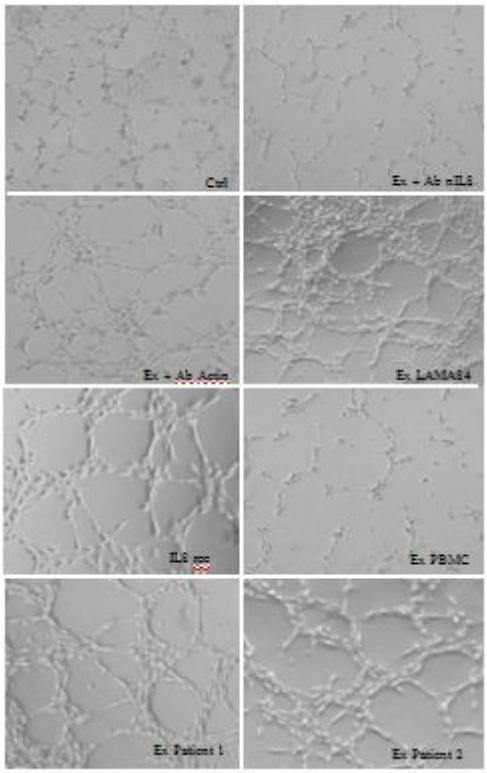

Fig. 26: Phase contrast micrographs showing that exosomes induce an endothelial network formation on matrigel. No tube formation is observed when HUVEC are plated in low-serum medium or in the presence of 50 µg/ml of exosomes plus neutralizing antibody against IL8 or with exosomes from PBMC; the addition to HUVEC cells of 50µg/ml of LAMA84 exosomes or 50 µg/ml of exosomes plus a nonspecific antibody against actin or exosomes from CML patients caused the formation of capillary-like structures

dose-dependent manner. I obtained the same results with the addition of recombinant IL8 or with exosomes from CML patients, while the treatment with exosomes from PBMC had no effect. The anti-IL8 neutralizing antibody inhibited exosome-induced tube formation while treatment of cells with an anti-actin antibody had no effect.

In vivo Angiogenesis Assay

The angiogenic potential of LAMA84 exosomes was then assayed *in vivo* by examining the recruitment of vasculature into subcutaneously implanted Matrigel plugs containing exosomes. Figure 27 shows that the plugs containing LAMA84 exosomes and LAMA84 exosomes plus a non specific antibody (anti-actin antibody), became more vascularized than implants with PBS control or with exosomes plus an anti-IL8 neutralizing antibody. This suggests that IL-8 is critical for vascular recruitment and organization in this model. This is supported by the increased haemoglobin concentration in the exosomes-containing Matrigel (Table 1).

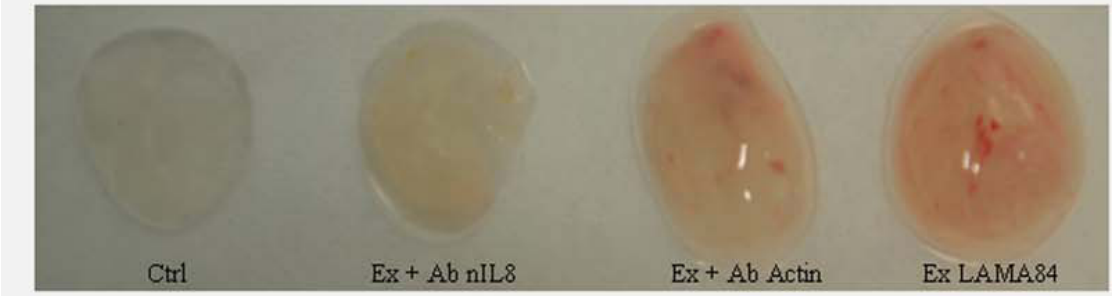

Fig. 27: Matrigel plug containing LAMA84 exosomes stimulate angiogenesis in nude mice. Ctrl: Negative control (Matrigel plus PBS), Ex + Ab nIL8: LAMA84 exosomes (100 µg) plus 10 µg/ml of an antibody neutralizing anti-IL8, Ex + Ab Actin: LAMA84 exosomes (100 µg) plus 10 µg/ml of a non specific antibody against actin, Ex LAMA84: LAMA84 exosomes (100 µg).

Table 1. Haemoglobin concentration in the exosomes-containing Matrigel

	ABS
Ctrl	0.040
EX+Ab nIL8	0.070
Ex+ Ab actin	0.247
Ex LAMA84	0.378

An important aspect of tumor progression is tumor vascularization, and tube formation of endothelial cells is one of the key steps of angiogenesis. Increased vascularization is seen in AML (224), ALL (225) and also CML (226) patients show an increase in the

number of blood vessels but little is know about angiogenesis and angiogenesis-related molecules in leukemia, while there are many data about the effect of exosomes on solid tumor angiogenesis. Skog et al. have showed that glioblastoma tumor cells release different types of microvesicles including exosomes that contain mRNA, miRNA and proteins that may stimulate endothelial cells to acquire an angiogenic phenotype (126). It has been demonstrated that exosomes of human SW480 colon carcinoma cells are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells, suggesting that microvesicles from cancer cells can be involved in tumor growth and metastasis by facilitating angiogenesis-related processes (227). The role of exosomes in metastasis has been also demonstrated in melanoma cells in a study by Hood and collaborators. They found that exosomes modulated both angiogenic and immunological cytokine signalling, thus serving as paracrine nanocarriers that might prepare distal sites for the arrest of metastatic cells (123).

Here, both *in vitro* and *in vivo* assays demonstrated that LAMA84 and CML patients exosomes stimulates endothelial cell proliferation and capillary-tube organization, which can be blocked by neutralizing anti-IL-8 Abs, reinforcing the role of IL-8 in exosomes-induced vascularization and angiogenesis. These data support the role of exosomes in leukemic microenvironment as important mediators of tumor growth, progression and angiogenesis.

Exosomes trigger phosphorylation of MAPK p42/44

I analyzed MAPK signalling after interaction between endothelial cells and purified exosomes to begin to understand the molecular pathways through which exosomes affect angiogenesis. MAPK is a key signalling pathway activated in endothelial cells after binding of angiogenic factors (228). Crosstalk between MAPK and other

signalling pathways can further stimulate angiogenesis (229). As shown in figure 28 stimulation of HUVEC with 50 µg/ml exosomes caused a time-dependent phosporylation of MAPK p42/44. The role of ERK activation in angiogenic growth factor signaling has been well established (230). ERK activation is probably required for the growth factor-induced secretion of angiogenic factors from tumor cells (231). The strong activation of ERK1/2 in endothelial cells early, after the exosomes addition suggest that these exosomes exert a specific stimulus for endothelial cell function.

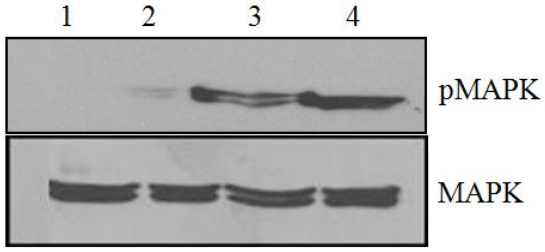

Fig. 28: : Western blot analysis of pMAPK and MAPK in HUVEC treated with 50 µg of LAMA84 exosomes. HUVEC were starved for 3 hr with serum free medium and then treated with 50 µg of LAMA 84 exosomes for 15 min (lane 3) and 30 min (lane 4), or with low serum medium alone for 15 min (lane 1) and 30 min (lane 2) as control.

Effects of exosomes on γδ T cell function

The ability of tumor cells to evade or suppress an active immune response is considered to be a significant factor in the development and progression of tumors. It has been shown that cancer patients, in particular those with malignant effusions such as ascites, produce enormous amounts of exosomes *in vivo* and, instead of boosted anti-cancer immunity, they succumb to the cancer with a deranged immune system (48). Increasing clinical and experimental evidence shows that cancer cells produce exosomes which affect cytotoxic ability of NK- and T cells and thus assist cancers in their immune evasion. Consequently, tumor-derived exosomes might be vehicles for immunosuppression with negative impact on the immune system of cancer patients and their effects should be taken in consideration when designing treatment for cancer patients (232). It has been described that the immune escape of leukemia may be related to inadequate NK cell function such as low NK cell numbers and impaired cytotoxicity. In AML, microvesicles from patient's sera suppress NK cell activity (233) and represent a mechanism by which leukaemia escape form immune system. Most of the papers in literature investigated the effects of exosomes on NK cells, while to our knowledge no data exist on the possible role of exosomes on γδ T cells function. As NK cells, γδ T cells have been know to mediated killing of a broad range of tumor targets, including leukaemia blasts. As mentionated before, γδ T cells are able to kill CML cells when those cells are pretreated with ZA. Then the aim of this second part of my thesis is to study the effects of exosomes released from K562 CML cell line on γδ T cells to better understand the interaction between cancer-exosomes and the immune system. Before each experiment I checked the purity of γδ T cells after immunomagnetic separation of T cells. The purity of γδ T cells was assessed by flow cytometry. The percentage of purified γδ T cells was 96,7% (Figure 29)

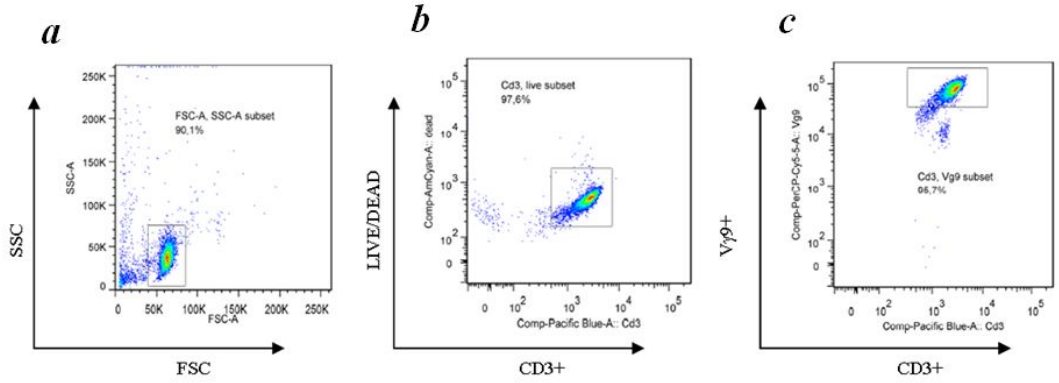

Fig. 29: Analysis of γδT cells isolated by magnetic-activated cell sorting (MACS) using CANTO II. a) gate of lymphocytes population based on FSC and SSC parameters; b) CD3+ live subset; c) subset of Vγ9+ cells

I tested the effect of exosomes shed by K562 and K562 after treatment with 5 μM of zoledronate on cytokines production, NKG2D, CD69 and CD25 expression by γδ T cells. Exosomes don't exert any effects on γδ alone (data not shown), but they are able to induce a down-regulation of activation marker, CD69 and CD25, and NKG2D expression when γδ T cells are activated with 10 nM of HMBPP and 20 U/ml of IL2. Exosomes affect also the IFNγ and TNFα release, suggesting that exosomes from CML have an inhibitory effect on γδ T lymphocytes.

Tumor exosomes inhibit IFNγ and TNFα release

γδ T cells is a subset of lymphocytes able to provide an early source of IFNγ and TNFα that are important to kill transformed cells and contribute to the prompt generation of adaptive immune responses with αβ T lymphocytes. I investigated the effect of exosomes on cytokine release. γδ T cells from healthy donors were treated with different doses of K562 and K562zol exosomes (1,5,10 μg/ml) in presence of 10 nM of HMBPP and 20 U/ml of IL2. After overnight incubation, lymphocytes were treated with

brefeldin for 4 hr and analyzed for IFNγ and TNFα expression (gating scheme is shown Fig. 30).

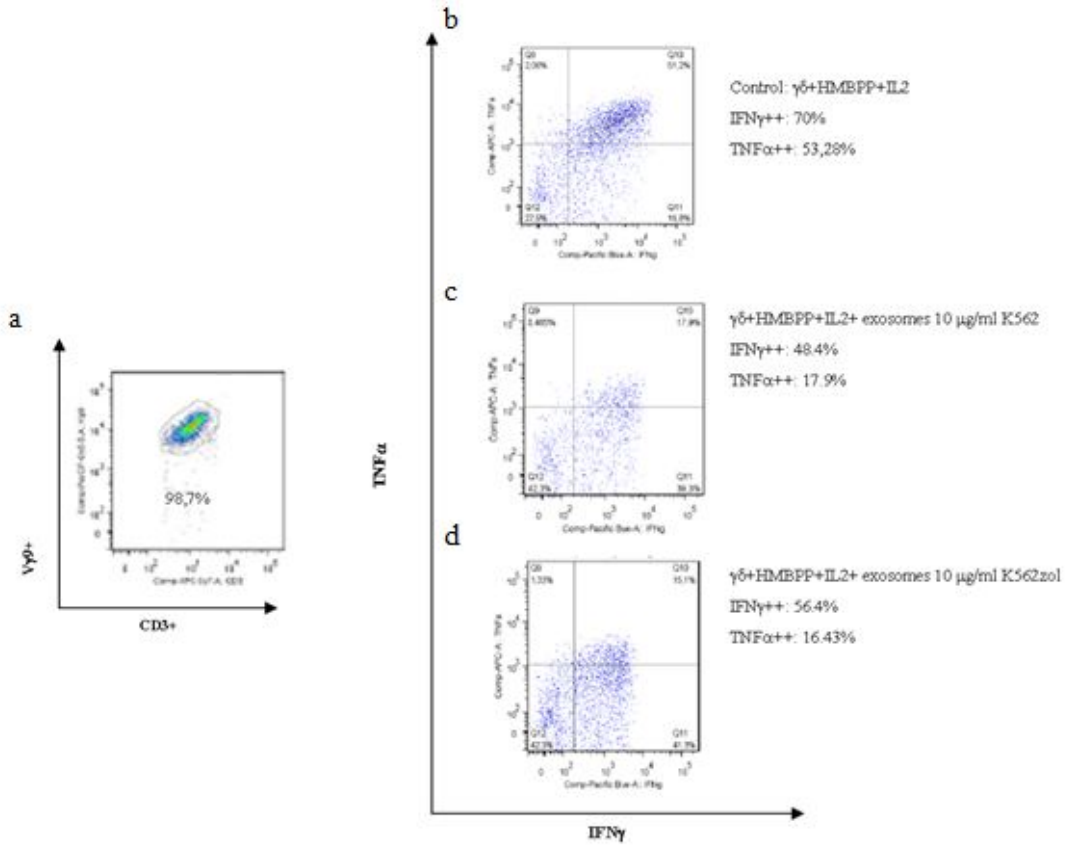

Fig. 40: Schematic representation of the multicolor flow cytometry gating scheme used to analyze lymphocyte IFNγ and TNFα expression. a) Vγ9+ CD3+ cells gated; b) γδ+HMBPP+IL2 used as control shows 70% IFNγ double positive cells and 53,28% TNFα positive cells; c)γδ+HMBPP+IL2+10 µg/ml of K562 exosomes shows a decrease in both cytokines expression (48,4% for IFNγ and 17,9% for TNFα); d) γδ+HMBPP+IL2+10 µg/ml of K562zol exosomes shows a decrease as K562 exosomes without treatment (56,4% for IFNγ and 16,43% for TNFα);

Treatment with K562 and K562zol exosomes induces an impairment in both IFNγ and TNFα secreting cells. In figure 31, the graph shows a dose-dependent decrease in IFNγ release compared to control.

Also for TNFα the treatment with exosomes induces a dose-dependent decrease (fig.32).

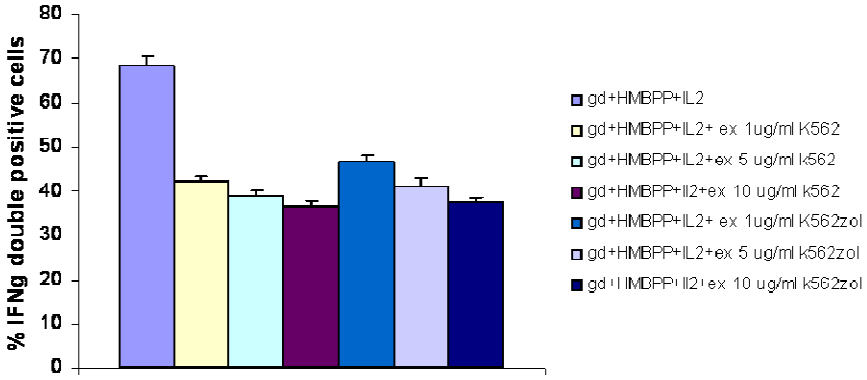

Fig. 31: Treatment of γδ+HMBPP+IL2 with 1,5,10 µg/ml of exosomes from K562 and K562 treated with 5 µM zoledronic acid induce a dose-dependent decrease in IFNγ release, compared to control (γδ+HMBPP+IL2). Data represent mean ± SD of three independent experiments.

Interestingly, the treatment with K562zol exosomes seems have a less effect on the decrease of TNFα production compared to treatment with K562 exosomes, especially with the dose of 1 µg/ml (23,8% of TNFα after treatment with 1 µg/ml of K562 exosomes and 29,6% of TNFα after treatment with 1 µg/ml of K562zol).

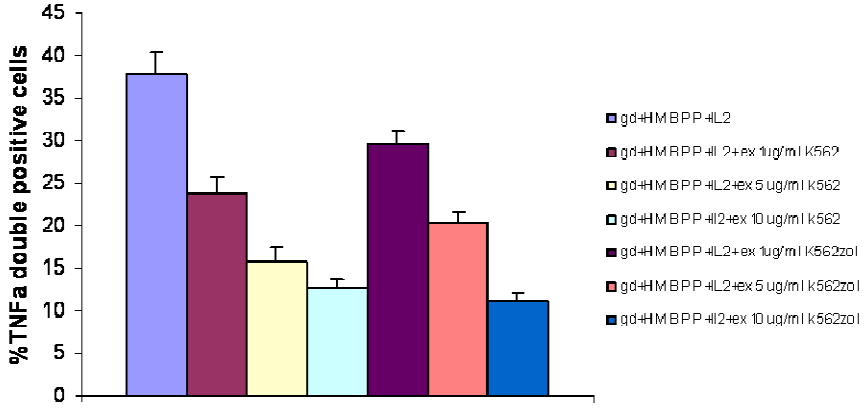

Fig. 32: Treatment of γδ+HMBPP+IL2 with 1,5,10 µg/ml of exosomes from K562 and K562 treated with 5 µM zoledronic acid induce a dose-dependent decrease in TNFα release, compared to control (γδ+HMBPP+IL2). Data represent mean ± SD of three independent experiments.

IFNγ and TNFα produced by γδ T cells are critical cytokines for protective immune responses against tumors. The treatment with exosomes induces an inhibition on cytokines production in γδ T cells, then impair the ability of these cells to provide an

early source of IFNγ in tumor immunosurveillance, and probably on cytotoxicity activity of γδ T cells. These data suggest that tumor exosomes express specific molecules that mediate the inhibition of γδ T cell activation. The identification of these molecules will be of importance in the future design of successful cancer immune therapy.

Exosomes downregulate NKG2D receptor

NKG2D receptor is an activatory receptor of NK, CD8+ and γδ T cells. Elevated levels of NKG2D ligands have been documented in a range of epithelial and other malignancies (234), and are important in tumor immune surveillance (235). These ligands include members of the MICA and UL16-binding protein (ULBP) families, molecules that have highly restricted expression patterns in health, but are readily up-regulated following viral infection or genotoxic stress (236). The presence of these ligands essentially making cancer cells as attractive targets for NK cells (237), γδ T cells (238) and CD8+ lymphocytes (239), through NKG2D-mediated lymphocyte activation via PI3K and IFNγ release. Clayton et al, described that exosomes from prostate cancer and mesothelioma express NKG2D ligands and are able to downregulate NKG2D receptor on NK cells (106). I investigated the effects of exosomes from K562 leukaemia cell line on NKG2D receptor on γδ T cells, in order to explain the decrease on cytokine production. γδ T cells plus 10 nM of HMBPP and 20 U/ml of IL2 were treated with 1,5,10 μg/ml of K562 exosomes and K562 zol exosomes. After overnight incubation, expression of NKG2D was analyzed by multicolour flow cytometry. Tumor-exosome treatment resulted in a significant reduction in cell surface NKG2D expression as shown in figure 33. Exosomes treatment don't change the percentage of NKG2D positive cells.

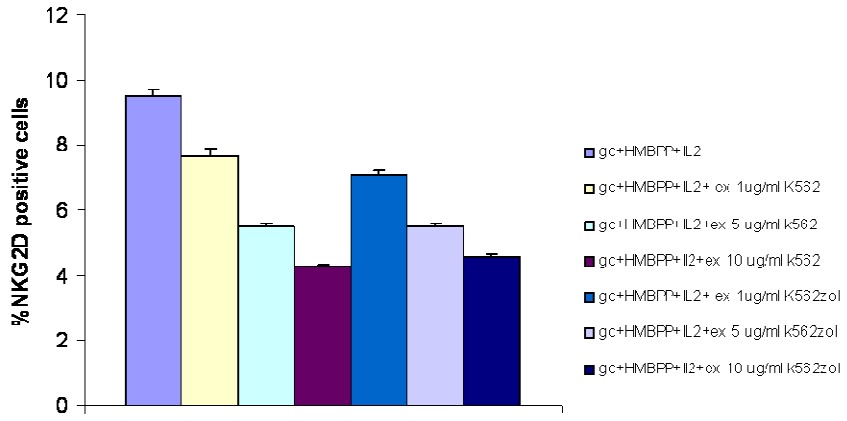

Fig. 33: Treatment of γδ+HMBPP+IL2 with 1,5,10 µg/ml of exosomes from K562 and K562 treated with 5 µM zoledronic acid induce a dose-dependent downregulation of NKG2D receptor, compared to control (γδ+HMBPP+IL2). Data represent mean ± SD of three independent experiments.

This data show that exosomes drive a down-modulation of NKG2D expression, and this can be an important mechanism by which tumor exosomes may suppress this key tumor cell recognition and lymphocyte activation pathway. It was described that K562 CML cell line express MICA, MICB, ULBP-1, and ULBP-2 ligands at the cell surface and in CML patient's sera was found a soluble form of MICA at high levels (240), while such molecules are not detectable in healthy donors sera. The production of NKG2D-ligand-bearing exosomes, therefore, may be a novel mechanism for tumor cell immune evasion, and further demonstrates the complexity of interactions possible between exosomes and the immune system.

CML exosomes downregulate CD69/CD25 expression

Purified γδ T cells rapidly express activation antigens CD25 (high-affinity IL2Rα chain) and CD69 following stimulation with HMBPP or IPP. CD69 and CD25 are involved in lymphocytes proliferation and activation. CD69 has a functional role in redirected lysis mediated by activated NK and γδ T cells. In figure 34, the graph show that the treatment

with exosomes induce a dose-dependent decrease in γδ T cells expressing both activation marker.

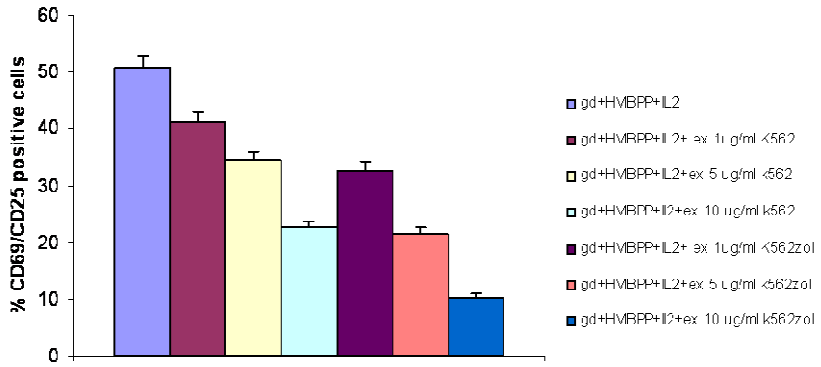

Fig. 34: Treatment of γδ+HMBPP+IL2 with 1,5,10 µg/ml of exosomes from K562 and K562 treated with 5 µM zoledronic acid induce a dose-dependent downregulation of CD69/CD25 positive cells, compared to control (γδ+HMBPP+IL2). Data represent mean ± SD of three independent experiments.

It was described that downregulation of NKG2D don't affect the expression of CD69, responsible also for NK cells activation, infact in NK cells, treatment with mesothelioma exosomes induces upregulation of CD69 (106). Here, treatment with K562 exosomes induces a downregulation of this marker and maybe responsible of the lack of γδ T cells activation. Normally, IL2 induces the expression of CD25, that is important for lymphocytes proliferation, and in the presence of tumor exosomes, this CD25 induction is inhibited, suggesting that exosomes are able to impair activation of γδ T cells.

Conclusion

The data obtained in this thesis have touched upon some issues in exosomes biology, particularly on the role of exosomes released from CML cell lines on angiogenesis and γδ T cell function. Angiogenesis plays an important role in the development and progression of CML. The bone marrow of patients with CML exhibit marked neovascularization and increased number of endothelial cells; but little is known about how CML cells induce the angiogenic phenotype. Recently, exosomes are described as new components that modulate the tumor microenvironment, promoting angiogenesis and tumor progression. This thesis provides insights into the role of exosomes in angiogenesis process. The data obtained indicate that chronic myeloid leukemia cells lines, release exosomes characterized by the presence of HSC70, CD63 and acetylcholinesterase. LAMA84 exosomes affect several steps of angiogenesis, expression of angiogenic factors, chemoinvasion and adhesion. The data showed that treatment of HUVEC cells with increasing doses of LAMA84 exosomes and CML patients exosomes induce an increase in cell-adhesion molecules ICAM1 and VCAM1, increase in production of IL8, a cytoskeletal reorganization with a concomitant translocation of VE-cadherin and β-catenin from cell surface to cytoplasm and nuclei, that can contribute to angiogenic process. Interestingly, here was found that IL8 play an important role in CML exosomes-mediated angiogenesis. It was demonstrated an increased mRNA and protein expression of interleukin-8, in exosomes-stimulated HUVEC cells. An ELISA assay evaluated the release of IL8 into HUVEC conditioned medium and the data confirmed that there is an increase in the release of IL8 from endothelial cells after treatment with exosomes. IL8 was found also in LAMA84 exosomes. To evaluate the angiogenic potential of LAMA84 exosomes it has been

performed an *in vitro* and *in vivo* angiogenesis assays. LAMA84, CML patients and rIL8 exosomes, induced tubular differentiation of HUVECs and stimulated vascularization of Matrigel plugs implanted into nude mice. The addition of IL-8 neutralizing antibodies to CML exosomes in both assays inhibited the process of angiogenesis thus reinforcing the role of IL-8 in exosomes-induced vascularization. The results here described contribute to understand tumor-host interaction in CML, with a prominent role of IL8 in angiogenic phenotype. For the first time, exosomes released from CML cells have been involved as important components leading to endothelium activation and angiogenesis.

On the other hand, the initial study on the effects of γδ T cell shows that exosomes from K562, and exosomes from K562 after treatment with zoledronate, are able to inhibit some γδ T cells function. For the first time it was demonstrated that exosomes from CML cell lines are able to downregulate NKG2D receptor, CD69/CD25 activation marker and the production of IFNγ and TNFα, suggesting a role of these exosomes in immunosuppression. These data provide few but interesting informations about the potential role of exosomes on leukemia immune escape mechanism. To better understand how exosomes are able to perform this inhibition will be useful assess the presence of FASL, MICA and MICB or other ligands on exosomes surface and evaluate the effects of exosomes on γδ T cells proliferation. In order to confirm that exosomes impair activaction marker and cytokine release, will be very interesting to evaluate the cytotoxic activity of γδ lymphocytes against CML cells targets following exosomes treatment. Another important observation is the effects of zoledronic acid on the release of exosomes from K562 cell line. After treatment with 5μM of this drug, the amount of exosomes is reduced about 50% compared to exosomes from cells untreated,

but the effects on γδ T cells function is the same.

These initial findings on the role of CML exosomes on γδ T cell shed new light on the immune-escape mechanism exosomes-mediated. Focus on the mechanism of action of zoledronate in the release of exosomes and if after treatment there are change in the quality of exosomes could be important to find some new approaches for CML therapy.

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