Characterization of Clever-1 expressing extracellular vesicles and their effect on T lymphocyte proliferation.

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Abstract

Immune checkpoint inhibitors (ICIs) are the new milestone for the management of advanced solid cancers. Unfortunately, most patients are not responsive to these treatments, since they may develop mechanisms that suppress the quality and quantity of antitumor T-cell responses. The tumor microenvironment (TME) is a key cellular component of tumors. It includes immunomodulatory cells that display important roles in the resistance to ICIs. Among these cells, tumor-associated macrophages (TAMs) from the M2-like phenotype have been described to favor tumor growth and downregulate local and systemic immune responses.

A subpopulation of these TAMs expresses Clever-1 (also known as Stabilin-1), which support the formation of an immunosuppressive TME and T-cell dysfunction. The depletion of Clever-1 both genetically and immunotherapeutically has been shown to activate the adaptive immune system and consequently, reduce tumor growth and metastasis. Recently, Clever-1 expression was detected in body fluids (plasma and lymph). However, how Clever-1 is secreted in the body and its functional consequences in T-cell function is poorly understood.

Here, we identify by using differential centrifugation process of body fluids and immortalized monocytic conditioned medium that Clever-1 is expressed in both extracellular vesicles (EVs) and non-vesicular (NV) formats. Further, we show using in vitro assays that Clever-1^{+}EVs functionally target T-cells affecting their proliferation status. Future studies are required to prove the process is dependent on Clever-1 and further investigate in the mechanistic routes involved in the inhibition mechanisms of Clever-1^{+}EVs. In conclusion, this study describes the expression of suppressive proteins (e.g., Clever-1) in EV compartments with their inhibitory effects on T-cells in body fluids.

Keywords: cancer; Clever-1; immunotherapy resistance; tumor-associated macrophages (TAMs); extracellular vesicles (EVs); T lymphocyte

Contents

1. Introduction

Tumors may escape the immune system through the immunosuppressive tumor microenvironment (TME). One way to harness the immune system is by suppressing the immune checkpoint pathways to unleash the power of effector T-cells (Teffs) to recognize and attack tumors. Immune checkpoint inhibitors (ICIs) became a viable immunotherapy option by releasing the natural brake on T-cells, therefore disrupting the inhibitory T-cell signaling. Although those therapies were successful in maintaining an activation state of tumor-specific immune responses, only a small proportion of patients respond to these inhibitors due to ICI resistance.

One of the immunotherapy resistance mechanisms is mediated by the accumulation of immunosuppressive cell populations in the TME, such as tumor associated macrophages (TAMs) expressing Clever-1, which supports the formation of an immunosuppressive TME and contributes to T-cell dysfunction. The depletion of Clever-1 both genetically and pharmacologically using inhibitory antibodies has shown to activate the adaptive immune system and consequently, reduce tumor growth and metastasis.

These effects have been related to Clever-1 expression in TAMs, and more recently, to its secretion in body fluids (plasma and lymph). However, how Clever-1 is secreted in the body and its functional consequence in T-cell function is poorly understood. The possibilities include its secretion in extracellular vesicles (EVs) consisting of either exosomes (EXOs), microvesicles (MVs), or a soluble non-vesicular (NV) format. EVs are secreted by almost every cell in the body and enable intercellular communication by acting as a cargo for functional signaling molecules. They have immune regulatory nature and therefore, may either mediate immune activation or suppression.

The objective of the study is to define the link between poor T-cell antitumor immune responses and the significantly elevated levels of a soluble form of Clever-1 in the plasma of cancer patients (unpublished observations). In addition, characterize Clever-1^{+}EVs derived from human biological fluids such as blood and lymph and from cellular sources, such as monocytes. Moreover, evaluate Clever-1^{+}EVs capacity to target and bind T-cells. Lastly, investigate if Clever-1^{+}EVs have suppressive effects on T-cell proliferation.

2. Scientific background

2.1 Overview of the immune system

The immune system has a pivotal role in protecting the human body from antigens during its lifetime. An antigen is a term used to define any foreign substance to the human body. Antigens may include viruses, bacteria, fungi, chemicals, toxins, cancerous cells, and drugs. Recognition of an antigen or "non-self" protein followed by its eradication through an effector response are two critical functions of the immune system. This requires the orchestration between the immune system organs, specialized cells, signaling proteins, cytokines, and other soluble bioactive molecules(Abbas et al., 2018)

The lymphatic system which is a part of the immune system consists of lymphoid organs which is subclassified to generative and peripheral lymphoid organs. The generative, or primary lymphoid organs consist of the bone marrow and thymus. During a process called hematopoiesis, that occurs in the bone marrow, the blood immune cells are produced from hematopoietic stem cells (HSCs) precursors. They are then committed into two different lineage pathways either the lymphoid or myeloid lineage. Immune cells such as T-cells, B-cells, Natural Killer (NK) cells and innate lymphoid cells (ILCs) generate from the lymphoid lineage. Whereas the myeloid lineage develops into monocytes, which are later differentiated into macrophages and dendritic cells (DCs). In addition, it generates granulocytes (such as basophils, eosinophils, and neutrophils). (Abbas et al., 2018).

Maturation of all immune cells occurs in the bone marrow except for T-cells which mature in the thymus. Then, after maturation both naïve B-cells and T-cells migrate from their sites of origin to the peripheral, or secondary lymphoid organs which include the lymph nodes and spleen and there they get activated by antigens. The fluid that circulates in the lymphatic system is called the lymph, which consists of tissue fluids and blood plasma containing mainly water along with other proteins and dissolved substances. Moreover, it contains a high number of lymphocytes that fight against pathogens(Abbas et al., 2018).

There are two different types of sequential and coordinated responses that mediate the immune response: the innate immune response and the adaptive immune response. The innate immune system also referred to as the natural or native immunity mediates the early defense against microbes. It invokes a rapid immune response immediately after infection

during the first few hours or days. It constitutes of different components including: 1) a physical barrier such as the skin and the mucous membrane surface, which forms a protective shield between the interior and external environment; 2) phagocytic cells (i.e., macrophages, monocytes, neutrophils), DCs, NKs, mast cells and other innate lymphoid cells (ILCs); and 3) blood proteins and other inflammatory mediators(Abbas et al., 2018).

The innate immune cells detect via the pattern recognition receptors (PRRs) expressed on their cell surface both pathogen-associated molecular patters (PAMPs) accompanying pathogen infection and damage-associated molecular patterns (DAMPs) which are released from damaged or dying cells. Then, the professional antigen presenting cells (APCs) capture part of these peptide patterns and express them via the major histocompatibility complex (MHC I or MHC II) molecules to T lymphocytes and consequently, linking the responses of the innate and adaptive systems.

The innate immune system has two main characteristics: 1) lack of antigen specificity; and 2) 'trained immunity' which is a term proposed to verify that the innate immunity displays immunological memory to previous pathogenic encounter(Netea et al., 2011). In contrast, the adaptive immune system is characterized by a more specific immune responses against pathogens. However, the response is slower and usually takes a few days to peak after the innate immune system has recognized the antigen.

The adaptive immune responses are mediated by both T-cells and B-cells, which express specialized receptors, so called the T-cell receptors (TCRs) and B-cell receptors (BCRs), respectively. During their development, these receptors are genetically rearranged in a process called somatic recombination. This provides an enormous diversity in the antigen binding capacity of the receptors, which gives the adaptive immune system its distinctive trait. (Abbas et al., 2018).

The BCR receptor can bind to free antigens considering its conformational structure. Whereas the TCR binding capability is restricted to peptides presented via MHC molecules of the APCs. A second hallmark of the adaptive immune system is its ability to have immunological memory, which provides faster responses during the second encounter of the antigen and therefore, offers a life-long protection for the host against certain pathogens(Abbas et al., 2018).

2.2 Cancer Immunobiology

2.2.1 The hallmarks of cancer

To help understand the biology of cancer and establish a robust base for our understanding of the disease, Douglas Hanahan and Robert A. Weinberg identified six hallmarks of cancer which distinguishes it from normal cells. These core features provide exceptional characteristics to cancer cells allowing them to grow and metastasize(Hanahan and Weinberg, 2011).

The first hallmark of cancer is the maintenance of a proliferative signaling by interrupting the growth promoting signals and thus, dysregulating the growth and division cycle of the cell. Cancer cells can trigger an autocrine proliferation by expressing cognate receptors to self-produced growth factors or they activate the normal cells within the tumor stroma to produce various growth factors which further stimulate their proliferative ability(Cheng et al., 2008); (Bhowmick et al., 2004).

Alternatively, cancer cells may become hyperresponsive to certain growth factors due to the upregulation of their cell surface receptors. Also, proliferation may be stimulated independent of ligand binding either due to the structural variations that alter the cell surface receptors or can be facilitated by the previously activated elements within the downstream signaling pathways of these receptors (Hanahan and Weinberg, 2011).

A second hallmark is the ability of cancer cells to circumvent growth suppressors and therefore, halt the negative proliferation machinery. For example, when a defect occurs in the pathway of certain growth suppressor proteins, such as TP53 and RB (retinoblastoma), the cell then retains a continuous state of proliferation (Burkhart and Sage, 2008; Sherr and McCormick, 2002).

The third hallmark is the apoptosis resisting mechanisms of cancer cells. Normally, the cell maintains a protective mechanism against the development of cancer through a programmed cell death machinery called apoptosis (Adams and Cory, 2007; Lowe et al., 2004);(Evan and Littlewood, 1998). The most popular mechanism that the cancer cell develop is the loss of the tumor suppressor function of TP53 (Rivlin et al., 2011). Also, it may upregulate the expression of survival signals (such as Igf1/2) or induce the expression

of anti-apoptotic regulators (such as Bcl-2) and ultimately bypassing apoptosis (Warren et al., 2019; Weroha and Haluska, 2012). Another mechanism that supports the cancer cell to resist cell death is the downregulation of pro-apoptotic factors (such as Puma) (Yu and Zhang, 2008).

A fourth hallmark is the enabling replicative immortality of cancer cells in opposition of normal cell lineages, which undergoes a restrictive number of cell growth and division cycles (Blasco, 2005; Shay and Wright, 2000). Studies have shown that telomeres, which are DNA-protein complexes located at the very end of the chromosomes have a key role in producing the immortalized nature of cancer cells.

Generally, telomeres act as a protective cap to help reduce the loss of DNA that normally occurs with each cell division. They shorten gradually during chromosome replication and at some point, lose their protective capacity. Then, the cell may either enter an irreversible growth arrest state called senescence or a crisis leading to cell death. However, in cancer, increased telomerase enzyme activity extends the eroded telomeric DNA and thus, overcome the limited replicative state associated with normal cells (Okamoto and Seimiya, 2019).

The fifth trait is the ability of cancer to induce angiogenesis, which is the sprouting of new vessels from already developed ones. The neovasculature supplies the tumors with the outgrow demands of nutrients and oxygen. Indeed, the 'angiogenic switch' is activated for short periods during normal physiological processes, such as wound healing and menstrual cycles. However, in cancer, the activation is continuous leading to the extension of neoplastic growth (Hanahan and Folkman, 1996).

Due to tumor associated hypoxia and oncogenic signaling, a well-known angiogenesis inducer gene called vascular endothelial growth factor (VEGF) is upregulated (Carmeliet, 2005; Ferrara, 2009; Mac Gabhann and Popel, 2008). The levels of VEGF may exceed the capacity of thrombospondin-1 (TSP-1) which is an angiogenic inhibitor acting as a modulator and as an intrinsic barrier to angiogenesis and tumor progression(Kazerounian et al., 2008). Moreover, a growth factor that is constantly upregulated in cancer is the fibroblast growth factor (FGF), which induces proangiogenic signals (Baeriswyl and Christofori, 2009).

Activation of invasion and metastasis is the sixth complex hallmark capability of cancer. Cancer cells undergo alterations in the way they are attached to the extracellular matrix

(ECM) and with other cells during the process of metastasis and invasion. An adhesion molecule called E-cadherin, which is responsible for mediating cell-cell adhesions as well as cell junctions was observed to be downregulated in tumor cells. Multiple studies have correlated the decreased expression of E-cadherin with the ability of cancer cells to dissociate from its primary sites, invade neighboring tissues, and migrate to distant sites. Thus, E-cadherin is identified as a key regulator to tumor invasion and metastasis(Berx and van Roy, 2009; Cavallaro and Christofori, 2004).

Another two hallmarks of cancer were added in 2011 by the same authors including: 1) Reprogramming Energy Metabolism and 2) Avoiding Immune Destruction (Hanahan and Weinberg, 2011). The first added hallmark of cancer is accompanied by an increased demand of energy, which is supplied through the modifications that occur in the energy metabolic pathways in tumors. In a normal cell, the energy is supplied through two pathways either by mitochondrial oxidative phosphorylation (OXPHOS) under normoxic conditions or by anaerobic glycolysis under hypoxic conditions.

However, a cancer cell even with the abundance of oxygen still relies on glycolysis for its energy supply under a process called 'Warburg effect' or 'aerobic glycolysis' (Vander Heiden et al., 2009). This process produces only 2 ATP molecules from each glucose as compared with the OXPHOS metabolic pathway that yields 36 molecules of ATP from one glucose molecule. This necessitates an increased uptake of glucose by the tumor cell, which is achieved by the upregulation of glucose transporters (such as GLUT1). This hallmark was studied by using a tracer within tumors and visualizing the increased glucose uptake by the positron-emission tomography (PET) (DeBerardinis et al., 2008; Gambhir, 2002; Hsu and Sabatini, 2008).

The last added key feature of cancer is evading immune destruction, ultimately leading to cancer progression and metastasis. Even though the immune system is constantly destructing any nascent tumor cells under the concept of immune surveillance, tumors may escape immune killing by halting components of the immune system responsible for initiating anti-tumor immune responses. Based on experiments conducted on genetically engineered mice, the immune system is proved to act as a barrier to the formation of some non-virus induced cancers.

Indeed, tumors arose more frequently and more rapidly in mice genetically engineered to lack certain components of the immune system relative to immunocompetent mice. Moreover, the failure in the development of $CD8^+$ T lymphocytes, $CD4^+$ Th1 cells, or NK cells effector functions causes increase in tumor incidence. Furthermore, mice were more prone to cancer progression when they had deficiencies in both T-cells and NK cells indicating the involvement of both the innate and the adaptive immune systems in tumor eradication (Hanahan and Weinberg, 2011; Kim et al., 2007).

2.2.2 Cancer immunoediting

The immune system protects the body from cancer by detecting and eliminating nascent tumor cells under a process called immune surveillance(Schreiber et al., 2011). Studies carried out on both tumor mouse models and cancer patients demonstrate that both the innate and the adaptive immune cells along with other signaling pathways and effector molecules function as extrinsic tumor-suppressor mechanisms(Dighe et al., 1994; Girardi et al., 2001; Kaplan et al., 1998; Shankaran et al., 2001; Smyth et al., 2000a; Smyth et al., 2000b; Street et al., 2002; Vesely et al., 2011). But, beside the host protective function of the immune system it may also promote tumor progression(Zamarron and Chen, 2011). These dual functions of the immune system are referred to as the cancer immunoediting, which progresses through three different phases: elimination, equilibrium, and escape(Schreiber et al., 2011).

In the elimination phase, the innate and the adaptive immune systems collaboratively work to fully recognize and eradicate any transformed cells that have evaded the cell-intrinsic mechanisms of tumor suppression before they become clinically apparent. However, if tumor subclones succeeded in evading the immune system by resisting its elimination processes, they precede to the equilibrium phase. In this phase, the net tumor growth is limited and may overtime be hindered(Schreiber et al., 2011).

Nevertheless, due to the genetic instability of tumor cells and the continual pressure from the adaptive immune system certain mutations may develop allowing the tumor cells to evade the immune system destruction(Schreiber et al., 2011). These mechanisms may include the loss of antigen presentation, lack of sensitivity to immune effector mechanisms, induction of negative immune regulators or decrease*d* IFN-γ (interferon-gamma) secretion by T-

cells(Sharma et al., 2017). Immunoedited tumor cells which are usually less immunogenic are then selected and progress to the escape phase. In this phase, the tumor growth is unrestricted and the disease can be detected clinically(Schreiber et al., 2011).

2.2.3 Tumor Microenvironment

Previously, studies have focused on exploring the main factors inducing the genetic mutations causing cancer initiation and progression. However, the cellular environment surrounding cancer cells defined as the TME also vitally contributes to cancer development. Indeed, anti-cancer therapeutics alone are yet unsuccessful in providing the desired durable clinical outcomes. The TME constitutes of various components beyond tumor cells that include immune cells, adipocytes, fibroblasts, tumor vasculature, extracellular matrix (ECM), and soluble signaling proteins (Binnewies et al., 2018).

The immune cells within the TME comprise of immunosuppressive cells such as TAMs, myeloid-derived suppressor cells (MDSCs), and regulatory T (Treg) cells. It is also infiltrated with anti-tumor effector cells, such as cytotoxic $CD8^+$ T-cells, $CD4^+$ Th1 (T helper 1) and NK cells. Apart from cellular components, measures such as the pH level, oxygen saturation, metabolites, and amount of nutrients (glucose, fatty acids, and amino acids etc.) all compose the TME (Binnewies et al., 2018).

Most of the TME components evolve during their interactions with cancer cells creating a metabolically stressed and immunosuppressive microenvironment. Those interactions are mediated either via cell-cell contact or through the release of mediators including signaling proteins, cytokines, chemokines, growth factors, and EVs. The tumor evades the immune system by increasing tumor cells stemness, promoting angiogenesis and metastasis (Fiaschi and Chiarugi, 2012). Moreover, they recruit immunosuppressive cells that facilitate the immune surveillance escape (Liu and Cao, 2016).

Therefore, the TME is considered an intricate battlefield between the host immune system and the tumor cells. Current studies focus on developing strategies to target cells, molecules, and pro-tumorigenic processes in the TME that hijack the immune system (Galon and Bruni, 2019). ‘Hot’ and ‘cold’ TME are two simple subclasses of the TME. These descriptions are defined based on the infiltration of the T-cells and the production of proinflammatory cytokines. A hot inflamed TME is characterized by anti-tumor immune

signatures and an activated T-cell state. Therefore, reflecting a better prognosis and improved therapeutic intervention.

Conversely, a cold non-inflamed TME features inactivated dysfunctional T-cells devoid of inflammation. Therefore, displaying poor prognosis and lower response rates to immunotherapies such as anti-programmed death ligand (PD-L)1/PD-1 therapy (Duan et al., 2020). Thus, approaches to convert the cold tumor to hot one is a promising way to activate the immune system and sensitize the tumors to immunotherapy (Galon and Bruni, 2019).

2.2.4 Tumor associated macrophages

Macrophages are phagocytic cells that have a role in maintaining homeostasis. They engulf and digest cellular debris and foreign substances. Being part of the innate immune system, they initiate non-specific immune responses as well as contribute to specific immune responses of the adaptive immune system (Hirayama et al., 2017). In cancer, they make a substantial contribution to either tumor inhibition or progression. There are two extreme phenotypes of macrophages which either initiate or dampen T-cell activation. They differ in their transcriptomic and proteomic profile and therefore, in the biological mediators and metabolites they produce. This results in different immune functions between the two phenotypes.

The first phenotype is defined as M1-like macrophages; they induce a pro-inflammatory state enhancing tumor destruction and angiogenesis inhibition. However, the second phenotype defined as M2-like macrophages; induce an anti-inflammatory state promoting tumor initiation and progression (Chanmee et al., 2014; Malyshev and Malyshev, 2015). Fifty percent of the immune mass in solid tumors, such as breast cancer, constitute of macrophages(Poh and Ernst, 2018). Therefore, they significantly influence the immune activation state of the patient and their therapeutic outcomes.

Macrophages that reside in the TME are defined as TAMs, they originate from two sources: circulating monocytes in the blood or from resident macrophages in the tissue. They are recruited to the TME depending on several stimuli such as cytokines and chemokines produced by both malignant cells and non-malignant immune cells(Ge and Ding, 2020). TAMs acquire M2-like features due to, among other mechanisms, lactate-induced TME

acidification produced by the high glycolytic activity of cancer cells.(Colegio et al., 2014; Mu et al., 2018).

Several studies have revealed different pathways by which TAMs induce immunosuppression and proangiogenic functions. A major mechanism is by secreting cytokines such as IL-6, IL-8, and IL-10. IL-6 is additionally secreted by tumor cells, immune cells, and stromal cells within the TME(Ge and Ding, 2020; Kumari et al., 2016; Noy and Pollard, 2014). Its increased expression in tumor correlates with poor prognosis of cancer patients(Ahmad et al., 2018; Bellone et al., 2006; Chen et al., 2012; Lesina et al., 2011; Li et al., 2008; Matsuo et al., 2009; Tang et al., 2005).

IL-6 induces the expression of signal transducer and activator of transcription 3 (STAT3) gene on tumor cells, which in turn promotes the expression of IL-6 gene establishing a forward feedback loop. STAT3 gene encodes the production of tumor proliferative and survival proteins (such as cyclin D1 and BCL-x_L)(Johnson et al., 2018). Moreover, the expression of other proangiogenic immunosuppressive factors like VEGF, IL-10 and transforming growth factor β (TGF-β) are induced by STAT3(Rébé and Ghiringhelli, 2019).

Apart from tumor cells, STAT3 positively modulates immune cells like Tregs and MDCS populations and negatively influences anti-tumor NK cells and Teffs and thus, contributes to an immunosuppressive microenvironment(Rébé and Ghiringhelli, 2019). Similarly, elevated IL-8 levels in the blood indicates poor therapeutic benefit from immunotherapies as immune checkpoint blockade (ICB) (Schalper et al., 2020). IL-8 induces the expression of STAT3 and another protein called engulfment and cell motility 1 (ELMO) on cancer cells, which mediates tumor metastasis(Fu et al., 2015). In addition, IL-8 can interfere in the antigen presenting function and polarization properties of macrophages and APCs(Valeta-Magara et al., 2019).

In addition to the production of cytokines, TAMs express various receptors and ligands that contribute to immunosuppression. PD-1 is normally expressed by T-cells and when binding to its ligand PD-L1 on APCs, halts the T-cell from launching an immune response. This is a mechanism by which the immune system self-regulates its immune responses to prevent tissue damage and autoimmune diseases(Boussiotis et al., 2014). However, tumor cells as well as TAMs can express PD-L1, which mediates the PD-1/L1 signaling pathway.

Therefore, limiting the Teffs functions and eventually, escaping the immune surveillance system(Gordon et al., 2017; Katsuya et al., 2016; Yu et al., 2015a).

CD47 molecule is another receptor expressed on tumor cells that exhibits the 'do not eat me' signal to its ligand SIRPα expressed on TAMs. Therefore, the SIRPα/CD47 signaling enhances the occurrence of tumor by preventing the phagocytosis of cancer cells by macrophages(Chao et al., 2012). Another mechanism underscored to suppress immunity by TAMs is through the fusion of EVs (such as EXOs). TAMs derived EVs associated with miR-21-5p and miR-29a-3p cause imbalance in the Treg/Th17 (T helper 17) cells and inhibit the STAT3 pathway leading to epithelial ovarian cancer progression(Zhou et al., 2018; Zhou et al., 2020). Moreover, TAM-EVs expressing miR-223 lead to the development of drug resistance by activating the PTEN-PI3K/AKT pathways in epithelial ovarian cancer (Zhu et al., 2019). All these studies among others disclose TAMs as a suppressor cellular population in the TME that when targeted may lead to better therapeutic outcomes.

2.2.5 Cancer Immunotherapy

Any treatment that enhances and boosts the immune system to fight against cancer is referred to as cancer immunotherapy. It emerges as a fundamental strategy in treating metastatic solid cancers along with the already well-established methods including cytotoxic chemotherapy, radiation, surgery, and other targeted therapies(Farkona et al., 2016). The T-cell activation is negatively regulated by the so called 'checkpoint' molecules. They tightly tune the immune response to prevent hyperactivation and autoimmune disorders. However, they still preserve its role in fighting against pathogens. The well-known examples for these molecules are the Cytotoxic T lymphocyte Antigen 4 (CTLA4) and PD1 (Fife and Bluestone, 2008).

When CD28 protein was discovered to have a role in mediating the co-stimulation of T-cells, further efforts were directed towards the identification of other immune regulators which eventually lead to the discovery of CTLA-4 (Brunet et al., 1987; Dariavach et al., 1988). The receptor has similarities to CD28 protein in terms of structure and therefore, they both bind to the same ligands expressed on APCs of B7-1 (also called CD80) and B7-2 (also called CD86). However, CTLA-4 molecule possesses higher affinity and avidity to the B7 ligands and therefore, exerts different biological activity and even an oppositional immune

function to CD28 (Linsley et al., 1991; Linsley et al., 1994). For example, the CTLA-4 protein halts the immune response by acting as a brake on T-cells, whereas CD28 drives the initiation of an immune response(Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1994).

James P Allison was the pioneer in proving this theory when he blocked the CTLA-4 receptor with an antibody that unleashed the effector immune responses against cancer in experimental models(Kwon et al., 1997; Leach et al., 1996). This discovery further led to the development of Ipilimumab, the first in a class of drugs known as ICIs. The drug showed promising results especially for late-stage metastatic melanoma patients and was FDA approved in 2011 for its proved results in causing tumor necrosis and improving the overall survival (OS) of melanoma patients(Grosso and Jure-Kunkel, 2013; Hodi et al., 2003; Hodi et al., 2010; Schadendorf et al., 2015).

Studies show that CTLA-4 exhibits its inhibitory effect on T-cell through multiple mechanisms. It could directly compete with the CD28 ligand and associates with the TCR receptor. Thus, preventing the immune conjugate formation while helping in the recruitment of other inhibitory effects(Intlekofer and Thompson, 2013). It may also prevent the binding of CD28 to its ligands by processing their internalization which in turn causes a reduction in IL-2 secretion and T-cells proliferation(Greenwald et al., 2001; Krummel and Allison, 1995; Qureshi et al., 2011). Moreover, it can have a role in the disruption of the immunological synapse between the T-cell and APC by reorganizing the cytoskeleton(Schneider et al., 2006).

Finally, phosphatases (such as SHP2 and PP2A) mediate the inhibitory effect on T-cells when they are recruited and bind to the cytoplasmic tail of CTLA-4(Chuang et al., 2000; Chuang et al., 1999; Marengère et al., 1996). Tregs also express CTLA-4 that has role in maintaining the immunosuppressive and anti-inflammatory effects of these cells(Jain et al., 2010; Wing et al., 2008). The CTLA-4 targeted agent is likely to produce a therapeutic response based on an already available T-cell response. Beside enhancing the Teffs responses, it is also reported to diminish intratumoural Tregs from the TME and helping in reverting the immunosuppression in mouse models. However, further studies are required to have a conclusive data about this phenomenon(Peggs et al., 2009; Sharma et al., 2019). Also,

a synergistic effect in anti-tumoral activity is observed by the specific blocking of CTLA-4 in both populations of T-cells and Tregs(Peggs et al., 2009).

Generally, previous studies confer that the ratio of T-cells to Tregs that infiltrates the tumor site is a crucial factor in determining the clinical benefit(Grosso and Jure-Kunkel, 2013; Yang et al., 1997). Another receptor analogous to CTLA-4 that was identified to regulate the immune system is the PD-1 protein. It was first recognized as a mediator of apoptosis; however, later it was proved that it restricts the hyperactivation of the immune system(Ishida et al., 1992). The loss of PD-1 orthologue named pdcd1 was observed to cause the development of autoimmunity in an in-vivo mouse model(Nishimura et al., 1999). Also, splenomegaly was detected when C57BL/6 mice lacked the functional protein PD-1(Nishimura et al., 1998).

After T-cell stimulation through the TCR receptor, the PD-1 is expressed on the cell surface and then it interacts with the B7 ligands (such as PD-L1 and PD-L2) present on APCs which then deactivates the T-cell(Freeman et al., 2000; Latchman et al., 2001). Moreover, the PD-L1 expressed on APCs has a role in controlling both the differentiation and suppressive activity of Tregs(Francisco et al., 2009). The tumor cells evade the immune system by inducing the expression of PD-1 ligands that generates an immunosuppressive TME and develops dysfunctional T-cells(Wherry and Kurachi, 2015).

It was observed that PD-1 ligands were overexpressed in cancer cell lines and negatively influenced the anti-tumoral activity of $CD8^+$ T-cells. Also, when PD-1 was blocked in syngeneic animals, the growth of transplanted myeloma cells was restricted(Hirano et al., 2005; Iwai et al., 2002). Several preclinical studies have correlated the high expression of PD-1 and negative immune regulation. Efforts were then directed to interfere with the PD-1/PD-L1 pathway to develop a biomarker and a cancer treatment, which then led to the development of anti-PD-1 mAbs (monoclonal antibodies) such as Pembrolizumab and Nivolumab.

They were the first FDA approved and fully humanized anti-PD-1 used for refractory and unresectable melanoma(Berman et al., 2015; Gong et al., 2018). PD-L1 mAb named Atezolizumab was also developed and proved effective in the treatment of urothelial carcinoma and was therefore, given FDA approval in 2016(Rosenberg et al., 2016).

Beside the development of ICIs, the Adoptive T-cell (ATC) therapy emerged as a successful and viable option for cancer immunotherapy. This treatment relies on infusing either autologous or allogenic T-cells into cancer patients(Waldman et al., 2020). ATC with tumor-infiltrating lymphocytes (TILs) depends on the isolation of lymphocytes from a cancer biopsy. The cells along with a large bolus of IL-2 are then reinfused intravenously to the same patient after their expansion using IL-2(Rosenberg et al., 1988; Rosenberg et al., 1994).

However, many cancer types are not responsive for ATC therapy since the tumor must allow infiltration of anti-tumoral Teffs, which is not always the case(Perica et al., 2015). Also, there have been challenges during the expansion of tumor-specific T cells in vitro, which further led to the development of engineered lymphocytes. The lymphocytes are first modified to express synthetic chimeric antigen receptors (CARs). These receptors allow the T-cells to directly exert cytotoxic activity on surface antigens without the necessity of presenting the tumor antigens by the MHC molecules. Therefore, this strategy bypasses the MHC downregulation challenge induced by the tumor cells(Garrido et al., 2016).

In addition, therapeutic and prophylactic cancer vaccines emerged as another cancer immunotherapy-based approach. The vaccines are used to either harness the immune system to eliminate pre-existing tumor cells expressing neoantigens or to prevent the future development of cancer(Guo et al., 2013). An example of a therapeutic vaccine is the bacillus Calmette–Guérin vaccine that was initially used as a prophylactic tuberculosis vaccine, but its administration was then repurposed to prevent the incidence of bladder vaccine(Morales et al., 1976). In addition, the occurrence of both hepatocellular carcinoma and cervical cancer by oncogenic viruses is decreased by administering the hepatitis B and human papillomavirus prophylactic vaccines, respectively(Guo et al., 2013).

Combining several cancer treatments with distinct mechanisms is now emerging as a strategy to control the complex immunopathogenesis of cancer. For example, the combinatory treatment of Ipilimumab and Nivolumab was FDA approved due to improved survival observed in both metastatic melanoma and advanced renal cell carcinoma patients(Motzer et al., 2018; Wolchok et al., 2017). Another example which has shown significant synergistic outcome with recalcitrant tumors is combining radiation therapy with immune checkpoint blockade (Twyman-Saint Victor et al., 2015). Moreover, many new negative T-cell regulators targets (such as LAG3, TIM3, VISTA and TIGIT) have been

revealed and may be developed as promising adjuvant checkpoint blockers(Burugu et al., 2018; Donini et al., 2018).

2.2.6 Mechanisms of immunotherapy resistance

Due to the dynamic and continuous evolving nature of immune responses in cancer patients, molecular mechanisms of immunotherapy resistance may be present at the initial stage of tumor presentation reflecting primary resistance or can develop when the tumor starts to protect itself by adapting to the immune attack signifying adaptive resistance. Certain environmental elements, treatment interventions, and genetic factors may also play a role in developing immunotherapy resistance. Also, acquired resistance to immunotherapy develops when the patient initially responds to immunotherapy but soon after develop resistance leading to tumor relapse and progression. Each type of immunotherapy resistance (primary, adaptive, and acquired) is further categorized into tumor cell intrinsic and extrinsic factors(Sharma et al., 2017).

Tumor cell intrinsic factors associated with primary and adaptive immunotherapy resistance are mainly linked with the increased or decreased expression of certain genes in tumor cells that have a role in influencing the immune cells infiltration and function within the TME (Sharma et al., 2017). For example, the MAPK pathway activated during oncogenic signaling serves to produce inhibitory proteins such as VEGF and IL-8, which negatively disturbs the T-cell function and recruitment process(Liu et al., 2013). Moreover, the enhancement of PI3K signaling pathway which is considered a key regulator of cancer through the downregulation of PTEN was correlated with resistance to anti-cancer therapy such as immune checkpoints(Peng et al., 2016). Notably, in T-cell-inflamed tumors, the rate of deletions and mutations associated with PTEN was lower in comparison to the non-T-cell inflamed tumors.

In addition, a decrease in $CD8^+$ T-cell infiltration into tumors and lower expression of granzyme B (GrB) and IFN–γ genes along with PTEN loss was observed in the melanoma dataset of the Cancer Genome Atlas (TCGA) (Sharma et al., 2017). A study conducted by Spranger et al. (2015) identified the WNT/β-catenin signaling pathway to induce T-cell exclusion in melanoma and develop resistance to PD-1/CTLA-4 immunotherapies. Interestingly, in a murine tumor model, the reduced expression of the chemokine CCL4

which is responsible for recruiting $CD103^{+}$DCs, a subset of DCs, was spotted in tumors with higher b-catenin levels. Furthermore, an effective response to immune checkpoint therapy was observed in murine tumors lacking b-catenin conversely to tumors possessing b-catenin. Also, it was observed that the TME deprived from T-cells and $CD103^{+}$ DCs was associated with increased expression of b-catenin signaling genes (Spranger et al., 2015; Hu-Lieskovan et al., 2015).

The expression of PD-L1 and PD-L2 ligands on the surface of tumor cells result in disrupting T-cell immune attack. The genes encoding for these surface ligands along with the Janus kinase 2 (JAK2) located on chromosome 9 is known as the PDJ amplicon(Ansell et al., 2015). A genetic amplification of the PDJ amplicon occurs in Hodgkin's disease specifically in Reed-Sternberg cells and effective responses to anti-PD-1 therapy is achieved in more than 80% of chemotherapy-refractory patients with Hodgkin's disease(Ansell et al., 2015; Green et al., 2010). The expression of PD-L1 in cancer cells can be induced by several other mechanisms including the deletions of PTEN, mutations accompanying PI3K and/or AKT, disruption in CDK5 (Cyclin Dependent Kinase 5), and the overexpression of the proto-oncogene MYC(Casey et al., 2016; Dorand et al., 2016; Lastwika et al., 2016; Parsa et al., 2007).

It is uncertain whether the increased expression of PD-L1 ligands on cancer cells will result in improved or poorer responses with anti-PD-1 and PD-L1 therapies. However, it is apparently responsible for the lack of anti-tumor T-cell immune responses, ultimately interfering with the potential effect of other cancer immunotherapies. Another key intrinsic factor leading to immunotherapy resistance is the loss of the IFN-γ signaling pathway. The IFN-γ pathway has a major role in modulating anti-tumor immune responses. The produced IFN-γ by T-cells enhances tumor specific T-cell responses by increasing the expression of the molecules involved in antigen presentation such as MHC molecules.

Moreover, the produced IFN-γ helps in the recruitment of other immune cells, exhibits an anti-proliferative effect on tumor cells, and induces their apoptosis(Platanias, 2005; Shankaran et al., 2001). However, due to the prolonged exposure of cancer cells to IFN-γ they may develop mechanisms to escape its anti-tumor effects. For example, the IFN-γ receptor chains (such as JAK1 and/or JAK2) may undergo mutations or downregulation by the tumor cells. Thus, disrupting the IFN-γ signaling pathway (Darnell et al., 1994).

Interestingly, a higher genetic mutation in the IFN-γ receptors 1 and 2, interferon regulatory factor 1 (IRF1), and JAK2 was observed in the tumors of cancer patients who lacked a therapeutic response to anti-CTLA-4 antibody, Ipilimumab. These mutations inhibit IFN-γ signaling and therefore, provide an opportunity for tumor cells to escape immune attack of T-cells and the development of primary immunotherapy resistance(Gao et al., 2016).

The primary and adaptive immunotherapy resistance developed by the tumor-cell intrinsic factors is mainly related to the tumor cell itself whereas the extrinsic factors involve other elements in the TME that inhibit anti-tumor immune responses. Those elements include immune inhibitory cells such as MDSCs, Tregs, TAMs, and other immune inhibitory checkpoints such as CTLA-4 and PD-L1. The human MDSC expressing markers such as $CD11b^{+}$ and $CD33^{+}$ are defined as a modulator of immune responses in several diseases involving cancer(Wesolowski et al., 2013).

Studies show that MDSCs contribute to tumor cell invasion, angiogenesis, and metastases(Yang et al., 2004; Yang et al., 2008) Moreover, the presence of MDSCs is correlated with decreased survival rates in breast and colorectal cancer patients and with poor therapeutic outcomes using immunotherapies(Solito et al., 2011). Thus, recent studies focus on eliminating and reprogramming MDSCs to improve the patient's clinical outcomes.

Rudensky (2011) stated that self-tolerance is maintained by Tregs, which are other immune regulatory cells specified by the expression of the transcription factor FoxP3(Rudensky, 2011). Also, several studies have shown that several inhibitory cytokines including TGF-β, IL-10 and IL-35 produced by Tregs inhibit the anti-tumor function of Teffs cells (Sakaguchi et al., 2008).Depleting Tregs from the TME in mice helps to restore the anti-tumor immunity(Linehan and Goedegebuure, 2005; Viehl et al., 2006). Also, the ratio of Teffs to Tregs in murine models is increased in response to anti-CTLA-4 therapy(Quezada et al., 2006).

Moreover, TAMs can as well contribute to the inhibition of the anti-tumor immune response. They are associated with poor prognosis and therapeutic resistance in cancer patients(Hu et al., 2016). In hepatocellular carcinoma, TAMs directly suppress the effect of Teffs cells through the PD-L1 receptor(Kuang et al., 2009). Eliminating TAMs in lung

adenocarcinoma mouse model helps to reduce tumor growth due to the inactivation of CCL2 and/or CCR2 signaling(Fritz et al., 2014).

The same signals that enhance the immune responses against cancer tend to activate the immune checkpoints (such as CTLA-4 and PD-1) immunoinhibitory signaling pathways and therefore, result in the downregulation of immune responses against tumors. For example, the CTLA-4 checkpoint expression is upregulated after the initial activation of the T-cell through the co-stimulation of the TCR and CD28 receptor(Leach et al., 1996). Likewise, the expression of PD-L1 protein is increased on several cells including T-cells, tumor cells, and macrophages by the increased production of IFN-γ from Teffs. The PD-L1 then interacts with the PD-1 receptor on T-cells and inhibits the immune activation against cancer(Chen, 2004; Dong et al., 2002).

Several ongoing studies have identified other inhibitory immune checkpoints in the TME (such as LAG-3, TIGIT, VISTA) that may have a role in the extrinsic mechanisms causing immunotherapy resistance(Topalian et al., 2015). Apart from immune regulatory cells and immune checkpoints, there are other immune suppressive elements such as the cytokines secreted by either tumor cells or macrophages. For example, the increased levels of the cytokine termed TGF-β is reported to be associated with poor prognosis in several cancers(Lin and Zhao, 2015; Massagué, 2008). It has a role in promoting angiogenesis and suppressing the immune system by activating Tregs(Lebrun, 2012).

Moreover, the cytokine adenosine suppresses T-cell proliferation by interacting with the A2A receptor(Zhang et al., 2004). Also, it interacts with the A2B receptor expressed on tumor cells and promotes metastasis(Mittal et al., 2016). The enzyme CD73 forms adenosine through the dephosphorylation of adenosine monophosphate (AMP) and thus, has a role in inhibiting immune attack, promoting angiogenesis, and stimulating metastasis(Stagg et al., 2010).

Several data suggest the correlation between high expression of CD73 in several cancer patients with poor clinical outcome(Loi et al., 2013; Turcotte et al., 2015) . Also, the high expression of CD73 corresponds with limited efficacy of anti-PD-1 therapy(Beavis et al., 2015).Moreover, the tumor cells produce chemokines such as CCL5, CCL7, and CXCL8 that bind to their corresponding receptors (CCR1 OR CXCR2) expressed on subtypes of MDSCs and Tregs. Then, they direct the trafficking of these cells to the TME(Highfill et al., 2014).

The T-cell anti-tumor immune responses could be then enhanced through the inhibition of these chemokine receptors.

Despite the continuous immunotherapy with anti-CTLA-4 or anti-PD-1 the patients receive. They experience relapse at some point. This is attributed to the development of acquired resistance due to several mechanisms that may include loss of the effector function of T-cells, lack of tumor antigen presentation due to the loss of the antigen presenting machinery, and the development of mutations enabling immune escape(Sharma et al., 2017).

For example, within months after receiving the ACT of T-cells engineered to recognize melanoma antigens (such as MART01, gp100) and other cancer testis antigens (NY ESO-1) patients may experience tumor relapse(Chodon et al., 2014; Morgan et al., 2006; Robbins et al., 2011). Studies show that the anti-tumor T-cells may start losing their cytotoxic activity due to a change in their functional phenotype(Ma et al., 2011). Also, some patients develop acquired resistance to IL-2 and tumor-infiltrating lymphocytes (TILs) ACT due to the loss of B2M, which is a common component of all the HLA class I molecules. This leads to the absence of $CD8^+$ T-cell recognition since B2M is essential for the surface expression of the antigen presenting machinery HLA class I. Remarkably, a mutation in B2M was recognized in a patient with late acquired resistance to anti-PD-1 therapy.

Also, the functional mutations that occur within JAK1 or JAK2 allow the cancer cells to escape the anti-proliferative effects of IFN-γ and ultimately, lead to tumor relapse(Zaretsky et al., 2016). Moreover, the deletions of neoantigens that are recognized by anti-tumor cells may lead to acquired resistance. For example, in some patients with acute lymphocytic leukemia (ALL), the selective deletion of the epitope CD19 that is recognized by the CAR T-cell ACT may lead to the development of acquired resistance and ultimately cancer progression(Ruella et al., 2016; Sotillo et al., 2015).

2.2.7 Clever-1 and immune suppression

Clever-1 is a large type H scavenger receptor weighing 270-300 kDa, designated common lymphatic endothelial and vascular endothelial receptor-1. It is also referred to as Stabilin-1 or Feel-1, coded by the Stab1 gene. The structure of Clever-1 contains extracellular, intracellular, and transmembrane parts. The extracellular portion is large, consisting of seven fasciclin-like domains, one x-link homology region, and several

epidermal growth factor-like domains (EGF) (Kzhyshkowska et al., 2006; Murphy et al., 2005). However, the intracellular portion is a small tail which directs the endosomal trafficking of Clever-1 upon its internalization by interacting with intracellular protein adaptors(Adachi and Tsujimoto, 2010; Kzhyshkowska et al., 2004).

Clever-1 expression is induced by inflammatory and proangiogenic stimuli on continuous vascular endothelia. Moreover, it is expressed in post-capillary venous structures termed high endothelial venules as well as in the discontinuous sinusoidal endothelia in the bone marrow, lymph nodes, liver, spleen, and renal cortex(Goerdt et al., 1993; Goerdt et al., 1991). In addition, Clever-1 is expressed in a subset of human blood monocytes as well as in the placenta, lymph nodes, colon, skin, and stomach specialized tissue macrophages(Goerdt et al., 1993; Mosig et al., 2009; Palani et al., 2011).

Clever-1 has mainly three important functions based on the location it is expressed in. First, on endothelial cells it acts as a cell adhesion molecule and as a regulator of leukocyte trafficking through both the lymphatic and vascular endothelium. Secondly, on sinusoidal endothelial cells and macrophages, which are both considered specialized scavenger cells, it functions as a scavenger receptor by internalizing acetylated and oxidized LDL, Gram-positive and Gram-negative bacteria, and phosphatidylserine located on apoptotic host cell surface(Hollmén et al., 2020; Karikoski et al., 2009; Salmi et al., 2004).

Beside acting as a scavenger receptor and adhesion molecule, Clever-1 also mediates the transportation and intercellular sorting of its ligands including placental lactogen, SPARC (secreted protein, acidic and rich in cysteine) and SI-CLP (Stabilin-1-interacting, chitinase-like protein) through different endosomal pathways. Under homeostatic conditions, Clever-1 is expressed in a subset of human blood monocytes and is a well-established marker for M2 macrophages both in vitro and in vivo(Hollmén et al., 2020). The expression of Clever-1 is upregulated when monocytes are cultured in-vitro along with M2-polarizing factor dexamethasone and glucocorticoid analogues. However, the expression is down regulated by the addition of M1 polarizing stimuli such as IFN-γ(Palani et al., 2016).

The anti-inflammatory phenotype of both monocytes and macrophages is related to Clever-1 expression. For example, in normal pregnancy, in the placenta, Clever-1 maintains an immunosuppressive environment. However, it was observed that in pre-eclampsia Clever-1 is significantly decreased. Cancers that have increased levels of macrophages expressing

Clever-1 have poor prognosis. The process by which Clever-1 regulates cancer behavior is not fully understood yet. A study showed that mice models lacking Clever-1 had smaller primary and metastatic tumors as compared to wild type controls. In addition, tumor progression in wild type mouse models was inhibited when the animals received anti-Clever-1 antibody treatment(Karikoski et al., 2014).

Moreover, it was observed that Clever-1 blockade reactivates $CD8^+$ T-cell anti-tumor responses combined with α-PD1(Viitala et al., 2019). Also, it was proved that Clever-1 suppresses B-cells antibody production(Dunkel et al., 2018). Palani and colleagues showed that Clever-1 positive monocytes, when silenced or treated with anti-clever-1 Ab, results in improved Th1 cells antigen specific immune responses(Palani et al., 2016). Therefore, these findings highlight Clever-1/STAB1 as a promising target for cancer adjuvant immunotherapy.

However, the mechanism by which Clever-1 regulates Th1 dependent inflammatory responses need to be further investigated. Preliminary unpublished data obtained by the host research group revealed that cancer patients express higher levels of free Clever-1 in the plasma compared with normal donors, suggesting that extracellular Clever-1 may have a functional role in the modulation of patients' immune responses.

2.3 Immune signaling networks in the context of cancer

2.3.1 Cytokines

Cytokines are proteins secreted by different immune cell types having molecular weight below 30 kDa. They are produced in response to an immune stimulus and carry different signals to other cells that may include inflammatory, anti-inflammatory, growth, activation, or differentiation signals. They exert either an autocrine, paracrine, or endocrine effects. Since they have a short half-life in the circulation, they exert their biological action only for a short-defined period. They modify gene transcription following the intracellular signaling upon binding to high affinity receptors on the cell surface membrane. Therefore, they regulate different immune responses and manage immune cell trafficking.

They either produce cell-mediated, humoral, cytotoxic (antiviral and anticancer), or allergic immune responses. Interestingly, each cytokine may have pleiotropic functions

depending on several factors including cellular source, target cell, concentration, and the stage of immune response in which it is secreted(Abbas et al., 2018). For example, low concentrations of IL-2 promote tumor growth. However, high IL-2 concentrations activate specific immune cells such as $CD8^+$, $CD4^+$, and NK cells. Then, these cells secrete proinflammatory cytokines as IFN-γ which aid in eradicating tumor cells(Valle-Mendiola et al., 2016).

Cytokines are classified into type-1 and type-2 cytokines based on the type of immune response they produce. For example, type-1 cytokines that include IL-2, IL-12, IL-15, and IFN-γ mediate Th1 immune responses, which produce proinflammatory cell-mediated immunity. They activate both $CD8^+$ cytotoxic T lymphocytes and NK cells. Moreover, they promote antigen presentation by upregulating MHC expression on the APCs. However, Th2 immune responses involve type-2 cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13 which produce anti-inflammatory immunosuppression responses by inhibiting Th1 cytokine production(Xu et al., 2016). TGFβ1, a cytokine which is secreted by another subtype of Th cells called Tregs promotes tumor escape by antagonizing IL-2 function. It also induces mesenchymal transition therefore, promoting tumor progression(Jakowlew, 2006; Wrzesinski et al., 2007).

2.3.2 Extracellular Vesicles

The immune system consists of a network of different cell types. The communication between cells is maintained through different pathways one of which is through shuttling of nano sized EVs. They mediate the processing of several physiological and pathological pathways. They behave as vehicles of biomolecules including proteins, nucleic acids, and lipids. Based on their origin and morphology, they are categorized into three groups: EXOs, MVs, and apoptotic bodies (ABs) as shown in Figure 1. If the vesicles are formed from multivesicular endosomes they are called EXOs. Once endosomal compartments merge with the plasma membrane they are then secreted outside the cell. However, MVs are released by the direct shedding of the cell membrane. When EVs are released, they exert their effects either when taken up by different distant target cells or by binding to certain cell-surface receptors(Colombo et al., 2014).

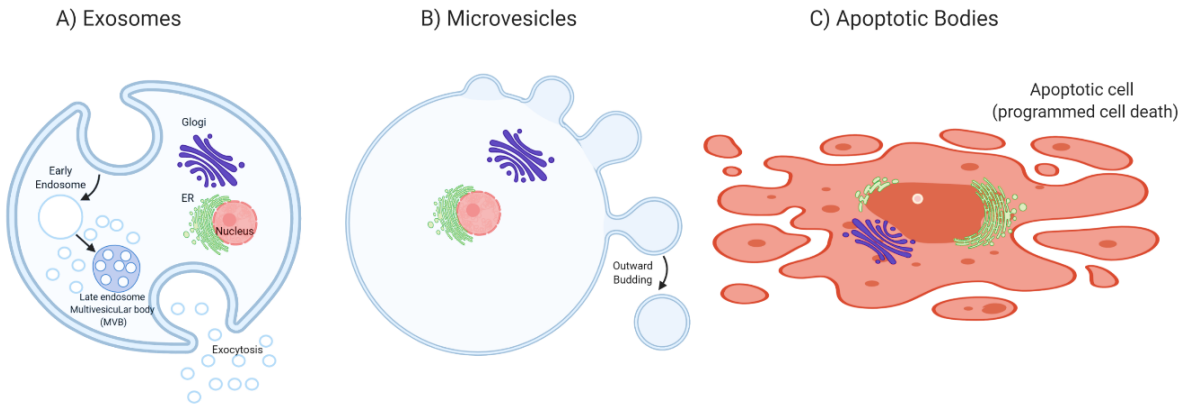

Figure 1. *Schematics representing the extracellular vesicles (EVs) formation by stromal cells of the tumor microenvironment (TME). A) Exosomes (EXOs), B) Microvesicles (MVs), and C) Apoptotic bodies (ABs). Figure "created with Biorender.com".*

EVs play an important role in immune response regulation. They have the capacity to induce both pro-inflammatory and anti-inflammatory effects by releasing their cargo, which in turn modulates certain immune responses. For example, EVs carrying microbial antigens or pathogen-associated molecular patters (PAMPs) can initiate inflammatory responses by activating macrophage Toll-like receptors (TLRs) (Bhatnagar et al., 2007). Also, immature DC-derived EVs express reduced amounts of costimulatory molecules, MHC complexes, and integrins which explain their immune-inhibitory effects(Segura et al., 2005). T-cell proliferation is also inhibited by the EXOs derived from both IL-10-stimulated DCs and the growth factor TGFβ stimulated DCs(Cai et al., 2012; Kim et al., 2005). EVs derived from M1-like macrophages have miRNA content resembling the M1 signature which can initiate an immune response(Garzetti et al., 2014).

In addition, EVs transport different types of cytokines either on their membrane surface or inside their lumen, which could then kill targeted cells. The tumor necrosis factor (TNF) and CD95 ligand (CD95L) carried on EVs secreted by both NKs cells and DCs initiate a cytotoxic response against tumor cells(Munich et al., 2012). Synthesis of leukotriene B4 (LTB4) and LTC4 is also induced by the enzymes carried by DC-derived and macrophage-derived exosomes(Esser et al., 2010). Alongside, EVs promote immunosuppressive responses through different routes to preserve a homeostatic immune profile and thus, preventing systemic inflammation. EVs derived from M2 like macrophages have miRNA content resembling the M2 signature that could suppress immune responses and induce cancer metastasis(Garzetti et al., 2014).

Therefore, understanding the biological effects of EVs derived from M2 macrophages is considered a potential key for the development of adjuvant strategies that can restore the immune response. TAMs often express the M2 functional marker Clever-1, and we want to evaluate if Clever-1^{+}EVs would target and potentially have a suppressive effect on T-cells activation. Then, Clever-1 blockade could represent a suitable strategy to inhibit the immunosuppressive effects triggered by M2-like derived EVs.

3. Objectives

The ultimate objective of this thesis is to characterize the expression of Clever-1 in EVs and their potential interaction with T lymphocytes (Figure 2*).*

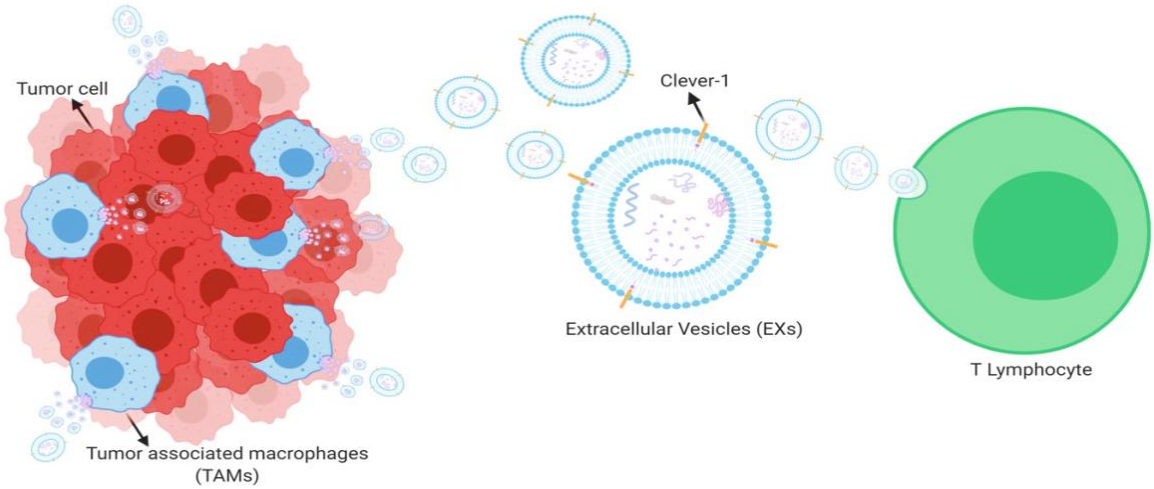

Figure 2. *The main hypothesis of the study is that Clever-1^{+}extracellular vesicles (EVs) secreted from TAMs and monocytes interact with T lymphocyte and disrupt its functional activation. Figure "created with Biorender.com".*

<u>The goals of the thesis are as follows:</u>

1) Characterize Clever-1 expression in EVs derived from different human biological fluidics and monocytic cellular sources.

2) Quantify the interaction and characterize the spatial distribution of macrophage/monocyte derived EVs on T lymphocytes.

3) Evaluate strategies to investigate whether and how EVs functionally interact with T lymphocytes.

Based on previous literature, Clever-1 is considered as an immune suppressor molecule. It supports tumor growth, metastasis, and the maintenance of the immunosuppressive phenotype of tumor infiltrating $CD8^+$ T-cells(Viitala et al., 2019). Preliminary unpublished data suggest that extracellular Clever-1 is significantly increased in the plasma of cancer patients as compared with normal donors.

Although among all cancer patients increased level of Clever-1 was associated with lower survival rates. One potential source of soluble Clever-1 are the EVs secreted by monocytes or TAMs. EVs secreted by both immune and non-immune cells mediate several intercellular communications. They act as a cargo to either immune stimulatory or inhibitory molecules. Therefore, they contribute greatly to the level of immune activation in cancer patients and to their therapeutic outcomes. The aims and methodological approaches of this study are represented in Figure 3.

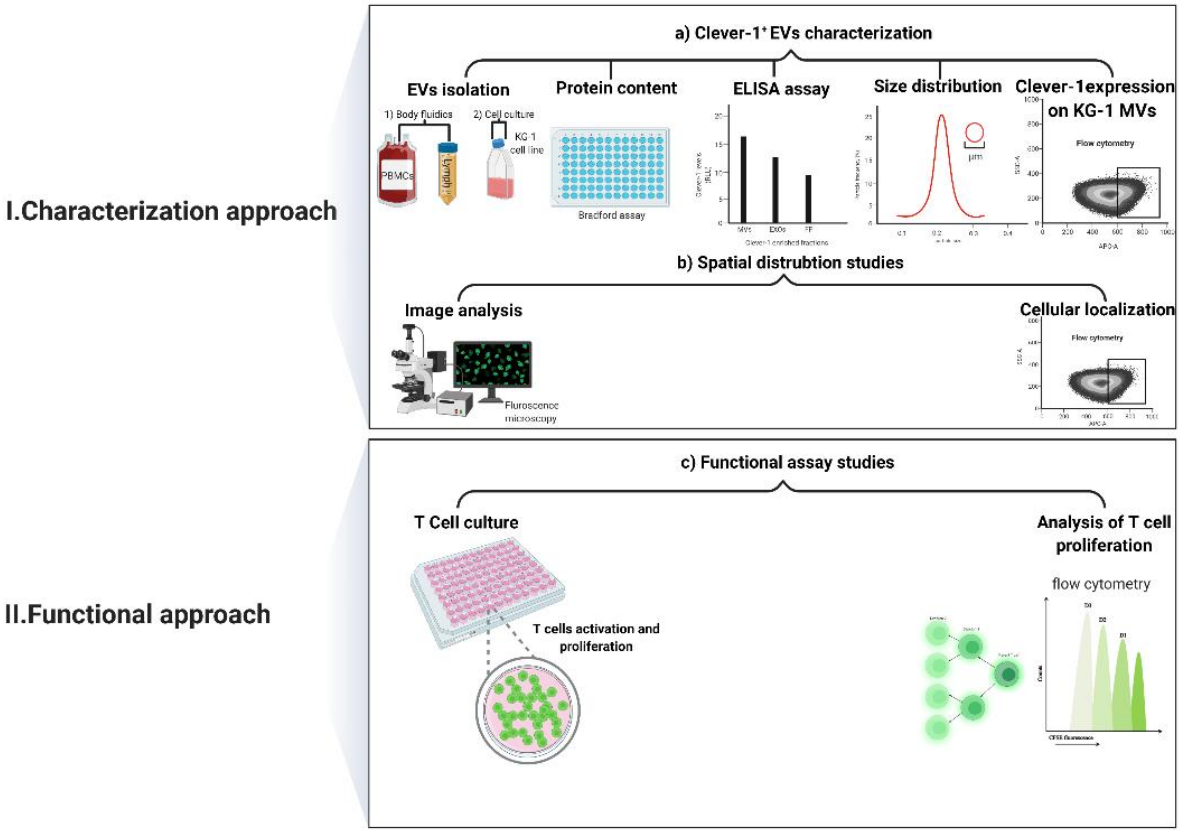

Figure 3. *Representative diagram of the approaches used in the studies of this thesis. Figure "created with Biorender.com".*

The first objective of this study was to characterize the expression of Clever-1 in different human biological fluidics and cellular sources derived EVs. This would help to better understand the diagnostic value of Clever-1^+EVs in human body fluids. Recently

published data show that placental purified Clever-1 interacts with lymphocytes, mostly B-cells and $CD8^+$T-cells (Tadayon et al., 2019). In addition, it was shown that blocking Clever-1 reactivates antitumor $CD8^+$ T-cell response in vivo(Viitala et al., 2019).

Therefore, the second aim was to investigate whether and to what extent Clever-1^+EVs has the capability to interact with T lymphocytes. These immune cells are considered major drivers of a successful immune response against tumors. When a T-cell efficiently recognizes an antigen, it starts to expand and differentiate into specific Teffs cells that can track down and eradicate the antigen expressing tumor cell. However, this process can be impaired if the T-cells are suppressed by any tolerogenic mechanism in the TME, including binding of T-cells with suppressive cytokines or EVs carrying these cytokines.

Therefore, the third aim was to validate whether the binding of Clever-1^+EVs to T lymphocyte is functionally relevant by investigating the proliferative T-cell responses through dye-based proliferation assays. In general, this project was developed through two main approaches: 1) The characterization approach, with the isolation and characterization of EVs derived from different biological sources, and 2) The functional approach, where the functional relevance of Clever-1 expressing EVs is evaluated in the context of T-cell functions (proliferation).

4. Materials and Methods

4.1 Cell lines and culture conditions

KG-1 (ATCC® CCL-246™) and Jurkat, Clone E6-1 (ATCC® TIB-152™) cell lines were used in the study. KG-1 cell line provides continuous culture when grown in a suspension of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS. Whereas the base medium for Jurkat T cell line is RPMI-1640 supplemented with 10% FBS.

For cryopreservation of the immortalized cells, a freezing media was formulated using complete growth medium supplemented with 10% (v/v) of DMSO. Briefly, cells were harvested from culture, washed 1x in PBS, and then resuspended in 1 mL of freezing media in a cryotube and kept overnight at -80°C in Mr. Frost container with enough isopropanol. Then, the next day it was relocated in the -80°C freezer or stored in liquid nitrogen.

4.2 Isolation, culture, and activation of human T lymphocytes from PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained from consented donors by density gradient centrifugation. First, 9 ml of blood (EDTA-tubes) was mixed with 10 ml of HBSS. The blood/HBSS mixture was then poured carefully on the top of a 15 ml Ficoll-Paque Plus without mixing in a falcon tube. The tube was then centrifuged for 20 min at 2000 rpm without brake. The lymphocytes were then collected with a Pasteur pipette between the Ficoll and HBSS layers.

Then, 20-30ml of HBSS was added to the collected lymphocytes followed by 2x washes with HBSS. Then, using $CD8^+$ T-cell Isolation Kit, a LS column, and a separator the $CD8^+$T-cells were isolated from the single-cell suspension as previously described (Finney et al., 2004). The cells were then fluorescently stained with the Miltenyi Biotec (MC) $CD8^+$ T-cell Cocktail and finally analyzed by Fortessa flow cytometry (BD, New Jersey, USA). Based on scatter signals or propidium iodide fluorescence, cell debris and dead cells were excluded from the analysis.

For the T-cell functional studies, Human T-Activator CD3/CD28 Dynabeads® were used for the activation and expansion of the T-cells following the manufacturer's instructions(Trickett and Kwan, 2003). The two antibodies deliver primary and co-stimulatory signals enhanced for the efficient expansion and activation of T-cells. Stimulation of the T-cell population expansion was achieved by using recombinant IL-2 in RPMI media.

4.3 Extracellular vesicles purification from cell cultures and body fluidics

Culture supernatant from 50-70% confluent cell cultures (KG1, previously activated or not with PMA) was collected. For viscous biological fluids as blood and lymph, they were first diluted with an equal volume of PBS (1:1). Then, the same steps were followed as dealing with the tissue culture conditioned media. The samples were centrifuged by an optimized routine proposed by Théry et al. (2006) as shown in (figure 4) without further filtration process for EXOs purification. Since this application is suggested when using larger preparation volumes (> 1L) and for clinical applications rather than laboratory use (Théry et al., 2006).

The samples were first centrifuged at 300×g for 10 min at 4°C using Sorvall RT6000B refrigerated centrifuge for the removal of cells. Then, cells were discarded, and the supernatant was collected and centrifuged at 2000×g for 10 min at 4°C using the same centrifuge for the removal of cell debris and apoptotic bodies (ABs). Supernatant was then again collected and centrifuged at 20000×g for 30 min at 4°C using rotor SS-34, Sorvall RC5C centrifuge.

The MVs pellet was then collected and suspended in 100µl-200µl of PBS then stored at -20 C for the characterization and functional assays. For EXOs purification, the supernatant was transferred to ultracentrifuge tubes and then centrifuged at 100000×g for 70 min at 4°C using Beckman coulter, Optima L-90K ultracentrifuge. The EXOs pellet was then collected and suspended in 100µl-200µl of PBS then stored at -20 C. The supernatant which is the last fraction from the ultracentrifugation process depleted from EVs (dEVs) was stored as well at -20°C to quantify the expression of the non-vesicular free format of Clever-1 protein.

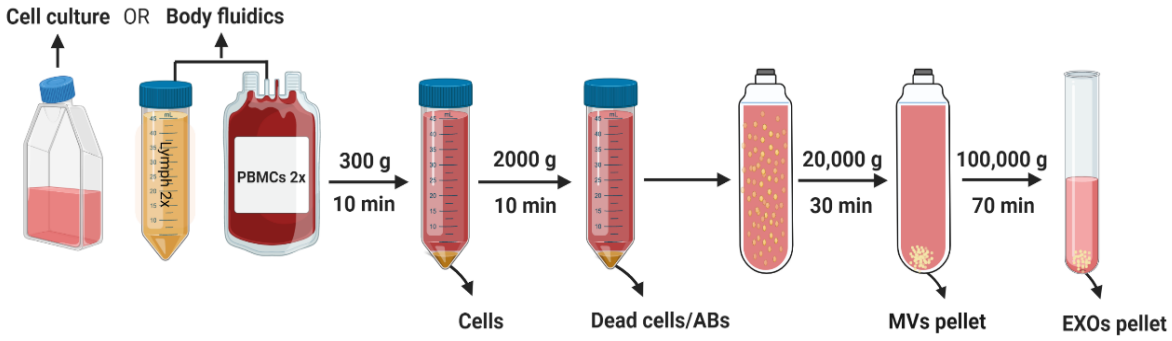

Figure 4. *Simplified schematics describing the isolation process of EVs from body fluidics and cell cultures media. Figure "created with Biorender.com".*

4.4 Clever-1 ELISA protocol for body fluidics and KG-1 monocytic cell line

First, the ELISA assay plate (Nunc Maxisorp white) was coated using antibody FUMM 9-11 (in-vivo) diluted with 0,1M NaHCO3 pH 9,6 to a concentration of 10µg/ml. Then, 100µl of the antibody solution was pipetted into the wells. The plate was then stored at room temperature overnight protected from light. After the incubation period, the plate was then washed six times with a washing buffer containing 0,1% tween in PBS using a plate washer.

Then, the plate was blocked using the blocking buffer consisting of 1% milk powder and 1% gelatin in PBS (200µl/well) for 1hr in room temperature. Then, the plate was again

washed six times with the same washing buffer. During the blocking incubation time, the samples were diluted in duplicates 10x using dilution buffer of PBS on a sample dilution LBS coated Spectra Plate (Transparent 96-well, PerkinElmer).

The lymph standard was also diluted to 1:10, 1:15, 1:20, 1:40, 1:80, 1:160 with PBS. To obtain Clever-1 depleted lymph, the lymph was immunodepleted 3x with 9-11 antibody bound to CnBr activated beads, so it does not contain anything else other than the lymph and it is diluted the same way as the other samples. After the plate was washed following the blocking incubation period, the samples and standards (100µl) were transferred from the sample dilution plate to the coated and blocked assay plate and was then incubated at room temperature for 1hr.

After the sample incubation, the plate was washed 6x with the washing buffer and then 100µl of the biotinylated FP-1305 detection antibody (diluted in blocking buffer to 1,5µg/ml) was applied to the wells. The plate was then incubated for 1hr at room temperate and then washed 6x with the same washing buffer. Then, 100µl of Eu-Labeled Streptavidin (Perkin Elmer) diluted 1000-fold in the assay buffer (Ready-for-use Tris-HCL Buffered NaCL solution) (pH 7.8), containing <0.1% NaN3, BSA, Bovine gamma globulins, Tween 40, diethylenetriaminepentaacetic acid (DTPA), and an inert red dye, Perkin Elmer) was applied to the wells and incubated for half an hour at room temperature protected from light.

Following the incubation period, the plate was again washed 6x with the washing buffer and then 100µl of pre tempered to room temperature enhancement Solution (Ready-for-use solution with Triton X-100, acetic acid, and chelator, Perkin Elmer) was applied to the wells and protected from light. Time-resolved fluorescence, Europium was then measured immediately with Viktor.

4.5 Quantification of KG-1 derived MVs using flow cytometry

First, MVs were isolated from KG-1 monocyte conditioned media. Thereafter, the MVs were labelled using 100nM – 10 µM CFDA and kept for 30 to 45 min at 37°C in the dark. CFSE solution was diluted to 10mM in PBS. After the cleavage of the CFDA acetate groups by esterases present in both the cells as well as in the secreted MVs, CFDA had a peak of excitation of 494nm and a peak of emission of 521nm.

MVs were then centrifuged at 10.000 g to 20.000 g for 10 min and the supernatant was discarded. The MVs pellets were then resuspended in 200µl of PBS. Then, 50µl of the MVs solution was collected and diluted in 500µl of PBS in a FACS tube. Data acquisition was acquired by Fortessa flow cytometer. The 488-nm (blue) laser excitation was used and 535/35 band pass filter for detection.

Log scale was set for both forward scatter (FSC) and side scatter (SSC) voltage parameters and the lowest thresholds allowed by the cytometer was selected for each (FSC=200 and SSC=200) (Inglis et al., 2015; Marcoux et al., 2016). MQ-H_2O was run in a fast mode in Fortessa cytometer for 5 min to clean the Cell-Flow from the residual particles. Finally, by gating the MVs, the entire volume of MVs sample preparation was obtained.

4.6 Protein quantification assay

The following kit was used for protein quantification: DC Bio-Rad Protein Assay Kit (Cat.500-0115). The working reagent was prepared by adding 20µl of reagent S to each ml of reagent A that is needed for the run. Then, dilutions of a protein standard containing from 0 mg/ml to about 1.5 mg/ml protein was arranged for preparing the standard curve. Each time the assay was run a standard curve was prepared. 5µl of standards with a similar volume from samples were pipetted into a clean, dry microtiter plate. Afterwards 25 µl of reagent A and 200µl of reagent B was added into each well. After 15 min, absorbances were read at 750nm using the Tecan microplate reader. Nunc 96-well transparent bottom plate was used for obtaining raw data.

4.7 Particle analysis using computer imaging analysis

To analyze the particles size and quantify their frequency across different images, an image algorithm for particle analysis was used in NIS-Elements Advanced Research platform (Nikon, Tokyo). Images of CFDA stained MVs were acquired using a 60X magnification objective using immersion oil in a fluorescence microscope (Leica, Germany). Images were uploaded in NIS-Elements software and calibrated for 10 µm scale bar.

Automated measurements were performed using the pixel classifier plugin applied in channel green (FITC) with the following settings: Low (21), high (255), smooth 1x, clean 1x, size varying accordingly with calibrated image (0.2µm – 1µm) and circularity 0.5. With

these settings, only round shape objects of different sizes were quantified, excluding eventual debris, which usually have an asymmetric structure. The number of threshold selected objects was then quantified in the entire fields in the best of three independent slide preparations. Size normality distribution was then analyzed using Shapiro-Wilk and Kolmogorov-Smirnov tests in GraphPad Prism 7 software.

4.8 In-vitro binding assay of KG-1 MVs on T-cell

First, $8x10^4$ T-cells were seeded in a Nunc Transparent 96-well Plate in a final volume of 100µL of complete RPMI media. Then KG-1 MVs that is protein quantified by DC Bio-Rad Protein Assay Kit was added to the cell culture at 37 °C overnight. The next day, cells were harvested and washed 1x with PBS. T-cells were then incubated with a blocking solution including PBS/BSA 2.5%, FBS 2.5% and 30% of a specific specie serum in case of fluorescent secondary usage or alternatively, a Human TruStain FcX Fc Receptor Blocking Solution can be used.

Then, the cells were surface stained with 20µl of PE Mouse Anti-Human CD3 (BD Pharmingen, Cat 555340) and 2µl of 911-AF647 anti-Clever1 antibody. The cells were then analyzed for Clever-1 expression using BD FACSDiVa™ software version 8. For the binding inhibition assay FP-1305 functional antibody (50µg/mL) was used to treat the MVs for 1h at 37 °C prior incubation with T-cells.

4.9 Immunofluorescence imaging of T-cell and MVs

For immunofluorescence of the T-cell and MVs, the following protocol was used. Briefly, T-cells were harvested from MVs co-culture binding assay and centrifuged at 2000 rpm for 5 min. Further, the cells were washed in PBS and resuspended in Hoechst 33342 (Thermo scientific, 20mM). 0.5µl of Hoechst was diluted in 400µl of PBS and was used for staining the cells for at least 30 min in ice. Then, the cells were centrifuged again at 2000 rpm for 5 min. The cell pellet is then vortexed for no more than 2 seconds and then suspended in 100µl of PFA 4% for 10 min at room temperature.

The cells were further washed 1x in PBS and resuspended in 8µL of Alexa 546 Phalloidin (200U/ml) diluted in 400µl of PBS in ice for 20 min. The cells were further washed 1x with PBS. And for the slide preparation, 10µL of the cell suspension was seeded on the

surface of a glass coverslip and put to dry in the hood protected from light. Immediately after drying, 7µL of ProLong™ Gold antifade reagent (Invitrogen) by Thermo Fisher Scientific was added on the top of the cells and the slide was mounted using a round shape glass coverslip and sealed with a nail polish. The slide is then put to dry in the hood protected from light for 10 min and then imaged under a fluorescent microscopy (BX60, Olympus, USA).

4.10 In-vitro T-cell proliferation functional assays

First, T-cells were stained with CFDA green probe at a concentration of 1 µM according to manufacture instructions. Then, $8x10^4$ T-cells were incubated with Dynabeads CD3/CD28 and IL-2 (30U/mL) in a 96-well plate in a final volume of 200µL of complete RPMI media. EVs including MVs, EXOs, and dEVs isolated from blood, lymph, and KG-1 monocytic cell medium were then added at protein concentration of 125µg, and the culture was maintained for 3 to 6 days. Then, T-cells were harvested, washed, and stained after using the blocking solution with 20µl of PE Mouse Anti-Human CD3 (BD Pharmingen, Cat 555340) and 20µl of APC Mouse Anti-Human CD8 (BD Pharmingen, Cat 555369).

Also, Dynabeads CD3/CD28 were removed when present using a magnetic field. The CFDA fluorescence was then used to measure and track cell divisions using Fortessa flow cytometer. When the cell divides and proceeds to next generations, the CFDA intensity is decreased since the dye is halved by each cell division which allows differentiating between the cell generations. The proliferation percentage was then calculated by the number of proliferating and non-proliferating generations observed using the BD FACSDiVa™ software version 8.

4.11 Statistical analysis

Bar graphs presenting mean±SEM of the data were created. Additional individual data points are illustrated in the bar graphs. The frequency of different MVs particle size range was evaluated in a normality, using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Most of the characterization approach includes qualitative and descriptive analysis and data show the best of at least three repetitions. For the flow cytometry CFDA proliferation assay, different treatment conditions were evaluated and compared with negative and positive controls. The assay protocol was optimized for the first time after different trial conditions.

To compare between different groups the Mann-whitney unpaired, two-tailed t-test was used and data was considered statistically significant if $P < 0.05$. The GraphPad Prism 7 software was used for the statistical analysis. In the functional approach, data show the combination of two or three best biological repetitions, including standard errors.

5. Results

5.1 Clever-1 is expressed in different fractions of human blood and lymph

Recently, our group observed that soluble Clever-1 is present in the plasma of cancer patients in higher levels as compared with normal donors (Supplementary Figure 1). Normally, human cells secrete proteins by different routes either within EVs or through the passive and active secretion of the free form(Maas et al., 2017). Therefore, we sought to investigate the possible forms of Clever-1 protein that can be present in human biological fluids such as blood and lymph.

So, EVs fractions including both MVs and EXOs were isolated from the blood and lymph of healthy volunteers based on the protocol previously described in the methods section and represented in the schematics of (Figure 4). In addition, the supernatant depleted from EVs that is the last fraction from the ultracentrifugation process was collected to examine if Clever-1 could be as well expressed in its native free form and we so-called this fraction the depleted extracellular vesicles (dEVs). Then, all isolated fractions (MVs, EXOs, and dEVs) from both blood and lymph were normalized at the same protein level of 1ug/uL in the assay using DC Bio-Rad Protein Assay Kit. They were then analyzed for Clever-1 expression by ELISA assay technique following the protocol described in the methods section. And a human lymph purified Clever-1 titration curve was used as the reference curve (Figure 5a).

It was observed that both blood and lymph were expressing Clever-1 protein within both EVs and dEVs fractions. As shown in (Figure 5b), the dEVs fraction of blood was expressing the highest level of Clever-1. Whereas in the lymph, the EXOs fraction was expressing the highest level of Clever-1 (Figure 5c). These results suggest that Clever-1 can be present in body fluidics (blood and lymph) in three different forms including MVs, EXOs and non-vesicular free format as observed in the dEVs fraction.

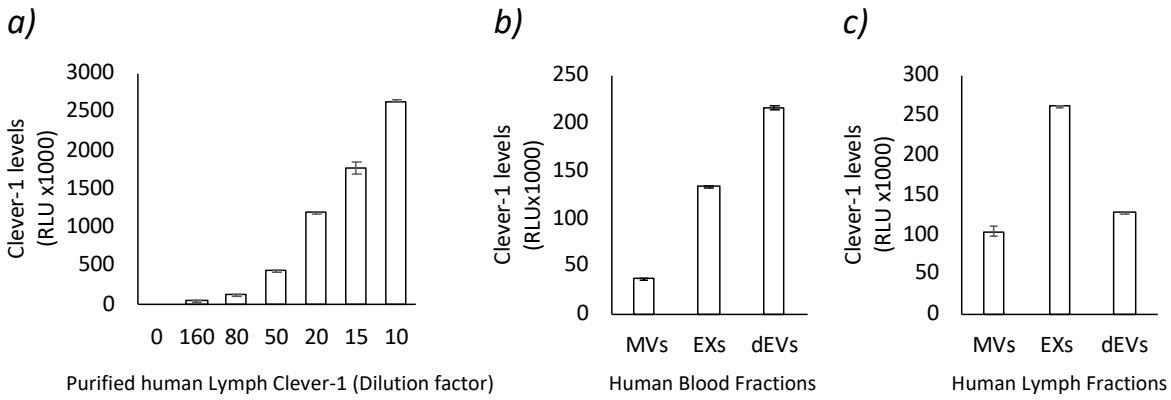

Figure 5. *Clever-1 quantification by ELISA in isolated extracellular vesicles (EVs) and depleted EVs (dEVs) fractions from different body fluidics.* ***a)*** *Represents the reference curve obtained from a human lymph purified Clever-1 titration curve.* ***b)*** *Clever-1 expression levels in human blood fractions.* ***c)*** *Clever-1 expression levels in human lymph fractions. All isolated fractions were normalized to 1ug/uL of protein in the assay. The graphs represent the measures of one independent assay with three technical replicates.*

5.2 Clever-1 is expressed in different fractions of KG-1 monocytic cell line conditioned medium

Monocytes, M2 macrophages, vascular endothelial cells, and lymphatic endothelium are all well-known potential sources of Clever-1 where it normally plays a role in maintaining homeostasis. Generally, diseased sites contain accumulated macrophages that are usually originating from circulating monocytes. And since it was previously observed that antitumor immune responses are restored in the context of depleted/blocked Clever-1 macrophages (Viitala et al., 2019), we sought to investigate how Clever-1 is secreted from human monocytes using KG-1 monocytic cell line.

KG-1 is an immortalized human myeloid leukemia monocytic cell line which naturally expresses high levels of Clever-1 and can differentiate into macrophage-like cells when treated with PMA (Teobald et al., 2008). The high constitutive expression of Clever-1 in these cells make them a suitable model to evaluate the expression of Clever-1 in different compartments of secreted EVs from monocytes. Fractions including MVs, EXOs, and dEVs were isolated from KG-1 conditioned medium following the same protocol represented in (figure 4).

The isolated fractions were then normalized to 1ug/uL of protein in the assay using the DC Bio-Rad Protein Assay Kit and further analyzed for Clever-1 expression by ELISA assay technique. The result shows that both MVs and EXOs fractions were expressing different levels of Clever-1. Moreover, Clever-1 was detected in the isolated fraction of different soluble components 'dEVs' (Figure 6a). This result suggests that EVs derived from monocytes/macrophages may represent an important source of Clever-1 in the human body fluidics, which may potentially target immune cells including circulating T lymphocytes.

Since the MVs fraction is relatively easier to isolate as compared with the EXOs fraction and given the fact that the EXOs are already characterized as well-controlled secretory machinery that may carry immunosuppressive proteins other than Clever-1, and thus, could mask Clever-1 effects on T-cells. Thus, the KG-1 MVs fraction containing Clever-1 was chosen to carry out the in-vitro functional assays with the T lymphocytes.

We further performed particle characterization of the MVs fraction obtained from KG-1 cells using a fluorescence microscopy approach. KG-1 derived MVs from 7 days cell culture were isolated, washed, and stained with a fluorescence CFDA probe as described in the methods section. An aliquot of 10 µL from the concentrated MVs fraction was used to make different slides and were imaged using a fluorescent microscope. Based on the analysis of images acquired, KG-1 derived MVs were showing good integrity as CFDA positivity indicates enzyme activation which is essential for its fluorescence activation. Additionally, it signifies a good integrity of the MVs membrane which is necessary for the intravesical entrapment of the fluorescent product.

Then, using a nano particle analysis algorithm developed by NIS-Elements Advanced Analysis Solution Software, the size and frequency of different MVs obtained were evaluated. The particle detection threshold strategy in the software quantifies the particles size (diameter) ranging from 0 – 10 µm. It was observed that 60% of KG1 derived MVs were having a size ranging from 0.4 µm to 0.6 µm diameter (Figure 6b, cyan threshold and Figure 6c). Particles bigger than 1 µm were also found but in a lower frequency of 1.7% (Figure 6b, purple threshold and Figure 6c).

Moreover, the frequency of different particle size range was evaluated in a normality, where the null hypothesis is rejected for the Shapiro-Wilk and Kolmogorov-Smirnov tests, with P values of 0.19 and >0.1, respectively (Figure 6c, bottom). This result suggests that the particle characterization represents a characteristic population of different MVs particles obtained from KG-1 cells, predominantly with the size of 0.4-0.6 µm. Also, the particle size of KG-1 MVs obtained is consistent with the previous literature which reports the MVs size can vary from 0.1µm to 1µm (Chen et al., 2018).

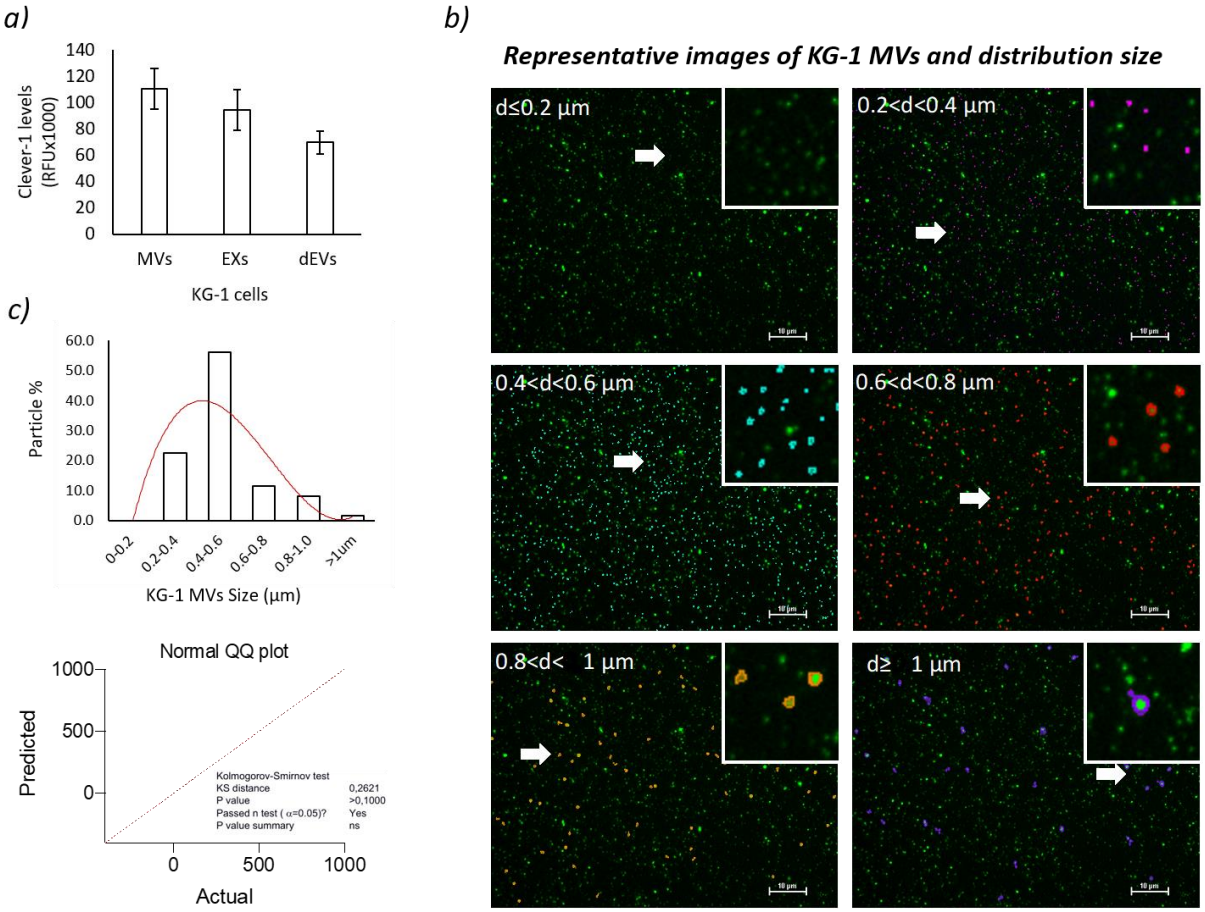

Figure 6. *Characterization of KG1 cells derived Clever-1^{+}MVs.* ***a)*** *ELISA for quantification of Clever-1 expression in different fractions from KG-1 cells derived conditioned media.* ***b)*** *Digital size characterization of CFDA^{+}MVs obtained from KG1 cells. Scale bars represent 10 µm and particles size was quantified using a digital threshold strategy for nano particles in NIS-Elements. No particles were detected in the size range of 0-0.2 µm.* ***c)*** *Frequency of different MVs size secreted by KG-1 cells. Particles were digitally quantified in NIS-Elements software and expressed in percentage of all particles accounted ranging from 0 to 10 µm diameter.*

Further, we sought to quantify not only the efficiency of the CFDA staining of KG-1 MVs, but the frequency of Clever-1 positivity among these vesicles using a flow cytometry

approach. Briefly, KG-1 MVs were co-stained with CFDA and 911-AF647 fluorescent Clever-1 antibody to detect the levels of Clever-1. The flow cytometer was adjusted for a small threshold acquisition strategy (SSC/FSC adjusted to 200 threshold) and the log scale was used instead of the linear scale in the FACs plots.

Then, using a trypan blue quenching strategy we removed the CFDA fluorescence as a negative control (Figure 7a). It was found that 72.4% of the acquired MVs are positive for the CFDA probe. Amongst the CFDA^{+}MVs, 45.8% of them were expressing Clever-1 (Figure 7b).

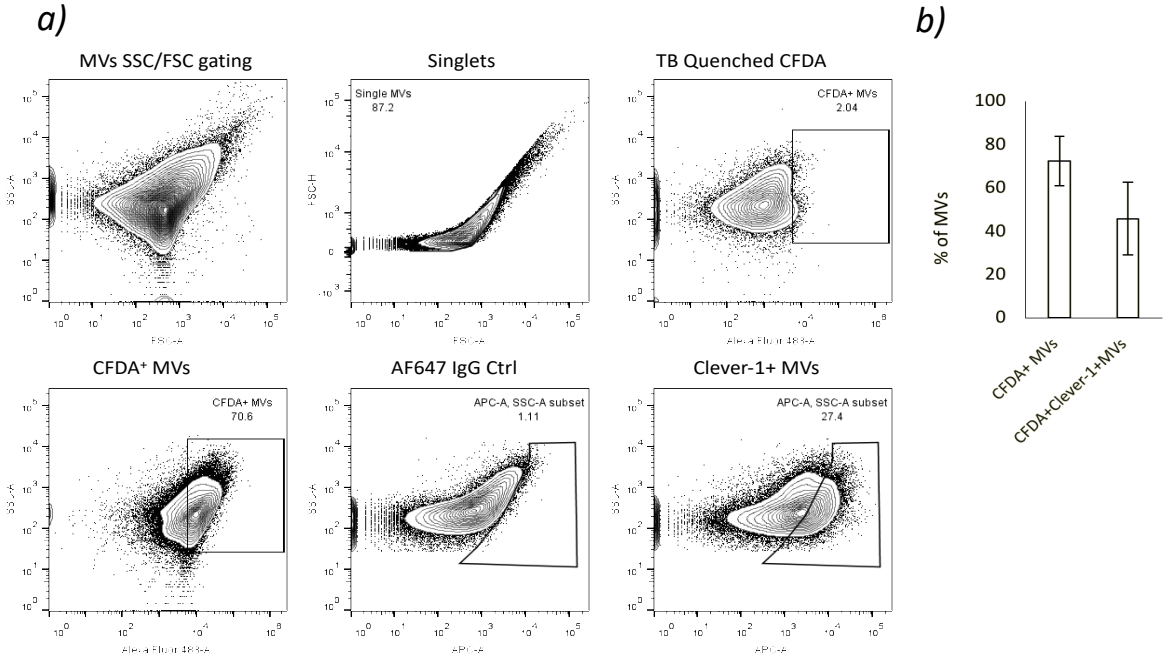

***Figure** 7. a) Flow cytometry gating strategy for quality (CFDA^{+} staining) and quantify Clever-1 expression in KG-1 derived MVs cultured for 7 days. b) The bar graph shows that approximately 72.4% of KG-1 derived MVs were CFDA positive amongst them 45.8% were expressing Clever-1. The data are represented from two independent assays.*

5.3 Quantitative characterization of KG-1 derived Clever-1^{+}MVs binding on T lymphocytes

Since EVs from different cellular sources can carry immune suppressive molecules that may not only bind to T-cells but internalize and interact with different molecular targets and suppress these cells (Aiello et al., 2017; Shi et al., 2020).We sought to investigate if MVs

expressing Clever-1 derived from monocytes/macrophages could target and bind T lymphocytes.

Therefore, a robust quantitative characterization of Clever-1^{+}MVs binding on both Jurkat T cells and human purified CD8^{+} T-cells populations using in-vitro binding assays was performed as described in the methods section. Jurkat was chosen as an appropriate model since it is a well-established prototypical T-cell line used to study multiple events within T-cell biology.

Briefly, T-cells were incubated with isolated fraction of KG-1 Clever-1^{+}MVs overnight and then the cells were collected, washed, and stained for the detection of the cell surface expression of Clever-1 using the 911-AF647 Clever-1 antibody. Then, cells were analyzed in the Fortessa flow cytometer. We observed a dose dependent binding of MVs ranging from 43.3% for 1:1 dilution, 23.3% for 1:2 dilution, and 3.19% for 1:4 dilution on Jurkat T cells (Figure 8). This data suggest that macrophages/monocytes derived Clever-1^{+} MVs can bind T-cells.

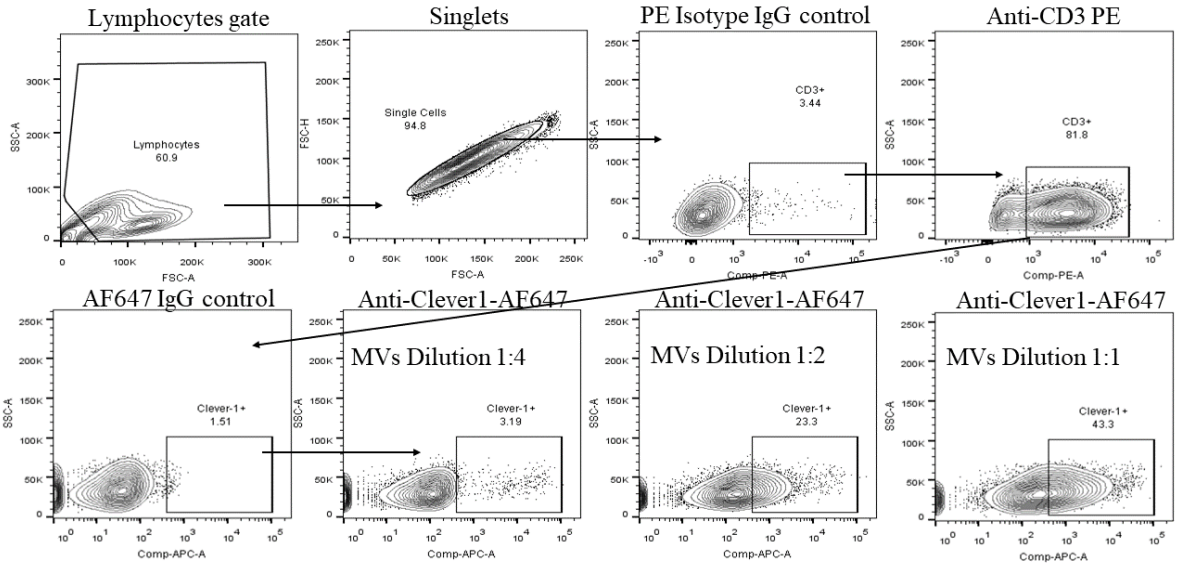

Figure 8. *Flow cytometry quantification of Clever-1^{+}MVs binding on Jurkat T cells. Different dilutions of KG-1 MVs were incubated overnight with Jurkat T cells. Result shows that Jurkat T cells bind KG-1 MVs expressing Clever-1 in a dose dependent manner.*

Further, we evaluated the possibility of the therapeutic anti-Clever-1 antibody FP-1305 to inhibit the binding of Clever-1^{+}MVs on T cells. Therefore, primary CD8^{+}T-cells freshly obtained from healthy donor were incubated with 1:1 dilution fraction of KG-1 MVs overnight. Then, one group was stained with the non-functional anti-Clever-1 911-AF647

antibody while the other group was treated with FP-1305-PE (phycoerythrin) antibody. Lower binding frequency of Clever-1$^+$MVs was observed with the group treated with FP-1305 (Data not shown). This result suggests that FP-1305 may reduce the binding of Clever-1$^+$ MVs on T cells. However, our group observed that 911-AF647 antibody usually binds better than FP-1305. Therefore, the difference in Clever-1$^+$CD8$^+$ T-cells percentages between the two groups may be due to variation in the staining intensity.

Thus, the experiment protocol was optimized by pre-incubating one aliquot of KG-1 MVs with FP-1305 functional antibody (50µg/mL) for 1h at 37 °C prior incubation with CD8$^+$ T-cells while the other KG-1 MVs aliquot was left untreated. The two different aliquots (pre-treated and non-treated KG-1 MVs) were then added to two different groups of CD8$^+$ T-cells. The cells from the two groups were collected and stained for the cell surface expression of Clever-1 using the same 911-AF647 antibody.

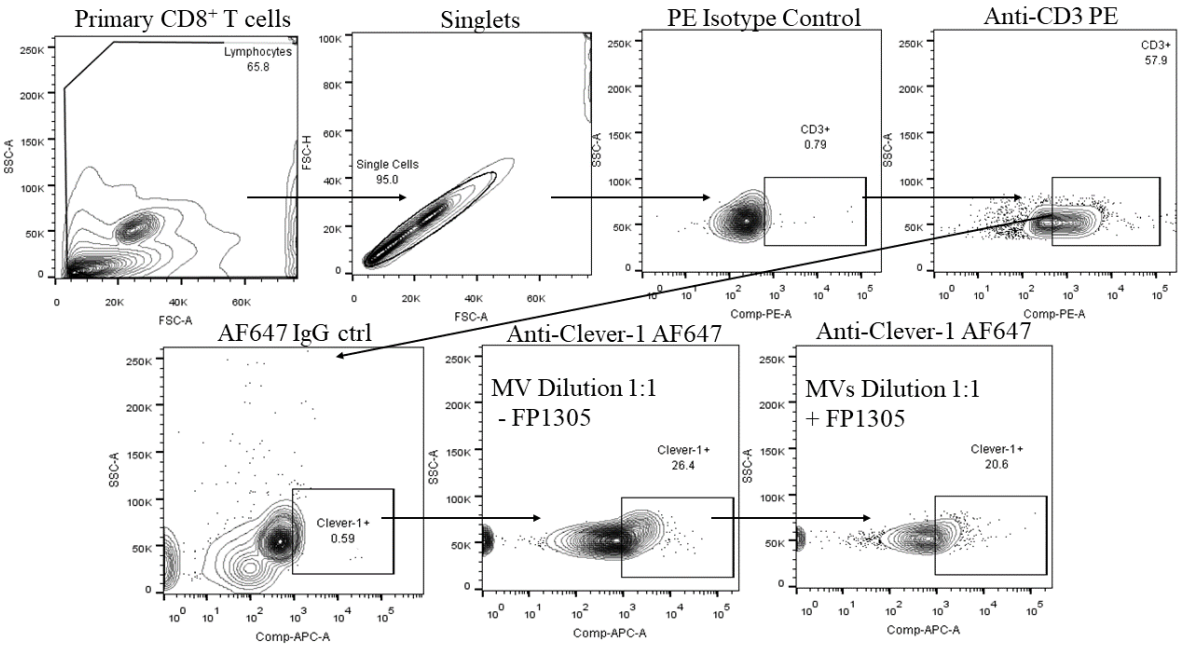

Figure 9. *Human CD8$^+$T-cells bind Clever-1$^+$MVs, but this process is disturbed with previous Clever-1$^+$MVs incubation with FP-1305 (50 µg/mL) for 1h at 37 °C. Results show that 26.4% of CD3$^+$CD8$^+$ T-cells were binding Clever-1$^+$MVs, and this percentage was reduced to 20.6 % following the pre-treatment with FP-1305 antibody which represents a 21.5% of reduction in Clever-1$^+$MVs binding.*

Again, the percentage of Clever-1$^+$CD8$^+$ T-cells incubated with pre-treated KG-1 MVs was lower as compared with the group incubated with non-treated KG-1 MVs. The results show that 26.4% of CD3$^+$CD8$^+$ T-cells were binding Clever-1$^+$MVs, and this percentage was

reduced to 20.6% following the pre-treatment of KG-1 MVs with FP-1305 antibody representing a reduction of 21.5% in the MVs binding on T cells (Figure 9).

5.4 Qualitative characterization of KG-1 derived Clever-1^{+}MVs distribution on T lymphocytes

We wanted to observe how these KG-1 derived Clever-1^{+}MVs bind to the T cells. Therefore, we carried out spatial distribution assays by incubating KG-1 MVs pre-stained with CFDA with Jurkat T cells. The culture was established for 24h then the cells were collected and co-stained with Hoechst and Phalloidin-PE. Then, cells were used to mount immunofluorescence glass slides to analyze the different levels of MVs binding on T-cells. The images obtained using the fluorescent microscopy show that Jurkat T cells were partially binding KG1 MVs suggesting the binding of vesicles which may contain different functional relevant proteins, including Clever-1.

The results show that amongst the cells binding the MVs, different levels of binding were observed. Almost 19.9% of the total cells were showing at least one MV located in the surface level of the cell (MVs^{low}, Figure 10a), while 12.3% of the cells were having at least two or more interacting MVs in the cell surface (MVs^{high}, Figure 10a). Also, almost 67.8% of the cells were not binding CFDA^{+}MVs (Figure 10b).

The differences in binding may be due to the variations in the target levels those vesicles are binding to or because of different metabolic needs which is related with the cell cycle, amongst other factors not known. The percentages of Jurkat T cells that were negative and positive for CFDA^{+}KG-1 MVs were quantified from at least three different fields from three independent experiments (Figure 10b). Also, during the quantification using ImageJ cell counter tool, only Hoechst and Phalloidin-PE positive cells were counted.

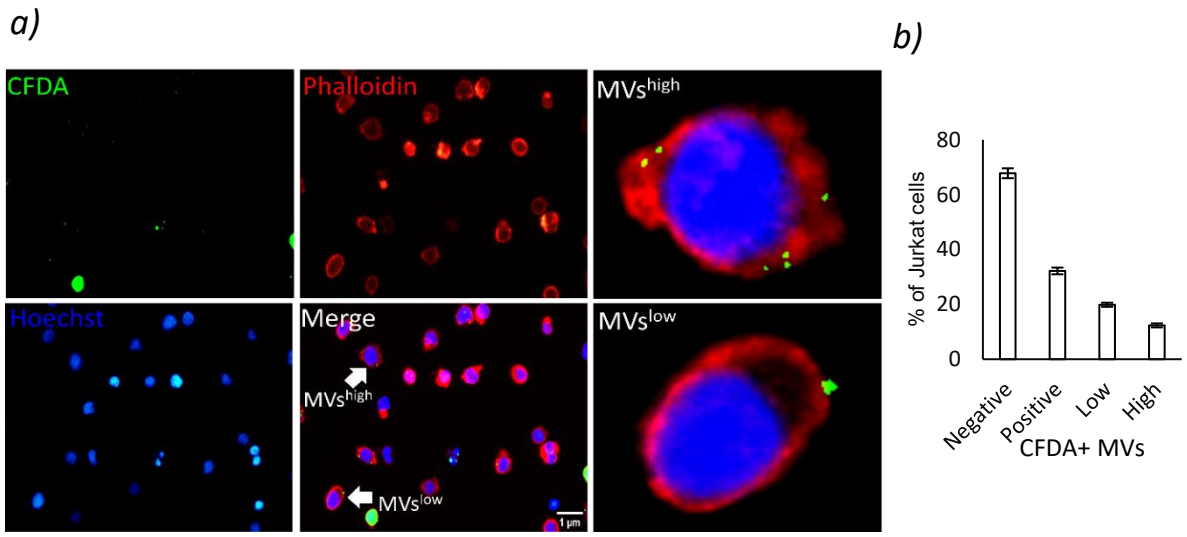

Figure 10. a) *Spatial distribution of KG-1 derived MVs on Jurkat T cells and the different cellular interacting levels (low and high), as indicated in the insert, images were obtained using the fluorescence microscopy. The Jurkat T cells were stained with Hoechst and Phalloidin-PE and the KG-1 derived MVs were pre-stained with CFDA prior incubation with Jurkat T cells.* ***b)*** *Bar-graphs representing the percentages of CFDA$^+$MVs binding on Jurkat T cells. Quantification was performed using ImageJ cell counter quantification tool from at least three different fields of three independent experiments.*

5.5 KG-1 derived Clever-1$^+$EVs are not shuttled into Jurkat T cells in a co-culture

The binding of KG-1 derived Clever-1$^+$MVs on Jurkat T cells was observed when the fraction of MVs was first isolated from KG-1 conditioned medium and then added to the cell culture. However, the binding of Clever-1$^+$EVs on Jurkat T cells did not occur in a co-culture environment. Briefly, a constant number of Jurkat T cells were seeded at a concentration of $8x10^4$ per well with KG-1 cells at 3 different densities of $1x10^6$, $5x10^5$ and $2.5x10^5$.

At day 3 of the co-culture Jurkat T cells were harvested and stained for 911-488 fluorescent Clever-1 antibody to detect the levels of Clever-1 by Fortessa flow cytometer. The results show that KG-1 derived Clever-1$^+$EVs were not shuttling into Jurkat T cells in a well-defined in-vitro system (Supplementary Figure 2). This may imply that the secreted monocytes EVs repertoire in a culture system is much inferior to an isolated EVs fraction or may indicate the presence of cytokines and other cellular components that interfere in the mechanistic of this binding assay.

5.6 Clever-1$^+$EVs may inhibit CD8$^+$ T-cells proliferation

Since KG-1 derived Clever-1$^+$EVs did not bind Jurkat T cells in a co-culture system. The direct addition of isolated fractions of clever-1$^+$EVs were used to perform the T-cell proliferation functional studies as described in the methods section. Also, concentrated isolated fractions of Clever-1$^+$EVs would better simulate the abundancy of Clever-1 in the blood of cancer patients and may therefore better evaluate their immunomodulatory effects. The results showed that Clever-1$^+$EVs were not inhibiting the proliferation of Jurkat T cells (results not included in the thesis). Jurkat T cell model is an immortalized leukemia cell line with a high proliferative capacity which may exceed and mask the anti-proliferative effect of Clever-1 or any other immunosuppressive molecules carried on those vesicles. Thus, it was not a suitable model to study the hypothesized anti-proliferative effect of Clever-1$^+$EVs. Hence, only human T lymphocytes from peripheral blood mononuclear cells (PBMCs) were used for the proliferation assays.

We investigated whether Clever-1$^+$fractions including MVs, EXOs and dEVs could disrupt CD8$^+$ T-cells proliferation. CD8$^+$ T-cells pre-stained with CFDA were cultured at a density of $8x10^4$ cells per well in RPMI media supplemented with Human T-Activator CD3/CD28 Dynabeads® and IL-2 (30U/ml) with the Clever-1$^+$fractions isolated from three different sources including human fluidics (blood and lymph) and KG-1 monocytic medium. The Clever-1$^+$fractions volumes were added to achieve a concentration of 125µg of protein calculated using DC Bio-Rad Protein Assay Kit as mentioned in the methods section.

Then the dilution of the CFDA proliferation dye was measured using flow cytometry, as roughly represented in a gating strategy (Figure 11a). The total T-cell proliferation was combined from different assays and the frequency of proliferating cells was quantified for each treatment condition. The results show that Clever-1$^+$EVs were inhibiting the proliferation frequency of T-cells and significantly by the blood and KG-1 EVs (Figure 11b).

The proliferation score was subtracted from positive proliferation controls to obtain the inhibition indexes as follows: 34.3% (MVs), 53.6% (EXOs) and 49.7% (dEVs) for lymph fractions; 37.4% (MVs), 66.8% (EXOs) and 58.1% (dEVs) for blood fractions; and 59.1% (MVs), 52.6% (EXOs), and 57.4% (dEVs) for KG-1 cells. Although differences were observed in lymph fractions, they did not show significant differences to the positive

proliferation controls probably given to the low number of observations (n=2). For blood and KG-1 fractions, data represent at least three independent observations.

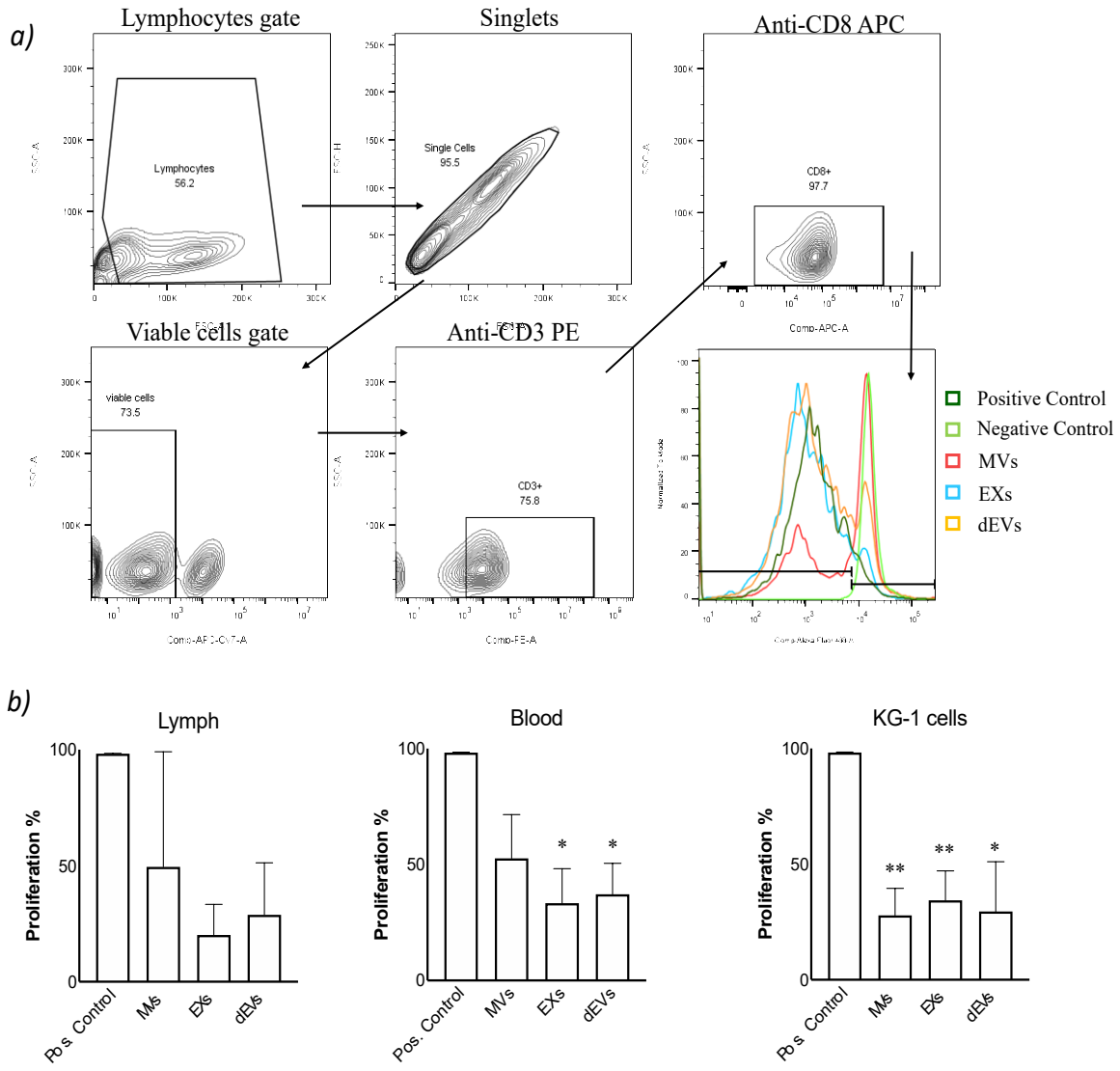

Figure 11. *Use of Clever-1$^+$EVs fractions in analyzing CD8$^+$ T-cells proliferation. CD8$^+$ T-cells proliferation assays were performed by culturing CFDA stained CD8$^+$ T-cells with different Clever-1$^+$EVs fractions normalized to the same protein levels isolated from blood, lymph, and KG-1 cell cultures in RPMI media supplemented with IL-2 with Human T-Activator CD3/CD28 Dynabeads® for 3 to 6 days.* ***a)*** *Gating strategy of this assay: lymphocytes, single cells, viable cells, CD3$^+$ positive cells, CD8$^+$ positive cells, proliferating and non-proliferating cells were gated* ***b)*** *Frequency of proliferating T-cells [gated in (A)] incubated with blood, lymph, and KG-1 derived MVs, EXOs, and depleted extracellular vesicles (dEVs). Data are representative of three independent experiments performed for blood and KG-1 cells derived EVs and two independent experiments were performed using lymph derived EVs. Mann-whitney unpaired, two-tailed t-test was used. *p<0.05 and **p<0.01 were considered significantly different from positive proliferation controls.*

6. Discussion

Based on the previous published observations, Clever-1 is a scavenger receptor expressed but not exclusively in TAMs. When highly expressed in cancer patients is associated with poor immune responses and lower overall survival (OS) (Boström et al., 2015; Kwon et al., 2019; Lin et al., 2019; Tervahartiala et al., 2017; Wang et al., 2020). Indeed, numerous studies have pinpointed the emerging role of TAMs expressing scavenger receptors in cancer prognosis and carcinogenesis(Yu et al., 2015b).

A scavenger receptor expressed in TAMs termed as SRA (also known as CD204) is reported to be associated with tumor aggressiveness and poor prognosis(Kawachi et al., 2018; Miyasato et al., 2017; Ohtaki et al., 2010; Seung et al., 2018; Shigeoka et al., 2013). Moreover, the downregulation of SRA/CD204 on DC enhanced the cross-presentation of cell-associated antigen and T-cell activation(Guo et al., 2012).Another study has highlighted the involvement of SRA/CD204 expressed by TAMs in causing outgrowth of EL4 lymphoma by downregulating the production of tumoricidal molecules such as IFN-β, IFN-γ, and nitric oxide(Komohara et al., 2009). Moreover, Neyen and his colleagues showed in vivo that TAMs expressing SRA/CD204 was essential for the invasion and metastasis of cancers such as ovarian and pancreatic tumors(Neyen et al., 2013).

Additionally, a scavenger receptor called CD163 was reported to be upregulated in macrophages undergoing polarization towards M2 phenotype(Fujimura et al., 2013; Komohara et al., 2008). Circulating CD163 in cancer patients was recognized as a prognostic biomarker for poor prognosis and accompanied increased activity of $CD163^{+}$ TAMs (No et al., 2013; Sugaya et al., 2012). Moreover, it was proved that in B16 melanoma, a higher number of tumor-reactive T-cells and improved therapeutic efficacy was observed when MARCO, a scavenger receptor on DCs was blocked(Matsushita et al., 2010). This current study along with others reflect the complexity of this network of receptors in modulating anti-tumor immune responses.

The membrane-bound form of Clever-1 expressed on macrophages is already identified to have a role in maintaining homeostasis by endocytosing bacteria, apoptotic cells, and several other proteins such as ac-LDL, placental lactogen, secreted protein acidic and rich in cysteine (SPARC)(Hollmén et al., 2020). However, the occurrence of an extracellular scavenger receptor such as Clever-1 in body fluidics suggests its potential to affect the

biology of circulatory effector immune cells (e.g., T lymphocytes). A similar proposition was described for the scavenger receptor CD163 expression in serum and other tissue fluids to have a role in inhibiting T-cell proliferation in-vitro(Högger and Sorg, 2001).

Immune cells as well as being initiators of immune responses, they maintain homeostasis by utilizing Tregs and secreting cell signaling proteins such as cytokines to self-regulate the immune system. However, the high systemic levels of certain cytokines correlate with poor survival of cancer patients. For example, the increased serum level of IL-10 in multiple myeloma, pancreatic, and thyroid cancer is associated with poor prognosis (Cunha et al., 2017; Feng et al., 2018; Wang et al., 2016). In addition, the elevated levels of IL-6 cause tumor progression and poor treatment outcome(Lippitz and Harris, 2016; Sansone et al., 2007; Sapochnik et al., 2017; Tchirkov et al., 2007).

Moreover, the elevated levels of the cytokine colony-stimulating factor (CSF-1) correlate with poor survival in early breast cancer patients(Aharinejad et al., 2013). Blocking the CSF-1 receptor on TAMs increases the recruitment of $CD8^+$ T-cells (Cannarile et al., 2017) . Another immune systemic inhibitory cytokine named VEGF was detected to limit T-cell infiltration to tumors and induce T-cell exhaustion(Lanitis et al., 2015; Voron et al., 2015). Accordingly, beside that Clever-1 protein has a natural and a normal functionality in terms of homeostasis. The role of its soluble increased levels observed in the plasma of cancer patients may negatively influence the quality and quantity of anti-tumor immune responses.

Extracellular proteins may be present in the human biological fluids in different forms that include soluble non-vesicular (NV) formats, EVs, and within immunocomplexes(Raposo and Stoorvogel, 2013). Generally, EVs secretion is considered an attribute of activated cells. They form a loop of communication by carrying bioactive molecules between cells. Several research groups have focused on studying cancer cells derived EVs and their effects on anti-tumor immune responses and patient's clinical outcome as reviewed (Becker et al., 2016).

However, the TME constitutes of several other cells i.e., immune cells that hugely contribute to the secretion of EVs in the TME and may therefore, have an influence on the disease prognosis(Balkwill et al., 2012). The observation that Clever-1 can be secreted in different extracellular forms has never been observed before. Indeed, immune proteins with suppressive potential that are secreted in the form of EVs by immunosuppressive immune cells have been previously described to affect the biology of tumor associated immune cells.

For example, previous data suggest that TAMs contribute to the immunosuppressive milieu in the TME by shuttling proteins and microRNAs (miRNAs) through EVs compartments. For example, TAM-EVs associated miR-21-5p and miR-29a-3p promote epithelial ovarian cancer progression by causing imbalance in the Treg/Th17 cells and inhibiting the STAT3 pathway(Zhou et al., 2018). Also, under hypoxic conditions TAMs derived EXOs carrying miR-223 activate the PTEN-PI3K/AKT pathways leading to drug resistance in epithelial ovarian cancer(Zhu et al., 2019).

In addition, the migration of gastric tumors is promoted by TAM-EXOs carrying Apolipoprotein E(Zheng et al., 2018). Moreover, TAM-EVs associated CD206 and CD163 were elevated in newly diagnosed multiple melanoma patients as compared with relapsed patients and those in remission(Kvorning et al., 2020). Therefore, TAM-EVs associated Clever-1 may as well have an important role in the pathology of tumor progression and resistance to immunotherapies.

The different profile of Clever-1 expression in body fluidics could have two possible explanations. First, due to the difference in the levels and biology of Clever-1 cellular sources found in each environment. For example, in the lymph, macrophages and lymphatic endothelium are the two main sources of Clever-1. While Clever-1 in the blood mainly infiltrates from liver sinusoidal endothelial cells, monocytes, and vascular endothelium. Secondly, because of the probable variations in the affinity of 911-AF647 Clever-1 antibody binding, due to different conformations, isoforms, or glycosylation of Clever-1 protein. Or due to presence of other interacting proteins that prevent the antibody binding to Clever-1.

A study suggested that the release of macrophage derived EVs expressing CD206 and CD163 outweigh the shedding of the free form during cancer development, suggesting the involvement of EVs in a well-defined interaction between TAMs and tumor associated immune or cancer cells (Kvorning et al., 2020). This result obtained raises some intriguing questions related to the current study including: "*Would the switch from the dEVs free form of Clever-1 to vesicle-bound form reflect the stage of cancer progression?* i.e., malignant transformation?" and "*Would there be any significant decrease in Clever-1^{+}EVs as compared to* the *dEVs free form of Clever-1 in patients in remission?*". Future studies can be considered by applying the methods described and developed in this study to identify Clever-1 levels in

both the dEVs free from and EVs in samples derived from cancer patients to correlate the findings with their clinical information.

Based on the previous studies of the group and in light of earlier observation that human placental purified Clever-1 binds T-cells(Tadayon et al., 2019). We hypothesized that monocyte derived Clever-1^{+}EVs could negatively impact T lymphocytes proliferation, which is a fundamentally important feature for proper anti-cancer immune response(Waldman et al., 2020). Therefore, we isolated fractions of Clever-1^{+}EVs obtained from KG-1 monocytic cell line to investigate the capability of these vesicles in binding to T-cells.

There are many proposed procedures for EVs isolation and to date we lack a universally agreed method. Thus, raising an uncertainty about the EVs proper definition and biological activity. However, we focused on a previously proposed procedure by (Théry et al., 2006) that relies on a series of sequential centrifugation steps. The author mentioned a final filtration process to increase the purity of the vesicles obtained. However, we did not opt for further EVs refinement since this is recommended for a more sophisticated characterization where the samples are going to be used for mass-spectrometry and we only aimed to check Clever-1 levels and whether they have biological activity.

While characterizing KG-1-MVs in terms of size, the diameter of mostly all vesicles ranged from 0.2µm-0.8µm suggesting a microvesical origin (Doyle and Wang, 2019). Particles bigger than 1µm formed only 1.7% and are potentially related to clusters of MVs which were not reduced following sonication. In addition, they are present in the third quartile of the size distribution curve. Therefore, they are not representative of the material obtained.

The current study proved that Clever-1^{+}EVs bind T-cell and may be involved in inhibiting its proliferation. However, additional studies are required to prove that the binding and suppression of T cells are dependent on Clever-1 since EVs may possibly carry other immunosuppressive proteins. This could be confirmed by knocking out Clever-1 or by using the functional Clever-1 antibody treatment FP-1305 as a negative control. This hypothesis was preliminary tested when KG-1 MVs pretreated with FP-1305 reduced the binding of Clever-1^{+}MVs on CD^{+}8 T cell. However, additional repeats are required for further validation.

Also, the mechanisms by which Clever-1^{+}EVs target and suppress T-cells are yet to be investigated. Since proliferation is the functionality affected, different mechanistic routes could be suggested to investigate in the inhibition mechanisms of Clever-1^{+}EVs. The proliferation assay carried out in this study depends on the activation with Human T-Activator CD3/CD28 Dynabeads that act on the TCR. Therefore, binding of Clever-1^{+}MVs to T-cell could possibly be related to pathways regulating T-cell proliferation dependent on TCR activation.

The vesicles could possibly have a similar mechanism of action of certain drugs such as rapamycin. A more refined mechanism of TCR dysfunction could potentially be that Clever-1^{+}EVs interact with targets expressed in the immunological synapse and thus, dysregulating the antigen-TCR activation signal and ultimately, suppressing T-cell proliferation. Another proposed mechanism could be that Clever-1^{+}EVs act as a carrier to biomolecular immunosuppressive cargo that could possibly alter the metabolism of T-cells once they are internalized and release their content either in the cytosol or endosomes.

To advance the current research and step forward, further studies are essential. The observed impact of dEVs free form of Clever-1 and Clever-1^{+}EVs in modulation of T-cell responses suggests that immune responses of cancer patients can be systemically modulated by extracellular soluble Clever-1 and may as well act as a significant and direct inhibitor to T-cell activating therapies by halting the proliferation and expansion of effector T-cells. The different forms of extracellular soluble Clever-1 were investigated in samples obtained from healthy volunteers. Having accessibility to cancer patients' samples would have strengthened the current study to examine closely if the profile of extracellular Clever-1 expression differs from healthy individuals.

Also, it would be of a similar interest to look if those Clever-1^{+}EVs fractions increase in body fluidics during different stages of cancer development (i.e., metastasis) which could then act as a prognostic cancer biomarker and provide a basis for liquid biopsy. This may then assist in evaluating the anti-tumor immune responses of cancer patients and better evaluate their responsiveness to the therapeutic anti-Clever-1 antibody (FP-1305, *bexmarilimab*) which is currently under phase I/II MATINS clinical trials.

In addition, we lack to date enough knowledge on the set of factors and other stimulus such as cytokines involved in inducing the expression of Clever-1 on immunosuppressive

macrophages and would be equally interesting to identify the triggering factors behind the shedding of extracellular Clever-1 either in its native free form or within EVs compartments. This may then help develop and evaluate better combinatory treatments that would represent an entirely new therapeutic approach for cancer patients. Moreover, the findings presented in this study would facilitate the current clinical development process of *bexmarilimab* immunotherapy.

In conclusion, this study unveiled a potential key that could possibly restore the immune response in cancer patients, by characterizing and better understanding the diagnostic value of Clever-1^{+}EVs derived from human biological fluids. Blocking Clever-1 associated with these vesicles may represent a promising strategy and adjuvant immunotherapy to inhibit the immunosuppressive effects triggered by M2-like derived EVs. The study will aid future directions in understanding the mechanisms of how Clever-1^{+}EVs control T-cell proliferation and functions, including the understanding of molecular targets involved in this functional interaction.

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UAE, 8th of April 2021

Nesrin Yasser

8. Abbreviation list

ATC	Adoptive T-cell
APC	Antigen Presenting Cell
CD	Cluster of Differentiation (for example CD4)
Clever-1	Common Lymphatic Endothelial and Vascular Endothelial receptor-1
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein -4
DCs	Dendritic cells
dEVs	Depleted extracellular vesicles
EVs	Extracellular vesicles
EXOs	Exosomes
FSC	Forward scatter
ICIs	Immune checkpoint inhibitors
IFN-γ	Interferon-gamma
IL	Interleukin (for example IL-2)
JAK	Janus kinase (for example JAK2)
MDSCs	Myeloid derived suppressor cells
MHC	Major Histocompatibility Complex
MVs	Microvesicles
NKs	Natural killer cells
PD-1	Programmed Death -1
PD-L1	Programmed Death Ligand-1
SSC	Side scatter
STAT3	Signal transducer and activator of transcription 3
TAMs	Tumor-associated macrophages
TCR	T-cell receptor
Teffs	Effector T-cells
TGF-β	Transforming growth factor beta
Th-1	T helper-1
TME	Tumor microenvironment
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor

9. References

Abbas, A.K., A.H. Lichtman, and S. Pillai. 2018. Cellular and molecular immunology / Abul K. Abbas, Andrew H. H. Lichtman, Shiv Pillai ; illustrations by David L. Baker, Alexandra Baker. Elsevier, Philadelphia, PA.

Adachi, H., and M. Tsujimoto. 2010. Adaptor protein sorting nexin 17 interacts with the scavenger receptor FEEL-1/stabilin-1 and modulates its expression on the cell surface. *Biochim Biophys Acta*. 1803:553-563.

Adams, J.M., and S. Cory. 2007. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*. 26:1324-1337.

Aharinejad, S., M. Salama, P. Paulus, K. Zins, A. Berger, and C.F. Singer. 2013. Elevated CSF1 serum concentration predicts poor overall survival in women with early breast cancer. *Endocr Relat Cancer*. 20:777-783.

Ahmad, N., A. Ammar, S.J. Storr, A.R. Green, E. Rakha, I.O. Ellis, and S.G. Martin. 2018. IL-6 and IL-10 are associated with good prognosis in early stage invasive breast cancer patients. *Cancer Immunol Immunother*. 67:537-549.

Aiello, S., F. Rocchetta, L. Longaretti, S. Faravelli, M. Todeschini, L. Cassis, F. Pezzuto, S. Tomasoni, N. Azzollini, M. Mister, C. Mele, S. Conti, M. Breno, G. Remuzzi, M. Noris, and A. Benigni. 2017. Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival. *Sci Rep*. 7:11518.

Ansell, S.M., A.M. Lesokhin, I. Borrello, A. Halwani, E.C. Scott, M. Gutierrez, S.J. Schuster, M.M. Millenson, D. Cattry, G.J. Freeman, S.J. Rodig, B. Chapuy, A.H. Ligon, L. Zhu, J.F. Grosso, S.Y. Kim, J.M. Timmerman, M.A. Shipp, and P. Armand. 2015. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 372:311-319.

Baeriswyl, V., and G. Christofori. 2009. The angiogenic switch in carcinogenesis. *Semin Cancer Biol*. 19:329-337.

Balkwill, F.R., M. Capasso, and T. Hagemann. 2012. The tumor microenvironment at a glance. *J Cell Sci*. 125:5591-5596.

Beavis, P.A., C.Y. Slaney, N. Milenkovski, M.A. Henderson, S. Loi, J. Stagg, M.H. Kershaw, and P.K. Darcy. 2015. CD73: A potential biomarker for anti-PD-1 therapy. *Oncoimmunology*. 4:e1046675.

Becker, A., B.K. Thakur, J.M. Weiss, H.S. Kim, H. Peinado, and D. Lyden. 2016. Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell*. 30:836-848.

Bellone, G., C. Smirne, F.A. Mauri, E. Tonel, A. Carbone, A. Buffolino, L. Dughera, A. Robecchi, M. Pirisi, and G. Emanuelli. 2006. Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunol Immunother*. 55:684-698.

Berman, D., A. Korman, R. Peck, D. Feltquate, N. Lonberg, and R. Canetta. 2015. The development of immunomodulatory monoclonal antibodies as a new therapeutic modality for cancer: the Bristol-Myers Squibb experience. *Pharmacol Ther*. 148:132-153.

Berx, G., and F. van Roy. 2009. Involvement of members of the cadherin superfamily in cancer. *Cold Spring Harb Perspect Biol*. 1:a003129.

Bhatnagar, S., K. Shinagawa, F.J. Castellino, and J.S. Schorey. 2007. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. *Blood*. 110:3234-3244.

Bhowmick, N.A., E.G. Neilson, and H.L. Moses. 2004. Stromal fibroblasts in cancer initiation and progression. *Nature*. 432:332-337.

Binnewies, M., E.W. Roberts, K. Kersten, V. Chan, D.F. Fearon, M. Merad, L.M. Coussens, D.I. Gabrilovich, S. Ostrand-Rosenberg, C.C. Hedrick, R.H. Vonderheide, M.J. Pittet, R.K. Jain, W. Zou, T.K. Howcroft, E.C. Woodhouse, R.A. Weinberg, and M.F. Krummel. 2018. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med*. 24:541-550.

Blasco, M.A. 2005. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet*. 6:611-622.

Boström, M.M., H. Irjala, T. Mirtti, P. Taimen, T. Kauko, A. Ålgars, S. Jalkanen, and P.J. Boström. 2015. Tumor-Associated Macrophages Provide Significant Prognostic Information in Urothelial Bladder Cancer. *PLoS One*. 10:e0133552.

Boussiotis, V.A., P. Chatterjee, and L. Li. 2014. Biochemical signaling of PD-1 on T cells and its functional implications. *Cancer J*. 20:265-271.

Brunet, J.F., F. Denizot, M.F. Luciani, M. Roux-Dosseto, M. Suzan, M.G. Mattei, and P. Golstein. 1987. A new member of the immunoglobulin superfamily--CTLA-4. *Nature*. 328:267-270.

Burkhart, D.L., and J. Sage. 2008. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer*. 8:671-682.

Burugu, S., A.R. Dancsok, and T.O. Nielsen. 2018. Emerging targets in cancer immunotherapy. *Semin Cancer Biol*. 52:39-52.

Cai, Z., W. Zhang, F. Yang, L. Yu, Z. Yu, J. Pan, L. Wang, X. Cao, and J. Wang. 2012. Immunosuppressive exosomes from TGF-β1 gene-modified dendritic cells attenuate Th17-mediated inflammatory autoimmune disease by inducing regulatory T cells. *Cell Res*. 22:607-610.

Cannarile, M.A., M. Weisser, W. Jacob, A.M. Jegg, C.H. Ries, and D. Rüttinger. 2017. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J Immunother Cancer*. 5:53.

Carmeliet, P. 2005. VEGF as a key mediator of angiogenesis in cancer. *Oncology*. 69 Suppl 3:4-10.

Casey, S.C., L. Tong, Y. Li, R. Do, S. Walz, K.N. Fitzgerald, A.M. Gouw, V. Baylot, I. Gütgemann, M. Eilers, and D.W. Felsher. 2016. MYC regulates the antitumor immune response through CD47 and PD-L1. *Science*. 352:227-231.

Cavallaro, U., and G. Christofori. 2004. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer*. 4:118-132.

Chanmee, T., P. Ontong, K. Konno, and N. Itano. 2014. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel)*. 6:1670-1690.

Chao, M.P., I.L. Weissman, and R. Majeti. 2012. The CD47-SIRPα pathway in cancer immune evasion and potential therapeutic implications. *Curr Opin Immunol*. 24:225-232.

Chen, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol*. 4:336-347.

Chen, Y., G. Li, and M.L. Liu. 2018. Microvesicles as Emerging Biomarkers and Therapeutic Targets in Cardiometabolic Diseases. *Genomics Proteomics Bioinformatics*. 16:50-62.

Chen, Y., M. Shi, G.Z. Yu, X.R. Qin, G. Jin, P. Chen, and M.H. Zhu. 2012. Interleukin-8, a promising predictor for prognosis of pancreatic cancer. *World J Gastroenterol*. 18:1123-1129.

Cheng, N., A. Chytil, Y. Shyr, A. Joly, and H.L. Moses. 2008. Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. *Mol Cancer Res*. 6:1521-1533.

Chodon, T., B. Comin-Anduix, B. Chmielowski, R.C. Koya, Z. Wu, M. Auerbach, C. Ng, E. Avramis, E. Seja, A. Villanueva, T.A. McCannel, A. Ishiyama, J. Czernin, C.G. Radu, X. Wang, D.W. Gjertson, A.J. Cochran, K. Cornetta, D.J. Wong, P. Kaplan-Lefko, O. Hamid, W. Samlowski, P.A. Cohen, G.A. Daniels, B. Mukherji, L. Yang, J.A. Zack, D.B. Kohn, J.R. Heath, J.A. Glaspy, O.N. Witte, D. Baltimore, J.S. Economou, and A. Ribas. 2014. Adoptive transfer of MART-1 T-cell receptor transgenic lymphocytes and dendritic cell vaccination in patients with metastatic melanoma. *Clin Cancer Res*. 20:2457-2465.

Chuang, E., T.S. Fisher, R.W. Morgan, M.D. Robbins, J.M. Duerr, M.G. Vander Heiden, J.P. Gardner, J.E. Hambor, M.J. Neveu, and C.B. Thompson. 2000. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity*. 13:313-322.

Chuang, E., K.M. Lee, M.D. Robbins, J.M. Duerr, M.L. Alegre, J.E. Hambor, M.J. Neveu, J.A. Bluestone, and C.B. Thompson. 1999. Regulation of cytotoxic T lymphocyte-associated molecule-4 by Src kinases. *J Immunol*. 162:1270-1277.

Colegio, O.R., N.Q. Chu, A.L. Szabo, T. Chu, A.M. Rhebergen, V. Jairam, N. Cyrus, C.E. Brokowski, S.C. Eisenbarth, G.M. Phillips, G.W. Cline, A.J. Phillips, and R. Medzhitov. 2014. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. 513:559-563.

Colombo, M., G. Raposo, and C. Théry. 2014. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 30:255-289.

Cunha, L.L., E.C. Morari, S. Nonogaki, M.A. Marcello, F.A. Soares, J. Vassallo, and L.S. Ward. 2017. Interleukin 10 expression is related to aggressiveness and poor

prognosis of patients with thyroid cancer. *Cancer Immunol Immunother*. 66:141-148.

Dariavach, P., M.G. Mattéi, P. Golstein, and M.P. Lefranc. 1988. Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. *Eur J Immunol*. 18:1901-1905.

Darnell, J.E., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*. 264:1415-1421.

DeBerardinis, R.J., J.J. Lum, G. Hatzivassiliou, and C.B. Thompson. 2008. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab*. 7:11-20.

Dighe, A.S., E. Richards, L.J. Old, and R.D. Schreiber. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity*. 1:447-456.

Dong, H., S.E. Strome, D.R. Salomao, H. Tamura, F. Hirano, D.B. Flies, P.C. Roche, J. Lu, G. Zhu, K. Tamada, V.A. Lennon, E. Celis, and L. Chen. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 8:793-800.

Donini, C., L. D'Ambrosio, G. Grignani, M. Aglietta, and D. Sangiolo. 2018. Next generation immune-checkpoints for cancer therapy. *J Thorac Dis*. 10:S1581-S1601.

Dorand, R.D., J. Nthale, J.T. Myers, D.S. Barkauskas, S. Avril, S.M. Chirieleison, T.K. Pareek, D.W. Abbott, D.S. Stearns, J.J. Letterio, A.Y. Huang, and A. Petrosiute. 2016. Cdk5 disruption attenuates tumor PD-L1 expression and promotes antitumor immunity. *Science*. 353:399-403.

Doyle, L.M., and M.Z. Wang. 2019. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells*. 8.

Duan, Q., H. Zhang, J. Zheng, and L. Zhang. 2020. Turning Cold into Hot: Firing up the Tumor Microenvironment. *Trends Cancer*. 6:605-618.

Dunkel, J., M. Viitala, M. Karikoski, P. Rantakari, R. Virtakoivu, K. Elima, M. Hollmén, S. Jalkanen, and M. Salmi. 2018. Enhanced Antibody Production in Clever-1/Stabilin-1-Deficient Mice. *Front Immunol*. 9:2257.

Esser, J., U. Gehrmann, F.L. D'Alexandri, A.M. Hidalgo-Estévez, C.E. Wheelock, A. Scheynius, S. Gabrielsson, and O. Rådmark. 2010. Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration. *J Allergy Clin Immunol*. 126:1032-1040, 1040.e1031-1034.

Evan, G., and T. Littlewood. 1998. A matter of life and cell death. *Science*. 281:1317-1322.

Farkona, S., E.P. Diamandis, and I.M. Blasutig. 2016. Cancer immunotherapy: the beginning of the end of cancer? *BMC Med*. 14:73.

Feng, L., Q. Qi, P. Wang, H. Chen, Z. Chen, Z. Meng, and L. Liu. 2018. Serum levels of IL-6, IL-8, and IL-10 are indicators of prognosis in pancreatic cancer. *J Int Med Res*. 46:5228-5236.

Ferrara, N. 2009. Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol*. 29:789-791.

Fiaschi, T., and P. Chiarugi. 2012. Oxidative stress, tumor microenvironment, and metabolic reprogramming: a diabolic liaison. *Int J Cell Biol*. 2012:762825.

Fife, B.T., and J.A. Bluestone. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev*. 224:166-182.

Finney, H.M., A.N. Akbar, and A.D. Lawson. 2004. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. *J Immunol*. 172:104-113.

Francisco, L.M., V.H. Salinas, K.E. Brown, V.K. Vanguri, G.J. Freeman, V.K. Kuchroo, and A.H. Sharpe. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 206:3015-3029.

Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 192:1027-1034.

Fritz, J.M., M.A. Tennis, D.J. Orlicky, H. Lin, C. Ju, E.F. Redente, K.S. Choo, T.A. Staab, R.J. Bouchard, D.T. Merrick, A.M. Malkinson, and L.D. Dwyer-Nield. 2014.

Depletion of tumor-associated macrophages slows the growth of chemically induced mouse lung adenocarcinomas. *Front Immunol*. 5:587.

Fu, X.T., Z. Dai, K. Song, Z.J. Zhang, Z.J. Zhou, S.L. Zhou, Y.M. Zhao, Y.S. Xiao, Q.M. Sun, Z.B. Ding, and J. Fan. 2015. Macrophage-secreted IL-8 induces epithelial-mesenchymal transition in hepatocellular carcinoma cells by activating the JAK2/STAT3/Snail pathway. *Int J Oncol*. 46:587-596.

Fujimura, T., Y. Kambayashi, S. Furudate, A. Kakizaki, and S. Aiba. 2013. Immunomodulatory effect of bisphosphonate risedronate sodium on CD163+ arginase 1+ M2 macrophages: the development of a possible supportive therapy for angiosarcoma. *Clin Dev Immunol*. 2013:325412.

Galon, J., and D. Bruni. 2019. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov*. 18:197-218.

Gambhir, S.S. 2002. Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer*. 2:683-693.

Gao, J., L.Z. Shi, H. Zhao, J. Chen, L. Xiong, Q. He, T. Chen, J. Roszik, C. Bernatchez, S.E. Woodman, P.L. Chen, P. Hwu, J.P. Allison, A. Futreal, J.A. Wargo, and P. Sharma. 2016. Loss of IFN-γ Pathway Genes in Tumor Cells as a Mechanism of Resistance to Anti-CTLA-4 Therapy. *Cell*. 167:397-404.e399.

Garrido, F., N. Aptsiauri, E.M. Doorduijn, A.M. Garcia Lora, and T. van Hall. 2016. The urgent need to recover MHC class I in cancers for effective immunotherapy. *Curr Opin Immunol*. 39:44-51.

Garzetti, L., R. Menon, A. Finardi, A. Bergami, A. Sica, G. Martino, G. Comi, C. Verderio, C. Farina, and R. Furlan. 2014. Activated macrophages release microvesicles containing polarized M1 or M2 mRNAs. *J Leukoc Biol*. 95:817-825.

Ge, Z., and S. Ding. 2020. The Crosstalk Between Tumor-Associated Macrophages (TAMs) and Tumor Cells and the Corresponding Targeted Therapy. *Front Oncol*. 10:590941.

Girardi, M., D.E. Oppenheim, C.R. Steele, J.M. Lewis, E. Glusac, R. Filler, P. Hobby, B. Sutton, R.E. Tigelaar, and A.C. Hayday. 2001. Regulation of cutaneous malignancy by gammadelta T cells. *Science*. 294:605-609.

Goerdt, S., R. Bhardwaj, and C. Sorg. 1993. Inducible expression of MS-1 high-molecular-weight protein by endothelial cells of continuous origin and by dendritic cells/macrophages in vivo and in vitro. *Am J Pathol*. 142:1409-1422.

Goerdt, S., L.J. Walsh, G.F. Murphy, and J.S. Pober. 1991. Identification of a novel high molecular weight protein preferentially expressed by sinusoidal endothelial cells in normal human tissues. *J Cell Biol*. 113:1425-1437.

Gong, J., A. Chehrazi-Raffle, S. Reddi, and R. Salgia. 2018. Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. *J Immunother Cancer*. 6:8.

Gordon, S.R., R.L. Maute, B.W. Dulken, G. Hutter, B.M. George, M.N. McCracken, R. Gupta, J.M. Tsai, R. Sinha, D. Corey, A.M. Ring, A.J. Connolly, and I.L. Weissman. 2017. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature*. 545:495-499.

Green, M.R., S. Monti, S.J. Rodig, P. Juszczynski, T. Currie, E. O'Donnell, B. Chapuy, K. Takeyama, D. Neuberg, T.R. Golub, J.L. Kutok, and M.A. Shipp. 2010. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood*. 116:3268-3277.

Greenwald, R.J., V.A. Boussiotis, R.B. Lorsbach, A.K. Abbas, and A.H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. *Immunity*. 14:145-155.

Grosso, J.F., and M.N. Jure-Kunkel. 2013. CTLA-4 blockade in tumor models: an overview of preclinical and translational research. *Cancer Immun*. 13:5.

Guo, C., M.H. Manjili, J.R. Subjeck, D. Sarkar, P.B. Fisher, and X.Y. Wang. 2013. Therapeutic cancer vaccines: past, present, and future. *Adv Cancer Res*. 119:421-475.

Guo, C., H. Yi, X. Yu, F. Hu, D. Zuo, J.R. Subjeck, and X.Y. Wang. 2012. Absence of scavenger receptor A promotes dendritic cell-mediated cross-presentation of cell-associated antigen and antitumor immune response. *Immunol Cell Biol*. 90:101-108.

Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 86:353-364.

Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-674.

Highfill, S.L., Y. Cui, A.J. Giles, J.P. Smith, H. Zhang, E. Morse, R.N. Kaplan, and C.L. Mackall. 2014. Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. *Sci Transl Med*. 6:237ra267.

Hirano, F., K. Kaneko, H. Tamura, H. Dong, S. Wang, M. Ichikawa, C. Rietz, D.B. Flies, J.S. Lau, G. Zhu, K. Tamada, and L. Chen. 2005. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res*. 65:1089-1096.

Hirayama, D., T. Iida, and H. Nakase. 2017. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *Int J Mol Sci*. 19.

Hodi, F.S., M.C. Mihm, R.J. Soiffer, F.G. Haluska, M. Butler, M.V. Seiden, T. Davis, R. Henry-Spires, S. MacRae, A. Willman, R. Padera, M.T. Jaklitsch, S. Shankar, T.C. Chen, A. Korman, J.P. Allison, and G. Dranoff. 2003. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A*. 100:4712-4717.

Hodi, F.S., S.J. O'Day, D.F. McDermott, R.W. Weber, J.A. Sosman, J.B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J.C. Hassel, W. Akerley, A.J. van den Eertwegh, J. Lutzky, P. Lorigan, J.M. Vaubel, G.P. Linette, D. Hogg, C.H. Ottensmeier, C. Lebbé, C. Peschel, I. Quirt, J.I. Clark, J.D. Wolchok, J.S. Weber, J. Tian, M.J. Yellin, G.M. Nichol, A. Hoos, and W.J. Urba. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 363:711-723.

Hollmén, M., C.R. Figueiredo, and S. Jalkanen. 2020. New tools to prevent cancer growth and spread: a 'Clever' approach. *Br J Cancer*. 123:501-509.

Hsu, P.P., and D.M. Sabatini. 2008. Cancer cell metabolism: Warburg and beyond. *Cell*. 134:703-707.

Hu, W., X. Li, C. Zhang, Y. Yang, J. Jiang, and C. Wu. 2016. Tumor-associated macrophages in cancers. *Clin Transl Oncol*. 18:251-258.

Hu-Lieskovan, S., B. Homet Moreno, and A. Ribas. 2015. Excluding T Cells: Is β-Catenin the Full Story? *Cancer Cell*. 27:749-750.

Högger, P., and C. Sorg. 2001. Soluble CD163 inhibits phorbol ester-induced lymphocyte proliferation. *Biochem Biophys Res Commun*. 288:841-843.

Inglis, H., P. Norris, and A. Danesh. 2015. Techniques for the analysis of extracellular vesicles using flow cytometry. *J Vis Exp*.

Intlekofer, A.M., and C.B. Thompson. 2013. At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. *J Leukoc Biol*. 94:25-39.

Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J*. 11:3887-3895.

Iwai, Y., M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A*. 99:12293-12297.

Jain, N., H. Nguyen, C. Chambers, and J. Kang. 2010. Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. *Proc Natl Acad Sci U S A*. 107:1524-1528.

Jakowlew, S.B. 2006. Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev*. 25:435-457.

Johnson, D.E., R.A. O'Keefe, and J.R. Grandis. 2018. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nat Rev Clin Oncol*. 15:234-248.

Kaplan, D.H., V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, and R.D. Schreiber. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A*. 95:7556-7561.

Karikoski, M., H. Irjala, M. Maksimow, M. Miiluniemi, K. Granfors, S. Hernesniemi, K. Elima, G. Moldenhauer, K. Schledzewski, J. Kzhyshkowska, S. Goerdt, M. Salmi, and S. Jalkanen. 2009. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *Eur J Immunol*. 39:3477-3487.

Karikoski, M., F. Marttila-Ichihara, K. Elima, P. Rantakari, M. Hollmén, T. Kelkka, H. Gerke, V. Huovinen, H. Irjala, R. Holmdahl, M. Salmi, and S. Jalkanen. 2014. Clever-1/stabilin-1 controls cancer growth and metastasis. *Clin Cancer Res*. 20:6452-6464.

Katsuya, Y., H. Horinouchi, T. Asao, S. Kitahara, Y. Goto, S. Kanda, Y. Fujiwara, H. Nokihara, N. Yamamoto, S. Watanabe, K. Tsuta, and Y. Ohe. 2016. Expression of

programmed death 1 (PD-1) and its ligand (PD-L1) in thymic epithelial tumors: Impact on treatment efficacy and alteration in expression after chemotherapy. *Lung Cancer*. 99:4-10.

Kawachi, A., H. Yoshida, S. Kitano, Y. Ino, T. Kato, and N. Hiraoka. 2018. Tumor-associated CD204. *Cancer Sci*. 109:863-870.

Kazerounian, S., K.O. Yee, and J. Lawler. 2008. Thrombospondins in cancer. *Cell Mol Life Sci*. 65:700-712.

Kim, R., M. Emi, and K. Tanabe. 2007. Cancer immunoediting from immune surveillance to immune escape. *Immunology*. 121:1-14.

Kim, S.H., E.R. Lechman, N. Bianco, R. Menon, A. Keravala, J. Nash, Z. Mi, S.C. Watkins, A. Gambotto, and P.D. Robbins. 2005. Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol*. 174:6440-6448.

Komohara, Y., K. Ohnishi, J. Kuratsu, and M. Takeya. 2008. Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. *J Pathol*. 216:15-24.

Komohara, Y., K. Takemura, X.F. Lei, N. Sakashita, M. Harada, H. Suzuki, T. Kodama, and M. Takeya. 2009. Delayed growth of EL4 lymphoma in SR-A-deficient mice is due to upregulation of nitric oxide and interferon-gamma production by tumor-associated macrophages. *Cancer Sci*. 100:2160-2166.

Krummel, M.F., and J.P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med*. 182:459-465.

Krummel, M.F., and J.P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med*. 183:2533-2540.

Kuang, D.M., Q. Zhao, C. Peng, J. Xu, J.P. Zhang, C. Wu, and L. Zheng. 2009. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med*. 206:1327-1337.

Kumari, N., B.S. Dwarakanath, A. Das, and A.N. Bhatt. 2016. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol*. 37:11553-11572.

Kvorning, S.L., M.C. Nielsen, N.F. Andersen, M. Hokland, M.N. Andersen, and H.J. Møller. 2020. Circulating extracellular vesicle-associated CD163 and CD206 in multiple myeloma. *Eur J Haematol*. 104:409-419.

Kwon, E.D., A.A. Hurwitz, B.A. Foster, C. Madias, A.L. Feldhaus, N.M. Greenberg, M.B. Burg, and J.P. Allison. 1997. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci U S A*. 94:8099-8103.

Kwon, M., S.C. Yeo, J.S. Lee, and J.J. Park. 2019. Not CD68 but stabilin-1 expression is associated with the risk of recurrence in patients with oral cavity squamous cell carcinoma. *Head Neck*. 41:2058-2064.

Kzhyshkowska, J., A. Gratchev, and S. Goerdt. 2006. Stabilin-1, a homeostatic scavenger receptor with multiple functions. *J Cell Mol Med*. 10:635-649.

Kzhyshkowska, J., A. Gratchev, J.H. Martens, O. Pervushina, S. Mamidi, S. Johansson, K. Schledzewski, B. Hansen, X. He, J. Tang, K. Nakayama, and S. Goerdt. 2004. Stabilin-1 localizes to endosomes and the trans-Golgi network in human macrophages and interacts with GGA adaptors. *J Leukoc Biol*. 76:1151-1161.

Lanitis, E., M. Irving, and G. Coukos. 2015. Targeting the tumor vasculature to enhance T cell activity. *Curr Opin Immunol*. 33:55-63.

Lastwika, K.J., W. Wilson, Q.K. Li, J. Norris, H. Xu, S.R. Ghazarian, H. Kitagawa, S. Kawabata, J.M. Taube, S. Yao, L.N. Liu, J.J. Gills, and P.A. Dennis. 2016. Control of PD-L1 Expression by Oncogenic Activation of the AKT-mTOR Pathway in Non-Small Cell Lung Cancer. *Cancer Res*. 76:227-238.

Latchman, Y., C.R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A.J. Long, J.A. Brown, R. Nunes, E.A. Greenfield, K. Bourque, V.A. Boussiotis, L.L. Carter, B.M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A.H. Sharpe, and G.J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*. 2:261-268.

Leach, D.R., M.F. Krummel, and J.P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 271:1734-1736.

Lebrun, J.J. 2012. The Dual Role of TGFβ in Human Cancer: From Tumor Suppression to Cancer Metastasis. *ISRN Mol Biol*. 2012:381428.

Lesina, M., M.U. Kurkowski, K. Ludes, S. Rose-John, M. Treiber, G. Klöppel, A. Yoshimura, W. Reindl, B. Sipos, S. Akira, R.M. Schmid, and H. Algül. 2011. Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer Cell*. 19:456-469.

Li, M., Y. Zhang, L.W. Feurino, H. Wang, W.E. Fisher, F.C. Brunicardi, C. Chen, and Q. Yao. 2008. Interleukin-8 increases vascular endothelial growth factor and neuropilin expression and stimulates ERK activation in human pancreatic cancer. *Cancer Sci*. 99:733-737.

Lin, R.L., and L.J. Zhao. 2015. Mechanistic basis and clinical relevance of the role of transforming growth factor-β in cancer. *Cancer Biol Med*. 12:385-393.

Lin, S.Y., F.F. Hu, Y.R. Miao, H. Hu, Q. Lei, Q. Zhang, Q. Li, H. Wang, Z. Chen, and A.Y. Guo. 2019. Identification of STAB1 in Multiple Datasets as a Prognostic Factor for Cytogenetically Normal AML: Mechanism and Drug Indications. *Mol Ther Nucleic Acids*. 18:476-484.

Linehan, D.C., and P.S. Goedegebuure. 2005. CD25+ CD4+ regulatory T-cells in cancer. *Immunol Res*. 32:155-168.

Linsley, P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med*. 174:561-569.

Linsley, P.S., J.L. Greene, W. Brady, J. Bajorath, J.A. Ledbetter, and R. Peach. 1994. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity*. 1:793-801.

Lippitz, B.E., and R.A. Harris. 2016. Cytokine patterns in cancer patients: A review of the correlation between interleukin 6 and prognosis. *Oncoimmunology*. 5:e1093722.

Liu, C., W. Peng, C. Xu, Y. Lou, M. Zhang, J.A. Wargo, J.Q. Chen, H.S. Li, S.S. Watowich, Y. Yang, D. Tompers Frederick, Z.A. Cooper, R.M. Mbofung, M. Whittington, K.T. Flaherty, S.E. Woodman, M.A. Davies, L.G. Radvanyi, W.W. Overwijk, G. Lizée, and P. Hwu. 2013. BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. *Clin Cancer Res*. 19:393-403.

Liu, Y., and X. Cao. 2016. Immunosuppressive cells in tumor immune escape and metastasis. *J Mol Med (Berl)*. 94:509-522.

Loi, S., S. Pommey, B. Haibe-Kains, P.A. Beavis, P.K. Darcy, M.J. Smyth, and J. Stagg. 2013. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. *Proc Natl Acad Sci U S A*. 110:11091-11096.

Lowe, S.W., E. Cepero, and G. Evan. 2004. Intrinsic tumour suppression. *Nature*. 432:307-315.

Ma, C., R. Fan, H. Ahmad, Q. Shi, B. Comin-Anduix, T. Chodon, R.C. Koya, C.C. Liu, G.A. Kwong, C.G. Radu, A. Ribas, and J.R. Heath. 2011. A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat Med*. 17:738-743.

Maas, S.L.N., X.O. Breakefield, and A.M. Weaver. 2017. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends Cell Biol*. 27:172-188.

Mac Gabhann, F., and A.S. Popel. 2008. Systems biology of vascular endothelial growth factors. *Microcirculation*. 15:715-738.

Malyshev, I., and Y. Malyshev. 2015. Current Concept and Update of the Macrophage Plasticity Concept: Intracellular Mechanisms of Reprogramming and M3 Macrophage "Switch" Phenotype. *Biomed Res Int*. 2015:341308.

Marcoux, G., A.C. Duchez, N. Cloutier, P. Provost, P.A. Nigrovic, and E. Boilard. 2016. Revealing the diversity of extracellular vesicles using high-dimensional flow cytometry analyses. *Sci Rep*. 6:35928.

Marengère, L.E., P. Waterhouse, G.S. Duncan, H.W. Mittrücker, G.S. Feng, and T.W. Mak. 1996. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science*. 272:1170-1173.

Massagué, J. 2008. TGFbeta in Cancer. *Cell*. 134:215-230.

Matsuo, Y., N. Ochi, H. Sawai, A. Yasuda, H. Takahashi, H. Funahashi, H. Takeyama, Z. Tong, and S. Guha. 2009. CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer. *Int J Cancer*. 124:853-861.

Matsushita, N., H. Komine, A. Grolleau-Julius, S. Pilon-Thomas, and J.J. Mulé. 2010. Targeting MARCO can lead to enhanced dendritic cell motility and anti-melanoma activity. *Cancer Immunol Immunother*. 59:875-884.

Mittal, D., D. Sinha, D. Barkauskas, A. Young, M. Kalimutho, K. Stannard, F. Caramia, B. Haibe-Kains, J. Stagg, K.K. Khanna, S. Loi, and M.J. Smyth. 2016. Adenosine 2B Receptor Expression on Cancer Cells Promotes Metastasis. *Cancer Res*. 76:4372-4382.

Miyasato, Y., T. Shiota, K. Ohnishi, C. Pan, H. Yano, H. Horlad, Y. Yamamoto, M. Yamamoto-Ibusuki, H. Iwase, M. Takeya, and Y. Komohara. 2017. High density of CD204-positive macrophages predicts worse clinical prognosis in patients with breast cancer. *Cancer Sci*. 108:1693-1700.

Morales, A., D. Eidinger, and A.W. Bruce. 1976. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J Urol*. 116:180-183.

Morgan, R.A., M.E. Dudley, J.R. Wunderlich, M.S. Hughes, J.C. Yang, R.M. Sherry, R.E. Royal, S.L. Topalian, U.S. Kammula, N.P. Restifo, Z. Zheng, A. Nahvi, C.R. de Vries, L.J. Rogers-Freezer, S.A. Mavroukakis, and S.A. Rosenberg. 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 314:126-129.

Mosig, S., K. Rennert, S. Krause, J. Kzhyshkowska, K. Neunübel, R. Heller, and H. Funke. 2009. Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+ CD16+ monocytes in detoxification of oxidized LDL. *FASEB J*. 23:866-874.

Motzer, R.J., N.M. Tannir, D.F. McDermott, O. Arén Frontera, B. Melichar, T.K. Choueiri, E.R. Plimack, P. Barthélémy, C. Porta, S. George, T. Powles, F. Donskov, V. Neiman, C.K. Kollmannsberger, P. Salman, H. Gurney, R. Hawkins, A. Ravaud, M.O. Grimm, S. Bracarda, C.H. Barrios, Y. Tomita, D. Castellano, B.I. Rini, A.C. Chen, S. Mekan, M.B. McHenry, M. Wind-Rotolo, J. Doan, P. Sharma, H.J. Hammers, B. Escudier, and C. Investigators. 2018. Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. *N Engl J Med*. 378:1277-1290.

Mu, X., W. Shi, Y. Xu, C. Xu, T. Zhao, B. Geng, J. Yang, J. Pan, S. Hu, C. Zhang, J. Zhang, C. Wang, J. Shen, Y. Che, Z. Liu, Y. Lv, H. Wen, and Q. You. 2018. Tumor-derived lactate induces M2 macrophage polarization via the activation of the ERK/STAT3 signaling pathway in breast cancer. *Cell Cycle*. 17:428-438.

Munich, S., A. Sobo-Vujanovic, W.J. Buchser, D. Beer-Stolz, and N.L. Vujanovic. 2012. Dendritic cell exosomes directly kill tumor cells and activate natural killer cells via TNF superfamily ligands. *Oncoimmunology*. 1:1074-1083.

Murphy, J.E., P.R. Tedbury, S. Homer-Vanniasinkam, J.H. Walker, and S. Ponnambalam. 2005. Biochemistry and cell biology of mammalian scavenger receptors. *Atherosclerosis*. 182:1-15.

Netea, M.G., J. Quintin, and J.W. van der Meer. 2011. Trained immunity: a memory for innate host defense. *Cell Host Microbe*. 9:355-361.

Neyen, C., A. Plüddemann, S. Mukhopadhyay, E. Maniati, M. Bossard, S. Gordon, and T. Hagemann. 2013. Macrophage scavenger receptor a promotes tumor progression in murine models of ovarian and pancreatic cancer. *J Immunol*. 190:3798-3805.

Nishimura, H., N. Minato, T. Nakano, and T. Honjo. 1998. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int Immunol*. 10:1563-1572.

Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 11:141-151.

No, J.H., J.M. Moon, K. Kim, and Y.B. Kim. 2013. Prognostic significance of serum soluble CD163 level in patients with epithelial ovarian cancer. *Gynecol Obstet Invest*. 75:263-267.

Noy, R., and J.W. Pollard. 2014. Tumor-associated macrophages: from mechanisms to therapy. *Immunity*. 41:49-61.

Ohtaki, Y., G. Ishii, K. Nagai, S. Ashimine, T. Kuwata, T. Hishida, M. Nishimura, J. Yoshida, I. Takeyoshi, and A. Ochiai. 2010. Stromal macrophage expressing CD204 is associated with tumor aggressiveness in lung adenocarcinoma. *J Thorac Oncol*. 5:1507-1515.

Okamoto, K., and H. Seimiya. 2019. Revisiting Telomere Shortening in Cancer. *Cells*. 8.

Palani, S., K. Elima, E. Ekholm, S. Jalkanen, and M. Salmi. 2016. Monocyte Stabilin-1 Suppresses the Activation of Th1 Lymphocytes. *J Immunol*. 196:115-123.

Palani, S., M. Maksimow, M. Miiluniemi, K. Auvinen, S. Jalkanen, and M. Salmi. 2011. Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. *Eur J Immunol*. 41:2052-2063.

Parsa, A.T., J.S. Waldron, A. Panner, C.A. Crane, I.F. Parney, J.J. Barry, K.E. Cachola, J.C. Murray, T. Tihan, M.C. Jensen, P.S. Mischel, D. Stokoe, and R.O. Pieper. 2007. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med*. 13:84-88.

Peggs, K.S., S.A. Quezada, C.A. Chambers, A.J. Korman, and J.P. Allison. 2009. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med*. 206:1717-1725.

Peng, W., J.Q. Chen, C. Liu, S. Malu, C. Creasy, M.T. Tetzlaff, C. Xu, J.A. McKenzie, C. Zhang, X. Liang, L.J. Williams, W. Deng, G. Chen, R. Mbofung, A.J. Lazar, C.A. Torres-Cabala, Z.A. Cooper, P.L. Chen, T.N. Tieu, S. Spranger, X. Yu, C. Bernatchez, M.A. Forget, C. Haymaker, R. Amaria, J.L. McQuade, I.C. Glitza, T. Cascone, H.S. Li, L.N. Kwong, T.P. Heffernan, J. Hu, R.L. Bassett, M.W. Bosenberg, S.E. Woodman, W.W. Overwijk, G. Lizée, J. Roszik, T.F. Gajewski, J.A. Wargo, J.E. Gershenwald, L. Radvanyi, M.A. Davies, and P. Hwu. 2016. Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy. *Cancer Discov*. 6:202-216.

Perica, K., J.C. Varela, M. Oelke, and J. Schneck. 2015. Adoptive T cell immunotherapy for cancer. *Rambam Maimonides Med J*. 6:e0004.

Platanias, L.C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. 5:375-386.

Poh, A.R., and M. Ernst. 2018. Targeting Macrophages in Cancer: From Bench to Bedside. *Front Oncol*. 8:49.

Quezada, S.A., K.S. Peggs, M.A. Curran, and J.P. Allison. 2006. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J Clin Invest*. 116:1935-1945.

Qureshi, O.S., Y. Zheng, K. Nakamura, K. Attridge, C. Manzotti, E.M. Schmidt, J. Baker, L.E. Jeffery, S. Kaur, Z. Briggs, T.Z. Hou, C.E. Futter, G. Anderson, L.S. Walker, and D.M. Sansom. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 332:600-603.

Raposo, G., and W. Stoorvogel. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 200:373-383.

Rivlin, N., R. Brosh, M. Oren, and V. Rotter. 2011. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes Cancer*. 2:466-474.

Robbins, P.F., R.A. Morgan, S.A. Feldman, J.C. Yang, R.M. Sherry, M.E. Dudley, J.R. Wunderlich, A.V. Nahvi, L.J. Helman, C.L. Mackall, U.S. Kammula, M.S. Hughes, N.P. Restifo, M. Raffeld, C.C. Lee, C.L. Levy, Y.F. Li, M. El-Gamil, S.L. Schwarz, C. Laurencot, and S.A. Rosenberg. 2011. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol*. 29:917-924.

Rosenberg, J.E., J. Hoffman-Censits, T. Powles, M.S. van der Heijden, A.V. Balar, A. Necchi, N. Dawson, P.H. O'Donnell, A. Balmanoukian, Y. Loriot, S. Srinivas, M.M. Retz, P. Grivas, R.W. Joseph, M.D. Galsky, M.T. Fleming, D.P. Petrylak, J.L. Perez-Gracia, H.A. Burris, D. Castellano, C. Canil, J. Bellmunt, D. Bajorin, D. Nickles, R. Bourgon, G.M. Frampton, N. Cui, S. Mariathasan, O. Abidoye, G.D. Fine, and R. Dreicer. 2016. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet*. 387:1909-1920.

Rosenberg, S.A., B.S. Packard, P.M. Aebersold, D. Solomon, S.L. Topalian, S.T. Toy, P. Simon, M.T. Lotze, J.C. Yang, and C.A. Seipp. 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med*. 319:1676-1680.

Rosenberg, S.A., J.R. Yannelli, J.C. Yang, S.L. Topalian, D.J. Schwartzentruber, J.S. Weber, D.R. Parkinson, C.A. Seipp, J.H. Einhorn, and D.E. White. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst*. 86:1159-1166.

Rudensky, A.Y. 2011. Regulatory T cells and Foxp3. *Immunol Rev*. 241:260-268.

Ruella, M., D.M. Barrett, S.S. Kenderian, O. Shestova, T.J. Hofmann, J. Perazzelli, M. Klichinsky, V. Aikawa, F. Nazimuddin, M. Kozlowski, J. Scholler, S.F. Lacey, J.J. Melenhorst, J.J. Morrissette, D.A. Christian, C.A. Hunter, M. Kalos, D.L. Porter, C.H. June, S.A. Grupp, and S. Gill. 2016. Dual CD19 and CD123 targeting prevents

antigen-loss relapses after CD19-directed immunotherapies. *J Clin Invest*. 126:3814-3826.

Rébé, C., and F. Ghiringhelli. 2019. STAT3, a Master Regulator of Anti-Tumor Immune Response. *Cancers (Basel)*. 11.

Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell*. 133:775-787.

Salmi, M., K. Koskinen, T. Henttinen, K. Elima, and S. Jalkanen. 2004. CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood*. 104:3849-3857.

Sansone, P., G. Storci, S. Tavolari, T. Guarnieri, C. Giovannini, M. Taffurelli, C. Ceccarelli, D. Santini, P. Paterini, K.B. Marcu, P. Chieco, and M. Bonafè. 2007. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest*. 117:3988-4002.

Sapochnik, M., M.R. Haedo, M. Fuertes, P. Ajler, G. Carrizo, A. Cervio, G. Sevlever, G.K. Stalla, and E. Arzt. 2017. Autocrine IL-6 mediates pituitary tumor senescence. *Oncotarget*. 8:4690-4702.

Schadendorf, D., F.S. Hodi, C. Robert, J.S. Weber, K. Margolin, O. Hamid, D. Patt, T.T. Chen, D.M. Berman, and J.D. Wolchok. 2015. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol*. 33:1889-1894.

Schalper, K.A., M. Carleton, M. Zhou, T. Chen, Y. Feng, S.P. Huang, A.M. Walsh, V. Baxi, D. Pandya, T. Baradet, D. Locke, Q. Wu, T.P. Reilly, P. Phillips, V. Nagineni, N. Gianino, J. Gu, H. Zhao, J.L. Perez-Gracia, M.F. Sanmamed, and I. Melero. 2020. Elevated serum interleukin-8 is associated with enhanced intratumor neutrophils and reduced clinical benefit of immune-checkpoint inhibitors. *Nat Med*. 26:688-692.

Schneider, H., J. Downey, A. Smith, B.H. Zinselmeyer, C. Rush, J.M. Brewer, B. Wei, N. Hogg, P. Garside, and C.E. Rudd. 2006. Reversal of the TCR stop signal by CTLA-4. *Science*. 313:1972-1975.

Schreiber, R.D., L.J. Old, and M.J. Smyth. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 331:1565-1570.

Segura, E., C. Nicco, B. Lombard, P. Véron, G. Raposo, F. Batteux, S. Amigorena, and C. Théry. 2005. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood*. 106:216-223.

Seung, B.J., H.Y. Lim, J.I. Shin, H.W. Kim, S.H. Cho, S.H. Kim, and J.H. Sur. 2018. CD204-Expressing Tumor-Associated Macrophages Are Associated With Malignant, High-Grade, and Hormone Receptor-Negative Canine Mammary Gland Tumors. *Vet Pathol*. 55:417-424.

Shankaran, V., H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, and R.D. Schreiber. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 410:1107-1111.

Sharma, N., J. Vacher, and J.P. Allison. 2019. TLR1/2 ligand enhances antitumor efficacy of CTLA-4 blockade by increasing intratumoral Treg depletion. *Proc Natl Acad Sci U S A*. 116:10453-10462.

Sharma, P., S. Hu-Lieskovan, J.A. Wargo, and A. Ribas. 2017. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell*. 168:707-723.

Shay, J.W., and W.E. Wright. 2000. Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol*. 1:72-76.

Sherr, C.J., and F. McCormick. 2002. The RB and p53 pathways in cancer. *Cancer Cell*. 2:103-112.

Shi, Y., J. Zhang, Z. Mao, H. Jiang, W. Liu, H. Shi, R. Ji, W. Xu, H. Qian, and X. Zhang. 2020. Extracellular Vesicles From Gastric Cancer Cells Induce PD-L1 Expression on Neutrophils to Suppress T-Cell Immunity. *Front Oncol*. 10:629.

Shigeoka, M., N. Urakawa, T. Nakamura, M. Nishio, T. Watajima, D. Kuroda, T. Komori, Y. Kakeji, S. Semba, and H. Yokozaki. 2013. Tumor associated macrophage expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma. *Cancer Sci*. 104:1112-1119.

Smyth, M.J., K.Y. Thia, S.E. Street, E. Cretney, J.A. Trapani, M. Taniguchi, T. Kawano, S.B. Pelikan, N.Y. Crowe, and D.I. Godfrey. 2000a. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med*. 191:661-668.

Smyth, M.J., K.Y. Thia, S.E. Street, D. MacGregor, D.I. Godfrey, and J.A. Trapani. 2000b. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med*. 192:755-760.

Solito, S., E. Falisi, C.M. Diaz-Montero, A. Doni, L. Pinton, A. Rosato, S. Francescato, G. Basso, P. Zanovello, G. Onicescu, E. Garrett-Mayer, A.J. Montero, V. Bronte, and S. Mandruzzato. 2011. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood*. 118:2254-2265.

Sotillo, E., D.M. Barrett, K.L. Black, A. Bagashev, D. Oldridge, G. Wu, R. Sussman, C. Lanauze, M. Ruella, M.R. Gazzara, N.M. Martinez, C.T. Harrington, E.Y. Chung, J. Perazzelli, T.J. Hofmann, S.L. Maude, P. Raman, A. Barrera, S. Gill, S.F. Lacey, J.J. Melenhorst, D. Allman, E. Jacoby, T. Fry, C. Mackall, Y. Barash, K.W. Lynch, J.M. Maris, S.A. Grupp, and A. Thomas-Tikhonenko. 2015. Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy. *Cancer Discov*. 5:1282-1295.

Spranger, S., R. Bao, and T.F. Gajewski. 2015. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. *Nature*. 523:231-235.

Stagg, J., U. Divisekera, N. McLaughlin, J. Sharkey, S. Pommey, D. Denoyer, K.M. Dwyer, and M.J. Smyth. 2010. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A*. 107:1547-1552.

Street, S.E., J.A. Trapani, D. MacGregor, and M.J. Smyth. 2002. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med*. 196:129-134.

Sugaya, M., T. Miyagaki, H. Ohmatsu, H. Suga, H. Kai, M. Kamata, H. Fujita, Y. Asano, Y. Tada, T. Kadono, H. Okochi, and S. Sato. 2012. Association of the numbers of CD163(+) cells in lesional skin and serum levels of soluble CD163 with disease progression of cutaneous T cell lymphoma. *J Dermatol Sci*. 68:45-51.

Tadayon, S., J. Dunkel, A. Takeda, O. Halle, M. Karikoski, H. Gerke, P. Rantakari, R. Virtakoivu, O. Pabst, M. Salmi, M. Hollmén, and S. Jalkanen. 2019. Clever-1 contributes to lymphocyte entry into the spleen via the red pulp. *Sci Immunol*. 4.

Tang, R.F., S.X. Wang, F.R. Zhang, L. Peng, Y. Xiao, and M. Zhang. 2005. Interleukin-1alpha, 6 regulate the secretion of vascular endothelial growth factor A, C in pancreatic cancer. *Hepatobiliary Pancreat Dis Int*. 4:460-463.

Tchirkov, A., T. Khalil, E. Chautard, K. Mokhtari, L. Véronèse, B. Irthum, P. Vago, J.L. Kémény, and P. Verrelle. 2007. Interleukin-6 gene amplification and shortened survival in glioblastoma patients. *Br J Cancer*. 96:474-476.

Teobald, I., D.J. Dunnion, M. Whitbread, S.J. Curnow, and M.J. Browning. 2008. Phenotypic and functional differentiation of KG-1 into dendritic-like cells. *Immunobiology*. 213:75-86.

Tervahartiala, M., P. Taimen, T. Mirtti, I. Koskinen, T. Ecke, S. Jalkanen, and P.J. Boström. 2017. Immunological tumor status may predict response to neoadjuvant chemotherapy and outcome after radical cystectomy in bladder cancer. *Sci Rep*. 7:12682.

Théry, C., S. Amigorena, G. Raposo, and A. Clayton. 2006. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. Chapter 3:Unit 3.22.

Topalian, S.L., C.G. Drake, and D.M. Pardoll. 2015. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*. 27:450-461.

Trickett, A., and Y.L. Kwan. 2003. T cell stimulation and expansion using anti-CD3/CD28 beads. *J Immunol Methods*. 275:251-255.

Turcotte, M., K. Spring, S. Pommey, G. Chouinard, I. Cousineau, J. George, G.M. Chen, D.M. Gendoo, B. Haibe-Kains, T. Karn, K. Rahimi, C. Le Page, D. Provencher, A.M. Mes-Masson, and J. Stagg. 2015. CD73 is associated with poor prognosis in high-grade serous ovarian cancer. *Cancer Res*. 75:4494-4503.

Twyman-Saint Victor, C., A.J. Rech, A. Maity, R. Rengan, K.E. Pauken, E. Stelekati, J.L. Benci, B. Xu, H. Dada, P.M. Odorizzi, R.S. Herati, K.D. Mansfield, D. Patsch, R.K. Amaravadi, L.M. Schuchter, H. Ishwaran, R. Mick, D.A. Pryma, X. Xu, M.D. Feldman, T.C. Gangadhar, S.M. Hahn, E.J. Wherry, R.H. Vonderheide, and A.J. Minn. 2015. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature*. 520:373-377.

Valeta-Magara, A., A. Gadi, V. Volta, B. Walters, R. Arju, S. Giashuddin, H. Zhong, and R.J. Schneider. 2019. Inflammatory Breast Cancer Promotes Development of M2 Tumor-Associated Macrophages and Cancer Mesenchymal Cells through a Complex Chemokine Network. *Cancer Res*. 79:3360-3371.

Valle-Mendiola, A., A. Gutiérrez-Hoya, M.e.C. Lagunas-Cruz, B. Weiss-Steider, and I. Soto-Cruz. 2016. Pleiotropic Effects of IL-2 on Cancer: Its Role in Cervical Cancer. *Mediators Inflamm*. 2016:2849523.

Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 324:1029-1033.

Vesely, M.D., M.H. Kershaw, R.D. Schreiber, and M.J. Smyth. 2011. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol*. 29:235-271.

Viehl, C.T., T.T. Moore, U.K. Liyanage, D.M. Frey, J.P. Ehlers, T.J. Eberlein, P.S. Goedegebuure, and D.C. Linehan. 2006. Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. *Ann Surg Oncol*. 13:1252-1258.

Viitala, M., R. Virtakoivu, S. Tadayon, J. Rannikko, S. Jalkanen, and M. Hollmén. 2019. Immunotherapeutic Blockade of Macrophage Clever-1 Reactivates the CD8. *Clin Cancer Res*. 25:3289-3303.

Voron, T., O. Colussi, E. Marcheteau, S. Pernot, M. Nizard, A.L. Pointet, S. Latreche, S. Bergaya, N. Benhamouda, C. Tanchot, C. Stockmann, P. Combe, A. Berger, F. Zinzindohoue, H. Yagita, E. Tartour, J. Taieb, and M. Terme. 2015. VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J Exp Med*. 212:139-148.

Waldman, A.D., J.M. Fritz, and M.J. Lenardo. 2020. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol*. 20:651-668.

Walunas, T.L., D.J. Lenschow, C.Y. Bakker, P.S. Linsley, G.J. Freeman, J.M. Green, C.B. Thompson, and J.A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1:405-413.

Wang, B., H. Huang, M. Yang, W. Yang, Z. Liu, W. Hou, H. Zeng, Z. He, T. Lin, and J. Huang. 2020. Microlocalization and clinical significance of stabilin-1. *World J Urol*. 38:709-716.

Wang, H., L. Wang, P.D. Chi, W.D. Wang, X.Q. Chen, Q.R. Geng, Z.J. Xia, and Y. Lu. 2016. High level of interleukin-10 in serum predicts poor prognosis in multiple myeloma. *Br J Cancer*. 114:463-468.

Warren, C.F.A., M.W. Wong-Brown, and N.A. Bowden. 2019. BCL-2 family isoforms in apoptosis and cancer. *Cell Death Dis*. 10:177.

Weroha, S.J., and P. Haluska. 2012. The insulin-like growth factor system in cancer. *Endocrinol Metab Clin North Am*. 41:335-350, vi.

Wesolowski, R., J. Markowitz, and W.E. Carson. 2013. Myeloid derived suppressor cells - a new therapeutic target in the treatment of cancer. *J Immunother Cancer*. 1:10.

Wherry, E.J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol*. 15:486-499.

Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 322:271-275.

Wolchok, J.D., V. Chiarion-Sileni, R. Gonzalez, P. Rutkowski, J.J. Grob, C.L. Cowey, C.D. Lao, J. Wagstaff, D. Schadendorf, P.F. Ferrucci, M. Smylie, R. Dummer, A. Hill, D. Hogg, J. Haanen, M.S. Carlino, O. Bechter, M. Maio, I. Marquez-Rodas, M. Guidoboni, G. McArthur, C. Lebbé, P.A. Ascierto, G.V. Long, J. Cebon, J. Sosman, M.A. Postow, M.K. Callahan, D. Walker, L. Rollin, R. Bhore, F.S. Hodi, and J. Larkin. 2017. Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med*. 377:1345-1356.

Wrzesinski, S.H., Y.Y. Wan, and R.A. Flavell. 2007. Transforming growth factor-beta and the immune response: implications for anticancer therapy. *Clin Cancer Res*. 13:5262-5270.

Xu, X., R. Wang, Q. Su, H. Huang, P. Zhou, J. Luan, J. Liu, J. Wang, and X. Chen. 2016. Expression of Th1- Th2- and Th17-associated cytokines in laryngeal carcinoma. *Oncol Lett*. 12:1941-1948.

Yang, L., L.M. DeBusk, K. Fukuda, B. Fingleton, B. Green-Jarvis, Y. Shyr, L.M. Matrisian, D.P. Carbone, and P.C. Lin. 2004. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*. 6:409-421.

Yang, L., J. Huang, X. Ren, A.E. Gorska, A. Chytil, M. Aakre, D.P. Carbone, L.M. Matrisian, A. Richmond, P.C. Lin, and H.L. Moses. 2008. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. 13:23-35.

Yang, Y.F., J.P. Zou, J. Mu, R. Wijesuriya, S. Ono, T. Walunas, J. Bluestone, H. Fujiwara, and T. Hamaoka. 1997. Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages. *Cancer Res*. 57:4036-4041.

Yu, G.T., L.L. Bu, C.F. Huang, W.F. Zhang, W.J. Chen, J.S. Gutkind, A.B. Kulkarni, and Z.J. Sun. 2015a. PD-1 blockade attenuates immunosuppressive myeloid cells due to inhibition of CD47/SIRPα axis in HPV negative head and neck squamous cell carcinoma. *Oncotarget*. 6:42067-42080.

Yu, J., and L. Zhang. 2008. PUMA, a potent killer with or without p53. *Oncogene*. 27 Suppl 1:S71-83.

Yu, X., C. Guo, P.B. Fisher, J.R. Subjeck, and X.Y. Wang. 2015b. Scavenger Receptors: Emerging Roles in Cancer Biology and Immunology. *Adv Cancer Res*. 128:309-364.

Zamarron, B.F., and W. Chen. 2011. Dual roles of immune cells and their factors in cancer development and progression. *Int J Biol Sci*. 7:651-658.

Zaretsky, J.M., A. Garcia-Diaz, D.S. Shin, H. Escuin-Ordinas, W. Hugo, S. Hu-Lieskovan, D.Y. Torrejon, G. Abril-Rodriguez, S. Sandoval, L. Barthly, J. Saco, B. Homet Moreno, R. Mezzadra, B. Chmielowski, K. Ruchalski, I.P. Shintaku, P.J. Sanchez, C. Puig-Saus, G. Cherry, E. Seja, X. Kong, J. Pang, B. Berent-Maoz, B. Comin-Anduix, T.G. Graeber, P.C. Tumeh, T.N. Schumacher, R.S. Lo, and A. Ribas. 2016. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *N Engl J Med*. 375:819-829.

Zhang, H., D.M. Conrad, J.J. Butler, C. Zhao, J. Blay, and D.W. Hoskin. 2004. Adenosine acts through A2 receptors to inhibit IL-2-induced tyrosine phosphorylation of STAT5 in T lymphocytes: role of cyclic adenosine 3',5'-monophosphate and phosphatases. *J Immunol*. 173:932-944.

Zheng, P., Q. Luo, W. Wang, J. Li, T. Wang, P. Wang, L. Chen, P. Zhang, H. Chen, Y. Liu, P. Dong, G. Xie, Y. Ma, L. Jiang, X. Yuan, and L. Shen. 2018. Tumor-associated macrophages-derived exosomes promote the migration of gastric cancer cells by transfer of functional Apolipoprotein E. *Cell Death Dis*. 9:434.

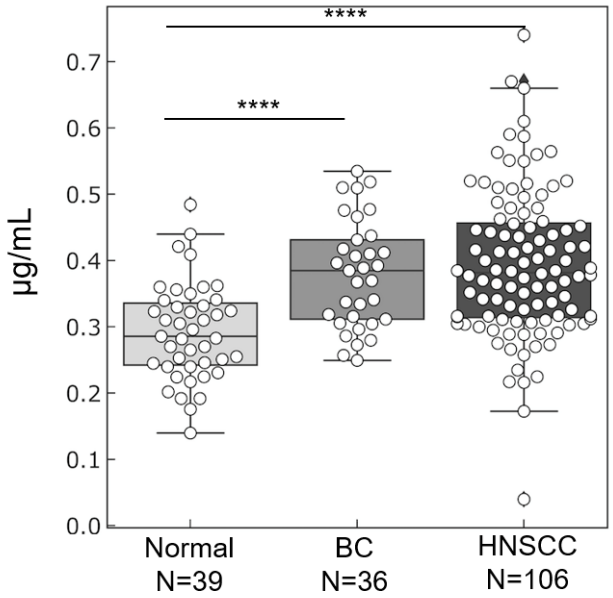
Zhou, J., X. Li, X. Wu, T. Zhang, Q. Zhu, X. Wang, H. Wang, K. Wang, and Y. Lin. 2018. Exosomes Released from Tumor-Associated Macrophages Transfer miRNAs That Induce a Treg/Th17 Cell Imbalance in Epithelial Ovarian Cancer. *Cancer Immunol Res*. 6:1578-1592.

Zhou, J., Z. Tang, S. Gao, C. Li, Y. Feng, and X. Zhou. 2020. Tumor-Associated Macrophages: Recent Insights and Therapies. *Front Oncol*. 10:188.

Zhu, X., H. Shen, X. Yin, M. Yang, H. Wei, Q. Chen, F. Feng, Y. Liu, W. Xu, and Y. Li. 2019. Macrophages derived exosomes deliver miR-223 to epithelial ovarian cancer cells to elicit a chemoresistant phenotype. *J Exp Clin Cancer Res*. 38:81.

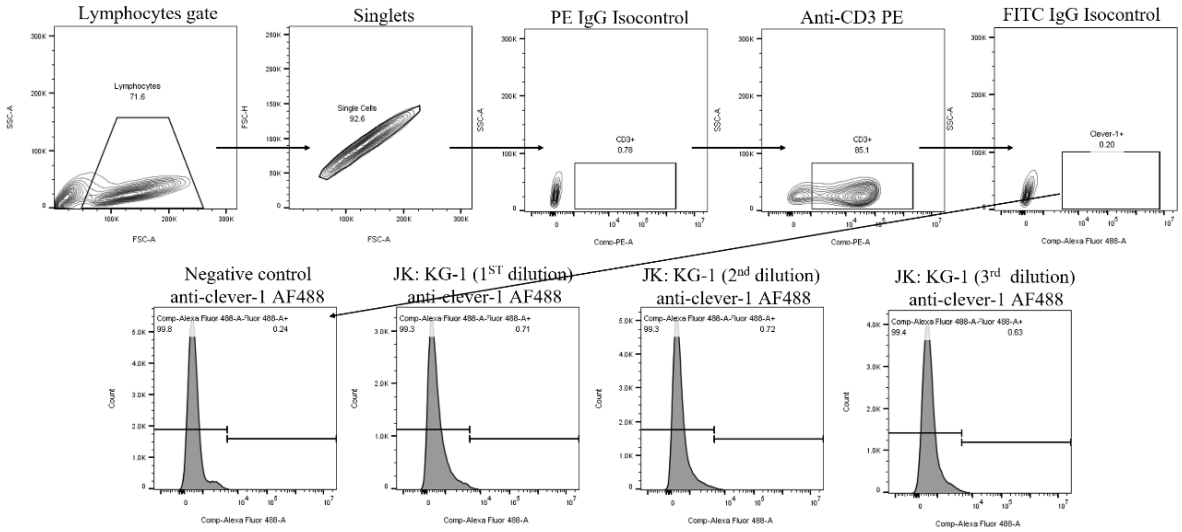
10. Appendices

10.1 Supplementary figure 1



Supplementary Figure 1: *Preliminary data generated in our group. Soluble Clever-1 levels are increased in cancer patient blood compared with blood of healthy donors. Soluble Clever-1 in plasma of normal healthy donors (n=44), breast cancer patients (BC, treatment naïve; n=33) and Head and neck squamous cell carcinoma (HNSCC) patients (n=106) measured by Time-resolved fluorescence immunoassay (TRFIA). Cancer samples are individually compared with normal blood using an unpaired, two-tailed t-test, where **** = p<0.0001 considered as significantly different from normal blood control.*

10.2 Supplementary figure 2



Supplementary figure 2*. Jurkat T cells were added in different dilutions of KG1 cells: 1st dilution (10^6 KG-1 cells); 2nd dilution (5 x 10^5 KG-1 cells), 3rd dilution (2.5 x 10^5 KG-1 cells). Clever-1 levels were measured by staining the Jurkat T cells with AF488 Clever1 antibody. Results from one pilot independent co-culture binding assay show that Jurkat T cells were negative for Clever-1expression.*