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DISSERTATION

# Immunoregulatory properties of cancer stem-like cells derived from carcinoma cell lines of the cervix uteri

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# CONTENTS

# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ALDH1 | Aldehyde dehydrogenase isoform 1 |
| APC | Allophycocyanin |
| APC | Antigen-presenting cell |
| BFA | Brefeldin A |
| bFGF | Basic fibroblast growth factor |
| BLCL | B-lymphoblastoid cell line |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CIN | Cervical intraepithelial neoplasia |
| CMV | Cytomegalovirus |
| CSC | Cancer stem cell |
| CTL | Cytolytic T lymphocyte |
| DC | Dendritic cell |
| DEAB | Diethylaminobenzaldehyde |
| DMEM | Dulbecco's modified eagled medium |
| EGF | Epidermal growth factor |
| FACS | Fluorescence activated cell sorter |
| Far Red | Far red dimethyldodecylamine oxide-succinimidyl ester |
| FBS | Fetal bovine serum |
| FITC | Fluorescein-isothiocyanate |
| HLA | Human leukocyte antigen |
| HNSCC | Head and neck squamous cell carcinoma |
| HPV | Human papillomavirus |
| IFN- γ | Interferon-γ |
| IL | Interleukin |
| LSC | Leukemia stem cell |
| MDC | Monolayer derived cell |
| MFI | Median fluorescence intensity |
| NOD/SCID | Non-obese diabetic/severe combined |

| | |
|---|---|
| | immune-deficient |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PE | R-phycoerythrin |
| PerCP | Peridinin chlorophyll protein |
| PI | Propidium iodide |
| RNA | Ribonucleic acid |
| RT-PCR | Real time-polymerase chain reaction |
| SC | Stem cell |
| SDC | Spheroid derived cell |
| TF | Transcription factor |
| TGF-β | Transforming growth factor-β |

# Zusammenfassung

**Hintergrund:** Krebsstammzellen (CSC, engl.: cancer stem cell) repräsentieren eine Subpopulation von Zellen in Tumoren, die als die Ausgangszellen eines Tumors angesehen werden. Sie werden für Tumorentstehung, Tumordifferenzierung, Tumorerhalt, Metastasierung und Tumorrezidivierung nach der Therapie verantwortlich gemacht. Es gibt zunehmend Hinweise darauf, dass CSCs neoplastisches Wachstum und Progression der Erkrankung zumindest teilweise initiieren und aufrecht erhalten, indem sie sowohl das angeborene als auch das adaptive Immunsystem durch eine Vielzahl von CSC-sezernierten Produkten und Zellen-Membran-Wechselwirkungen supprimieren. Dies wird Immuntherapie dieser Zellen beeinträchtigen. Bisher sind die immunologischen Eigenschaften der CSC noch wenig erforscht.

**Methode:** Für diese Untersuchungen wurden drei-dimensionale Zellkulturen (Sphaeroide) von drei Gebärmutterhalskrebszelllinien (CaSki, HeLa, MRIH215) erzeugt, um CSC anzureichern. Die Expression von Stammzell-Transkriptionsfaktoren (Sox2, Nanog, Oct3/4) und der Stammzellmarker ALDH1, CD44 und CD24 wurden zwischen CSC-angereicherten aus Sphaeroidkultur-abgeleiteten Zellen (SDC: engl.: spheroid culture-derived cells) und den entsprechenden aus Monolayerkultur-abgeleiteten Zellen (MDC: engl.: monolayer culture-derived cells) verglichen. Wir untersuchten die Wirkung von SDC oder MDC auf die Proliferation, Aktivierung und Funktion ruhender oder vorstimulierter T-Zellen in Transwell-Analysen.

**Ergebnisse:** Wir fanden, dass CSC-angereicherte SDC Populationen einen höheren Anteil an ALDH1 exprimierenden Zellen, an $ALDH1^{+}CD44^{+}CD24^{-}$ Populationen und der Expression der Stammzell-/Vorläuferzellmarker (Sox2, Oct3/4, Nanog) im Vergleich zu den entsprechenden MDCs zeigen. Ausserdem hatten SDCs der Gebärmutterhalskrebszelllinien eine stärkere supprimierende Wirkung auf T-Zell-Proliferation, -Aktivierung, -Zytokin-Produktion und zytotoxische T-Zell-Effektorfunktionen als MDC. Die Proliferationsrate der mit SDC ko-kultivierten T-Zellen (zwischen 0,11 bis 11,17%), war statistisch signifikant geringer als diejenige der mit MDC (zwischen 9,91 bis 43,59%) ko-kultivierten T-Zellen. Der Anteil aktivierter T-Zellen, nach Bestimmung der Oberflächenmarkerexpression von CD69, CD137 und CD154, war signifikant geringer wenn sie mit SDC (jeweils MFI 15.71-26.33, 21.82-54.62, 12.37-32.6,) ko-kultiviert worden waren, als diejenige der mit MDC ko-kultivierten (jeweils MFI 23.08-34.82, 34.49-87.79, 15.03-48.39; $P<0.05$). Die Expression der Zytokinproduktion von IFN-γ, IL-2 und TNF-α war signifikant geringer von den mit SDC (jeweils MFI 24.55-107.13, 32.67-221.75, 12.24-41.3) ko-kultivierten T-Zellen als diejenige der mit MDC

ko-kultivierten T-Zellen (jeweils MFI 23.56-152.16, 35.52-252.44, 16.33-48.38; P<0.05).

Bei Effektorzell: Zielzell-Verhältnissen von 10:1, war die zytotoxische Lyse durch CMV CTL in Gegenwart von SDC erheblich geringer (zwischen12,46% bis 53,8%) als die Lyse in Gegenwart von MDC (zwischen19,65% bis 60,29%; P<0.05).

**Zusammenfassung:** Wir haben konsistent in 3 Gebärmutterhalskrebszelllinien eine starke immunsuppressive Aktivität gefunden, die durch CSC ausgeübt wurde. Alle untersuchten T-Zell-Funktionen wurden erheblich stärker durch Ko-Kultur mit SDC unterdrückt als durch MDC. Das könnte ein wichtiger Escape-Mechanismus gegen Immunzellen sein, der anhaltendes Wachstum der Tumoren unterstützt und immuntherapeutische Strategien behindert.

# Summary

**Background:** Cancer stem cells (CSCs) represent a subpopulation of cells within tumors that are characterized as the original cells of a tumor which are responsible for tumorigenesis, tumor differentiation, tumor maintenance, metastasis, and tumor relapse following therapy. Increasing evidence suggests that CSCs might at least in part initiate and sustain neoplastic growth and disease progression by suppressing both the innate and adaptive immune systems by a variety of CSC-secreted products and cell-membrane interactions. This will challenge the targeting of these cells by immunotherapy. However, the immunologic properties of CSC are largely unexplored.

**Methods:** In this study, three-dimensional cultures (spheroids) were generated from three cervical cancer cell lines (CaSki, HeLa, and MRIH215) in order to enrich for CSCs. Stemness-related transcription factor expression (Sox2, Nanog, and Oct3/4) and stem cell markers ALDH1, CD44, and CD24 were compared between CSC-enriched spheroid culture-derived cells (SDC) and the corresponding monolayer culture-derived cells (MDC). We evaluated the effect on proliferation, activation and function of SDC or MDC on resting or pre-stimulated T cells in transwell assays.

**Results:** We show that CSC-enriched SDC populations exhibit a higher proportion of ALDH1-expressing cells, $ALDH1^{+}CD44^{+}CD24^{-}$ populations and expression of stem/progenitor cell markers (Sox2, Oct3/4, Nanog) as compared with corresponding MDCs. Moreover, SDCs from cervical cancer cell lines had a stronger suppressive effect on T cell proliferation, activation, cytokine production and cytotoxic T cell effector functions than MDC. The proliferation rate of T cells co-cultured with SDC (ranging from 0.11-11.17%) was significantly less than that of T cells co-cultured with MDC (ranging from 9.91-43.59%). The percentage of activated T cells, according to surface activation markers CD69, CD137, and CD154, when co-cultured with SDC (MFI 15.71-26.33, 21.82-54.62, 12.37-32.6, respectively) was significantly less than those co-cultured with MDC (MFI 23.08-34.82, 34.49-87.79, 15.03-48.39, respectively; $P<0.05$). The expression of cytokine production of IFN-γ, IL-2, and TNF-α by T cells co-cultured with SDC (MFI 24.55-107.13, 32.67-221.75, 12.24-41.3, respectively) was significantly less than that of T cells co-cultured with MDC (MFI 23.56-152.16, 35.52-252.44, 16.33-48.38, respectively; $P<0.05$). At effector: target ratios of 10:1, the CMV CTL cytotoxic lysis in the presence of SDC (the relative lysis ranged from 12.46% to 53.8%) was significantly less than the lysis in the

presence of MDC (the relative lysis ranged from 19.65% to 60.29%; $P<0.05$).

**Conclusion:** Consistently, we have found in 3 cervical cancer cell lines a strong immunosuppressive activity exerted by CSC. All T cell functions investigated were suppressed upon co-culture with SDC significantly more strongly than by MDC. This may be an important escape mechanism from immune cells supporting sustained growth of tumors and hampering immune therapeutic strategies.

# 1. Introduction

Cervical cancer is the major cause of death in women worldwide, and most cases are reported in less developed countries because of limited awareness and the absence of medical support. Despite recent advances in conventional treatments such as radical hysterectomy, surgical debulking or chemoradiation therapy, the prognosis for most patients with advanced cervical cancer remains poor. It is, therefore, desirable to develop a deeper understanding of the biology of this disease to adapt current therapeutic strategies and to develop therapies that are more effective.

CSCs are thought to be responsible for tumor maintenance, progression, and relapse of the disease due to, in part, an exhibition of multiple resistance mechanisms to chemotherapy and radiation [1]. Additionally, these CSCs can give rise to a wide variety of more “differentiated” cancer cells which comprise the bulk of the tumor and provide the basis for tumor heterogeneity [2]. Based on the CSC theory, any tumor therapy that fails to eradicate CSCs will result in recurrence or regrowth of the residual CSCs, resulting in eventual disease progression [3]. Hence, effective tumor therapy will require eradication of these cells.

The relationship between the development of cervical cancer and persistent infection with certain types of HPV (High risk HPV, hr-HPV) is well established [4]. HPVs are small DNA viruses that infect basal proliferating epithelial cells of either the skin or mucosa, and more than 150 HPV subtypes are known to date. On the basis of epidemiological and biochemical data, at least 14 genotypes ( types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), are designated as high-risk and typified by HPV16 and HPV18, and are associated with cervical cancers [5]. In the HPV infected squamous cell, E6 and E7 viral oncoproteins bind host regulatory proteins leading to degradation of p53 protein and inactivation of retinoblastoma (Rb) protein, two tumor suppressor gene products [6]. It is tempting to speculate that HPV, which primarily infects basal cells in the epithelium, indeed infects epithelial stem cells that are subsequently transformed to become CSCs [7]. This concept is consistent with the highly regulated replication and propagation strategy of these viruses.

The risk of progression to cancer is significantly higher for immunosuppressed patients [8]. Because of the immunological breaks that the HPV virus causes, eradication of infected cells does not occur, potentially leading to development of intraepithelial and invasive lesions [9].

However, cervical cancer is a promising tumor for targeted treatment using vaccine approaches due to the constitutive expression of tumor-specific viral antigens, i.e. HPV E6 and E7 oncogenes. Recently, various forms of HPV vaccines for cervical intraepithelial lesions and invasive cervical cancer have been described in experimental systems with promising results [10].

The identification and characterization of cervical CSCs may have important application in cancer therapy for cervical cancer patients. Therapies targeting cervical CSCs may help overcome the persistent cancer resistance to chemotherapies and radiation therapies. Moreover, cervical CSC-directed immunotherapeutic approaches might represent a promising strategy to stimulate potent antitumor immunity and treat cervical cancer patients. In the following, we will focus on the description of known and potential markers for CSC in cervical cancer and their potential use for immunotherapy.

## 1.1 Characteristics of CSCs

CSCs can be defined as cells in the tumor with a tumor initiating potential [2]. Many of the signaling cascades and interactions with stromal elements that orchestrate physiological stem cell behavior, and consequently normal development, have also been found to play important roles in the initiation and progression of tumors [11] and the proposed properties of CSCs may explain what is commonly known: a person with cancer can generally not be considered cured, even when his or her initial response to radiation or chemotherapy is encouragingly robust. Rare CSCs may be able to survive these therapeutic regimens, thus explaining why local recurrence is the almost-inevitable outcome of seemingly effective treatment of solid tumors by radiation or chemotherapy [12]. Consistent with these findings, some groups have demonstrated that CSCs are indeed more resistant to therapy than their progeny. Cheng and her colleagues showed that the residual breast tumor cell populations that survived after conventional treatment were enriched for the subpopulation of cells with tumor stem cell-like features [13]. CSCs in colorectal cancers are believed to be responsible for resistance to chemotherapeutic drugs [14]. Similarly, chronic myelogenous leukemia reveals the presence of a $CD34^-$ cell population with intrinsic resistance to imatinib [15].

To date, the existence of CSCs has been documented in a number of human cancers, including leukemia, breast cancer, prostate cancer, bladder cancer, lung cancer, head and neck cancer, liver cancer, ovarian cancer, colon carcinoma, malignant melanoma, cervical cancer, pancreatic cancer and

Ewing sarcoma [16-27]. The CSCs in these malignancies have the capacity for self-renewal, the potential to develop into any cell type in the tumors, increased drug resistance, and the proliferative capacity to drive continued expansion of the tumor population. Given these features, it is possible that CSCs may arise from normal stem cells. However, CSCs in primary tumors do not always display the properties classically used to define normal stem cells, cells with the ability to self-renew and -differentiate into multiple cell types [24]. Several lines of evidence suggest CSC could also arise from transformed progenitor cells [28]. However, to become a CSC, a progenitor cell must acquire mutations that cause it to regain the property of self-renewal. This raises the possibility that multiple pathways and processes can give rise to CSCs and contribute to tumor heterogeneity.

## 1.3 Immunologic properties of CSCs

Anti-tumor effects can be mediated early by the innate immune system (i.e., phagocytes, NK cells, NKT cells, cytokines, and complement proteins) and later by the adaptive immune system (i.e., B cells and T cells) [29]. However, increasing evidence suggests that only a restricted minority of malignant cells, namely CSCs, might initiate and sustain neoplastic growth and disease progression by suppressing both the innate and adaptive immune systems by a variety of CSC-secreted products and cell-membrane interactions, which will challenge the immunotherapy targeting of these cells. Recent findings that suggest a negative correlation between degrees of host immunocompetence and rates of cancer development suggest the possibility that CSC may possess the phenotypic and functional characteristics to evade host immuno-surveillance and immune-mediated rejection in immunologically intact individuals [30]. For example, solid organ transplant recipients on immunosuppressive medications and HIV-infected individuals following AIDS onset show a markedly increased risk of developing malignant neoplasms of diverse etiologies [31]. Similarly, in experimental model systems, a minority of undifferentiated cells (1 in $5 \times 10^3$ cells) isolated from leukaemic patients proved to be the only cells capable of reconstituting tumors on transfer into NOD/SCID (non-obese diabetic/severe combined immune-deficient) mice [29]. In addition, $CD34^+CD38^-$ acute myeloid leukemia cells are capable of initiating human AML in NOD/SCID mice [18], but not in the less severely immune-compromised SCID hosts [32]. Taken together with the findings of higher rates of cancer development in immune-compromised patients and animal models, these results lend support to the notion that an intact immune system might be able to control or eliminate the majority of tumors early in their development [33]. At the same time, only those cells that could avoid recognition and elimination would have the capability to progress to the neoplastic state. An immune-selection of CSC populations that would be expected to be more capable of

surviving in an immune-competent host compared to tumor bulk components might be especially relevant in a highly immunogenic cancer.

The immune regulatory mechanisms of CSCs may require engagement of the inhibitory molecule-programmed cell death-1 and secretion or induction of soluble immune-modulatory factors that are required for immunosuppression, such as transforming growth factor-β (TGF-β) and transmembrane glycoprotein, CD200 (OX-2) which have been shown to be important players in immunoregulation, tolerance and cancer prognosis [34-36]. Indeed, Levina et al. [37] have reported that lung CSCs selected by treatment with chemotherapeutic drugs produce higher levels of human angiogenic and growth factors, such as vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-8, and granulocyte colony-stimulating factor (G-CSF), than a parental cell line. Moreover, human CSCs growing in SCID mice stimulated murine stroma to produce elevated levels of angiogenic and growth factors. In addition, Kawasaki et al. [38] found that CSCs derived from breast, colon and brain cell lines showed an increased expression of the glycoprotein CD200.

Recently, macrophages were found to be closely related to the CSC microenvironment. Several studies have reported that tumor-associated macrophages (TAMs) are always found distributed around CSCs, and the number of infiltrating TAMs has been positively correlated with the histological grade of the malignancy and the number of CSCs found [39]. Among immune cells, macrophages constitute one of the major components of immune cell infiltrate observed in the tumor microenvironment (TME) of many types of malignancies [40]. Macrophages can be polarized by their microenvironment to mount specific functional activities relevant to different phases of inflammation [41]. Although various categories of classification have been proposed, macrophages are typically classified into two main groups: classically activated macrophages(M1) and alternatively activated macrophages(M2)[42]. M1 is triggered by T helper 1 (Th1) cytokines, such as interferon-γ, bacterial lipopolysaccharide (LPS), and TNF-α, while M2 is induced by T helper 2 (Th2) cytokines, such as IL-4, IL-13 and macrophage-colony stimulating factor (M-CSF) [43, 44]. Heterogeneity and plasticity are important features of macrophages. Under different stimuli, macrophages can polarize into different phenotypes. For example, during tumor progression, the macrophage phenotype changes from M1 to M2 [45]. In contrast, the macrophage phenotype changes from M2 to M1 in obesity [46]. Wu et al. reported that CSCs in glioma tissue induced macrophage infiltration and polarized the macrophages into an M2 phenotype because the macrophages secreted a large number of cytokines, such as TGF-

β 1, IL-10, and IL-23 [47]. This paper indicated that CSCs play a leading role in macrophage infiltration and polarization. Another article reported that the high expression of CD47 by malignant leukemia stem cells (LSCs) can reduce the macrophage-induced phagocytosis of LSCs and decrease the clearance by the innate immune system [48]. CD47, also known as integrin-associated protein (IAP), can inhibit the phagocytosis of macrophages by binding to signal regulatory protein alpha-chain of inhibitory receptor on macrophages (SIRP α) which is expressed on dendritic cells (DCs), and upon interaction with CD47 on human LSCs, it initiates a signal transduction cascade resulting in inhibition of DC activation [49]. Accordingly, the possibility of CSC-driven tumor escape from immune-mediated rejection has important implications for current cancer immunotherapy and might represent a resistance mechanism susceptible to therapeutic intervention.

## 1.4 Cervical CSC markers

The CSC hypothesis postulates that cells composing a tumor are hierarchically organized with respect to their potential to initiate and sustain tumor growth [50]. If these cancer cell populations, as it seems, exhibit different responses to cancer therapy, it would still be meaningful to identify and purify each population to investigate possible susceptibilities with regard to therapy and to understand their possibly unique biology. Many attempts have therefore been made to identify candidate markers that are either useful for the isolation and identification of cell populations for further investigation or for specific therapies. These markers could be cell-surface markers or molecules involved in specific metabolic or signaling pathways.
To date, no general CSC marker for solid tumors has been identified. Future research is needed to illustrate whether such a marker exists or not. Mounting evidence suggests
that stem cell markers are tumor-specific for the tissue of origin and the niche from where the tumor is growing. Table 1 gives a summary of currently used candidate markers of cervical CSCs and a number of other solid tumors. As it has been emerging that normal stem cells and CSCs share similar phenotypic and functional properties, further identification of more accurate CSC markers that can better distinguish CSC from normal stem cells remains one of the critical challenges facing stem cell studies. Signaling pathways such as Bmi-1 and Wnt have similar effects in normal and CSC self-renewal, which suggests that common molecular pathways regulate both populations [51]. Initially, the $CD24^{-/low}CD44^{+}$ cells were reported to exhibit properties of self-renewal in vitro, form tumors from very few cells, divide slowly, and were selectively resistant to chemotherapy, all of which are hallmarks of CSCs [52]. Afterwards a

number of markers have been proposed to identify and isolate CSCs including CD90, CD34, CD117, CD20, CD133 and aldehyde dehydrogenase 1 (ALDH1) [53, 54]. Currently, a growing body of evidence has been reported supporting the presence of cervical CSCs. For example, significantly elevated expression of Nanog was shown in squamous cervical carcinoma patients compared with dysplasia patients [55]. Similarly, Gong et al. [56] showed that the expression of Bmi-1, which is associated with self-renewal of stem cells, was higher in cervical carcinomas than in normal cervices. In addition, Feng et al. demonstrated that a population of cervical CSC displayed stem cell features [57], but a relatively high number of $CD44^{+}CK17^{+}$ cells ($10^{5}$ cells) were needed to initiate new tumors in immune-deficient mice. Moreover, ALDH1 has been suggested as a surrogate biomarker for CSCs in breast cancer, hepatocellular carcinoma and other tumors [58, 59]. Recent studies from our lab have shown that the spheroid-derived cells generated from a cervical cancer cell line (CaSki), which have been described to be able to enrich for CSC, exhibited higher expression of ALDH1 than the parental monolayer-derived cells [60]. In addition, Chen and his colleagues recently pointed out that in cervical cancer cell lines ( CaSki, HeLa, and SiHa), ALDH1- positive cells were significantly more tumorigenic and showed higher rates of cell proliferation compared to ALDH1-negative cells [61]. The ALDH1-positive cell population has a small overlap with the $CD44^{+}/CD24^{-}/lin^{-}$ phenotype. In breast carcinomas, the overlap represented approximately 1% or less of the total cancer cell population. However, the cells bearing both phenotypes appeared to be highly enriched in tumorigenic capability, being able to generate tumors from as few as 20 cells [62]. It remains to be determined if there is also a small overlap of stem cell markers in cervical cancer, and the exact surface phenotype of cervical CSCs continues to be a subject of debate.

**Table 1: Overview of CSC markers used in cervical cancer and other solid tumors.**

| CSC marker [Ref.] | Origin | Function/physiological role |
|---|---|---|
| CD24 [63] | Pancreas, lung, negative on breast | A cell adhesion molecule expressed at the surface of most B cells and differentiating neuroblasts. |
| CD44 [62] | Breast, cervical cancer, liver, head and neck, pancreas | A cell surface glycoprotein involved in cell − cell interaction, cell migration, and adhesion with multiple isoforms that has pleiotropic roles in signaling and homing. |
| $ALDH1^+$ [64] | head and neck, cervical cancer, breast | A member of the aldehyde dehydrogenase family of enzymes with roles in proliferation, differentiation, and survival. |
| Oct3/4 [65] | cervical cancer, HNSCC, lung, bladder, embryonic stem cells (ES) | A member of the family of POU-domain transcription factors, is expressed in pluripotent embryonic stem and germ cells. Oct3/4 mRNA is normally found in totipotent and pluripotent stem cells of pregastrulation embryos. Knocking out the Oct3/4 gene in mice causes early lethality due to the lack of ICM formation, indicating that Oct4 has a critical function for self-renewal of ES cells |
| Nanog [66] | cervical cancer, ES, many other | The transcription factor Nanog is a key determinant of pluripotency in embryonic stem cell. Nanog exhibits high variability from cell to cell. High levels of Nanog are associated with pluripotency, while low levels are associated with a tendency to differentiate. |
| Sox2 [67] | cervical cancer, melanoma tumor, breast cancer, HNSCC, ES, | A transcription factor essential to maintaining self-renewal properties of undifferentiated embryonic stem cells. Through a cooperative interaction, Sox2 and Oct3/4 have been described to drive pluripotent-specific expression of a number of genes. |
| Bmi-1 [68] | cervical cancer, leukemic, neuroblastoma, oligodendroglioma, prostate cancer | The polycomb gene Bmi-1 is a key regulator in several cellular processes including stem cell self-renewal and cancer cell proliferation. |

## 1.6 Cervical CSC: possible targets for immunotherapy

Many vaccines targeting solid tumors have been employed with varying success, both preclinically and clinically in the treatment of cancer. To induce long-lasting clinical responses by immunotherapy, CSCs need to be targeted [68]. Cytolytic effector cells, including $CD8^+$ T cells and natural killer (NK) cells, are thought to play a role in antitumor defense reactions [69]. Among them, NK cells represent the most efficient effectors against tumors and are considered suitable candidates for adoptive immunotherapy of both hematological and non-hematological malignancies [70]. NK cells have been reported to recognize CSCs. However, these immune cells belong to the innate immune system and do not recognize target cells in an antigen-specific manner. Thus, activation of these cells in vivo may not be more effective than CSC antigen-specific cytotoxic T lymphocytes (CTLs) [71]. CTLs are able to effectively detect and kill CSCs by targeting the specific antigens expressed by the CSCs [72]. Hence, it is important to identify as many antigens as possible on cervical CSCs which could serve as recognition targets for CTLs.

Feng et al. [57] isolated from 8 of 19 cervical cancer-derived cultures stem-like cells capable of self-renewal and extensive proliferation as clonal non-adherent spherical clusters. Sox2 staining was detected in the majority of tumor sphere cells isolated from fresh cervical cancer tissues but not from the differentiated cells. In a recent publication, CTLs raised against a Sox2-derived peptide are able to lyse Sox2-expressing glioma cells, suggesting that Sox2 is a possible target for immunotherapy [73]. Recent studies suggest that the spectrum of ES genes that can induce T cell immunity is not restricted to Sox2, and may be broad. For example, Oct3/4-specific T cells can be readily detected in freshly isolated T cells from most healthy donors [68]. Interestingly, these responses are deficient in patients with newly diagnosed germ-cell tumors (GCT). However, chemotherapy of GCT leads to rapid induction of these responses. Liao et al. reported that the mRNA levels of Oct3/4 were found to be significantly elevated in the CSCs derived from the cervical cancer cell line (CaSki), showing that the ES genes might represent potential targets for immunotherapy of cervical CSCs [60].

The ALDH family is a cytosolic isoenzyme responsible for oxidizing intracellular aldehydes, thus contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation [74]. Increased ALDH1 activity has been found in stem cell populations in human multiple myeloma,

acute myeloid leukemia, brain cancer, and breast cancer, as well as in their normal counterparts, showing correlation with drug resistance and poor prognosis [75-77]. Recently, Visus et al. have reported that the ALDH1A1 isoform can mediate the recognition and lysis of $ALDH1A1^{+}$ squamous cell carcinoma of the head and neck (SCCHN) cell lines by cognate $CD8^{+}$ CTLs [78]. In addition, ALDH1A1-specific CTLs recognize neither normal differentiated cells, nor normal $CD34^{+}$ hematopoietic stem cells, suggesting the potential clinical use of ALDH1A1-specific CTL-based immunotherapy to target CSCs [79]. For cervical cancer, Gong et al. [56] reported that cervical dysplasia showed positive staining for ALDH1 comparable to normal cervical mucosa. ALDH1 expression and distribution parallels the degree of cervical dysplasia. These findings suggested that it might be an early marker for development of cervical cancer and an attractive target for immunoprevention as well as therapy of this disease.

## 1.7 Conclusion

Recently, evidence has been generated for the existence of molecularly defined CSCs in cervical cancer. Furthermore, numerous studies on the links between initiation, recurrence and metastasis of cancers and the presence of CSCs have emerged that underscore the importance of eliminating the CSCs in order to reduce both local and distant recurrence. The discovery of new and unique markers on cervical CSCs as well as CSC pathways will undoubtedly lead to the production of new CSC-targeted therapeutic modalities.

The impressive preclinical data for therapeutic HPV vaccines targeting cervical cancer suggests that immunotherapy may be a promising approach to the treatment of cervical cancer. Nevertheless, most of the immunotherapy research has largely ignored the CSC phenomenon in the context of immune responses. The finding that CSCs have immuno-suppressive functions presents a great challenge to immunotherapy. Multiple treatments with different immunotherapy strategies may provide more benefit to eliminate cervical cancer. Further improvements in understanding of cervical CSC biology, cervical CSC specific immunotherapy combined with other therapeutic strategies may eventually provide new rays of hopes for a curative treatment.

# 2 Aim of the study

The objective of this thesis was to identify cervical cancer-derived stem cells and to investigate their immunogenic and immunosuppressive potential. Therefore, the following aims were pursued:

1. To characterize the expression of ALDH1, CD44 and CD24 by flow cytometry analysis in monolayer-derived cervical cancer cells and their corresponding spheroid-derived cells.

2. To compare the expression of stemness-related transcription factor (Sox2, Nanog and Oct3/4) between monolayer-derived cervical cancer cells and their corresponding spheroid-derived cells

3. To assess the effect of monolayer-derived cervical cancer cells and their corresponding spheroid-derived cells on resting or pre-activated T cell-proliferation, activation, cytokine production, and CTL lytic function

4. To investigate the effects of ALDH1-sorted cervical cancer cells on T cell cytokine production

# 3 Material

## 3.1. Laboratory Equipment

| | |
|---|---|
| Axiovert 40 CFL | Carl Zeiss, Jena, Germany |
| Amaxa Nucleofector | Lonza, Basel, Switzerland |
| BD FACSCalibur System | BD Bioscience, Heidelberg, Germany |
| BioRad Chromo 4 | Bio-Rad, München, Germany |
| Freezer, -80°C | Heraeus, Hanau, Germany |
| Incubator, HERA cell 150 | Heraeus, Hanau, Germany |
| Multicentrifuge | Heraeus, Hanau, Germany |
| Pipettes | Eppendorf AG, Hamburg, Germany |
| Smart Spec$^{TM}$ Plus Spectrophotometer | BioRad, München, Germany |
| Thermocycler | Eppendorf AG, Hamburg, Germany |
| Vortexer | Scientific Industries, N.Y., USA |
| Nanodrop | Peqlab, Erlangen, Germany |

## 3.2 Chemicals, Reagents, Kits, Media, and RT-PCR primers

### Chemicals and Reagents

| | |
|---|---|
| Agarose | Biozym, Oldendorf, Germany |
| BD FACSFlow™ | BD Sciences, Franklin Lakes, USA |
| Bovine Serum Albumin (BSA) | Sigma, Steinheim, Germany |
| Brefeldin A (BFA) | BD Sciences, Franklin Lakes, USA |
| Carboxyfluorescein Succinimidyl Ester (CFSE) | Invitrogen, Carlsbad, CA, USA |
| Dimethyl Sulphoxide (DMSO) | Sigma, Steinheim, Germany |
| Ethanol, 70% | Sigma, Deisenhofen, Germany |
| Epidermal Growth Factor (EGF) | Biochrom, Berlin, Germany |
| Far Red dimethyldodecylamine oxide-succinimidyl ester (Far Red) | Invitrogen, Carlsbad, CA, USA |
| Fetal bovine serum (FBS) | Gibco BRL, Karlsruhe, Germany |
| Fibroblast Growth Factor-basic (bFGF) | Biochrom, Berlin, Germany |
| Ficoll-Paque™ Plus | GE Healthcare, Uppsala, Sweden |
| Interleukin (IL)-2 | ImmunoTools, Friesoythe, Germany |
| Interleukin (IL)-7 | ImmunoTools, Friesoythe, Germany |
| Penicillin/Streptomycin | Biochrom, Berlin, Germany |
| Phosphate-buffered saline (PBS) without $Mg^{2+}/Ca^{2+}$ | Biochrom, Berlin, Germany |
| Dynabeads® CD3/CD28 CTS™ | Invitrogen, Carlsbad, CA, USA |
| Trypsin/EDTA Solution | Biochrom, Berlin, Germany |
| Trizol Reagent | Invitrogen, Carlsbad, CA, USA |

**Cell Culture Media**

| | |
|---|---|
| Dulbecco's modified Eagle's Medium with GlutaMAX™-I (DMEM) | Invitrogen, Heidelberg, Germany |
| Quantum 263 medium | PAA, Cöllbe, Germany |
| RPMI 1640 | Invitrogen, Heidelberg, Germany |

**Kits and other Materials**

| | |
|---|---|
| BD Falcon™ Cell Culture Flasks | BD Bioscience, Franklin Lakes, USA |
| BD Falcon™ Polypropylene Conical Tubes (15 ml, 50 ml) | BD Bioscience, Franklin Lakes, USA |
| BD Falcon™ Polystyrene Round-Bottom Tubes (5 ml) | BD Bioscience, Franklin Lakes, USA |
| BD Falcon™ Tissue Culture Dish (100*20 mm) | BD Bioscience, Franklin Lakes, USA |
| Cell Culture Plates (48-well, 96-well) | BD Bioscience, Franklin Lakes, USA |
| Cluster Tubes, Polypropylene (1,2 ml) | Corning, NY, USA |
| Ultra-Low Attachment Cell Culture Plate (24 well) | Corning, NY, USA |
| HTS Transwell-24 well Permeable Supports | Corning, NY, USA |
| Aldefluor assay Kit | StemCell Technologies, NC, USA |
| Cytofix Fixation/Permeabilization Kit | BD Bioscience, San Diego, CA |
| RNeasy Mini kit | QIAGEN, Hilden, Germany |
| High Capacity RNA-to-cDNA Kit | Applied Biosystems, Foster City, CA, USA |
| Power SYBR Green Mix | Applied Biosystems, Foster City, CA, USA |

**Table 2: RT-PCR-Primers (5´⟶ 3´)**

| Transcript name | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| **Nanog** | AATACCTCAGCCTCCAGCAGATG | TGCGTCACACCATTGCTATTCTTC |
| **Oct3/4** | GACAGGGGGAGGGGAGGAGCTAGG | CTTCCCTCCAACCAGTTGCCCCAAAC |
| **Sox2** | GGGAAATGGGAGGGGTGCAAAAGAGG | TTGCGTGAGTGTGGATGGGATTGGTG |
| **GAPDH [81]** | AGCTCCCAAAAATAGACGCAC | TTCATAGCAGTAGGCACAAAGG |

## 3.3 Cell Lines and Culture Media

| | |
|---|---|
| MRIH215 (HPV45+) | DMEM or RPMI 1640 supplemented with 10% FBS, and 1% penicillin and streptomycin |
| HeLa (HPV18+) | |
| CaSki (HPV16+) | Quantum 263 medium supplemented with 10 ng/ml EGF and 10 ng/ml bFGF |

## 3.4 Antibodies and Fluorochromes

| | |
|---|---|
| 7AAD | BD Pharmingen, CA, USA |
| FITC mouse anti-human CD8 | ImmunoTools, Friesoythe, Germany |
| FITC mouse anti-human IFN-γ | BD Pharmingen, CA, USA |
| PE mouse anti-human TNF-α | BD Pharmingen, CA, USA |
| PE mouse anti-human IL-2 | BD Pharmingen, CA, USA |
| PE mouse anti-human CD24 | BD Pharmingen, CA, USA |
| PE mouse anti-human CD137 | MACS Miltenyi Biotec, Bergisch Gladbach, Germany |
| PerCP mouse anti-human CD69 | BD Pharmingen, CA, USA |
| PerCP mouse anti-human CD4 | BD Pharmingen, CA, USA |
| APC mouse anti-human CD154 | BD Pharmingen, CA, USA |
| APC mouse anti-human CD44 | BD Pharmingen, CA, USA |
| APC mouse anti-human TNF-α | BD Pharmingen, CA, USA |
| CMV Dextramer assay kit | Immundex, Copenhagen, Denmark |

# 4 Methods

## 4.1 Cell lines and cell culture

The following cervical cancer cell lines were obtained from the American Type Culture Collection (ATCC): HeLa (HPV18-positive), CaSki (HPV16-positive) and MRI-H215 (HPV 45-positive). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) or RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (FCS, Biochrom, Berlin, Germany)(heat-inactivated at 56°C for 30 min) at 37°C, 5% $CO_2$, and 95% air atmosphere. All of our experiments were performed on cultures that were 70% confluent.

## 4.2 Human peripheral blood mononuclear cells (PBMC) preparation

PBMCs were isolated from blood of healthy donors by Ficoll-Hypaque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and cultured in serum-free Quantum 263 medium (PAA), supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom), interleukin (IL)-2 and IL-7 (10 IU/ml; ImmunoTools, Friesoythe, Germany), and 1% penicillin and streptomycin.

## 4.3 Spheroid cell formation assay

Adherent monolayer cells were grown in normal 75 $cm^2$ culture flasks (BD Biosciences, Franklin Lakes, USA) in DMEM or RPMI 1640 containing 10% heat-inactivated FBS and 1% penicillin/streptomycin, until 70% confluency. Cells were washed with PBS without $Ca^{2+}$/$Mg^{2+}$ twice and detached using Trypsin/EDTA (Biochrom). The reaction was stopped by adding complete culture medium after 5 min digestion or when the cells detached. The cell suspension was transferred to 15 ml tubes. After centrifugation (200xg, 5 min) cells were washed twice with PBS without $Mg^{2+}$/$Ca^{2+}$. Cells were resuspended in Quantum 263 medium (PAA)

supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (Biochrom). To generate spheroids, single cells were plated in Corning* Ultra-Low Attachment plates (Corning, New York, USA) at a specific density of $2\times10^4$ cells/ml. Cells were kept in the incubator at 37°C in a humidified atmosphere with 5% $CO_2$. Every 3-4 days, half of the medium was replaced. The medium was aspirated slowly and filled into tubes with conical bottom. Cell suspensions were left for 10 min to sediment and the supernatant was carefully removed leaving behind the spheroids. The same volume of fresh medium was added and the spheroids were carefully resuspended. This suspension was put back into the plates for further culturing.

To passage the spheroids into next generations, a 40 µm mesh filter was used for collecting the cells. The cells were centrifuged at 1500 rpm for 5 min, 2 ml TE was added and resuspended. After incubation at 37°C at 5% $CO_2$ for 5 min, the cells were washed with PBS without $Mg^{2+}$/$Ca^{2+}$ twice before resuspending them in fresh culture medium. The cell culture was continued in Ultra-Low Attachment cell culture plates at a specific density of $2\times10^4$ cells/ml and kept in the incubator at 37°C in humidified atmosphere with 5% $CO_2$. For the experiments, 2nd and 3rd generation spheroids were used.

The morphology of spheroids was assessed and recorded using a HBO50 Microscope and a 5 × objective. Pictures were taken with an AxioCam MRC Zeiss Camera using the AxioVision Rel.4.8 Software.

## 4.4 Aldefluor analysis

The ALDH activity of spheroid- and monolayer-derived cells was determined by using the Aldefluor assay kit (StemCell Technologies, Durham, NC, USA; as shown in Fig. 1). Spheroids were collected using a 40 µm mesh and disaggregated into single cells by Trypsin/EDTA digestion for 3 min followed by 20 times up- and down pipetting using a 1000 µl pipette tip. Then the single-cell suspension was washed

twice in PBS without $Ca^{2+}$/$Mg^{2+}$, suspended in 1 ml ALDEFLUOR assay buffer containing 5 µl ALDH substrate (BAAA, 1 µmol/ml per $1\times10^6$ cells) and incubated for 40 min at 37°C in the dark. As a negative control, for each sample, an aliquot was treated with 5 µl diethylaminobenzaldehyde (DEAB; 50 mmol/l), a specific ALDH inhibitor. After additional staining and washing twice, cells were maintained in ALDH buffer on ice during all subsequent procedures.

Next, for cell surface antigen phenotyping, cells were resuspended in 100 µl Aldefluor incubation buffer and stained with 20 µl anti-CD24-PE, 20 µl anti-CD44-APC (BD bioscience) and 5 µl 7-AAD (BD bioscience) per $10^6$ cells. The cells were then incubated at 4°C for 15 min in the dark. Following incubation, cells were washed once with cold FACS buffer.

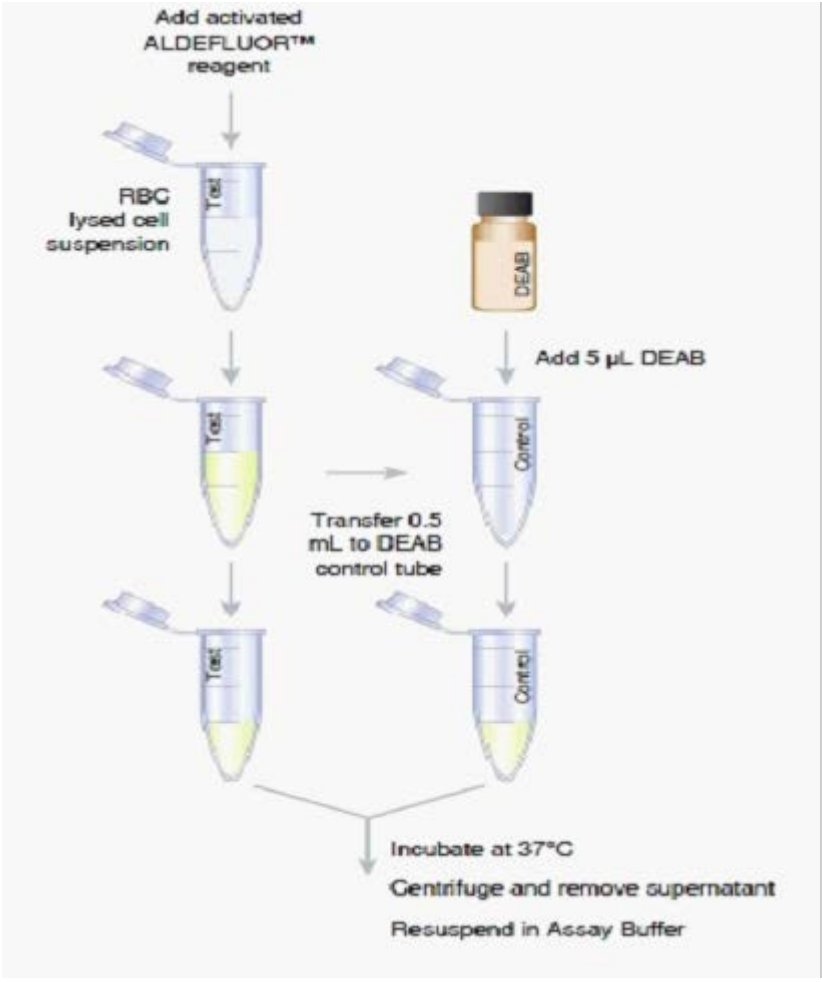



Figure 1. ALDEFLUOR™ assay

## 4.5 Co-culture of PBL with tumor cells

For transwell assays, spheroid- or monolayer-derived cells were seeded in the lower chamber and PBMCs in the upper chamber of a 24 well Transwell plate (Corning, NY) at a 1:5 ratio (cancer cell: PBMC). Thus, cell populations were physically separated by a semi-permeable membrane (0.4 μm pore size). In some experiments, PBMC proliferation was stimulated using the anti-CD3/CD28 dynabeads (Invitrogen). Stimulated PBMCs alone served as positive controls. Unstimulated PBMCs co-cultured with spheroid- or monolayer-derived cells served as negative controls for proliferation assays. PBMCs were co-cultured with spheroid- or monolayer-derived cells for 6 days and then subjected to further flow cytometry analysis.

## 4.6 CFSE-dilution proliferation assay

PBMCs were suspended in phosphate-buffered saline (PBS; $1 \times 10^6$/ml) and labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Grand Island, NY) for 7 minutes at 37°C. After labeling, the cells were washed three times in an equal volume of PBS and resuspended in serum-free Quantum 263 medium containing 10 ng/ml EGF, 10 ng/ml bFGF, IL-2 and IL-7 (10 IU/ml). PBMC proliferation was induced on day 0 by stimulating cells with anti-CD3/anti-CD28 T cell expander Dynabeads® (Invitrogen) at a bead:cell ratio of 2:1. The stained PBMCs were indirectly co-cultured with spheroid- or monolayer-derived cells as mentioned above. Proliferation of PBMCs was measured on day 6 by flow cytometry on the basis of CFSE dilution.

## 4.7 Intracellular cytokine analysis

To determine the function of $CD4^+$ T cells, stimulated PBMCs were stained for IL-2, TNF-α, and IFN-γ production. On co-culture day 5, 2 μl Brefeldin A (BFA; 10 μg/ml

final concentration) was added directly into the upper well and incubated at 37°C for 20 hours. Then PBMCs were harvested, fixed and permeabilized using the Cytofix Fixation/Permeabilization Kit as described in the manufacturer´s protocol. In brief, the harvested PBMCs were resuspended in 100 µl fixation/permeabilization solution (in the Kit) for 20 min at 4°C. After two washing steps in 250 µl 1:10 diluted Perm/Wash buffer (in the Kit), cells were stained 30 min on ice with PE-conjugated anti-human CD4, PerCP-conjugated anti-human IL-2, APC-conjugated anti-human TNF-α and FITC-conjugated anti-human IFN-γ antibodies. Stained cells were washed twice with 250 µl 1:10 diluted Perm/Wash buffer and then resuspended in 50 µl FACS buffer for flow cytometric analysis.

## 4.8 Flow-cytometric (FACS) analysis and sorting

Monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (Percp), or allophycocyanin (APC) against human CD4, CD8, CD69, CD137, CD154 (all BD Pharmingen, CA, USA), were used to characterize PBMCs. Briefly, $5\times10^4$ cells in 50 µl FACS buffer were incubated with mAbs according to the manufacturer´s instructions. Samples were stored at 4°C in the dark for 30 min. Then the stained cells were washed twice with FACS buffer and then resuspended in 100 µl FACS buffer for flow cytometric analysis, which was performed using a FACS Calibur (BD Bioscience) and analyzed using BD CellQuest Pro (BD Biosciences). Percentage of positive cells was determined, and surface density of selected markers was expressed as mean ratio fluorescence intensity, which represents the ratio between mean fluorescence intensity of cells stained with the selected mAb and that of unstained cells (negative control).

For FACS sorting, cells were resuspended in PBS buffer at $1\times10^7$ cells per ml and separated on an Aria cell sorter (BD Biosciences). The sorted cells were analyzed for expression of ALDH1 by FACS after 24 hours and after six days. The sorting gates were established, using as negative controls the cells treated with DEAB.

## 4.9 MHC-Dextramer analysis

The above co-cultured PBMCs ($1 \times 10^4$) from HLA-$A2^+$ donors were stained with 10 μl dextramer complexes of CMVpp65-derived peptide NLVPMVATV/HLA-A*0201-APC for 10 min in the dark at room temperature according to the manufacturer´s instructions (Immundex, Copenhagen, Denmark). All samples were then incubated with FITC-conjugated anti-CD8 mAb for 20 min at 4°C in the dark. The stained cells were washed twice with FACS buffer and then resuspended in 100 μl FACS buffer for flow cytometric analysis.

## 4.10 Generation of CD8+ CMV-specific CTLs

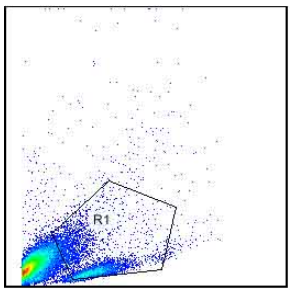
Freshly isolated PBMCs from 3 HLA-A2+ healthy donors were pulsed with 10 μg/ml CMV pp65 NLVPMVATV peptide for 24 hours at 37°C in RPMI 1640 medium supplemented with 10% FBS, IL-2 and IL-7 (10 IU/ml), and 1% Penicillin and Streptomycin at a cell concentration of $2.5 \times 10^6$ per ml. IL-2 and IL-7 (10 IU/ml) were added every 2–3 days. At 7-day intervals, cells were restimulated with peptide. An autologous BLCL was generated from each HLA-$A2^+$ healthy donors by infection of PBMC with supernatant from the Epstein- Barr virus–producing cell line B95-8 (American Type Culture Collection, Rockville, MD) + 1 ng/ml cyclosporin A. BLCL were maintained in RPMI 1640 medium containing 10% heat-inactivated bovine serum and supplemented with antibiotics.

## 4.11 CD8+ CMV-specific CTL Cytotoxicity Assay

The $CD8^+$ CMV-specific CTL cytotoxicity was assessed by VITAL-FR assay, a versatile fluorometric technique for assessing CTL-mediated cytotoxicity against multiple targets in vitro [63]. As target cells, Epstein-Barr virus-transformed B-cell lines (BLCL) from corresponding HLA-$A2^+$ healthy donors ($1 \times 10^6$) were incubated

with 10 μM CFSE or 5 μM Far Red for 5 min in Quantum 263 medium at 37°C. The reaction was terminated by addition of 20% FBS and the cells were thoroughly washed with standard culture medium. Cells stained with CFSE were incubated with 10 μg/ml of CMV pp65 $_{495–503}$ in Quantum 263 supplemented with 3% FBS for 5 days and thoroughly washed before being used as specific target cells. Effector T cells were titrated in 96-well V-bottom plates and $1\times10^3$ CFSE-labeled specific peptide-loaded and $1\times10^3$ Far Red-labeled control peptide-loaded target cells were added. Wells containing the target cells only served as a control. Final volumes were 200 μl of standard culture medium supplemented with 10 IU/ml IL-2. Cultures were incubated at 37°C and resuspended by pipetting once every 24 h. After up to 72 h, all cells were collected and immediately assessed by FACS. The entire target cell population was defined by a live gate in a forward scatter/side scatter dot plot. Specific target cells were denoted by regions in Fl-1 (CFSE)/Fl-4 (Far Red) dot plots and detected and enumerated as specific target cells as $CFSE^+$ (R3) and control target cells as $Far\ Red^+$ (R2) as shown in Fig. 2. Non-fluorochrome-labeled cells comprised the effector cell populations. Peptide-specific lysis was calculated from the ratio R3/R2 in cultures containing defined numbers (n) of effector T cells (R3/R2)n in comparison to control (co) wells without T cells (R3/R2)co using the formula:
$100\%-[(R3/R2)_n/(R3/R2)_{co}]\times100\%$.

**A**




B

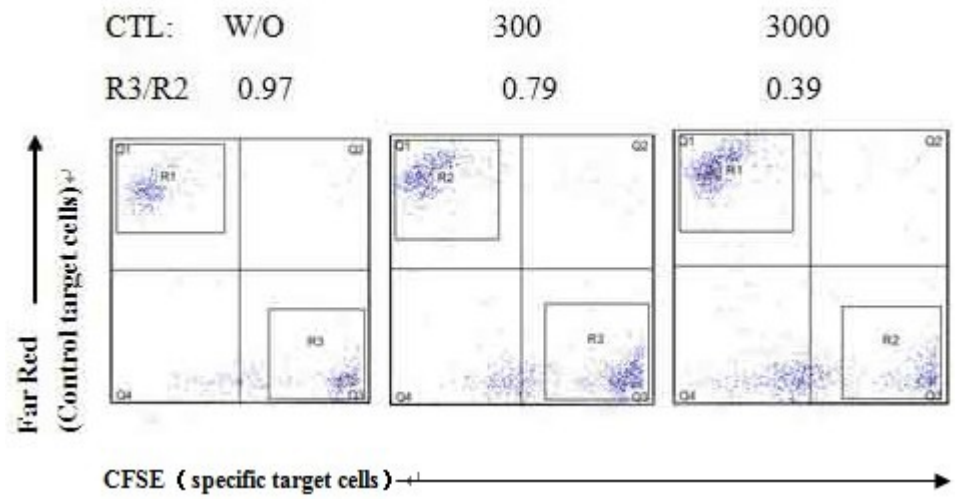



**Fig. 2: Flow cytometric analysis of target cell lysis by VITAL-FR assay.** Specific target cells were stained with CFSE and control target cells were labeled with Far Red. Mixtures of $10^3$ specific and $10^3$ control target cells were incubated either alone or in the presence of CMV specific CTL. After 72 hours the lysis was investigated by flow cytometry. (A) Cells were gated by FSC/SSC to generate G1 in R1 (B) Ratios of $CFSE^+$ (R4) and Far $Red^+$ (R3) labeled target cell numbers were directly determined and their relative amount defined the lysis within individual cultures. CMV-specific CTL-mediated target cell lysis was calculated in comparison to control cultures without CTL.

## 4.12 RNA extraction

Total RNA was extracted using TRIzol Reagent following the protocol below.

## a. Cell homogenization

**For monolayer:** Growth media was removed from culture dish; 1 ml Trizol reagent was added directly to the cells in the culture dish per 10 $cm^2$ of culture dish surface area; the cells were lysed directly in the culture dish by pipetting up and down several times. The lysed cells were transferred into a new Eppendorf tube.

**For spheroids:** The cells were harvested by 40 µm mesh. Then the cells were centrifuged at 1500 rpm for 5 min. Two ml TE was added to the cell pellet and the cells were resuspended. The cells were incubated at 37°C with 5% $CO_2$. Then the cells were washed with PBS without $Mg^{2+}$/$Ca^{2+}$ twice. After counting the cells, they were centrifuged at 1500 rpm for 5 min. The supernatant was removed and 1 ml per $10^6$ cells Trizol was added. The cells were lysed by pipetting up and down several times before being transferred into an Eppendorf tube.

## b. RNA isolation by Trizol

Chloroform (0.2 ml per 1 ml of Trizol reagent) was added. The tube was capped securely and shaken vigorously by hand for 15 sec. Then the tube was incubated on ice for 15 min. The tube was centrifuged at 12000xg for 15 min at 4°C. The aqueous phase of the sample was removed by angling the tube at 45° and pipetting the solution out. Intaking of any interphase or organic layer into the pipette when removing the aqueous phase was carefully avoided. The aqueous phase was placed into a new tube.
Per 1 ml used for homogenization, 0.5 ml of 100% isopropanol was added into the new tube with aqueous phase. The tube was incubated on ice for 10 min and then centrifuged at 12000xg for 10 min at 4°C.The supernatant from the tube was removed, leaving only the RNA pellet. The pellet was washed with 1 ml 75% ethanol per 1 ml of Trizol Reagent used in the initial homonization. The sample in the tube was

vortexed briefly then centrifuged at 7500xg for 5 min at 4°C. The supernatant was discarded. The RNA pellet was air dried for 5-10 min.

## 4.13 Reverse transcription and quantitative real-time PCR

Total RNA (1 µg) was converted to cDNA by RT-PCR using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time PCR was performed by the ABI Power SYBR Green mix and run on a BioRad Chromo 4 (Bio-Rad). PCR conditions were as follows: 95°C for 15 min, 40 cycles of 95°C for 2 min, 95°C for 15 sec and 72 °C for 1 min. Reactions were carried out in triplicate with RT controls, GAPDH was used as a reference gene, and data were analyzed using the modified delta delta Ct method.

## 4.14 Statistical Analysis

For statistical comparison, we used the SPSS software for Windows (version 15; SPSS, Chicago, IL, USA). Students t-Test was used to analyze statistical significance of differences in the data.

# 5 Results

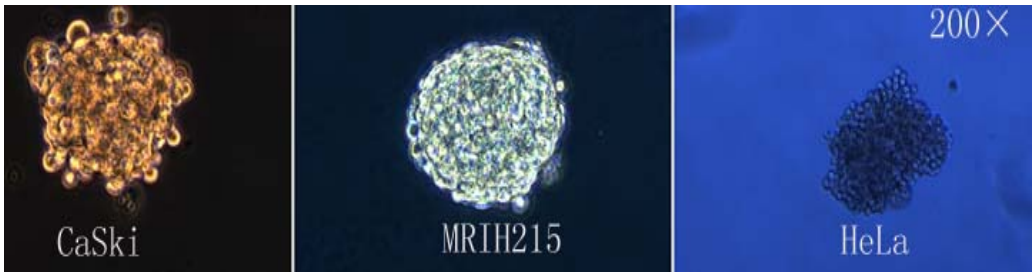
## 5.1 Sphere Formation and Phenotypic Characterization of MDC and SDC expanded from Cervical Cancer Cell Lines.

The three human cervical cancer cell lines CaSki, HeLa and MRIH215 were grown in suspension at low density in defined Quantum medium with bFGF and EGF for 7-10 days. They showed different ability of spheroid formation. CaSki and MRIH215 formed spheroids which were highly compact. HeLa formed only loose aggregates of cells. The cell-cell contacts established by these cultures were weak, and the aggregates could easily be dispersed mechanically by pipetting (Fig. 3A). Recently, the ALDEFLUOR assay has been successfully applied to detect $ALDH^{br}$ in CSCs from non-hematopoietic tumors [80]. In the present study, we measured ALDH1 enzymatic activity of the SDC of the three cervical cancer cell lines and their matched MDC to investigate the presence of a stem cell-like population (Fig. 3B). As control, cells incubated with ALDEFLUOR substrate (BAAA) together with the specific ALDH inhibitor (DEAB) were used to establish the baseline fluorescence and to define the ALDH1 positive population. As shown in Fig. 3B, the SDC from CaSki [(MDC: 10.80 ± 0.265%, SDC: 26.10 ± 1.049%) ($P<0.05$)] and HeLa [(MDC: 0.29 ± 0.212%, SDC: 4.19 ± 0.059%) ($P<0.05$)] had a significantly increased frequency of ALDH1 expressing cells as compared with their parental MDC. However, the SDC of the cervical cancer cell line MRIH215 which still showed a high capacity in spheroid formation contained less $ALDH1^{+}$ cells than its MDC [(MDC: 65.80± 1.348%, SDC: 45.08 ± 0.511%) ($P<0.05$)] (Fig. 3C).

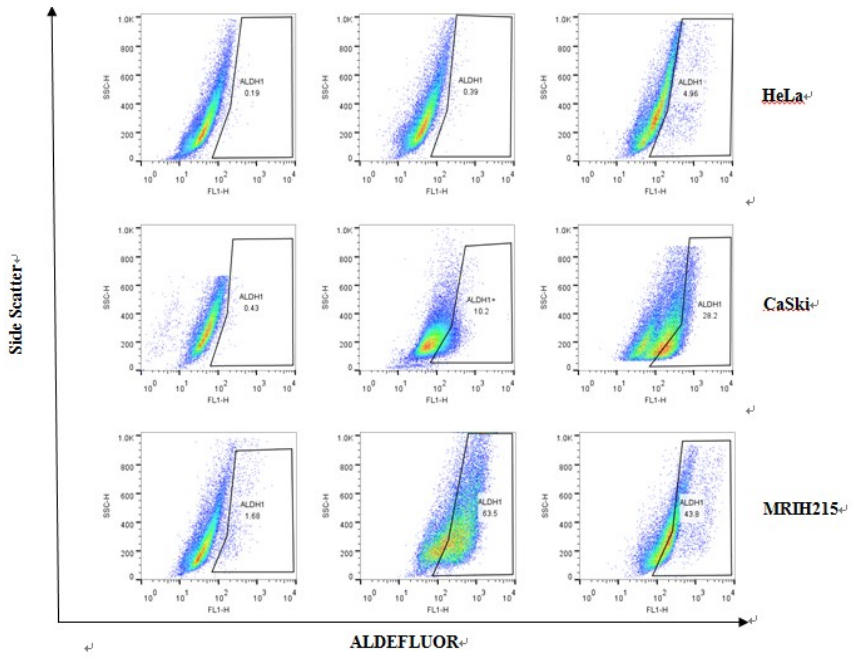
Since the combined use of different putative CSC markers may provide a more precise definition of stem cell-like populations and Ponti et al. reported that the combination of CD44/CD24 and ALDH1 was the most accurate method to identify CSC from breast cancer populations [6], we attempted to further characterize the

presence of a stem cell-like population by using this combination of markers. In our experiment, MDC and SDC of the MRIH215 cell line showed the highest expression of ALDH1$^{+}$/CD44$^{+}$/CD24$^{-}$cells (MDC: 8.60 ± 0.488%, SDC: 17.77 ± 0.346%) (P<0.05) as compared with HeLa (MDC: 0.14 ± 0.064%, SDC: 3.98 ± 0.068%) (P<0.05) and CaSki (MDC: 3.90 ± 0.173%, SDC: 6.85 ± 0.464%) (P<0.05). The data showed that the proportion of ALDH1+/CD44+/CD24－ cells was consistently enriched in SDC from all 3 cell lines (Fig. 3D).

**A**




**B**



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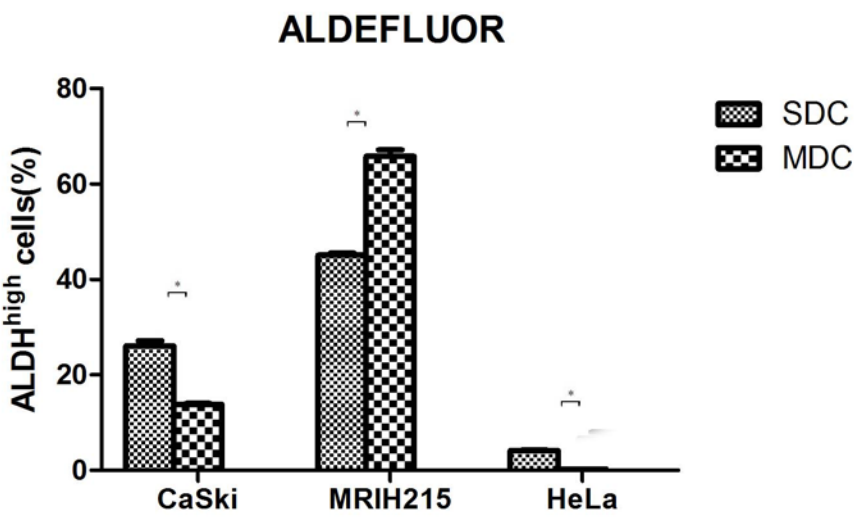



**D.**

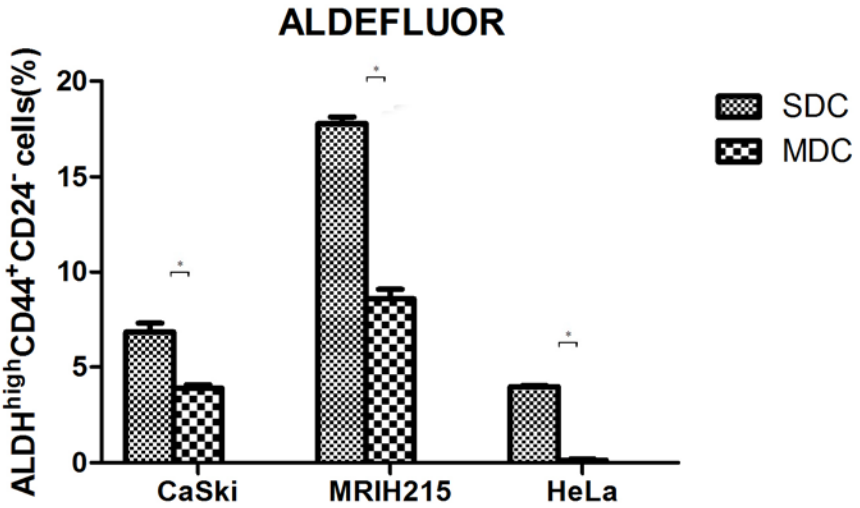



**Fig. 3: Examples of SDC from 3 cervical cancer cell lines in serum-free medium and comparison of ALDH1 and ALDH1$^+$/CD44$^+$/CD24$^-$ expression in MDC and SDC by flow cytometry.** (A) Example of SDC formed by CaSki, HeLa，and MRIH215 in suspension cultures in defined serum-free medium

supplemented with bFGF and EGF after 7-10 days in culture (magnification ×200). (B) An example of flow cytometric analysis of the expression of ALDH1 in SDC compared to MDC, and control cells treated with DEAB, a specific inhibitor of ALDH1. (C) Mean percent of $ALDH1^+$ cells in SDC and MDC of the 3 cell lines. (D) Mean percent of $ALDH1^+/CD44^+/CD24^-$ cells in SDC and MDC of the 3 cell lines.

## 5.2 Stemness Marker Gene Expression by SDC and MDC

It was reported that Sox2, Oct3/4, and Nanog, which form a self-organized core of transcription factors (TF), maintain pluripotency and self-renewal capacity of human embryonic stem cells [81, 82]. To investigate if SDCs also share this feature of TF expression with embryonic stem cells, we quantitatively compared the mRNA expression of these TF between SDC and parental monolayer-derived cells (Fig. 4). The mRNA levels of Sox2, Nanog, and Oct3/4 were all found to be increased in the SDC of all 3 cell lines. The highest increase was observed in HeLa SDC, where a 81.63-fold increase in Sox2 expression was found as compared to MDC. By comparison, the smallest change, a 1.58-fold increase in Oct3/4 expression, was seen in MRIH215 SDC.

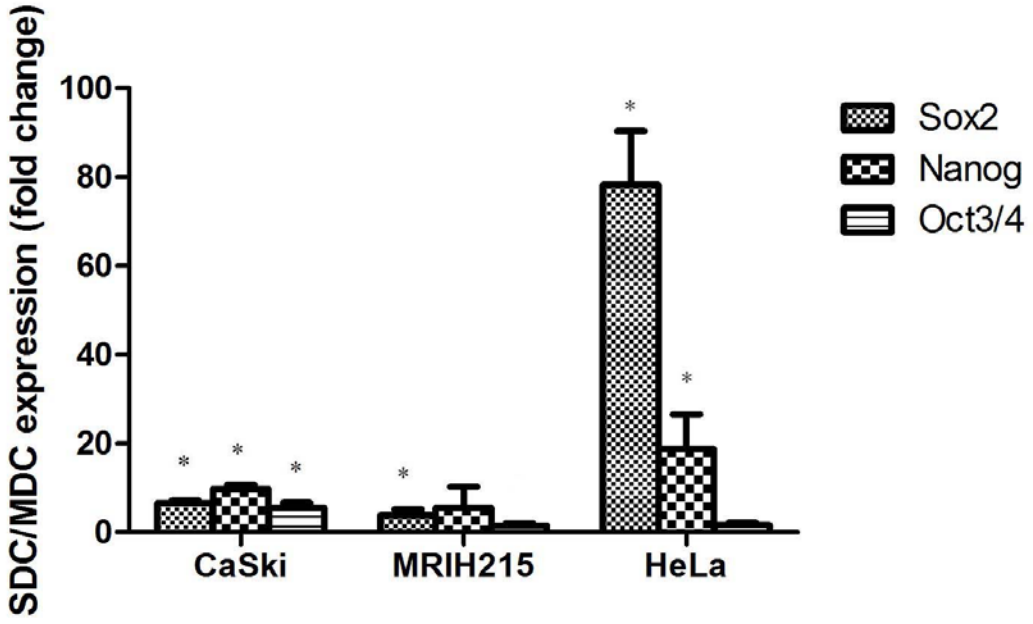


**Fig. 4: Quantitative PCR analysis of mRNA expression of stemness-related transcription factors (TF).** Messenger RNA isolated from SDC and MDC was quantified for expression of the indicated TF. The ratio of expression in SDC to MDC is shown. The mRNA level of Sox2, Nanog, and Oct3/4 was increased remarkably in SDC from all 3 cell lines. Mean values ± SD of three determinations. Significant differences are * $P < 0.05$.

## 5.3 T cell Proliferation in serum-free Quantum 263 medium

To examine whether T cells can be cultured in Quantum 263 medium, CFSE-labeled T cells were stimulated with anti-CD3/anti-CD28 and then allowed to expand in plates for 6 days. The proliferation rate of the T cells was observed every two days by FACS. RPMI 1640 supplemented with 10% FCS was used as control medium. As shown in Fig. 5, T cells cultured in Quantum 263 medium have a proliferation efficacy similar to that of T cells grown in RPMI 1640 supplemented with 10% FCS.

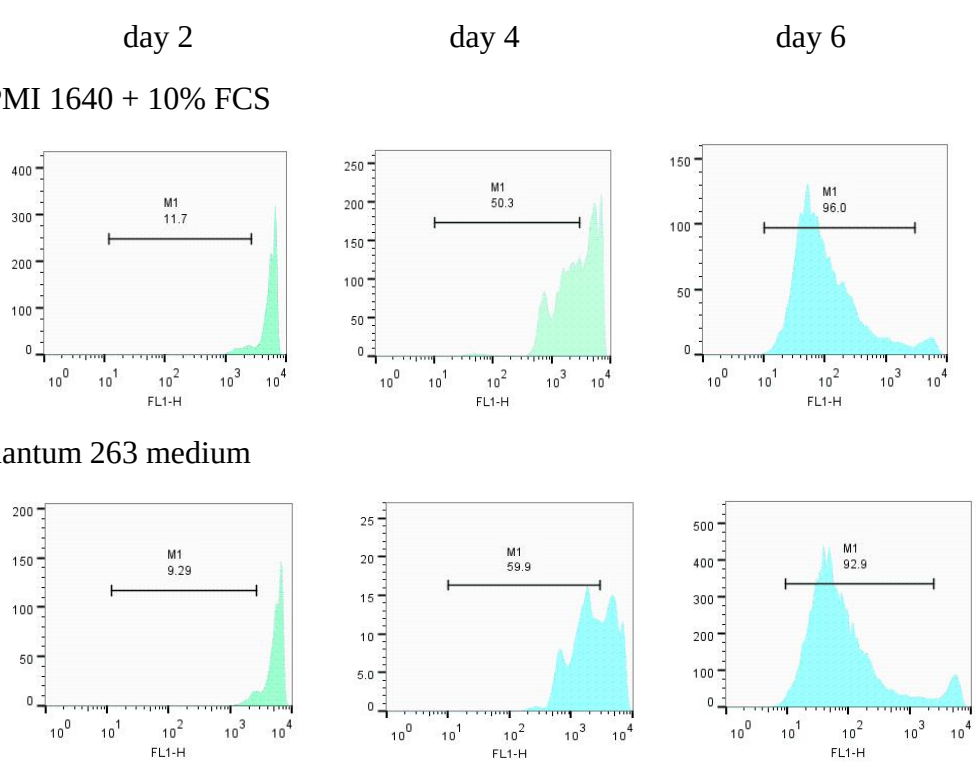


**Fig 5: Comparison of T cell proliferation in different media conditions.** The proliferation of T cells in Quantum 263 medium or in RPMI 1640 supplemented with 10% FCS was compared on day 2, day 4 and day 6.

## 5.4 Inhibition of T cell Proliferation

CSCs have been described to have a lower susceptibility for immunologic recognition. We therefore initially investigated their influence on T cell proliferation in a CFSE dilution experiment. The SDC and MDC were co-cultured with CFSE-labeled T cells in a 1:5 ratio. SDC and MDC were plated into the lower transwell chamber (2 x $10^4$cells/well), T cells ($10^5$ cells) were added to the inner chamber. After 6 days of co-culture with anti-CD3/anti-CD28 stimulus, proliferation of T cells was measured using flow cytometry. In co-cultures, both SDC and MDC from all 3 cell lines inhibited the proliferation of anti-CD3/anti-CD28-stimulated T cells from three independent donors (Fig. 6A). Moreover, SDC showed significantly higher capacity to suppress stimulated T cells than MDC ($P<0.05$).

To investigate whether SDC or MDC could suppress pre-activated T cell proliferation, SDC and MDC were added in a 1:5 ratio to 4 day-old cultures of T cells pre-stimulated with anti-CD3/anti-CD28 in the transwell insert system. As shown in Figure 6B, the proliferation of pre-activated T cells from three donors was significantly inhibited by the subsequent addition of SDC and MDC from all 3 cell lines. In addition, co-culture with SDC had a significantly higher suppressive potential on activated T cells than MDC.

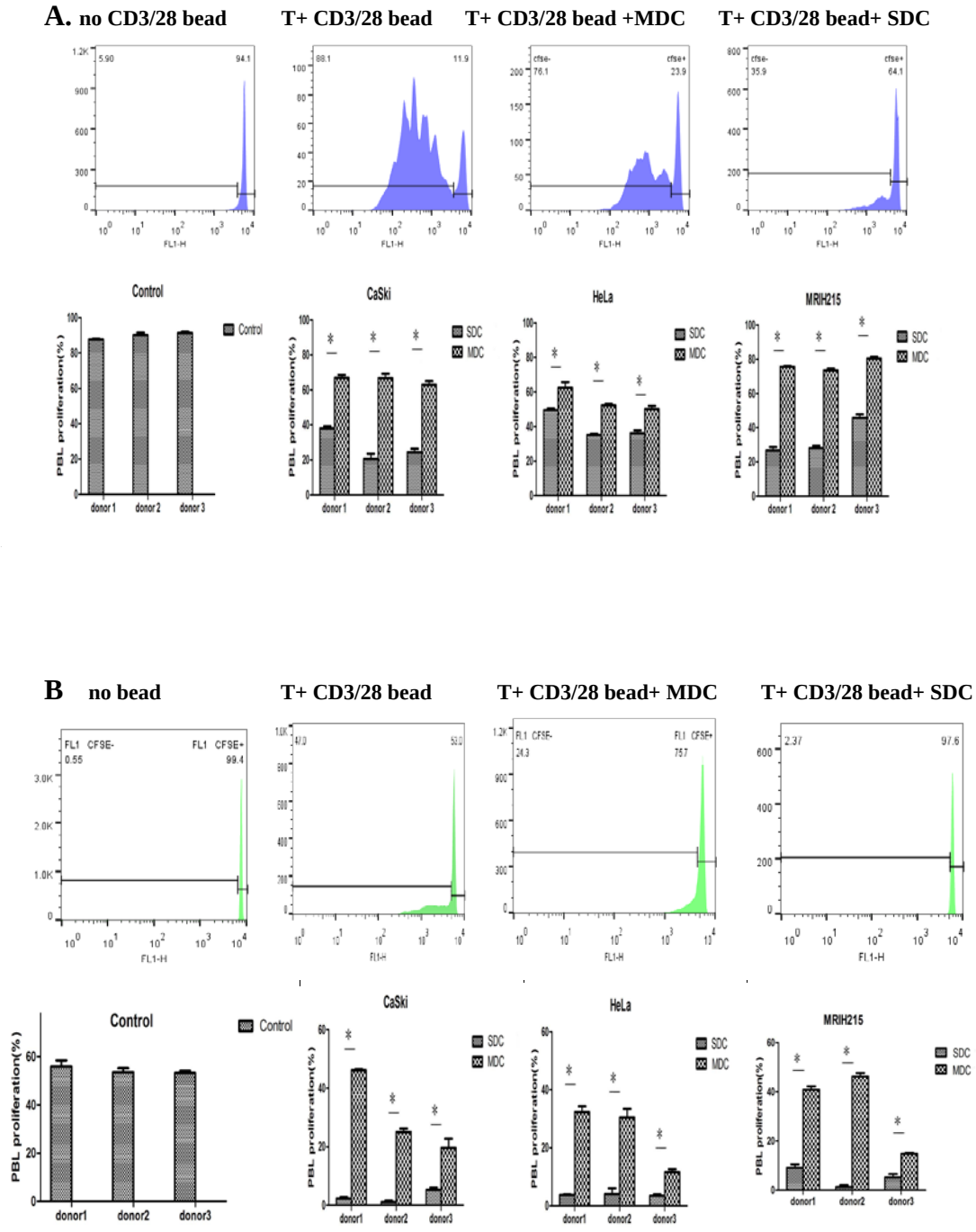


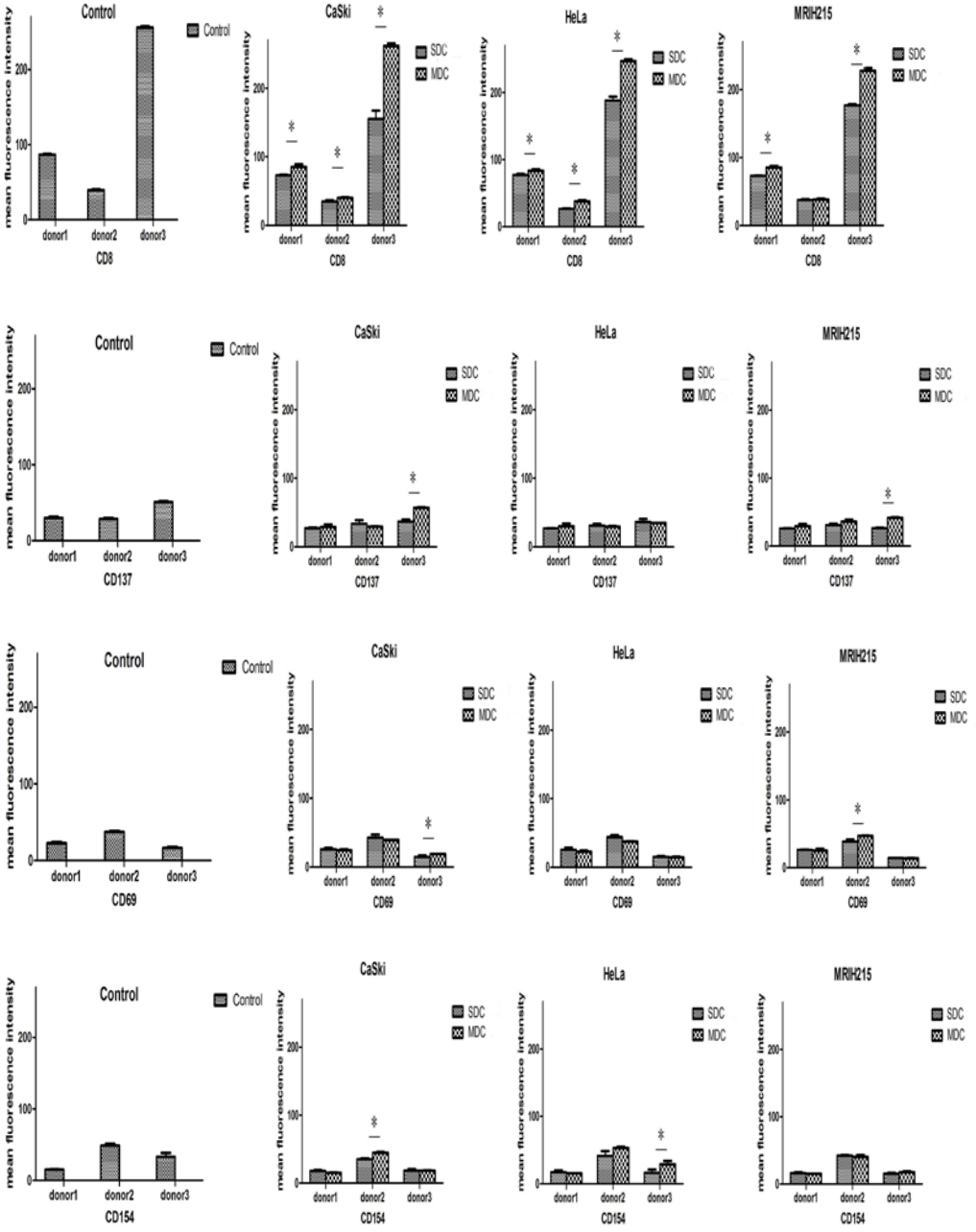

**Fig 6: SDC and MDC inhibit the proliferation of T cells.** (A) T cells from 3 donors were stained with CFSE and stimulated with anti-CD3/CD28 coated Dynal beads in the presence of SDC or MDC. SDC and MDC were plated into the lower transwell chamber (2 x $10^4$cells/well), T cells ($10^5$ cells) were added to the inner chamber. After 6 days of co-culture, T cell proliferation was assessed by CFSE dilution. FACS plots

are one representative of 3 experiments with different blood donors and identical design. Bar graphs show mean data of triplicate in all 3 cell lines tested with PBL of 3 independent donors. T cells stimulated with and without anti-CD3/CD28 bead in the absence of cancer cells for 6 days were used as positive and control, respectively. (B) Same as in (A) set, T cells were pre-activated for 4 days, then co-cultured with SDC or MDC in a transwell assay for another 6 days. The proliferation of T cells was measured on day 10. Statistical significance: *P < 0.05.

## 5.5 Inhibition of T-cell Activation

To identify the effect of SDC and MDC on functional activation of T cells, the expression of surface activation markers CD8, CD69, CD154, and CD137 were investigated. The SDC and MDC were co-cultured with T cells in the presence or in the absence of anti-CD3/anti-CD28 for six days. Among unstimulated T cells, no significant difference was seen between the MFI of activation-antigen CD69, CD154, and CD137 on T cell surfaces co-cultured with MDC and those with SDC in most experiments. However, the CD8 expression of resting T cells co-cultured with MDC was significantly higher than those with SDC (Table 2). When T cells were stimulated with anti-CD3/anti-CD28, the MFI of CD69, CD154, CD137, and CD8 in T cells from most donors co-cultured with MDC was significantly higher than from those cultured with SDC (Table 3).

**A**


Control
CaSki
HeLa
MRIH215
Control
SDC
MDC
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0
100
200
donor1
donor2
donor3
CD8
CD137
CD69
CD154
*

**B**

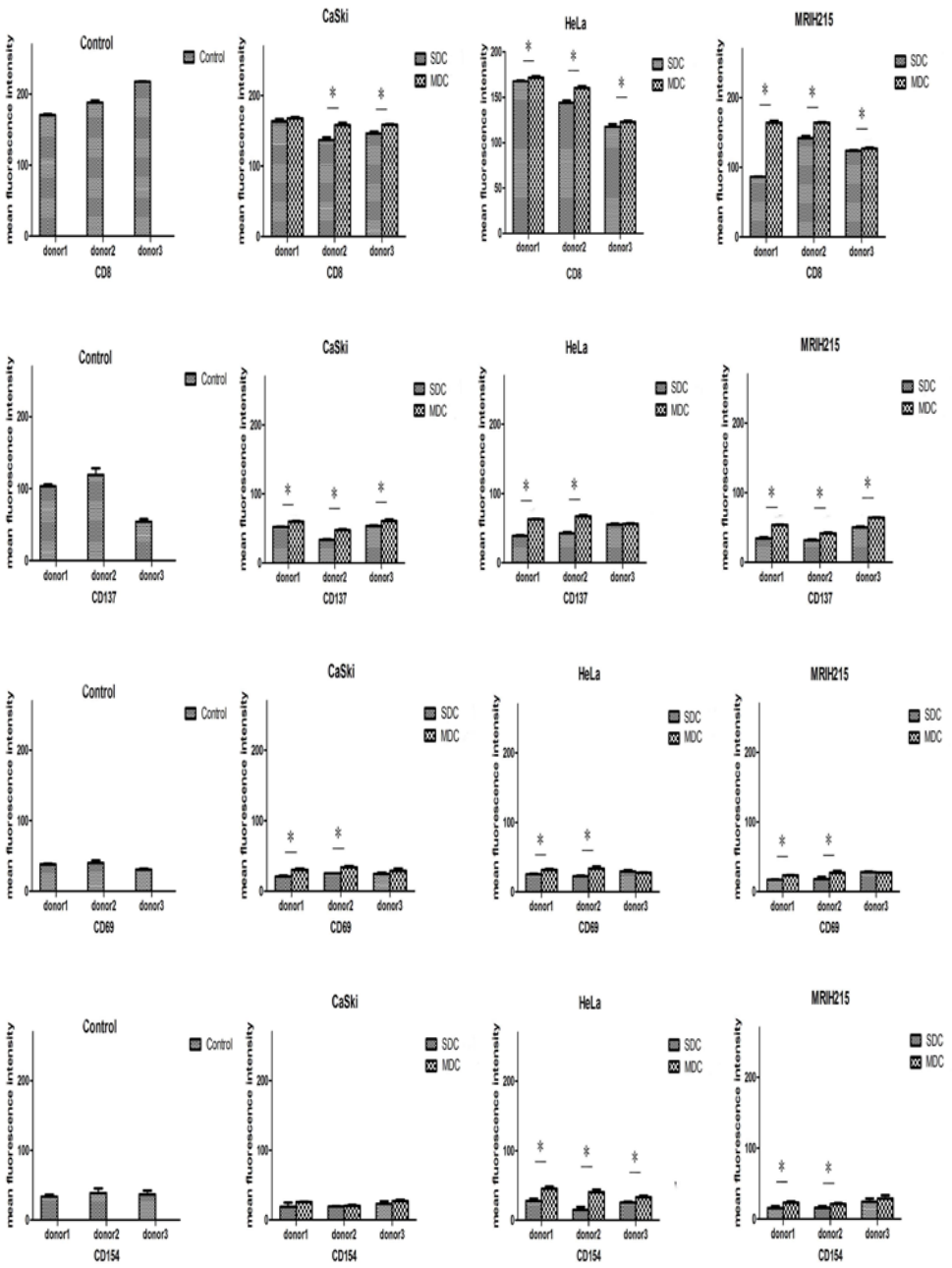


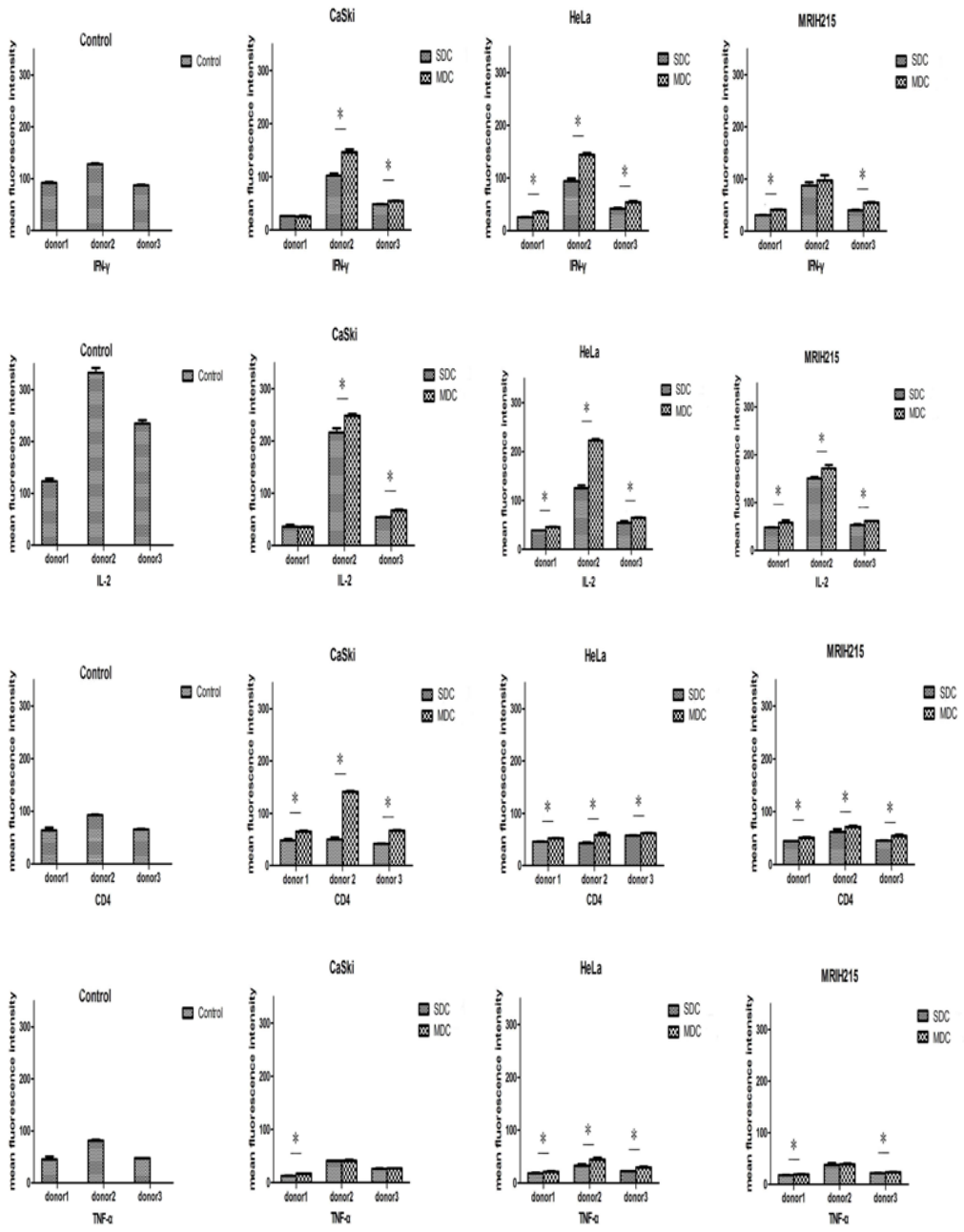

**Fig. 7: Suppression of activation marker expression by CSC.** Mean Fluorescence Intensity (MFI) of CD8, CD137, CD69, and CD154 expression on T cells from 3 donors co-cultured with SDC or MDC of 3 cell lines at a 5:1 ratio in transwell cultures is shown. (A) MFI of CD8, CD137, CD69, and CD154 expression of resting T cells which were co-cultured with SDC or MDC for 6 days. (B) MFI of CD8, CD137, CD69, and CD154 expression of anti-CD3/anti-CD28-activated T cells which were co-cultured with SDC or MDC for 6 days. Statistical significance: *P < 0.05.

## 5.6 Effects of SDC or MDC on cytokine production by T cell

To further investigate the effect of SDC and MDC on effector T cell function, we measured by intracellular staining the expression of IFN-γ, IL-2, and TNF-α by $CD4^+$ T cells which were the major producers of these cytokines. After 6 days of stimulation with anti-CD3/anti-CD28 beads, IFN-γ, IL-2, CD4, and TNF-α expression was monitored by FACS in T cells from 3 donors co-cultured at a 5:1 ratio with SDC or MDC derived from the 3 cell lines. As shown in Fig. 8A, stimulated T cells from most donors co-cultured with MDC showed significantly higher MFI of expression of IFN-γ, IL-2, CD4, and TNF-α than those with SDC.

Next, we assessed whether SDC or MDC could also inhibit cytokine production by pre-activated T cells. To this end, T cells from 3 donors were stimulated for 4 days, co-cultured with SDC or MDC from 3 cervical cancer cell lines for another 6 days, and then the expression was measured by FACS. As shown in Fig. 8B, co-culture in the presence of cervical cancer cell-derived SDC as compared to MDC showed decreased expression of IFN-γ, IL-2, CD4, and TNF-α in activated T cells, this differs significantly from most analyses.

**A**


Control
CaSki
HeLa
MRIH215
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SDC
MDC
mean fluorescence intensity
donor1
donor2
donor3
IFN-γ
IL-2
CD4
TNF-α

**B**

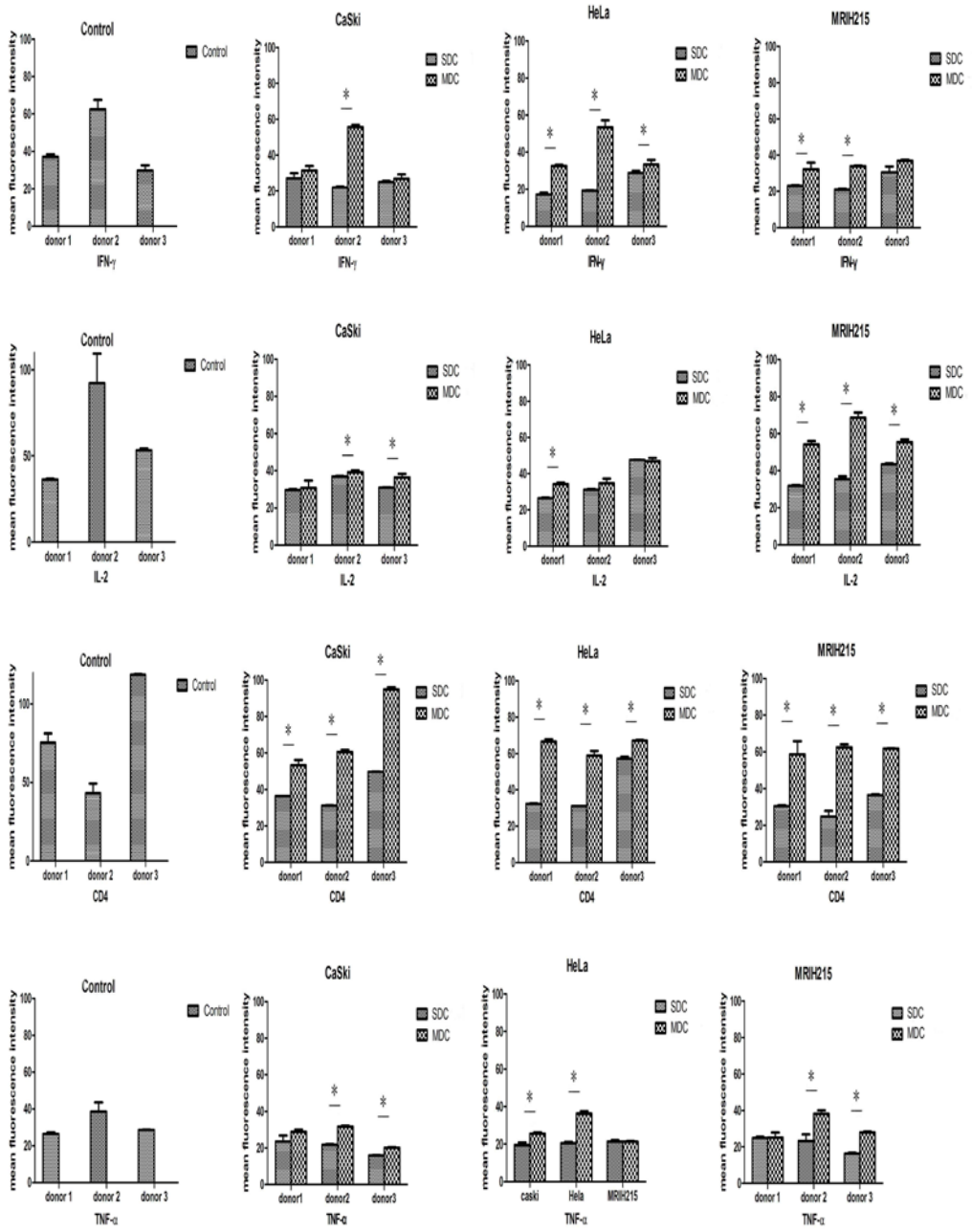



**Fig. 8: Suppression of cytokine expression by CSC.**

MFI of IFN-γ, IL-2, TNF-α, and CD4 expression in T cells from 3 donors co-cultured with SDC and MDC of 3 cell lines at a 5:1 ratio in a transwell assay. (A) T cells stimulated with anti-CD3/anti-CD28 beads and concurrently co-cultured with SDC and MDC for 6 days. (B) T cells stimulated with anti-CD3/anti-CD28 beads for 4 days and then subsequently co-cultured with SDC and MDC for another 6 days. Statistical significance: *P < 0.05.

## 5.7 Effects of SDC or MDC on the cytolytic function of cytomegalovirus (CMV)-specific T cells

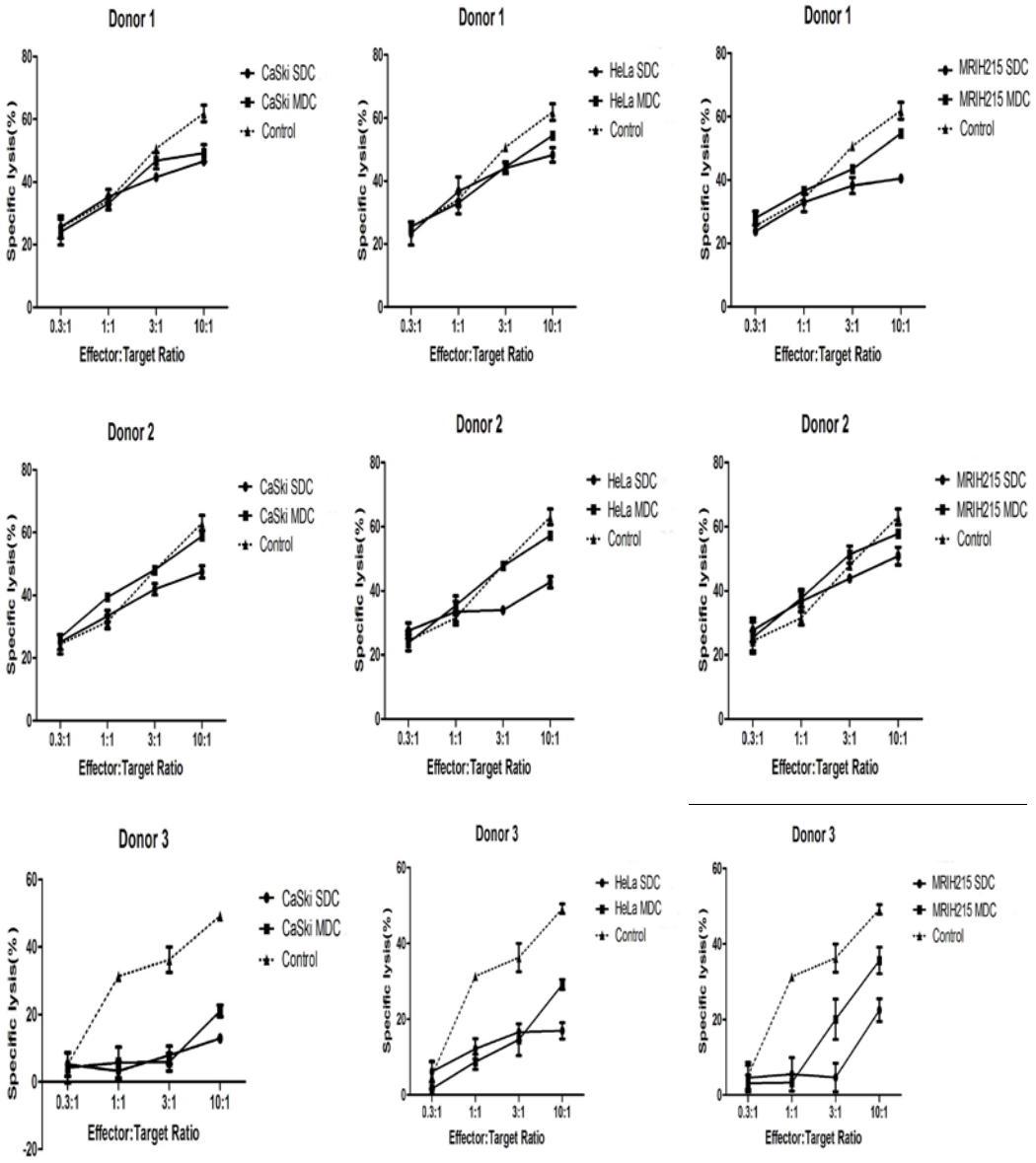
To evaluate whether SDC or MDC could have a suppressive effect on CTL lytic function, we performed a VITAL-FR assay. Activated CMV-specific CTL derived from 3 HLA-A2-positive donors were used as effector cells and co-cultured with SDC or MDC from 3 cell lines for 3 days. On day 3, CFSE-labeled autologous B-LCL pulsed with CMV $pp65_{495\text{-}503}$ peptide defined as specific target cells or Far Red-labeled autologous B-LCL defined as control target cells were co-incubated with effector CTL in the presence of SDC or MDC for another 3 days to measure the CMV $pp65_{495\text{-}503}$-specific target cell lysis.

As a control, 300 HLA-A2/CMV $pp65_{495–503}$ tetramer$^{+}$ effector cells relating to an effector : target (E:T) ratio of 1:3 mediated about 25% peptide-specific lysis after 72 h and addition of more CTL resulted in enhanced killing. At E:T ratios of 3:1 and 10:1, CMV-specific CTL co-cultured with SDC or MDC displayed decreased cytotoxic lysis in most experiments compared with the control group. Moreover, the CMV CTL cytotoxic lysis in the presence of SDC from 3 cell lines was significantly diminished compared with the lysis in the presence of MDC. Comparatively, there was no significant difference between the CMV CTL cytotoxic lysis in the presence of SDC and in the presence of MDC at the E:T ratio of 0.3:1 and 1:1 from most donors (Fig. 9), but it was significant at the E:T ratio 10:1. These data demonstrate that both SDC and MDC could suppress the ability of activated CMV-specific CTL to lyse pp65-pulsed autologous BLCL at a high ratio of effector cells to target cells. Again SDC showed greater suppressive activity on the cytotoxic T cell effector functions of established CMV-specific T cells than MDC as visible at the E:T ratio 10:1.

Having observed suppressive effects of SDC and MDC on CMV-specific CTL cytotoxic lysis, we then investigated the frequency of CMV-specific CD8$^{+}$ T cells

from one donor in the presence of SDC or MDC at a ratio of 5:1 on day 3 and day 6 of co-culture. The CMV-specific $CD8^+$ T cells cultured alone were taken as control group, that had a frequency of 3.09±0.017% at the start of the experiment and a frequency of 2.84±0.152% at day 3 and 2.86±0.12% at day 6. One representative dot plot of CD8 versus CMV MHC-dextramer is shown in Fig. 9B. On day 3 the frequency of CMV-specific CD8+ T cells in the presence of MDC (2.67±0.3279%) was significantly higher than that in the presence of SDC (1.09±0.06%)($p<0.05$, Figure 9B). On day 6, we also observed significantly higher frequency of CMV-specific $CD8^+$ T cells in the presence of MDC (2.4067±0.5201%) compared with that in the presence of SDC (1.1±0.3551) ($p<0.05$, Fig. 9B). This shows that not only the activation status but also the frequency of specific CTL is reduced by co-culture with CSC.

**A**


Donor 1
Specific lysis(%)
Effector:Target Ratio
0.3:1
1:1
3:1
10:1
CaSki SDC
CaSki MDC
Control
Donor 1
HeLa SDC
HeLa MDC
Control
Donor 1
MRIH215 SDC
MRIH215 MDC
Control
Donor 2
CaSki SDC
CaSki MDC
Control
Donor 2
HeLa SDC
HeLa MDC
Control
Donor 2
MRIH215 SDC
MRIH215 MDC
Control
Donor 3
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Donor 3
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HeLa MDC
Control
Donor 3
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MRIH215 MDC
Control

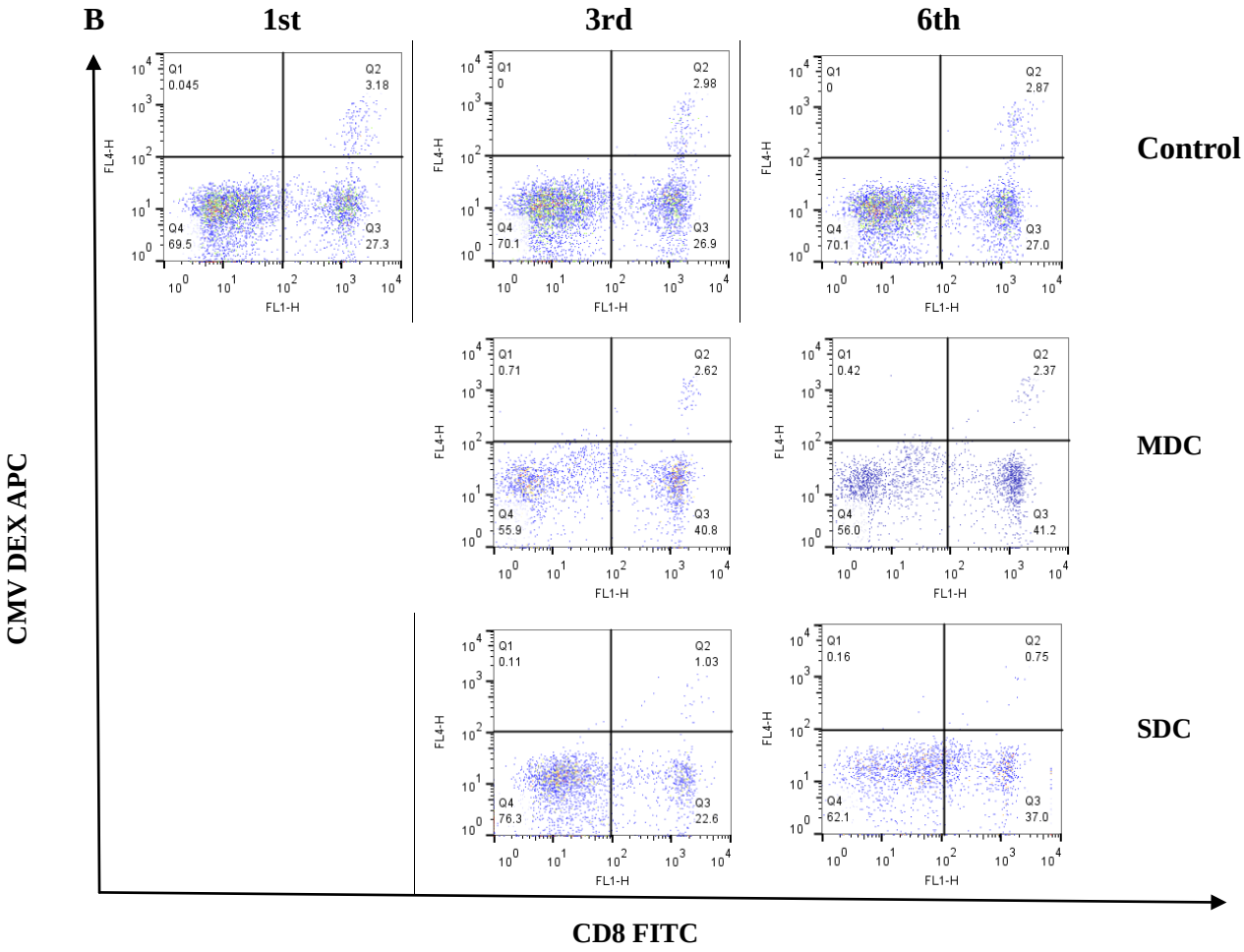



**Fig. 9: Functional suppression of CTL by CSC.**

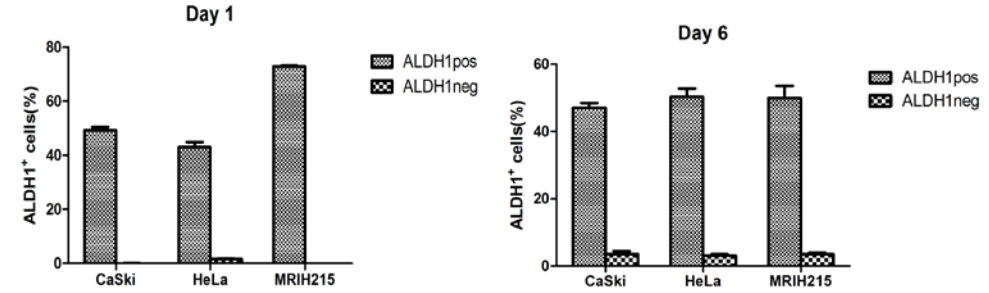
The specific lysis of target cells by CMV-specific T cells from 3 donors in the presence of SDC or MDC of 3 cell lines at a 5:1 ratio in a transwell assay is shown. CFSE-labeled autologous B-LCLs pulsed with CMV $pp65_{495\text{-}503}$ peptide were defined as specific target cells or Far Red-labeled autologous B-LCL were defined as control target cells. Effector cells (E) were co-cultured with SDC or MDC from 3 cell lines for 3 days. Then the two target cells (T) and effector cells (E) were incubated in the presence of SDC or MDC for another 3 days at different E:T ratios and autologous CMV-specific CTL lysis was assessed by flow cytometry. (A) The relative autologous CMV-specific CTL cytotoxic lysis of CMVpp65-pulsed BLCL at different E:T ratios in the presence of SDC or MDC is shown. Representative results for three independent donors are presented with mean values ± SD of three determinations. (B) The frequency of CMV-specific, Dextramer reactive $CD8^+$ T cells in the presence of SDC or MDC at a ratio of 5:1 on day 3 and day 6.

## 5.8 Effects of ALDH1-sorted cervical cancer cells on cytokine production by T cell

It is reported that CSCs can self-renew to generate additional CSCs and differentiate to generate phenotypically diverse cancer cells with limited proliferative potential. In addition, since spheroids are mixed cell populations, we wanted to enrich for CSC by ALDH-specific cell sorting. In the following experiment, the purity of the ALDH1-sorted cells was assessed by FACS on day 1 and day 6. As shown in Fig. 10A , 24 h after sorting, the ALDH1 expression of $ALDH1^+$-sorted cells decreased in all three cell lines (CaSki: 49.30 ± 1.206%, HeLa: 49.06 ± 1.8336%, MRIH215: 72.87 ± 0.2901% ) and that of $ALDH1^-$sorted cells increased in all three cell lines (CaSki: 0.06 ± 0.0116%, HeLa: 1.54 ± 0.2303%, MRIH215: 0.04 ± 0.0153% ). However, on day 6 after sorting, the ALDH1 levels of $ALDH1^+$-sorted cells were further decreased in two cell lines (CaSki: 47.00 ± 1.4746%, HeLa: 50.33 ± 2.4294%, MRIH215: 49.95 ± 3.6412% ) and $ALDH1^-$sorted cells further increased in all three cell lines (CaSki: 3.53 ± 0.9139%, HeLa: 3.10 ± 0.3704%, MRIH215: 3.53 ± 0.3926% ).

To further investigate the effect of $ALDH1^+$ cells and $ALDH1^-$ cells from 3 cell lines on effector T cell functions, the sorted $ALDH1^+$ and $ALDH1^-$ cells were co-cultured with T cells at a 5:1 ratio. After 6 days of co-culture with anti-CD3/anti-CD28 stimulus, the production of TNF-α, IFN-γ, and IL-2, and the expression of CD4 on T cells were measured by FACS. As shown in Fig. 10B, stimulated T cells co-cultured with $ALDH1^-$ cells showed significantly higher MFI of expression of TNF-α, IFN-γ, IL-2 and CD4 than those co-cultured with $ALDH1^+$cells.

A




B

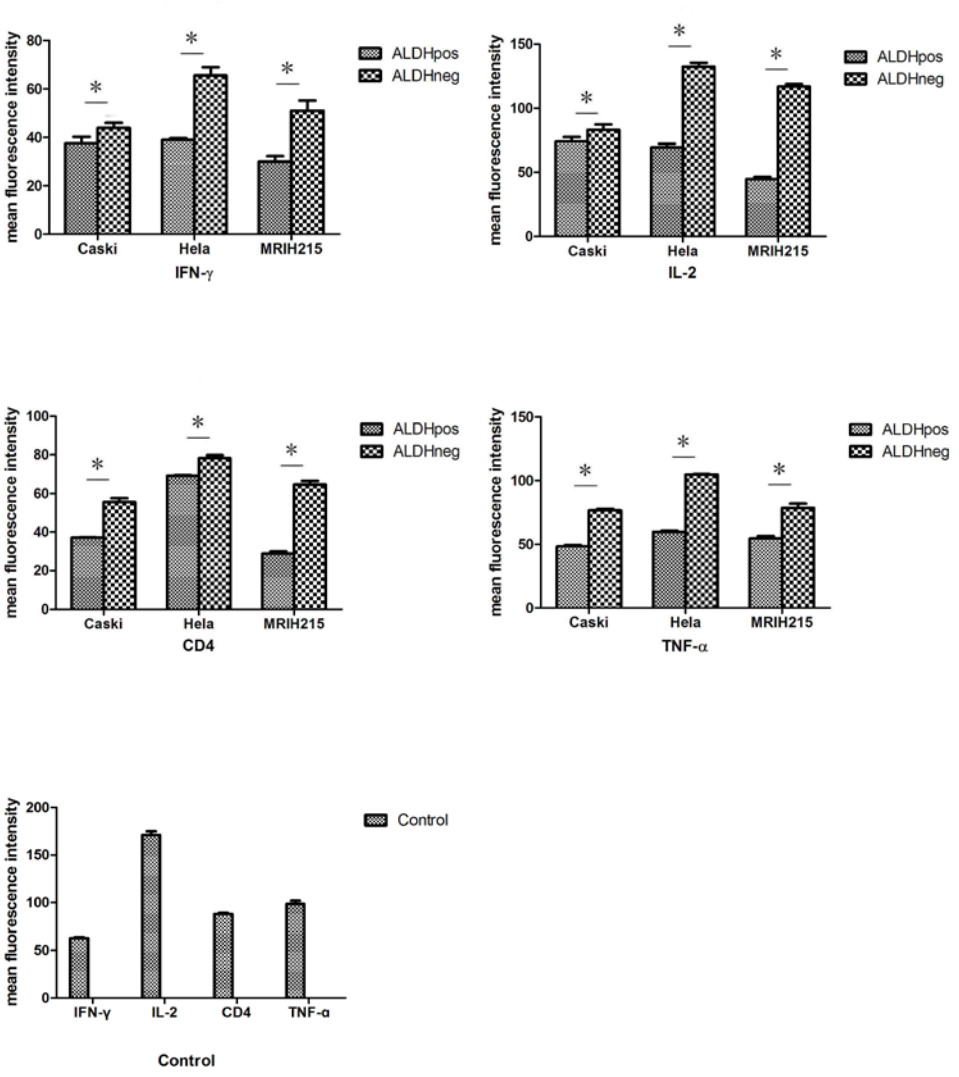



**Fig. 10: Enhanced suppressive activity by ALDH1-positive CSC.**

(A)Comparison of ALDH1 expression in $ALDH1^+$ cells and $ALDH1^-$ cells after

sorting by flow cytometry measured on day 1 and day 6. (B) MFI of IFN-γ, IL-2, TNF-α, and CD4 expression in T cells stimulated with anti-CD3/anti-CD28 and co-cultured with FACS-sorted $ALDH1^+$ cells or $ALDH1^-$ cells for 6 days. Statistical significance: *P < 0.05.

# 6. Discussion

The primary objective of this study was to evaluate some immunologic features of enriched CSCs from cervical cancer cell lines. In this study, we enriched CSC-like cells derived from spheroids grown from cervical cancer cell lines by the method described previously [83]. The stem cell-like characteristics of these cells were analyzed by comparing surface antigen expression and the expression of embryonal TF that are markers of stemness. ALDH1 has been considered to be a marker for CSCs. As exemplified in breast cancer, for example, Ginestier et al. [62] reported that cells with high ALDH activity containing the tumorigenic cell fraction are able to self-renew and to recapitulate the heterogeneity of the parental tumor. Ricardo et al. [64] showed that the $ALDH1^{+}CD44^{+}CD24^{low/-}$cells are highly tumorigenic and that studying the expression of CD44/CD24 and ALDH1 was the most accurate method to identify CSC from breast cancer cell populations. In our study, we found that SDC contained a significantly higher number of $ALDH1^{+}$ and $ALDH1^{+}/CD44^{+}/CD24^{-}$cells than corresponding MDCs. Cancer and normal stem cells (SCs) share proliferative properties of self-renewal and expression of key transcription factors (TFs). Nanog, Oct4, and Sox2 are the core regulators of mouse embryonic stem cell pluripotency and they cooperatively maintain the regulatory network responsible for self-renewal and pluripotency [65]. In our study, the expression of Oct3/4, Sox2, and Nanog was up-regulated in SDC derived from the three cervical cancer cell lines. This demonstrates that spheroids subcultivated from cancer cell lines exhibit CSC characteristics and are therefore useful for CSC research.

One of the challenges in developing a viable immune-based therapy of cancer is the impact a tumor and the tumor microenvironment plays in suppressing the immune system. A tremendous amount of progress has been made in evaluating the mechanisms by which cancer cells are able to avoid and/or suppress immune detection [66], including the production of immunosuppressive factors, lowering the level of expression of tumor antigens or eliminating it altogether, or the infiltration of

suppressor cell populations such as $CD4^+$ $CD25^{(high)}$ $Foxp3^+$ regulatory T cells (Tregs). However, a growing body of evidence shows that cancers are heterogeneous - both in their pathology and in their molecular profiles [84]. In the initial landmark paper by Al-Hajj and colleagues, they identified and isolated the tumorigenic cells as $CD44^+CD24^{-/low}$ $Lineage^-$ in eight of nine patients. They found that as few as 100 cells with this phenotype were able to form tumors in mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors [20]. Similar observations had been made for various human tumor types, including acute myeloid leukaemia and colon, brain and pancreatic tumors [29, 80, 85, 86]. However, few reports had been published on the immunological properties of this subpopulation of cells. Therefore, the aim of the present study was to evaluate the immunological characteristics of cervical cancer stem cells.

The immunosuppressive properties of CSCs had been confirmed in other tumor types. For example, Chikamatsu et al. [87] reported that $CD44^+$cancer stem-like cells in squamous cell carcinoma of the head and neck showed not only stronger inhibition of the proliferation of T cells activated with anti-CD3/CD28 mAb, but also more efficient induction of Treg cells and myeloid-derived suppressor cells than $CD44^-$ cells. A culture supernatant of $CD44^+$ cells contained significantly higher levels of IL-8, G-CSF, and TGF-β than those of $CD44^-$ cell cultures. Similarly, Tomaso et al. [88] reported that allogeneic glioblastoma multiforme CSCs but not their paired FBS cultured non-CSC tumor lines could inhibit mitogen-induced (PHA + concanavalin A) proliferation of T cells from healthy donors. Our results demonstrated that SDC from cervical cancer cell lines consistently showed greater suppressive activity on the proliferation of T cells irrespective of their activation status, i.e., unprimed and pre-activated effector T cells, compared to MDC.

Moreover, as for T cell activation, our results showed that SDC, when compared with MDC, inhibited more strongly CD3/CD28 polyclonal activation and the expression of CD69, CD137 and CD154, which are considered as T cell activation marker molecules. Further studies were conducted to investigate the inhibition of cytokine

production of T cells by SDC. Consistent with the observations above, SDC suppressed more strongly CD3/CD28 induced production of IFN-γ, IL-2, and TNF-α in resting and pre-activated effector T cells in most experiments. Moreover, our study demonstrated that both $CD4^+$ and $CD8^+$ T cells were equally inhibited by SDCs and MDCs, irrespective of their activation status (resting and already activated effector T cells), and SDC consistently showed greater suppressive activity. These findings indicated that SDC from the investigated cervical cancer cell lines had a more immunosuppressive effect on T cell proliferation, activation, and cytokine production than MDC.

VITAL-FR is an extension to the VITAL assay, a flow cytometry-based assay system assessing CTL frequency and function [63]. In co-cultures with CTL and different fluorescence-labeled target cells, lysis can be determined by the ratio of the remaining viable control and target cells, which are quantified by flow cytometry after a certain incubation time. The sensitivity and reproducibility of the VITAL-FR assay has been described before, proving that it is a highly sensitive and flexible flow cytometry-based in-vitro assay for clinically relevant specific CTL functions. Here, we adapted the VITAL assay to quantify and compare the efficacy of CMV-specific T cell-mediated target cell killing function in the presence of SDC or MDC. We found that CMV-specific CTL co-cultured with SDC showed stronger functional inhibition and a decrease in the frequency of CMV-specific $CD8^+$ T cells than those co-cultured with MDC. Consistent with this observation, SDC showed greater suppressor activity on the cytotoxic T cell effector functions of established CMV-specific T cells than MDC. As mentioned above, $CD44^+$ cancer stem-like cells in HNSCC showed more efficient induction of Treg cells than $CD44^-$cells. Supernatants of $CD44^+$ cells had significantly higher levels of TGF-β than those of $CD44^-$cell cultures. Mempel et al. reported that Treg cells, TGF-β, and probably other suppressive factors inhibited CTL degranulation and cytolysis [89]. Therefore, the possible explanation for the stronger suppression of CTL cytolytic function induced by cervical CSCs might be the more efficient induction of Treg cells and the enhanced secretion of immunosuppressive

cytokines like TGF-β than by their more differentiated bulk cancer cell counterparts. ALDH was investigated as a specific marker for identifying and isolating normal and malignant human colon SC. In addition, ALDH is more specific for stemness than CD44 ($CD44^+/ALDH^-$ cells are non-tumorigenic), based on tumorigenic potential [90]. To further confirm whether or not cervical CSCs suppressed more efficiently than the bulk cancer cells, the $ALDH1^+$ population and $ALDH1^-$population were isolated by FACS sorting and co-cultured with T cells polyclonally stimulated by CD3/CD28 activation. On co-culture day 1 (24 h after sorting) already, the expression of ALDH1 of the 3 sorted cells lines was found to be obviously decreased from the putative close to 100% pure cell population from between 50 and 70%. On co-culture day 6, a further decrease of the percentage of $ALDH1^+$ cell was observed. These results are consistent with the notion that CSCs are a cell population within a tumor that is able to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor [91]. Moreover, after 6 days of co-culture, activated T cells co-cultured with $ALDH1^-$ cells showed significantly higher MFI of expression of TNF-α, IFN-γ, IL-2, and CD4 than those co-cultured with $ALDH1^+$ cells. The result further confirmed that CSCs showed stronger inhibition of T cell function than the corresponding bulk cancer cells.

# Conclusion:

The CSC population within the tumor displays a high immunosuppressive activity, protecting itself from immune attack. Accordingly, the development of novel immunotherapeutic strategies to fight CSC-driven immune suppression and escape is strongly called for.

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# **8** Affidavit

"I, [Jiaying, Lin] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Immunoregulatory properties of cancer stem-like cells derived from carcinoma cell lines of the cervix uteri] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date 12022013 Signature Jiaying Lin

## Declaration of any eventual publications

[Jiaying Lin] had the following share in the following publications:

**J Lin**, J Xu, AE Albers, AM Kaufmann. New developments in therapeutic HPV vaccines. Current Obstetrics and Gynecology Reports. 2012 September; 1(3) :106-115. Responsible for drafting the manuscript.

Signature, date and stamp of the supervising University teacher

Andreas Kaufmann

Signature of the doctoral candidate

Jiaying Lin

# 9 Curriculum Vitae and Publications

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

# Publications

**J Lin**, J Xu, AE Albers, AM Kaufmann. New developments in therapeutic HPV vaccines. Current Obstetrics and Gynecology Reports. 2012 September; 1(3) :106-115

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