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DISSERTATION

Immunogenicity of putative cancer stem-like cells derived from carcinoma of the cervix uteri and the head and neck area

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ABBREVIATIONS AND ACRONYMS

ALDH1	Aldehyde Dehydrogenase isoform 1
APC	Allophycocyanin
APC	Antigen-Presenting Cells
BFA	Brefeldin A
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CSC	Cancer Stem Cells
CTL	Cytolytic T Lymphocytes
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 Carcinoma
HPV	Human Papilloma Virus
IFN- γ	Interferon-γ
IL	Interleukin
MACS	Magnet-Activated Cell Sorting
MHC	Major Histocompatibility Complex
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	R-Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PI	Propidium Iodide
RT-PCR	Real Time-Polymerase Chain Reaction
TF	Transcription Factors

Zusammenfassung

Tumorrezidive und Metastasen werden auf Tumorstammzellen (TSZ) zurückgeführt, welche gegenüber der Wirkung etablierter Tumortherapien, wie Chemo- und Strahlentherapie resistenter sind. Eine neue Möglichkeit TSZ direkt zu behandeln, wäre eine Antitumor-T-Zell-Antwort anzuregen. Jedoch ist die Empfindlichkeit von TSZ gegenüber den zytotoxischen T-Zellen (ZTL) des Wirts weitgehend unerforscht. In dieser Arbeit haben wir die Empfindlichkeit von TSZ, generiert aus Kopf-Hals-Tumor- und Zervixkarzinomzelllinien, gegenüber immunologischer Erkennung und Abtötung durch alloantigen-spezifische T-Zellen untersucht. Wir zeigen, dass Zellpopulationen aus TSZ-angereicherten Spheroidkulturen eine erhöhte Expression von ALDH1, ICAM1 und Stamm-/ Progenitormarkern (SOX2, Oct3/4, Nanog) in allen 3 untersuchten Tumorzelllinien zeigen. Lediglich die MHC-Klasse-I-Expression war in der Zervixkarzinomzelllinie CaSki niedriger. Die MHC-Klasse-I-Expression konnte durch IFN-γ Behandlung heraufreguliert werden. Außerdem waren TSZ-Populationen, im Vergleich zu den jeweiligen Monolayerzellen, weniger empfindlich gegenüber MHC-Klasse-I restringierter allogener $CD8^+$ Zytotoxizität. Dies könnte auf ihre niedrigere MHC-Klasse-I-Expression, dem wichtigsten Molekül für eine immunologische Erkennung und Antwort, zurückgeführt werden. IFN-γ Vorbehandlung konnte die allogene $CD8^+$ zytotoxische Lyse verbessern. Bemerkenswert war, dass bei Zellen aus Spheroidkulturen eine größere Verbesserung der Lyse als in Monolayerzellen zu beobachten war. Schließlich zeigte die Untergruppe der $ALDH1^{hoch}$-exprimierenden Zellpopulation eine größere Sensitivität gegenüber ZTL-Tötung als die $ALDH1^{niedrig}$-exprimierende Zellpopulation.

Durch die Etablierung eines In-vitro-Systems mit dem alloantigen-spezifische Antworten gegen TSZ getestet werden können, stellen wir ein Wergzeug zur Verfügung, um Substanzen zu testen, die verwendet werden können, um Immunantworten zu regulieren und zu untersuchen. Dieses Methode erlaubt die Entwicklung und Optimierung der Zusammensetztung von Immunadjuvantien für zukünftige gezielte Impfungen gegen CSC (unabhängig von der Verfügbarkeit von CSC-spezifischen Antigenen).

Weitere Untersuchungen der Interaktionen zwischen TSZ-Subpopulationen und dem humanen Immunsystem könnten eine Basis für die Entwicklung von ZTL-basierten Antitumorimpfstoffen gegen TSZ legen, um diese tumorerhaltende Zellpopulation zu eleminieren.

SUMMARY

Tumor recurrence and metastasis has been attributed to cancer stem cells (CSC), which are more resistant to the effect of established tumor treatments such as chemotherapy or radiation. A novel approach to target CSC directly, is to stimulate a T cell-mediated antitumor immune response. However, the CSC susceptibility to a host's cytotoxic T lymphocyte (CTL)-mediated immune system is largely unexplored. In this study, we compared the susceptibility to immunologic recognition and killing by allo-antigen specific $CD8^+$ CTL of cell lines from head and neck cancer and cervical cancer with CSC-like cells generated from these lines. We showed that CSC-enriched spheroid culture cell populations exhibit higher expression levels of ALDH1, ICAM1 and of stem/progenitor cell markers (SOX2, Oct3/4, Nanog) on all 3 tumor cell lines investigated but lower MHC class I expression on the cervical cancer cell line CaSki. The MHC class I expression could be upregulated by IFN-γ treatment. Furthermore, these CSC populations were less sensitive to MHC class I-restricted allogeneic $CD8^+$ CTL cytotoxicity compared with their matched monolayer cells. This may be attributable to their lower expression of MHC class I, the most important molecule in cytotoxic T cell recognition and response. IFN-γ pretreatment could enhance the allogeneic-$CD8^+$ CTL cytotoxic lysis. Importantly, in spheroid-derived cells, a stronger enhancement of lysis was observed than in monolayer-derived cells. Finally, the subset of the $ALDH1^{high}$ cell population presented more sensitivity towards $CD8^+$ CTL killing than the $ALDH1^{low}$ cell population.

By setting up an in vitro-system to test allo-antigen specific response to CSC, we provide a tool to test substances that can be used to regulate and investigate the immune response. This tool makes it possible to develop and optimize the composition of immune-adjuvants for future CSC-targeted vaccines (independently of the availability CSC-specific antigens). Further investigation of the interaction between cancer stem-like subpopulations and the human immune system may provide a basis for developing CTL-based antitumor vaccines targeting CSC to eliminate this tumor sustaining cell population.

1. Introduction

1.1 Immune Response

The concerted action of cellular and molecular components that constitute the immune system is called immune response. A prerequisite for a successful immune response is the recognition of the target which is then followed by the elimination of the target.

1.1.1 Innate and Adaptive Immunity

In higher eukaryotes there are two defense lines: innate immunity and adaptive immunity. The components of innate immunity consist of cellular and biochemical defense mechanisms that are able to react directly when being activated by an appropriate signal or target. This response has no memory component. In contrast, before an immune response can be launched by the adaptive arm of the immune system, stimulation by exposure to an appropriate target is required together with “danger signals” generated by the innate immune system that lead to inflammatory responses. Recurrent exposure to the same target leads to an increase in magnitude and defense capabilities. Because this form of immunity develops as a response to a specific target and adapts to the target, it is called adaptive immunity, and has a long-term memory.

1.1.2 Cell-Mediated Adaptive Immunity

Two types of adaptive immune responses are known. One branch is called humoral immunity and the other is called cell-mediated immunity, they are mediated by different components of the immune system and function to eliminate different types of substances. Cell-mediated immunity, also called cellular immunity, is mediated by T lymphocytes (also called T cells). Defense against intracellular substances, such as tumor cells is a function of T cell-mediated immunity, which promotes the destruction of agents residing within cells by the killing of targeted cells to eliminate reservoirs of such agents.

1.1.3 Mechanisms of Cell-Mediated Immunity

The first phase of cell-mediated immunity is the recognition of antigens by T cells. T cells recognize protein antigens of intracellular substances that are displayed on the surfaces of antigen-presenting cells (APC) as peptides bound to major histocompatibility complex (MHC) molecules. MHC class I presents peptides to $CD8^+$ cytolytic T lymphocytes (CTL), and MHC class II to $CD4^+$ helper T cells. Apart from antigen recognition, CTL also require for successful target lysis additional stimuli that are provided by costimulators expressed on APC, such as B7.1 (CD80), B7.2 (CD86), and ICAM-1 (CD54) as well as lymphokines like Interleukin-2 (IL-2) (Fig. 1). After naive T cells encounter antigen and costimulators, they start to proliferate, building a large population of lymphocytes with the potential capacity to differentiate into effector cells (Fig. 2). To trigger the effector phase of cell-mediated immunity, effector T cells have to come into contact with target cells presenting the antigens that initiated the response. When effector T cells migrate to sites of target cells or antigen challenge and recognize the antigen for which they are specific, the cells are activated to perform their effector functions. In cell-mediated immunity, the principal effector function of $CD4^+$ T cells is to stimulate the microbicidal activities of macrophages and other leukocytes, and the effector functions of $CD8^+$ CTL are to kill targeted cells and activate phagocytes.

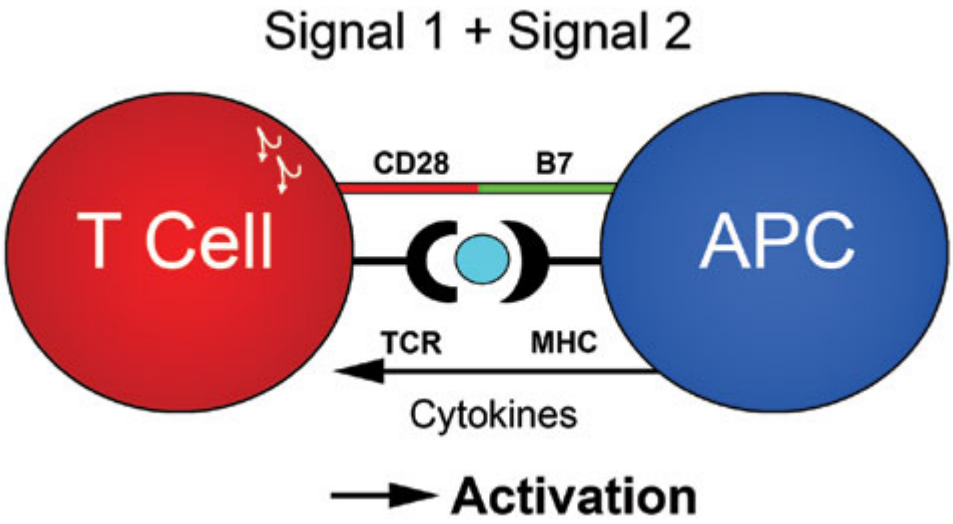

Fig. 1: Two signals for T cells activation. Signal 1 is the binding of the peptide-MHC complex of the APC to the T-cell receptor. Signal 2 is the binding of the costimulators to their receptors, for example, the B7 molecule on the APC and its CD28 receptor in the T cell [1].

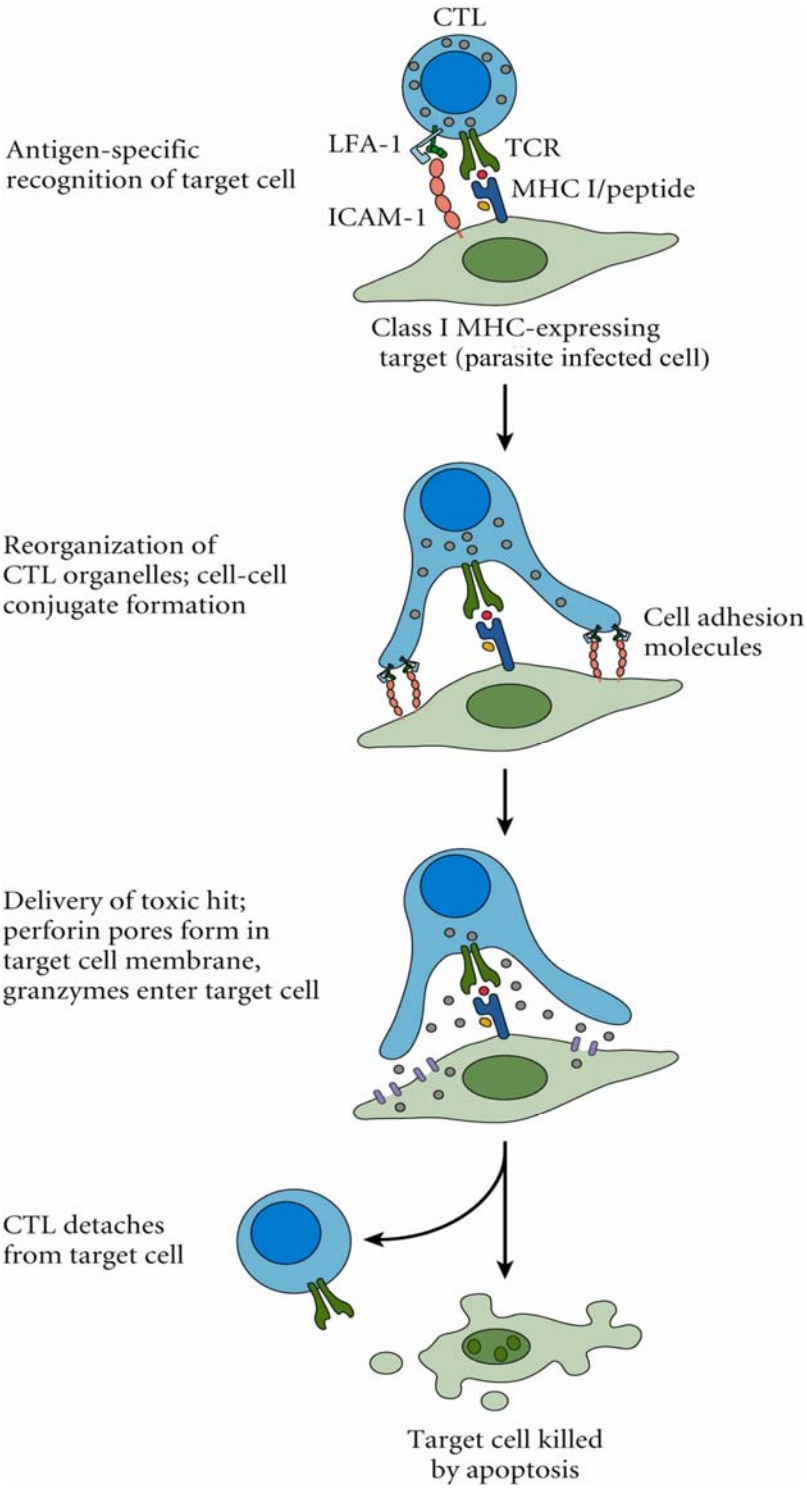

Fig. 2: Process of cell-mediated CTL cytotoxicity. First, antigens are processed and presented by APC as peptides-MHC complexes and become targets for CTL. Secondly, CTL inserts perforin protein into the cell membrane of the target cell presenting the antigen, creating a pore-like hole in the membrane. The CTL releases granzymes (specialized digestive enzymes) into the target cell, which induces apoptosis to occur. Finally, the CTL detaches from its target cell and moves on for another target cell [2].

1.2 Allogeneic Immune Response

Transplantation is the process of taking cells, tissues, or organs, called a graft, from one individual and placing them into a different individual. The individual who provides the graft is called the donor, and the individual who receives the graft is called either the recipient or the host. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft (or allograft). The major limitation in the success of transplantation is the immune response of the host to the donor tissue. The molecules that are recognized as foreign on allografts are called alloantigens, the lymphocytes and antibodies that react with alloantigens are described as allogeneic response. Here, we used irradiated CD80-positive human tumor cell lines presenting alloantigens to stimulate healthy donors' peripheral blood mononuclear cells to induce allogeneic T cells and then measure the allogeneic response to the tumor cell.

1.3 Tumor Immunology

Cancer is one of the most important and major health problems worldwide causing morbidity and mortality in humans. Cancers arise from the uncontrolled growth and invasion of clones of transformed cells. The growth of malignant tumors is determined to a large part by the proliferative and invasive capacity of the tumor cells and partly by the ability to evade the immune surveillance of the tumor-bearing host. It is now clear that the immune system does react against many tumors and exploit these reactions to specifically destroy tumors. However, immune responses frequently fail to prevent the growth of tumors for several reasons. First, tumor cells are derived from host cells and most tumors express only a few antigens that may be recognized as non-self. As a result, most tumors tend to be only weakly immunogenic. Second, the rapid growth and spread of tumors may overwhelm the capacity of the immune system to eradicate tumor cells. In addition, tumors have the ability of evading or overcoming the mechanisms of host defense. To understand the interaction of tumor antigens and immune responses, it is fundamental to study tumor immunity and develop strategies for cancer immunotherapy [3, 4].

1.3.1 Evasion from Immune Response by Tumors

Many malignant tumors possess mechanisms that enable them to evade or resist host immune responses. A major focus of tumor immunology is to understand the ways in which tumor cells evade immune destruction, with the hope that interventions can be designed to increase the immunogenicity of tumors and the responses of the host. The process of evasion, often called tumor escape, may be a result of several mechanisms as follows: a) MHC class I expression may be down-regulated on tumor cells so that they cannot be recognized by CTL, b) tumors may lose expression of antigens that elicit immune responses, c) tumors may fail to induce CTL because most tumor cells don't express costimulatory molecules or MHC molecules, d) the products of tumor cells may suppress anti-tumor immune responses, e) tumor antigens may induce specific immunologic tolerance [4].

1.4 Immunotherapy of Malignant Epithelial Tumors

Some cancers can be cured by conventional treatments including chemotherapy, radiotherapy and surgery. But some metastasizing tumors may recur after treatment, therefore, potential novel treatments using immunologic approaches have been explored by immunologists and cancer biologists for many years. The main reason for an interest in an immunologic approach is that current therapies for cancer mainly rely on drugs that kill dividing cells or block cell division, and these treatments have severe effects also on normal proliferating cells in patients with cancer. As a result, the treatment of cancers causes significant morbidity and mortality. Moreover, the optimal dosage of these drugs may not be reached due to comorbidities. Immune responses to tumors are designed to be specific for tumor antigens to limit impact on normal cells. Therefore, immunotherapy has the potential of being the most tumor-specific treatment that can be devised.

1.5 Stem Cells

Stem cells are cells found in all multicellular organisms, that can divide through mitosis, differentiate into diverse specialized cell types and can self renew to produce more stem cells. The classical definition of a stem cell is that it possesses two properties: a) self-renewal, the ability to go through numerous cycles of cell division while maintaining the undifferentiated state, b) potency, the capacity to differentiate into specialized cell types.

1.5.1 Cancer Stem Cells

Cancer stem cells (CSC) are viewed as the result of the oncogenic process and the starting point for the formation of tumor and metastasis. The characteristics that define CSC are summarized in Table 1 [5]. CSC may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. According to the CSC hypothesis these cells persist in tumors as a distinct population, they are put aside in a special environment, the so called tumor stem cell niche, and they are quiescent. They are responsible for relapse and metastasis. The efficacy of cancer treatments is, in the initial stages of testing, often measured by the ablation fraction of the tumor mass. As CSC would form a very small proportion of the tumor, this may not necessarily select for drugs that act specifically on the stem cells. The theory suggests that conventional chemotherapies kill differentiated or differentiating proliferating tumor cells, which form the bulk of the tumor but are unable to infinitely sustain tumor growth. In contrast, the population of quiescent CSC could remain untouched or less susceptible and cause a relapse of the disease. Therefore, development of specific therapies targeted at CSC holds out hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.

Table 1: Properties attributed to putative CSC

- CSC initiate malignant tumors and drive neoplastic proliferation [6]
- CSC can recreate themselves by symmetric cell division [7]
- After transplantation to a suitable host, CSC recreate the heterogeneous phenotype of the originating tumor by asymmetric cell divisions [7]
- CSC are generally slow or non-dividing cells and thus relatively resistant to radiation and chemotherapeutic treatment [8]
- Compared to the “bulk”-tumor population, CSC express a distinct repertoire of biomarkers that can be used to define and isolate them [9]

1.5.2 Expanding the CSC Population

In order to study CSC, the first requirement is to have a system, in which they can be cultured and expanded. Initially, it was an urgent task to create new methods to culture CSC for multiple passages and to expand the stem cell-like population. Tumor cells are traditionally grown as adherent monolayer cultures in the presence of serum. In serum, tumor cells express high levels of differentiation markers, and CSC differentiate into different bulk tumor cell types. Therefore, these conditions are not desirable for CSC propagation. Spheroids are three-dimensional (spherical) clusters of tumor cells grown from one or several cell clones under non-adherent conditions that support CSC growth. As compared to cell doubling times measured in monolayer culture, the rate and pattern of spheroid growth *in vitro* better matches that observed in tumors *in vivo* [10]. Anchorage independent growth has been shown to be a property shared by normal tissue cells that exhibit stem cell properties [11]. For neurosphere stem cells (NSCs) it was discovered that they could be cultured in defined, serum-free medium supplemented with growth factors [12]. At low densities, the NSCs grow as non-adherent clonal spheroids, termed neurospheres. Dirks and coworkers also demonstrated that cells isolated from pediatric brain cancers were able to form neurospheres in a similar serum-free medium [13]. Neurospheres are heterogeneous aggregates derived from a single CSC or early progenitor cell. When dissociated, these cultures are capable of serial plating, in which a small percentage of the cells which can self-renew as stem cells can form secondary and tertiary neurospheres for many passages. Also CSC derived from melanoma [14], breast cancer [15], gliosarcoma [16], and head and neck cancer [17] could be propagated anchorage independently and display the phenotype of non-adherent spheroids. This serum-free, suspension 3-D culture method for expanding CSC has significant advantages for CSC enrichment and has drawn a great deal of attention for CSC research.

1.5.2.1 CSC in Cancer Cell Lines

The first evidence for the existence of CSC came from a subpopulation of acute myeloid leukemia that comprised of 0.01%-1% of the total population and that could induce leukemia when transplanted into immunodeficient mice while other populations could not [18, 19]. The self-renewal properties of these CSC are thus the real driving force behind tumor-growth. Further proof of the significance of CSC for tumor growth has been provided by demonstrating that selective killing of CSC can stop tumor growth [20]. Similar to physiologic tissues, cancers are composed of heterogeneous cell populations [21] that exhibit distinct morphologic and

functional phenotypes [22-25]. Many lines of evidence suggest that malignant solid tumors, such as breast cancers [26], head and neck squamous cell carcinoma [27] and cervical cancers [28], contain tissue-specific CSC. However, tumor tissue could contain both CSC and normal stem cells. Importantly, cloned cell lines derived from single cancer cells can not contain any contaminating normal stem cells.

We have investigated the ability of HNSCC cell lines to grow anchorage independently as spheroid-cultures. The cell lines could form spheroids in serum-free medium under non-adherent conditions at a low density (1000 cells per ml). Moreover, single cell suspensions of spheroids at a clonal density could form subspheroids after 10-14 days but the parental monolayer cell cultures grown under the same conditions could not form subspheroids at the same concentration even after 21 days of subculture. When transferred back to adherent culture in serum-supplemented media (monolayer cell culture condition), the spheroids adhered and formed a confluent monolayer which had a phenotype identical to that of the parental cell lines [17]. This showed that these tumor spheroids derived from tumor cell lines had self-renewing capacity. Established tumor cell lines could maintain the heterogeneity of the original tumor, including cells with different morphological phenotypes, variable proliferative and clonogenic potential together with the presence of cells with stem-like characteristics. Taken together, these findings make cell lines attractive models for investigating the characteristics of CSC.

1.5.3 Identification of CSC

Properties of CSC can be illustrated *in vitro*, using methods such as clonogenic assays, where single cells are characterized by their ability to differentiate and self-renew. Several groups have succeeded in separating CSC from cancers and cancer cell lines using their common characteristics, such as cell surface markers, side population (SP), and sphere formation assay.

1.5.3.1 Side Population

Recently, the detection of SP cells, which have the ability to strongly efflux Hoechst 33342 (Ho) fluorescence dye excluded by breast cancer resistance protein (BCRP) 1, has caught attention as a method to isolate stem cells [29, 30]. A number of research groups have shown that some established cancer cell lines, which have been maintained in culture for decades, such as head and neck squamous carcinoma (HNSCC) cell lines, hepatocellular carcinoma (HCC) cell lines, glioma cell lines, and tumors isolated from breast cancer, neuroblastoma and prostate tumor, contain a small SP [15, 31-36]. These studies have demonstrated that SP cells, but not non-SP

cells, could self-renew in culture, are resistant to anti-cancer drugs and responsible for malignancy in vivo. Thus the SP assay is a method to identify cells with characteristics of CSC in cancers and cancer cell lines.

1.5.3.2 Sphere Formation Assay

The sphere formation assay is another method utilized for isolating putative stem cells, expanding and thereby purifying them. This assay involves dissociation into single cell suspension of stem cell-containing tissues or cell lines and subsequent culture on 3-D non-adherent substratum in serum-free medium supplemented with EGF and/or bFGF until they form multicellular spheroids. The stemness of spheroids can be proven as follows: a) when suspended as single cells on matrigel or methylcellulose, the primary cells could generate the same growth factor-dependent spheres, (b) when re-plated on the above non-adherent substrata, single cells from the primary spheres could generate secondary and later passage spheres that express the same functional cell marker that could be evaluated by flow cytometry or immunohistochemistry, and (c) when cultured in the presence of serum on a collagen substratum which promotes their differentiation, single cells from the primary spheres could produce multilineage colonies [37]. Now the sphere formation assay is utilized to identify CSC, from e.g. mammary gland [38], skeletal muscle [39], or human melanoma [14].

1.5.3.3 Stem Cell Markers

Markers are commonly used to identify and isolate different cells types. A break-through for studying CSC came with the discovery of markers that could be used to sort cells by FACS or magnetic bead isolation. Several markers have been proposed for CSC.

Aldehyde Dehydrogenase isoform 1 (ALDH1)

ALDH1 is a cytosolic enzyme responsible for oxidizing a variety of intra-cellular aldehydes to carboxylic acids [40-42]. To date, 17 isoforms of ALDH have been described. Most of these isoforms are widely distributed in tissues with the highest expression level observed in the liver and the kidney. The cellular distribution of ALDH is also broad as the cytoplasm, the mitochondria, and endoplasmic reticulum all contain several isoforms. Most of the isoforms can catalyze the oxidation to a variable extent of many different aldehydes. In the liver, ALDH1 functions mainly as a retinoic acid biosynthetic enzyme where it catalyzes the conversion of retinol (vitamin A) to retinoic acid [43]. With the growing need for new markers to identify and

isolate stem cells, Aldefluor staining based on ALDH1 activity measured by FACS has been proposed to be a promising choice. Irrespective of their tissue of origin, stem cells share a common definition including high levels of DNA repair enzymes, absence of lineage markers, low uptake of Hoechst 33342, expression of gap junctions, and communication pathways [44]. Based on these similarities, it is understandable that various types of stem cells show elevated ALDH activity and that Aldefluor staining using FACS may be a useful means to isolate stem cells. Thus, the use of Aldefluor staining to isolate other types of stem cells than those already reported will be of great benefit for the stem cell research field. As such, ALDH1 activity has now been employed successfully as a stem cell marker in head and neck squamous cell carcinoma, prostate, cervical cancer, and breast cancer [17, 26, 45-49]. Moreover, mounting evidence suggests that CSC could be expanded from permanent cancer cell lines including HNSCC [45, 50] and cervical carcinoma cell lines [48, 51] based on expression of ALDH1, and it is the ALDH1 high population (AHP) that has the most potent tumor-initiating activity. It is also noteworthy that tissue levels of ALDH1 activity can also be observed by immunohistochemistry, western blotting, and fluorescence microscopy [44, 49, 52, 53]. In summary, ALDH1 activity is an effective stem and progenitor cell marker as demonstrated in several tissue types. The means of its detection are versatile, making it a powerful tool for stem cell detection and characterization in many tissues.

SOX2

SOX2, located in chromosome 3q26.3, is a member of the SOX (SRY-related high mobility group box) family, which all contain a high mobility group (HMG) domain very similar to that in the sex-determining gene SRY (for sex-determining region Y) [54]. So far, more than 20 members of the SOX gene family have been identified that play an essential role in stem cell biology, regulation of organ development, and cell type specification [55-57]. Several studies have demonstrated that the transcription factor SOX2 is related to several human malignant tumors [58-61]. These data and the known role of SOX2 in development and cell differentiation suggest that this transcription factor may be relevant to the aberrant growth of tumor cells. It has been discovered that SOX2 is expressed in neural stem cells of the mouse nervous system [62, 63]. Recently, a mount of evidences showed that SOX2 is overexpressed on CSC in diverse tissues including head and neck cancer, cervical cancer, melanoma tumor and breast cancer [17, 26, 64-67]. These data demonstrate that SOX2 may play an important role in tumorigenesis and suggest an alternative stem marker for stem cells identification.

Oct4

Oct4 (octamer-binding transcription factor 4), also known as Oct3 or POU5F1 (POU domain, class 5, transcription factor 1), is a protein that in humans is encoded by the POU5F1 gene. Oct4 activates transcription via octamer motifs, and Oct4 binding sites have been found in various genes, including FGF4 (fibroblast growth factor 4) and PDGF (platelet-derived growth factor) α receptor. It is a major transcription factor that is mandatory for the self-renewal and pluripotency characteristics of embryonic stem cells and germ cells [68]. Primordial germ cells lacking Oct4 expression have been shown to undergo apoptosis rather than differentiation [69]. Its expression has been shown also in human breast CSC, suggesting that it may be implicated in self-renewal and tumorigenesis via activation of its downstream target genes [15]. This suggests that Oct4 functions as a master switch during differentiation by regulating the pluripotent potentials of the stem cell.

Nanog

Nanog is a transcription factor critically involved in self-renewal of embryonic stem cells and is thought to be a key factor in maintaining pluripotency. It is a unique homeobox transcription factor and has a homeodomain with homology to members of the natural killer gene family. Indeed, it has a similar critical role in regulating the cell fate of the pluripotent ICM (inner cell mass) during embryonic development, maintaining the pluripotent epiblast and preventing differentiation [70, 71]. Nanog is enriched in pluripotent cell lines such as embryonic stem, embryonic germ and embryonic cancer cells, but it is not expressed in adult tissues; its expression is down-regulated in differentiated tissues [72, 73]. Increased levels of Nanog can maintain the mouse embryonic stem cell self-renewal ability independent of leukemia inhibitory factor and allow human embryonic stem cell growth in the absence of feeder cells [74]. Gene knockdown of Nanog promotes differentiation, thereby demonstrating its role in human embryonic stem cell self-renewal [75]. This suggests that Nanog may be involved in the development and regulation of tumor initiation and malignant progression.

1.5.4 CSC-Targeting Cancer Immunotherapy

The current knowledge of the existence of CSC has led to studies of their specific elimination (Figure 3 and Table 1). It is envisioned that the therapy targeting CSC in combination with the established therapeutic modalities such as radiation and chemotherapy, may decrease the frequency of recurrences and enhance the patient's long-term survival. Therefore, the development of strategies that target the CSC population directly is highly desirable. Elimination CSC leads to an abrogation of the replenishing pool of cancer cells and ultimately leads to petering out the tumor growth, as has been documented in animal experiments where removal of CSC and transplantation of only the non-CSC tumor cells did not lead to sustained tumor growth [4].

The development of a CSC targeted therapy has to overcome three major obstacles, that relatively to the bulk tumor population is increased (a) chemoresistance, (b) resistance to radiotherapy, and (c) immunescape mechanisms.

Since radio- and chemotherapies have already been optimized to the limits of clinical benefit and yet tolerable side effects, a very attractive alternative approach of specifically targeting CSC is to develop antitumor T cell vaccines. One of the possible reasons that these therapies lacked efficacy in past clinical trials could be attributed to the fact that bulk tumor rather than CSC has been targeted. This may change with the identification of tumor specific epitopes derived from CSC markers. One such a CSC model-target for head and neck cancer and others is a recently described CD8-defined T-cell epitope of ALDH1 [76]. Examples of other such CD8-defined T-cell epitopes are available for prostate stem cell antigen [77]. Less well-defined approaches include the development of a CSC-dendritic cell vaccine [78]. Recent studies using animal models for prostate cancer and malignant glioma demonstrated the potential of different vaccination strategies (dendritic cells, gene-gun, and virus) targeting CSC in cancer immunotherapy [79, 80]. It was suggested recently that stemness-related proteins expressed in CSC might also be a source for tumor antigens. Tumor types most dependent on CSC for their growth kinetics were identified to be the best suited for approaches targeting stem cell genes [81].

In several studies, the efficacy of potential therapies directed against stem cell-associated signalling pathways was tested. For example, T cell immunity against embryonic stem cells antigens SOX2 and SOX6 has been explored in glioma stem cells [82, 83]. Since the expression of stemness-related genes is a common feature of stem cells and CSC, the question of vaccine-induced autoimmunity to stem cells will have to be addressed by scientists following

this path. One example is the vaccination with embryonic stem cells (ES) or induced pluripotent stem cells (iPS) that has been shown to induce protective immunity in colon carcinoma [84]. Another group used dendritic cells (DC) generated from mouse and human ES or iPS as a means for anti-cancer immunotherapy [85].

Success of these potential therapies will depend on how well immunological responses to CSC can be modulated for example by vaccine adjuvants upregulating antigen processing and presentation. Recently, a reduced activity of the 26S proteasome in breast cancer cells and in gliomas was observed as a feature of CSC [86]. Proteasomes are thought to play an important role in antigen processing and presentation of antigens in association with MHC I [87]. This may result in reduced antigen processing and presentation of peptides presented to the immune system on MHC I. Reduced proteasomal activity was also used to explain the high expression levels of known stem cell markers like BMI-1 and nestin in CSC, which are both substrates of the proteasome [88, 89].

The classification of conclusive CSC markers followed by the identification of defined T cell-recognized CSC epitopes in the future may lead to the clinical application of anti-CSC vaccination strategies. Several approaches are currently being evaluated (Table 2). Whether targeted therapies directed against stem cell-associated signalling pathways, which may be activated in stem cells and in CSC, will be of clinical use or be limited by undesirable side effects *in vivo* remains so far unresolved [4].

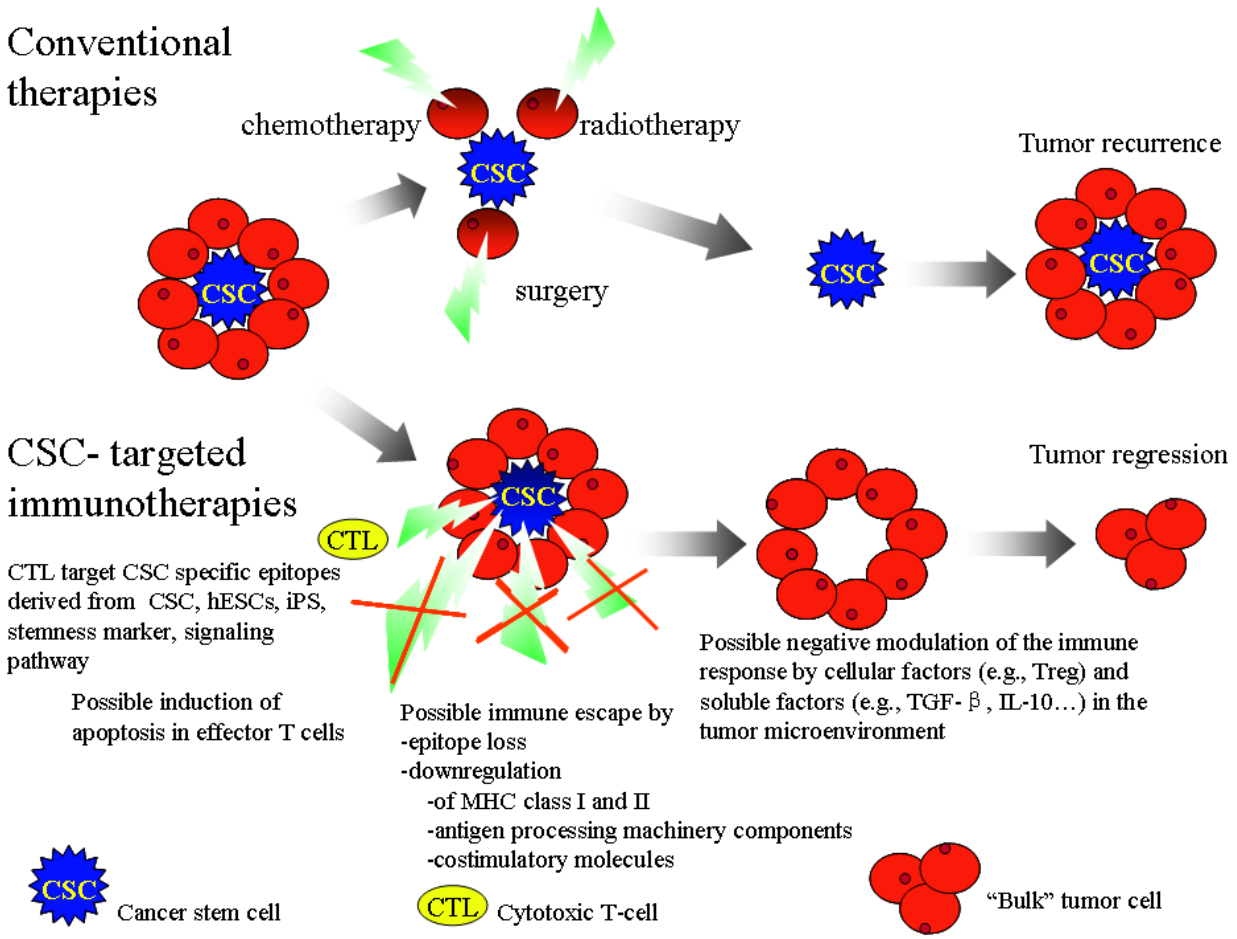

Fig. 3: Comparison of the effects of a failed conventional therapy and the outcome of a hypothetical CSC-targeted immunotherapy. Currently applied conventional therapies target bulk tumors cells that are less resistant than CSC. This leads to initial shrinking of the tumor mass but eventually regrowth from residual CSC. An immunotherapeutic approach targeting CSC directly would cut off rejuvenating supply of CSC and ultimately lead to tumor regression [4].

Table 2: Examples for studies targeting CSC with CTL

Target	Tissue	Ref
Dendritic cells loaded with CSC as antigen source	Glioblastoma	[78]
CD8 defined ALDH1-specific T cell epitope	HNSCC	[76]
Vaccination with murine prostate stem cell antigen encoding cDNA	Murine prostate cancer	[79]
Dendritic cells loaded with neurospheres from brain glioma cells	Murine glioma	[80]
Identification of two CD8 defined prostate stem cell antigen specific T cell epitopes	Prostate cancer	[77]
Vaccination with defined human embryonic stem cells (hESCs) or induced pluripotent stem (iPS) cells	Colon cancer	[90]
CD8 defined SOX2-specific T cell epitopes	Glioma	[82, 83]

1.6 Objectives

The aim of this project was to investigate the susceptibility of CSC to CTL-mediated lysis in comparison to monolayer-derived tumor cells.

Recently it was shown by others that human brain tumor stem cells (BTSC) were subject to immunologic recognition and elimination by $CD8^+$ CTLs using luciferase-based cytotoxicity assays *in vitro* and biophotonic imaging confirmation after tumor xenograft *in vivo*. Compared to $CD133^{low}$ tumor cells and established glioma cell lines, BTSC were equivalent with respect to expression levels of HLA class I and ICAM-1 (CD54), and equally susceptible to CTL-mediated cytolysis [91]. Until now, the susceptibility of CSC-derived from head and neck cancer or cervical cancer to CTL-mediated killing is still rudimentary. It is well known that HNSCC [92] and cervical cancer [93] express low levels of MHC class I and this may be one mechanism of immune evasion. In the current study, we compared the expression of these CTL immune-associated cell surface molecules of both tumor cell line monolayer-derived cells and spheroid-derived cancer stem-like cells. Moreover, we aimed to investigate if CTL can effectively and specifically target the CSC population by CTL-mediated killing. Finally, we assessed the susceptibility of $ALDH1^{high}$ and $ALDH1^{low}$ cancer stem-like spheroid-derived cells from HNSCC and cervical cancer cell lines to CTL-mediated cytotoxic killing and compared their sensitivity to the respective parental monolayer cell lines. This study represents the first investigation into the immunogenicity of $ALDH1^{high}$ cancer stem-like spheroid-derived cells with implications for targeting CSC-antitumor immunotherapy of cervical and head and neck cancer.

2. Materials

2.1 Laboratory Equipment

Axiovert 40 CFL	Carl Zeiss, Jena, Germany
Amaxa Nucleofector	Lonza, Basel, Switzerland
BD FACSCalibur System	BD Sciences, Heidelberg, Germany
BioRad Chromo 4	BioRad, München, Germany
Dynal MPC™-6 Magnetic Particle Concentrator	Invitrogen, Heidelberg, 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™ Plus Spectrophotometer	BioRad, München, Germany
Thermocycler	Eppendorf AG, Hamburg, Germany
Vortexer	Scientific Industries, N.Y., USA

2.2 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, 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, 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
Interferon-γ (IFN- γ)	ImmunoTools, Friesoythe, Germany
Neomycin (G-418)	Biochrom, Berlin, Germany
Pan mouse anti-human immunoglobulin G paramagnetic beads	BD Sciences, Franklin Lakes, USA
Penicillin/streptomycin, 1%	Biochrom, Berlin, Germany
Phosphate buffered saline (PBS) without Mg^{2+}/Ca^{2+}	Biochrom, Berlin, Germany
Plasmid pBJ coding for CD80 (B7.1)	Gift from Dr. L. L. Lanier
Propidium Iodide (PI)	Sigma, USA
Trypsin/EDTA Solution	Biochrom, Berlin, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany

Cell Culture Media

Dulbecco's Modified Eagled Medium with GlutaMAXTM-I (DMEM)	Invitrogen, Heidelberg, Germany
Quantum 263 medium	Biochrom, Berlin, Germany
RPMI 1640	Invitrogen, Heidelberg, Germany

Kits and other Materials

BD Falcon™ Cell Culture Flasks	BD Sciences, Franklin Lakes, USA
BD Falcon™ Polypropylene Conical Tubes (15 ml, 50 ml)	BD Sciences, Franklin Lakes, USA
BD Falcon™ Polystyrene Round-Bottom Tubes (5 ml)	BD Sciences, Franklin Lakes, USA
BD Falcon™ Tissue Culture Dish (100*20 mm)	BD Sciences, Franklin Lakes, USA
Cell Culture Plates (48-well, 96-well)	BD Sciences, Franklin Lakes, USA
Cluster Tubes, Polypropylene (1.2 ml)	Corning, NY, USA
Ultra-Low Attachment Cell Culture Flask (75 cm^2)	Corning, NY, USA
Aldefluor assay Kit	StemCell Technologies, NC, USA
Cell Line Nucleofector® Kit R	Lonza, Basel, Switzerland
CD4/CD8-Positive Isolation Kit	Dynal Biotech ASA, Oslo, Norway
Cytofix Fixation/Permeabilization Kit	BD Sciences, San Diego, CA
RNeasy Mini kit	QIAGEN, Hilden, Germany
High Capacity RNA-to-cDNA Kit	Applied Biosystems, Carlsbad, USA
Power SYBR Green Mix	Applied Biosystems, Foster City, CA, USA

RT-PCR-Primers

Transcript name	Forward primer sequence	Reverse primer sequence
Nanog	AATACCTCAGCCTCCAGCAGATG	TGCGTCACACCATTGCTATTCTTC
Oct3/4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
GAPDH (reference)	AGCTCCCAAAAATAGACGCAC	TTCATAGCAGTAGGCACAAAGG

2.3 Cell Lines and Cultured Media used

UM (University of Michigan) –SCC 11B (HPV -)	DMEM supplemented with 10% FBS, and 1% penicillin and streptomycin
UD (University of Düsseldorf)-SCC 2 (HPV16 +)	
CaSki (HPV16 +)	Quantum 263 medium supplemented with EGF and bFGF

2.4 Antibodies

FITC mouse anti-human MHC II	BD Pharmingen, CA, USA
FITC mouse anti-human CD58	ImmunoTools, Friesoythe, Germany
FITC mouse anti-human CD8	ImmunoTools, Friesoythe, Germany
FITC mouse anti-human IFN-γ	BD Pharmingen, CA, USA
PE mouse anti-human CD44	BD Pharmingen, CA, USA
PE mouse anti-human TNF-α	BD Pharmingen, CA, USA
PE mouse anti-human CD137	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
PE-Cy5 mouse anti-human CD86	BD Pharmingen, CA, USA
PerCP mouse anti-human CD4	BD Pharmingen, CA, USA
PerCP mouse anti-human CD8	BD Pharmingen, CA, USA
APC mouse anti-human MHC I	BD Pharmingen, CA, USA
APC mouse anti-human CD54	ImmunoTools, Friesoythe, Germany
APC mouse anti-human CD80	BD Pharmingen, CA, USA
APC mouse anti-human MHC II	BD Franklin Lakes, USA
APC mouse anti-human CD4	BD Franklin Lakes, USA
APC mouse anti-human CD3	ImmunoTools, Friesoythe, Germany
APC mouse anti-human CD154	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
APC mouse anti-human TNF-α	BD Pharmingen, CA, USA
Purified CD80-specific monoclonal antibody	ImmunoTools, Friesoythe, Germany
Purified mouse monoclonal to MHC class I framework, blocking antibody W6/32	Produced in the lab

3. Methods

3.1 Cell Lines and Cell Culture

Three cell lines were used in the experiments. The two HNSCC cell lines were UD (University of Düsseldorf)-SCC 2 (HPV16 +) and UM (University of Michigan)–SCC 11B (HPV -) [94]. The suffixes B indicate that the cell line was derived from a metastasis. These immortal cell lines have been in culture from primary tumor for approximately 40-60 generations. One cervical carcinoma cell line CaSki (HPV16 +) was obtained from ATCC. All the cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (10,000 U/ml and 10,000 mg/ml, respectively) at 37°C in 5% CO_2.

3.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. First, the 50 ml Leucosep centrifugation tube was filled with 15 ml Ficoll-Paque Plus and the anticoagulated blood sample was transferred on top of it without mixing of the solutions. Next, for separation of the cells, they were centrifuged for 15 min at 400xg without a break. After centrifugation the sequence of layers was as follows (from top to bottom): a) Plasma, b) white enriched cell fraction consisting of PBMC, c) separation medium, d) porous barriers, e) separation medium, f) pellet (erythrocytes and granulocytes). The white cell ring fraction was collected and transferred to a new 50 ml tube. A contamination with platelets was carefully prevented. Finally, the cell fraction was washed with PBS without Mg^{2+}/Ca^{2+} twice and then cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C in 5% CO_2.

3.3 Spheroid Cell Formation and Culture

Spheroids were generated in 3-D non-adherent culture with cytokines supporting their growth. Adherent monolayer cells were grown in 75 cm^2 tissue culture flasks until 80% confluency. Cells were washed with PBS without Mg^{2+}/Ca^{2+} twice and detached using Trypsin/EDTA solution. The reaction was stopped by adding complete culture medium after 5 min digestion. After centrifugation (200xg, 5 min) and washing with PBS without Mg^{2+}/Ca^{2+} twice, cells were resuspended in serum-free Quantum 263 medium, supplemented with 10 ng/ml EGF and 10 ng/ml bFGF. To generate spheroids, single cells were plated in Corning* Ultra-Low Attachment flasks at a specific density of 2×10^4 cells/ml. Cells were kept in the incubator at 37°C in

humidified atmosphere with 5% CO_2 content. At day three, half of the medium was replaced. After 5 to 7 days, representative pictures were taken and spheroids were collected by filtration through a 40 µm mesh for the following experiments.

3.4 Aldefluor Analysis

The ALDH1 activity of spheroid and monolayer cells was determined by using the Aldefluor assay kit. 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 µm pipette tip. After quenching the reaction with the same volume of complete culture medium, the cells were pelleted. Then the single cell suspension was washed twice in PBS buffer (without Mg^{2+}/Ca^{2+}) and suspended in 1 ml ALDEFLUOR assay buffer containing 5 µl ALDH substrate (BAAA, 1 mol/per 1×10^6 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 staining and washing twice, cells were maintained in ALDH1 buffer on ice during all subsequent procedures.

3.5 Flow Cytometry Analysis

Spheroids, treated with or without interferon-γ (IFN-γ), were cultured for 7-10 days. Subsequently, a singe cell suspension of spheroid-derived cells (SDC) and monolayer-derived cells (MDC) was analyzed by flow cytometry to assess the expression of MHC class I, MHC class II, CD54, CD58, CD80, and CD86. A suspension of 10^5 cells was washed twice with FACS buffer (PBS with 0.1% BSA) and incubated on ice for 15 min with APC-conjugated anti-human MHC class I (clone G46-26, IgG1), CD54 (clone HA58, IgG1), FITC-conjugated anti-human MHC class II (clone TÜ39, IgG2a), PE-cy5-conjugated anti-human CD86 (clone FUN-1, IgG1), FITC-conjugated anti-human CD58 (clone MEM-63, IgG1), PE-conjugated anti-human CD44 (clone MEM-85, IgG2a), APC-conjugated anti-human CD80 (clone MEM-233, IgG2b) monoclonal antibodies, respectively. Stained cells were washed twice with FACS buffer and then resuspended in 100 µl FACS buffer for flow cytometric analysis.

For triple staining with the ALDEFLUOR assay and MHC class I, CD54, or MHC class II, 20 µl APC-conjugated anti-human MHC class I, MHC class II, or CD54 monoclonal antibodies (Ab) and 2 µg/ml Propidium Iodide (PI) were added and incubated on ice for 15 min, after 45 min of incubation with ALDH substrate and with or without ALDH inhibitor. Following incubation, cells were washed once with cold FACS buffer and analyzed by flow cytometry immediately.

The entire target cell population was defined by a live gate in a forward scatter/side scatter dot plot (Fig. 4A). The viability of cells was assessed in a FL-3 (PI)/side scatter dot plot (Fig. 4B). For analysis, acquired cells were subgated in FL-1 (ALDH1)/FL-4 (respective Ab) dot plots as shown in Fig. 4C, D.

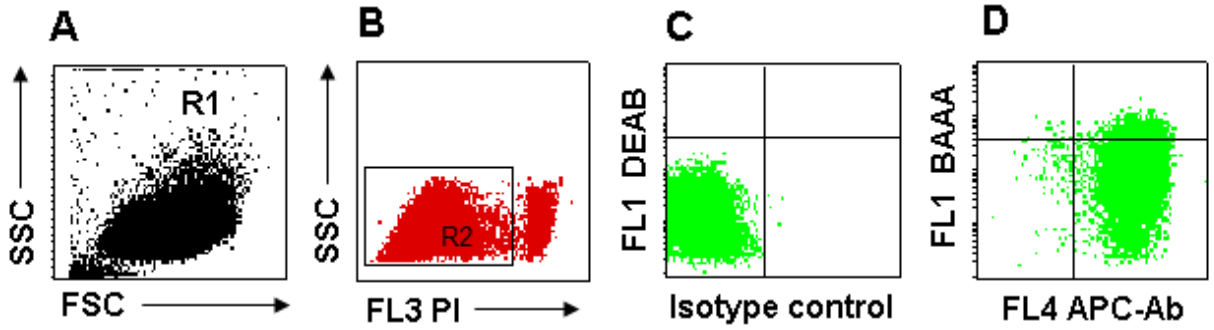

Fig. 4: Principle of flow cytometric analysis of MHC class I, MHC class II, or CD54 expression on ALDH1^{high} and ALDH1^{low} populations of SDC and MDC. (A) After 45 min of incubation with ALDH substrate and with or without ALDH inhibitor, cells were stained with MHC class I, MHC class II, and CD54 Ab. Double stained cells were collected in a live gate by flow cytometry. (B) To gate the viable cells, FL-3 (PI) dot plot was used to distinguish live cells and dead cells. (C) Cells incubated with ALDH inhibitor and isotype control were gated as negative control in a quadrant plot. (D) Cells stained with ALDH substrate and APC-Ab were the double positive cells.

3.6 Generation of Stably CD80-Expressing Cell Lines

For proper activation, T cells require a costimulatory signal, which is achieved by binding of CD28 to CD80 or CD86. For our experiments, we generated CD80-positive tumor cell lines acting as artificial APC to present tumor antigen and alloantigen (allogeneic MHC molecules as model antigens) to PBMC to induce activated T cell lines reactive with the tumor cell lines. Tumor monolayer cells were transfected by electroporation with the plasmid pBJ coding for CD80 (B7.1) in an Amaxa Nucleofector electroporator using Cell Line Nucleofector® Kit R and program A-28. Transfected cells were incubated with G418 (500 µg/ml) to select for stable transfectants. After 3-4 weeks, non-transfected tumor cells died and transfectants grew as tumor cell clones. A CD80-specific monoclonal antibody coupled to pan-mouse anti-human immunoglobulin G paramagnetic beads was used to enrich positive cells by magnet-activated cell sorting (MACS) for stable CD80 expression of the tumor cell clones. Briefly, the tumor cell clone pellet was mixed with purified CD80-specific monoclonal antibody at 2-8°C for 10 min. After washing in 1 ml FACS buffer and centrifugation for 8 min at 250xg, cells were incubated with 15 µl pan-mouse anti-human immunoglobulin G paramagnetic beads in 1 ml FACS buffer for 20 min at 2-8°C with gentle tilting and rotation. Then the cells were placed in a magnet for 2 min and washed in 5 ml FACS buffer in the magnet for 3-4 times. As determined by flow-cytometry, about 95% of the cells of this population was CD80-positive and the cell lines were now called CaSki-B7.1 and UM-SCC11B-B7.1. They were maintained in DMEM complete culture medium, and were subsequently used as source for target cells to present peptides to CTL.

3.7 Generation of Allogeneic T Cell Lines

PBMC (1-2x10^5) of three donors were cocultured with the irradiated (30 Gray) transfected tumor cells (5-10x10^3) CaSki-B7.1 and UM-SCC11B-B7.1 in a round-bottom 96-wells plate in RPMI 1640 supplemented with 10% FBS, 1% penicillin and streptomycin, 10 IU/ml IL-2 and 10 IU/ml IL-7 in a total volume of 200 µl for 7 days. Fresh medium was added every 3 days. Weekly, cultures were restimulated with freshly irradiated transfected tumor cell preparations as described above. After 3 weeks, cells were stained with a FITC-conjugated anti-human CD8 monoclonal antibody (clone RPA-T8) and an APC-conjugated anti-human CD4 monoclonal antibody (clone L200) to determine the percentage of $CD8^+$ T cells in the whole T cell population. In these cultures, frequencies of $CD8^+$ T cells were in the range of 20%-25% and these cell lines were used for $CD8^+$ T cell isolation. Activated $CD8^+$ T cells were allo-antigen

specific (tumor cell line MHC molecules) and were used to measure general susceptibility for CTL killing of CSC.

3.8 Determination of $CD8^+$ T Cell Function by Cytokine Staining

To determine the function of $CD8^+$ T cells, stimulated T cells were stained for CD137, TNF-α, and IFN-γ production. T cell lines were cultured with or without irradiated tumor-B7.1 cells in 200 µl RPMI complete medium in 96 well round-bottom plates. After 2 hours, 2 µl Brefeldin A (BFA; 10 µl/ml solution) was added directly into each well and incubated at 37°C for 16-20 hours. Then T cells were harvested, fixed and permeabilized using the Cytofix Fixation/Permeabilization Kit as described in the manufacturer's protocol. In brief, the harvested T cells pellet was resuspended in 100 µl fixation/permeabilization solution (in the Kit) for 20 min in 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 CD137, PerCP-conjugated anti-human CD8, 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.

3.9 Isolation of $CD4^+$ and $CD\,8^+$ T Cells by MACS

$CD4^+$ or $CD8^+$ T cells were obtained from stimulated allogeneic T cell lines as described above by positive selection using magnetic beads coated with an anti-CD4 or anti-CD8 antibody as described in the manufacturer's instructions. Briefly, the stimulated allogeneic T cells were resuspended in 1 ml PBS without Mg^{2+}/Ca^{2+} for 20 min at 4°C and Dynabeads (in the Kit) were added. After 3 times washing with 5 ml FACS buffer in a magnet, cells were detached by incubating with Detachabeads (in the Kit) for 45 min at room temperature. Following 3 times washing with 5 ml FACS buffer in a magnet, cells were stained with a CD4 or CD8-specific monoclonal antibody for 20 min on ice and then measured by FACS analysis. The isolated $CD4^+$ or $CD8^+$ T cells were 95%-99% positive for CD4 or CD8 and were subsequently used as effector cells (E).

3.10 $CD4^+$ or $CD8^+$ CTL Cytotoxicity Assay

The $CD4^+$ or $CD8^+$ CTL cytotoxicity was assessed by VITAL-FR assay, a versatile fluorometric technique for assessing CTL-mediated cytotoxicity against multiple targets *in vitro* [95]. As target cells (T), treated or untreated with IFN-γ, SDC and parental MDC (5-10x10^5) were labeled with 2 µl/ml CFSE and Far Red (5 µM stock concentration) respectively for 7 min in 2 ml DMEM without FBS at 37°C. After quenching the reaction by adding the same volume of FBS, cells were thoroughly washed with complete culture medium and immediately used in the cytotoxicity assay. To determine the MHC class I antigen restriction of the CTL reactivity, labeled target cells were incubated with MHC class I antigen-specific mAb W6/32 (final concentration 10 µg/ml) for 30 min at 37°C before addition of effector CTL. E was seeded with a constant number of CFSE/Far Red-labeled target cells (T) (1 x 10^3) at different E:T ratios (0.1:1, 1:1), respectively. In parallel, target cells were incubated alone to measure basal lysis. Cells were incubated in 48-well microplates in a total volume of 200 µl RPMI 1640 complete culture medium supplemented with 10 IU/ml IL-2 and in a 5% CO_2 atmosphere at 37°C. After up to 24 hours, two target cell mixtures were collected and incubated in FACS buffer containing 2 µg/ml PI for 10 min on ice, then cells were immediately assessed by flow cytometry. The entire target cell population was defined by a live gate in a forward scatter/side scatter dot plot. The vitality of cells was shown in a FL-3 (PI) dot plot. Specific target cells were denoted by regions (R) in FL-1 (CFSE)/FL-4 (Far Red) dot plots and detected and enumerated as specific target cells as $CFSE^+$ (R4) and control target cells as Far Red^+ (R3) as shown in Fig. 5. Unlabeled cells comprised the effector cell population. The percentage of specific lysis (PSL) was calculated from the ratio R4/R3 in cultures containing defined numbers (n) of effector T cells $(R4/R3)_n$ in comparison to control (co) wells without T cells $(R4/R3)_{co}$ using the formula: $1-[(R4/R3)_n/(R4/R3)_{co}] \times 100\%$.

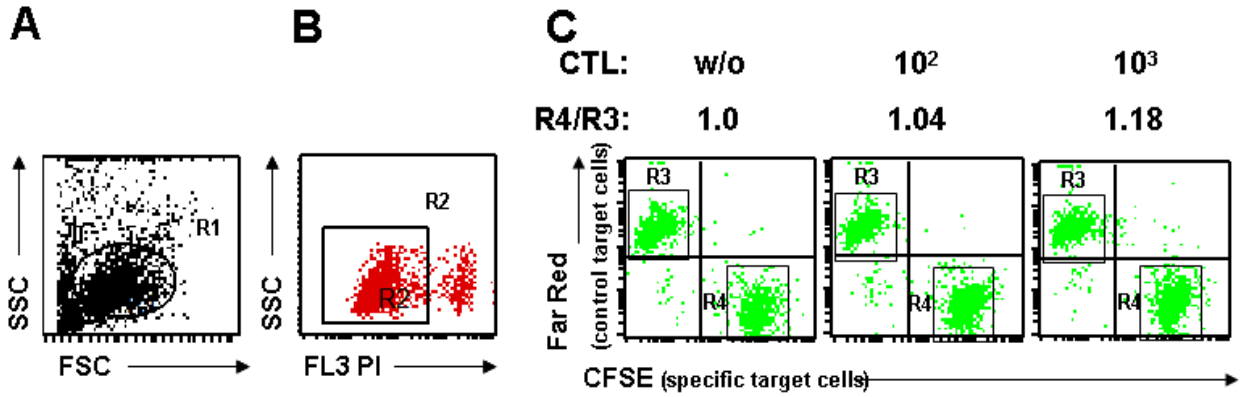

Fig. 5: Flow cytometric analysis of target cell lysis by VITAL-FR assay. (A) Specific target cells were stained with CFSE and control target cells were labeled with Far Red. Mixtures of 10^3 specific and 10^3 control target cells were incubated either alone or in the presence of titrated CTL numbers. After 24 hours target cells were acquired in a live gate (R1) by flow cytometry. (B) To gate the viable cells, a SSC/FL-3 (PI) dot plot was used to distinguish live cells and dead cells. (C) 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. Tumor-specific CTL-mediated target cell lysis was calculated in comparison to control cultures without CTL.

3.11 Allogeneic $CD8^+$ CTL Cytotoxic Lysis of $ALDH1^{high}$ and $ALDH1^{low}$ Cell Populations

As target cells, 1 x 10^5 spheroid-derived cells were cocultured with allogeneic $CD8^+$ CTL at different E:T ratios (0.1:1, 1:1). In parallel, target cells were incubated alone to measure basal ALDH1 expression level. Target cells were collected after 24 hours and stained with the ALDEFLUOR assay as described above. After 45 min of incubation with ALDH substrate and with or without ALDH inhibitor, 5 µl APC-conjugated anti-human CD3 mAb was added and incubated on ice for 15 min. CD3 mAb was used to gate out the T cells to prevent their contamination of the target cells. Following incubation, cells were washed once and stained with cold FACS buffer containing 2 µg/ml PI for 10 min on ice, then cells were immediately assessed by flow cytometry.

3.12 Quantitative Real-Time PCR

Total RNA was extracted by using a RNeasy Mini kit, then converted to cDNA by RT-PCR using a High Capacity RNA-to-cDNA Kit. Quantitative real-time PCR was performed by the ABI Power SYBR Green mix and run on a BioRad Chromo 4. 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.

3.13 Statistical Analysis

The extent of tumor-specific lysis was determined from the variation coefficients and lysis was considered significant when it was higher than the threefold standard deviation of the control wells with the target cells only. For statistical comparison, we used the SPSS software for Windows (version 15; SPSS, Chicago, IL, USA). Students *t*-Test was used to analyse statistical significance of the data.

4. Results

4.1 Sphere Formation and ALDH Activity of both MDC and SDC expanded from Carcinoma Cell Lines of the Head and Neck and Cervix Uteri.

Cells from three cell lines derived from human carcinoma of the head and neck (two) and cervix (one) were grown in suspension at low density in defined serum-free medium with bFGF and EGF for 7-10 days. All of them were able to form spheroids in suspension under non-adherent conditions (Fig. 6A). The self-renewing capacity of these spheroids has been confirmed by the *in vitro* spheroid colony formation method as shown previously [17]. High ALDH1 activity is a highly selective marker for CSC in HNSCC and cervical cancer cell lines [45, 48]. We measured ALDH1 enzymatic activity of the SDC of 3 cancer cell lines and their matched MDC to investigate the presence of a stem cell-like population. As controls, 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. 6B, MDC and SDC of UM-SCC11B showed the highest expression of ALDH1-positive cells (MDC: 20.70 ± 3.469%, SDC: 42.39 ± 8.325%) ($P<0.05$) as compared with UD-SCC2 (MDC: 4.55 ± 0.764%, SDC: 9.68 ± 1.115%) ($P<0.05$) and CaSki (MDC: 7.11 ± 1.533%, SDC: 27.97 ± 5.268%) ($P<0.05$). The data showed that SDC from all 3 cell lines had a significantly increased frequency of ALDH1-expressing cells as compared with parental MDC.

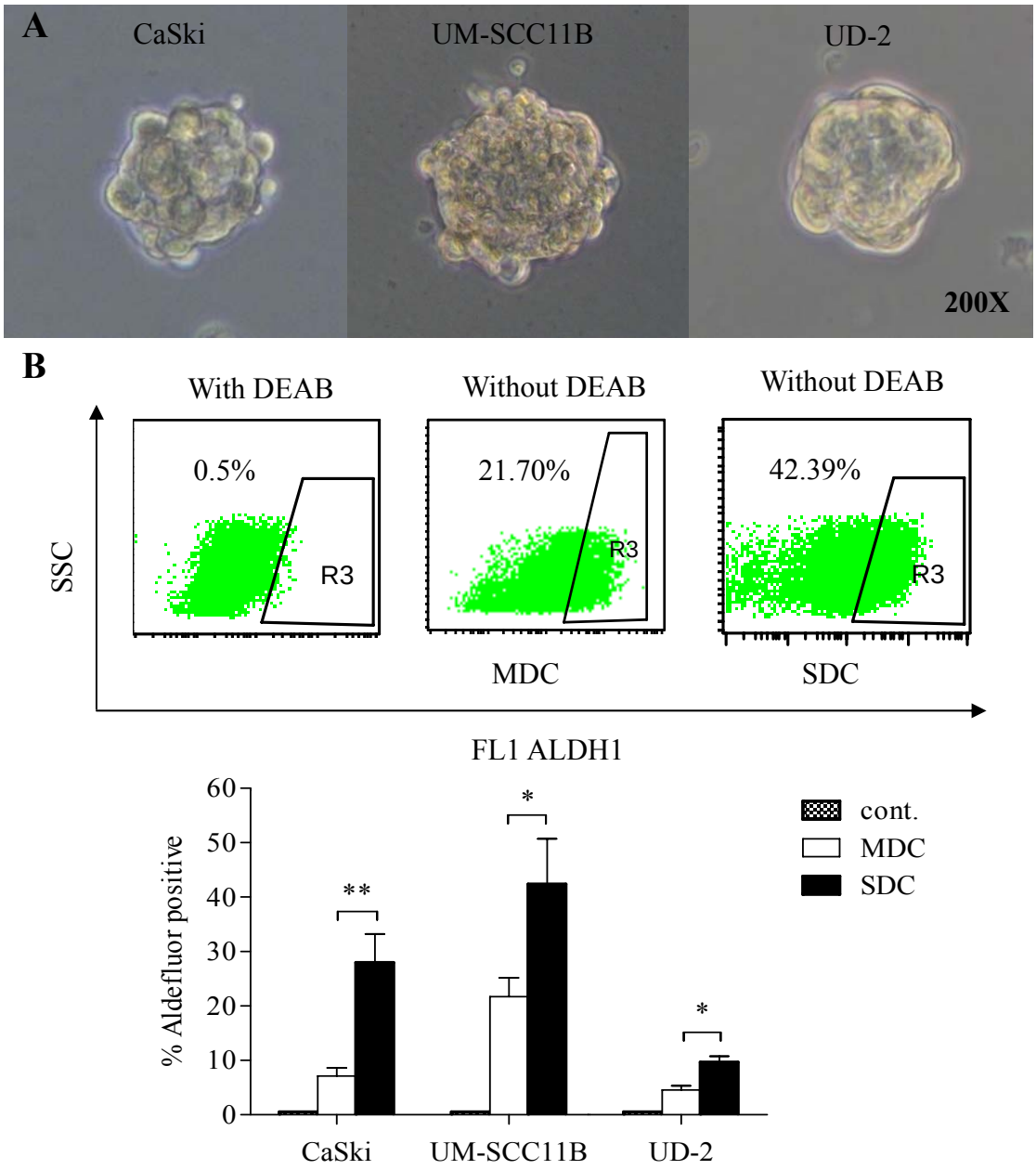

Fig. 6: Examples of SDC from 3 cancer cell lines in serum-free medium and comparison of ALDH1 expression in MDC and SDC by flow cytometry. (A) Example of SDC formed by CaSki, UM-SCC11B and UD-2 in suspension cultures in defined serum-free medium supplemented with bFGF and EGF after 7-10 days' seeding. (B) The expression of ALDH1 in SDC (black columns) compared to in MDC (open columns), control cells stained with DEAB, a specific inhibitor of ALDH1. The highest ALDH1 expression was found in UM-SCC11B MDC (20.70 ± 3.469) and SDC (42.39 ± 8.325). UD-2 had the lowest ALDH1 level, MDC and SDC were 4.55 ± 0.764 and 9.68 ± 1.115, respectively. All cell lines were evaluated in three independent experiments (mean % ± SD). The difference in ALDH1 expression between MDC and SDC were all statistically significant ($P<0.05$).

4.2 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 [96, 97]. To investigate if SDC also share this feature of TF expression with embryonic stem cells, we quantitatively compared the mRNA expression of these TF between SDC and parental MDC (Fig. 7). The mRNA levels of Oct3/4 and Nanog were found to be increased in the SDC of all 3 cell lines, whereas enhanced expression of SOX2 only was seen in UM-SCC11B and UD-2 SDC but was not significantly enhanced in SDC derived from CaSki cells. The highest increase was observed in UD-2, where a 16-fold increase in Nanog expression was found in SDC. By comparison, the smallest change, a 0.2-fold increase in SOX2 expression, was seen in CaSki SDC which had no significance.

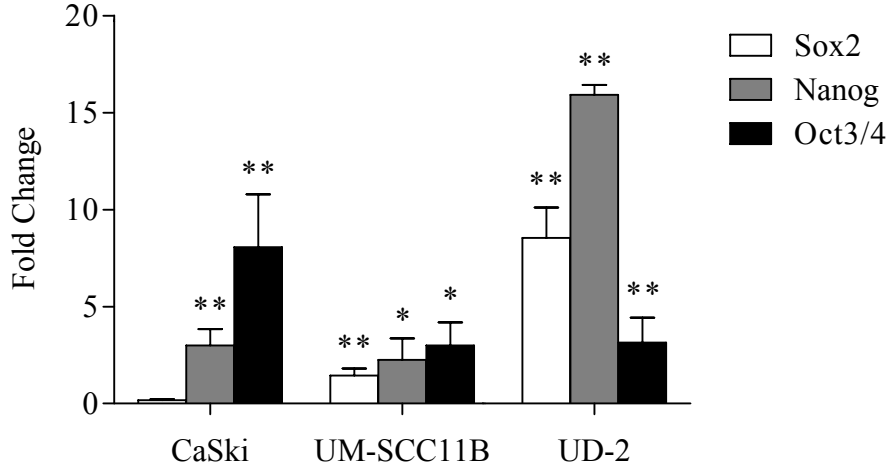

Fig. 7: Quantitative PCR analysis of mRNA expression of stemness-related transcription factors (TF). Messenger RNA isolated from SDC and MDC was quantified for expression of the indicated TF. The ratio of expression in SDC to MDC is shown. The mRNA levels of Nanog and Oct3/4 were increased in SDC from all 3 cell lines, the highest increase, nearly 16-fold, was Nanog expression in UD-2 SDC. The SOX2 mRNA level was enhanced in SDC of UM-SCC11B and UD-2, about 1.5-fold and 8.5-fold, respectively. Mean values ± SD of three determinations. Significant differences are * $P < 0.05$; ** $P < 0.01$.

4.3 Expression of T-cell/Tumor Immunology-Associated Cell Surface Molecules of SDC and MDC.

It is well documented that tumors can escape from T cell-mediated immune recognition by down-regulating molecules essential for antigen processing and presentation or costimulation [4]. Tumor cells may down-regulate MHC expression through epigenetic modification or other mechanisms to escape immune surveillance [78]. Although there are some reports regarding the basal level of MHC expression on glioma stem cell lines [98, 99], its expression on cervical and head and neck carcinoma stem cell are still elusive. To examine the distinct SDC and MDC for potential differences in molecules that are essential for T cell/tumor immune response, the expression of MHC class I and MHC class II and immune-recognition associated molecules was analyzed on SDC and MDC generated from the 3 cell lines. In addition we investigated if an upregulation by stimulation with IFN-γ could be achieved.

As shown in Fig. 8, we found that SDC and MDC from all 3 cell lines expressed MHC class I. Its expression on CaSki SDC was nearly two times lower than on MDC, but there was no change on UM-SCC11B or UD-2. CD54 was expressed on SDC and MDC of CaSki and UM-SCC11B but not UD-2. CD58 was positive only on SDC of CaSki and SDC and MDC of UD-2. In contrast, MHC class II, CD80 and CD86 were negative on SDC and MDC of all 3 cell lines. For CaSki, pretreating with IFN-γ had almost the same effect on both SDC and MDC. IFN-γ could upregulate the expression of MHC class I, MHC class II and CD54 about 2.6-fold, 98.2-fold, and 4.8-fold, respectively. The effect of IFN-γ on SDC and MDC of UM-SCC11B was also nearly the same. The expression of MHC class I and CD54 was upregulated about 4.2-fold and 3.8-fold, respectively. Only the expression of MHC class I could be upregulated about 3.9-fold by IFN-γ on both SDC and MDC of UD-2.

On the basis of above data, we examined MHC class I, MHC class II, and CD54 expression on $ALDH1^{high}$ (AHP) and $ALDH1^{low}$ (ALP) populations of SDC and MDC of 3 cell lines (Table 3, 4, 5). We also investigated if there was an upregulation by stimulation with IFN-γ. Both AHP and ALP of SDC and MDC of CaSki expressed MHC class I and CD54 but not MHC class II. Moreover, the level of MHC class I of both AHP and ALP of SDC was lower than MDC. For AHP and ALP of CaSki, IFN-γ could upregulate the expression of MHC class I about 1.6-fold on MDC and 2.2-fold on SDC, CD54 about 4.7-fold on MDC and 5.6-fold on SDC, MHC class II nearly 34.5-fold and 29-fold on MDC, 92.3-fold and 86.6-fold on SDC, respectively. However, there was no statistical significance in the difference of AHP and ALP of both SDC and MDC. For UM-SCC11B, AHP and ALP of both SDC and MDC were MHC class I and CD54 positive

but MHC class II negative. And they had nearly the same sensitivity to the treatment with IFN-γ. Pretreated with IFN-γ, the expression of MHC class I of both populations increased about 3.8-fold on MDC and 2.0-fold on SDC, CD54 increased about 4.4-fold on MDC and 4.7-fold on SDC. In contrast, AHP and ALP of both SDC and MDC of UD-2 did not express MHC class I, MHC class II, and CD54, and they also had nearly the same sensitivity to the treatment of IFN-γ. The level of MHC class I could enhance about 2.4-fold on MDC and 3.5-fold on SDC, MHC class II enhanced about 44-fold on MDC and 33-fold on SDC pretreated with IFN-γ.

Table 3: MHC class I expression of AHP and ALP on MDC (M) and SDC (S) pretreated with (+) or without (-) IFN-γ.

			MHC class I	
			- IFN-γ	**+ IFN-γ**
CaSki	M	AHP	1198.25 ± 77.87[a, #]	1833.61 ± 62.68*, #
		ALP	1149.33 ± 72.48[#]	1953.00 ± 60.12*, #
	S	AHP	439.62 ± 55.39	997.08 ± 59.59*
		ALP	449.18 ± 55.68	915.63 ± 58.64*
UM-SCC11B	M	AHP	81.13 ± 5.33	313.77 ± 34.05**, #
		ALP	73.69 ± 4.38	270.56 ± 33.89**, #
	S	AHP	87.43 ± 2.38	176.54 ± 5.67*
		ALP	80.37 ± 2.06	164.83 ± 5.54*
UD-2	M	AHP	26.52 ± 3.23	66.83 ± 3.41*
		ALP	31.83 ± 3.16	74.18 ± 3.33*
	S	AHP	21.30 ± 2.5	65.72 ± 0.81**
		ALP	18.29 ± 2.42	68.09 ± 0.73**

[a)] Mean values ± SD of mean fluorecence intensity of three independent determinations.

* represents statistically significant differences of the group "+ IFN-γ" compared to the group "- IFN-γ"

[#] represents statistically significant differences of AHP and ALP of M compared to those of S

Significant differences: *, [#] $P<0.05$; ** $P<0.01$.

Table 4: MHC class II expression of AHP and ALP on MDC (M) and SDC (S) pretreated with (+) or without (-) IFN-γ.

			MHC class II	
			- IFN-γ	**+ IFN-γ**
CaSki	M	AHP	12.44 ± 0.02[a)]	429.74 ± 15.87**, #
		ALP	13.19 ± 0.02	382.71 ± 14.95**, #
	S	AHP	12.24 ± 0.09	1130.12 ± 80.01**
		ALP	11.37 ± 0.08	984.95 ± 80.54**
UM-SCC11B	M	AHP	4.85 ± 0.28	10.00 ± 1.7*
		ALP	4.94 ± 0.3	8.22 ± 1.65
	S	AHP	5.90 ± 0.04	6.87 ± 0.63
		ALP	5.36 ± 0.04	6.33 ± 0.71
UD-2	M	AHP	6.43 ± 1.07	286.18 ± 42.27**
		ALP	5.21 ± 1.12	228.03 ± 41.54**
	S	AHP	8.23 ± 0.59	268.46 ± 9.69**
		ALP	7.39 ± 0.63	243.53 ± 9.5**

[a)] Mean values ± SD of mean fluorecence intensity of three independent determinations.
* represents statistically significant differences of the group "+ IFN-γ" compared to the group "- IFN-γ"

[#] represents statistically significant differences of AHP and ALP of M compared to those of S
Significant differences: *, [#] $P<0.05$; ** $P<0.01$.

Table 5: CD54 expression of AHP and ALP on MDC (M) and SDC (S) pretreated with (+) or without (-) IFN-γ.

			CD54	
			- IFN-γ	**+ IFN-γ**
CaSki	M	AHP	343.59 ± 63.56[a, #)]	1630.88 ± 363.1**, #
		ALP	260.49 ± 60.18[#]	1245.24 ± 360.82**, #
	S	AHP	658.45 ± 26.36	3684.44 ± 216.4**
		ALP	593.21 ± 25.74	3403.98 ± 215.5**
UM-SCC11B	M	AHP	62.94 ± 6.98	282.49 ± 56.85**
		ALP	50.18 ± 6.81	211.61 ± 55.49**
	S	AHP	43.44 ± 0.15	211.32 ± 25.47**
		ALP	41.86 ± 0.14	182.58 ± 24.32**
UD-2	M	AHP	22.67 ± 5.3	26.49 ± 1.72
		ALP	15.79 ± 5.21	23.89 ± 1.63
	S	AHP	21.03 ± 0.78	23.47 ± 0.53
		ALP	20.22 ± 0.68	21.91 ± 0.46

[a)] Mean values ± SD of mean fluorecence intensity of three independent determinations.
* represents statistically significant differences of the group "+ IFN-γ" compared to the group "- IFN-γ"

[#] represents statistically significant differences of AHP and ALP of M compared to those of S

Significant differences: *, [#] $P<0.05$; ** $P<0.01$.

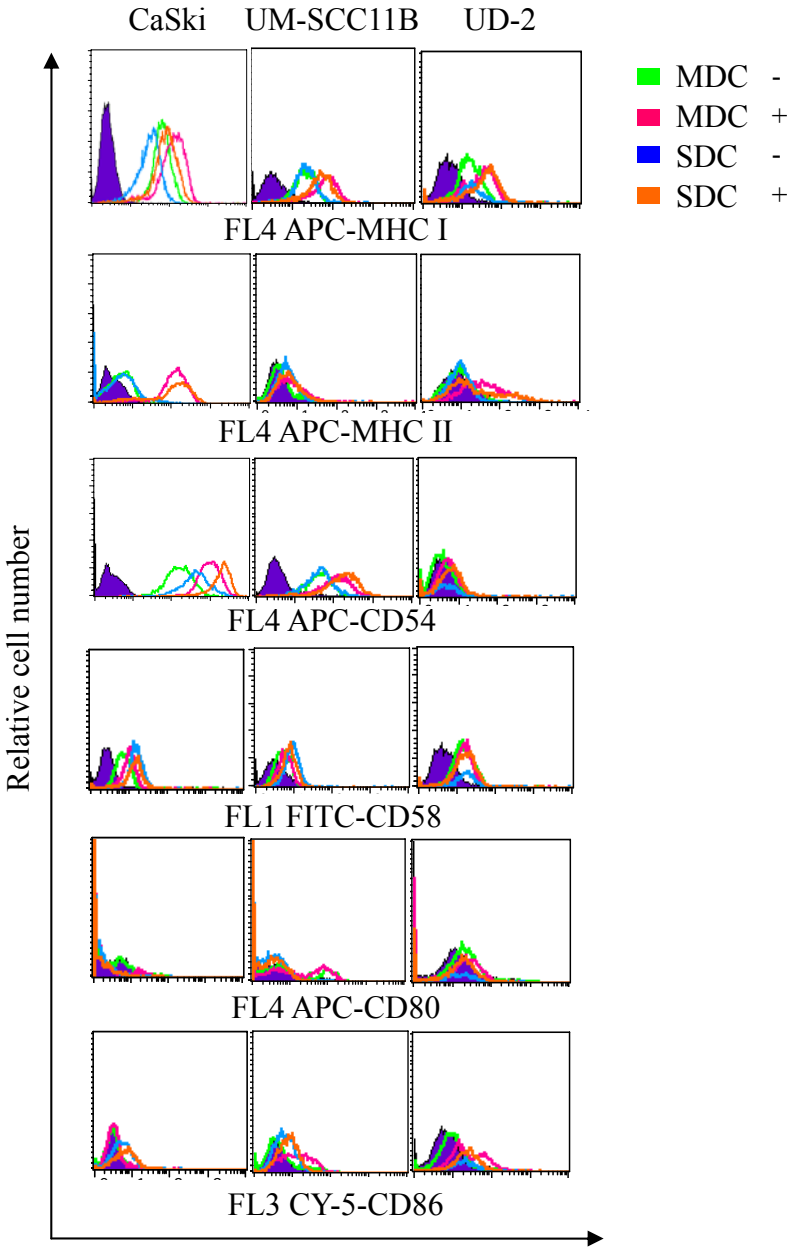

Fig. 8: Flow cytometric analysis of MHC class I, MHC class II and co-stimulatory molecule expression determined in monolayer-derived (M) and spheroid-derived (S) cells, either pretreated with (+) or without (-) IFN-γ. M (-), M (+) and S(-), S (+) cells were analyzed by flow cytometry using anti-HLA-A,B,C, anti-HLA-DR, anti-CD54, anti-CD58, anti-CD80, anti-CD86 (line) or isotype control antibodies (solid). Data are representative for three independent determinations.

4.4 CD80-positive Tumor-B7.1 Cell Lines

As shown in Fig. 8, all 3 tumor monolayer cell lines did not express CD80. To obtain the tumor cell lines stably expressing CD80, we transfected by electroporation three cell lines with the expression vector pBJ containing the SV40/HTLV1(SRα) promoter, and the CD80 gene plus the neomycin resistance gene. The transfected cells were selected for neomycin resistance. The CD80-positive cells were sorted using CD80-specific monoclonal antibody by FACS and passaged for a prolonged time in normal tumor cell culture. Two stably transfected cell lines, CaSki-B7.1 and UM-SCC11B-B7.1 could be generated. Repeated attempts failed to generate a stably B7.1-expressing cell line from UD-2. As shown in Fig. 9, the parental tumor cells (CaSki and UM-SCC11B) were negative for CD80 expression whereas the transfectants expressed the molecule on their surface. As described previously [100], stimulation of allogeneic PBMC with CD80-positive tumor cells resulted in proliferation of $CD4^+$ and $CD8^+$ lymphocytes. CD80 has been shown to be sufficient and important for activation of a primary T cell response to tumor cells. The newly generated CD80-positive tumor cell lines were subsequently used to present peptides and allogeneic MHC molecules to T cells and to generate activated primary alloantigen-specific T cell lines.

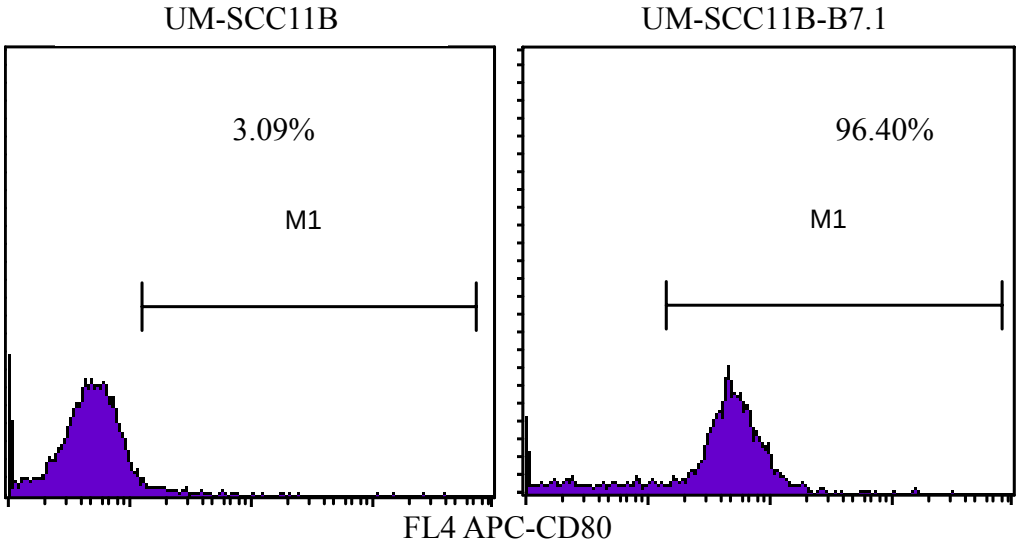

Fig. 9: Examples of CD80 expression by pBJ-transfected tumor-B7.1 cell lines. Tumor monolayer cells were transfected by electroporation with the plasmid pBJ coding for CD80 (B7.1) and were selected by G418 (500 ug/ml) for enrichment of stable transfectants. Transfectants were selected by CD80-specific monoclonal antibody for CD80 expression. As determined by flow-cytometry, about 95% of the cells of this population were CD80-positive (M1). Left: non-transfected parental tumor cells (3.09% CD80 positive in M1). Right: pBJ-transfected tumor-B7.1 cells (96.04% CD80-positive in M1).

4.5 Frequencies of $CD4^+$ and $CD8^+$ T cells in Allogeneic T Cell Lines

The T cell lines which were activated by CaSki-B7.1 and UM-SCC11B-B7.1 were composed of both CD4- and CD8-positive cells. To determine the proportion of $CD4^+$ and $CD8^+$ lymphocytes of the growing T cell lines, the PBMC were restimulated once a week for two weeks with irradiated tumor-B7.1 cells supplemented with IL-2 (10 IU/ml) and examined by FACS analysis. As identified by double staining, the CaSki-B7.1-stimulated PBMC contained 69.83% CD4 and 22.54% CD8 T cells, while UM-SCC11B-B7.1-stimulated PBMC had 68.6% $CD4^+$ T cells and 24.87% $CD8^+$ T cells (Fig. 10 A). For further experiments, we isolated $CD4^+$ and $CD8^+$ T cells by MACS and assessed purity by FACS analysis. The isolated $CD4^+$/$CD8^+$ T cells were 95%-99% positive for CD4 or CD8, respectively (Fig. 10 B), and were subsequently used as effector cells (E).

4.6 Identification of the Function of $CD8^+$ CTL

To determine whether the CD80-expressing tumor cells can directly activate T cells, and to verify that our stimulation protocol had primed naive T cells to react against allo-antigens, we stimulated the PBMC once a week for two weeks with irradiated tumor-B7.1 tumor cells supplemented with IL-2 and stained the proliferating T cells for cytokine production. As identified by four color staining as shown in Fig. 11, the proliferating $CD8^+$ T cells expressed CD137, TNF-α and IFN-γ with time. After one week (first stimulation), the expression of CD137, TNF-α and IFN-γ was 1.08%, 0.18%, and 0.04%, respectively. After two weeks (second stimulation), the expression increased to 12.48%, 1.84% and 4.27% respectively. These data indicate that stimulation of allogeneic PBMC with tumor-B7.1 tumor cells results in activation and proliferation of allogeneic $CD8^+$ CTL.

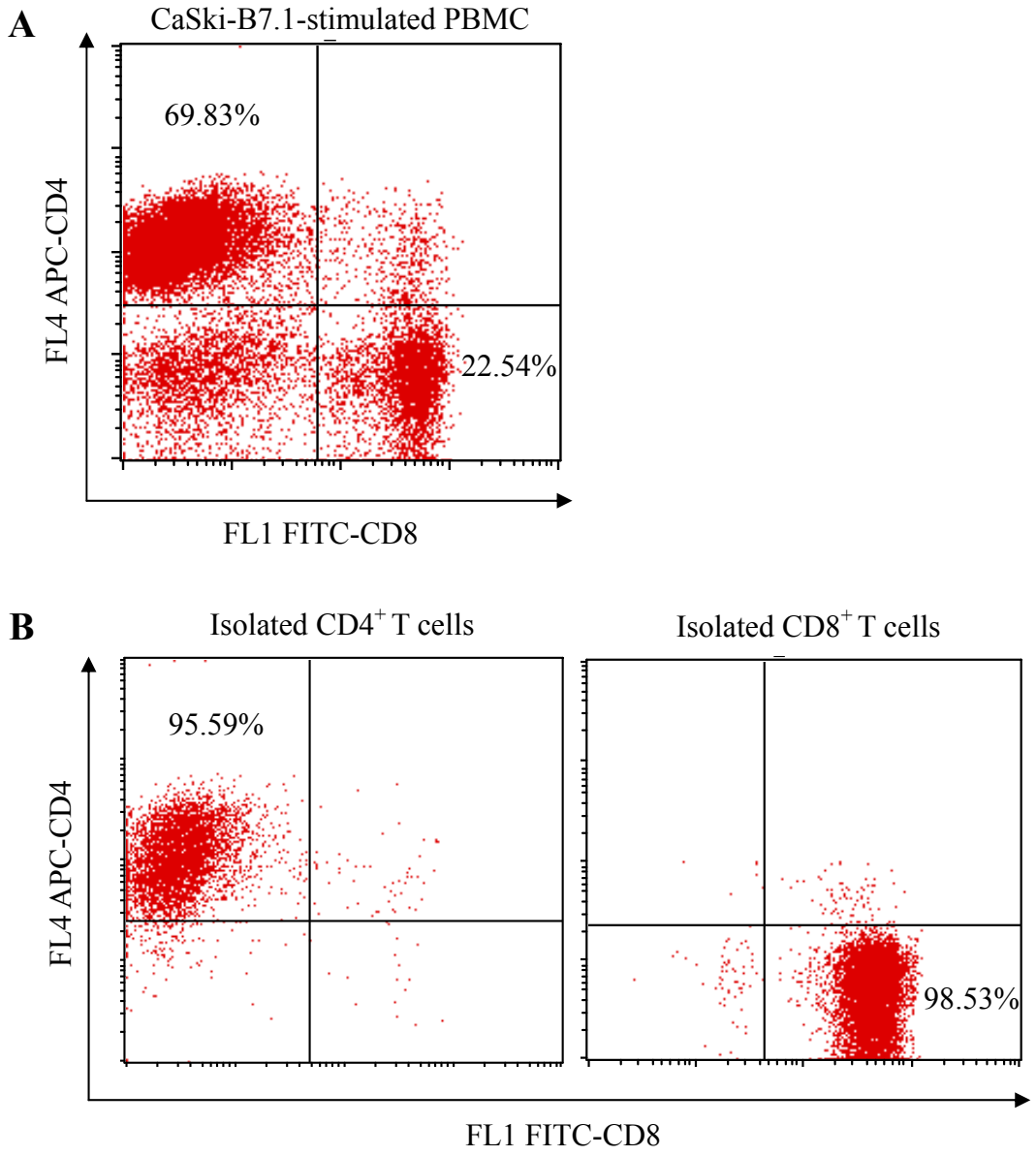

Fig. 10: Example of $CD4^+$ and $CD8^+$ T cell frequency in allogeneic T cell lines. PBMC were restimulated once a week for two weeks with irradiated tumor-B7.1 cells supplemented with IL-2 (10 IU/ml) and then the frequency of CD4- and CD8-positive cells examined by FACS analysis. (A) As identified by double staining, CaSki-B7.1-stimulated allogeneic T cells expressed 69.83% $CD4^+$ and 22.54% $CD8^+$ T cells, respectively. (B) Allogeneic T cells were isolated by MACS for $CD4^+$ and $CD8^+$ T cells and assessed by FACS analysis. The isolated $CD4^+$ and $CD8^+$ T cells were 95.59% and 98.53% positive for CD4 or CD8, respectively.

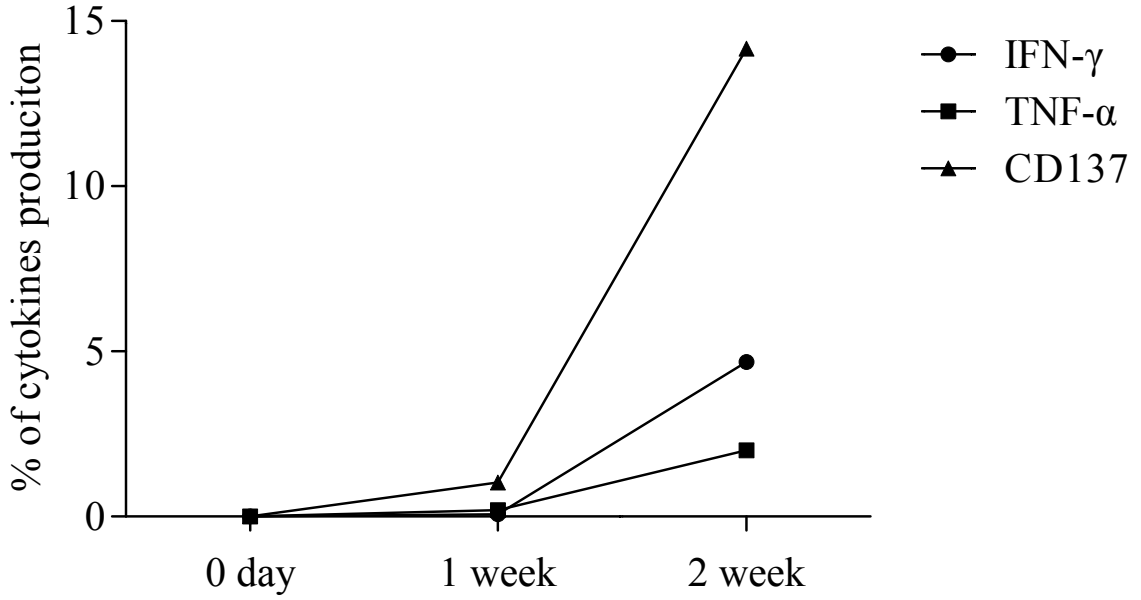

Fig. 11: Flow-cytometric cytokine expression analysis of $CD8^+$ CTL stimulated with tumor-B7.1 tumor cells. After being restimulated once a week for two weeks with irradiated CD80-positive tumor-B7.1 tumors cells supplemented with IL-2, PBMC were stained for CD8, CD137, TNF-α, and IFN-γ. $CD8^+$ CTL expressed 1.08% CD137, 0.18% TNF-α, and 0.04% IFN-γ after one stimulation (1 week), but 12.48%, 1.84% and 4.27%, respectively, after the second stimulation (2 weeks).

4.7 Sensitivity of SDC and MDC to Allogeneic $CD8^+$ CTL Cytotoxic Lysis determined by VITAL-FR Assay

Based on the data above, we assessed allogeneic $CD8^+$ CTL cytotoxic lysis of SDC and their parental MDC of CaSki and UM-SCC11B cells by VITAL-FR cytotoxicity assay. CFSE-labeled SDC defined as specific target cells or Far Red-labeled MDC defined as control target cells were co-incubated with effector cells to measure their relative amount to define the different sensitivity to allogeneic $CD8^+$ CTL lysis. As low as 100 CTL could induce the allogeneic lysis and more CTL resulted in enhanced killing. The allogeneic $CD8^+$ CTL cytotoxic lysis of CaSki SDC was less than the lysis of MDC at different E:T ratios in three independent donors, which was 4.3 ± 1.131%, 3.95 ± 1.768%, and 3.2 ± 1.980% at the 0.1:1 E:T ratio and 18.075 ± 6.852%, 8.5 ± 3.196%, and 16.225 ± 7.517% at the 1:1 E:T ratio, respectively. Comparatively, the allogeneic $CD8^+$ CTL cytotoxic lysis of UM-SCC11B SDC was nearly the same as the lysis of MDC, which was 1.055 ± 0.205%, 1.035 ± 0.319%, and 2.14 ± 0.424% at the 0.1:1 E:T ratio and 1.755 ± 1.195%, 1.1 ± 2.828%, and 2.9 ± 0.212% at the 1:1 E:T ratio, respectively (Fig. 12A, B). The data showed that CaSki SDC was less sensitive than MDC to the killing of allogeneic $CD8^+$ CTL at 1:1 E:T ratio. In contrast, UM-SCC11B SDC and MDC had nearly the same susceptibility to the killing of allogeneic $CD8^+$ CTL at different E:T ratios. The lysis of UM-SCC11B SDC and MDC was much lower than that of CaSki SDC and MDC.

To verify that the lysis was MHC class I antigen-restricted CTL reactivity, the target cells were preincubated and blocked with HLA-A,-B,-C antigen-specific mAb W6/32 before incubating with effector cells. After blocking, the relative lysis nearly completely decreased from 12.335 ± 2.510% to 3.095 ± 3.231% at the 0.1:1 E:T ratio, and from 30.51 ± 15.061% to 4.585 ± 2.609% at the 1:1 E:T ratio, respectively (Fig. 13A). This blocking experiment verified that the lysis measured in these experiments was indeed due to T cell-mediated MHC class I-restricted cytolysis.

As shown above, IFN-γ could upregulate some immune-related molecules of SDC and MDC. Therefore, we investigated the IFN-γ enhancement on the immune recognition and response against SDC and MDC of CaSki cells. Pretreatment with IFN-γ resulted in 1.785% and 13.540% increase of the relative lysis at the 0.1:1 and 1:1 E:T ratio, respectively. This demonstrates that IFN-γ treatment could enhance the allogeneic $CD8^+$ CTL cytotoxic lysis especially at 1:1 E:T ratio. Importantly, in SDC a stronger enhancement of lysis was observed than in MDC (Fig. 13B).

Since the SDC cultures consist of heterogenous populations of cells with more or less differentiated/stem cell phenotype, an important question is the sensitivity of true cancer stem cells characterized by ALDH1 expression. While CaSki SDC were more resistant to the killing by allogeneic $CD8^+$ CTL than MDC, the discrimination of AHP and ALP of SDC in sensitivity to the T cell killing was important. As shown in Fig. 14, the AHP of SDC were stronger killed than ALP on both CaSki ($P<0.01$) and UM-SCC11B ($P<0.05$) cell lines in three independent donors.

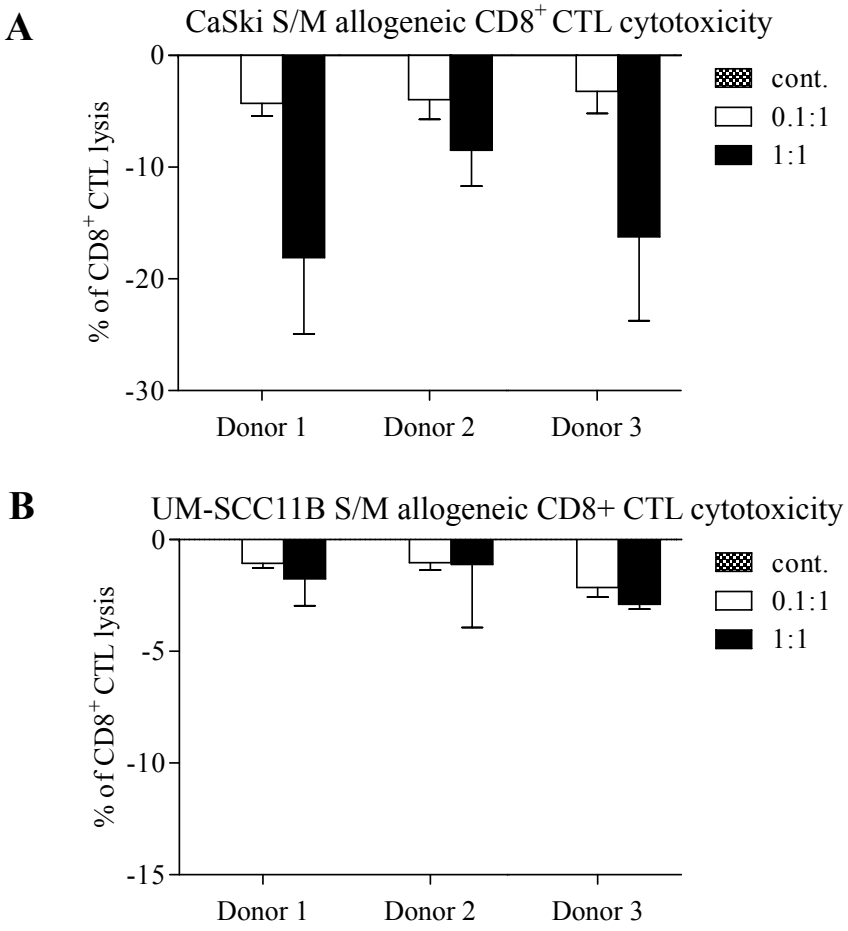

Fig. 12: Allogeneic $CD8^+$ CTL cytotoxicity of SDC and MDC. SDC and MDC of CaSki and UM-SCC11B were labeled with CFSE or Far Red to be used as target cells, respectively. The two target cells (T) and effector cells (E) were incubated for 24 hour at different E:T ratios respectively and allogeneic $CD8^+$ CTL lysis was assessed by flow cytometry together. (A) The relative allogeneic $CD8^+$ CTL cytotoxic lysis of SDC and MDC of CaSki at different E:T ratios. (B) The relative allogeneic $CD8^+$ CTL cytotoxic lysis of SDC and MDC of UM-SCC11B at different E:T ratios. Representative for three independent donors. Mean values ± SD of three determinations.

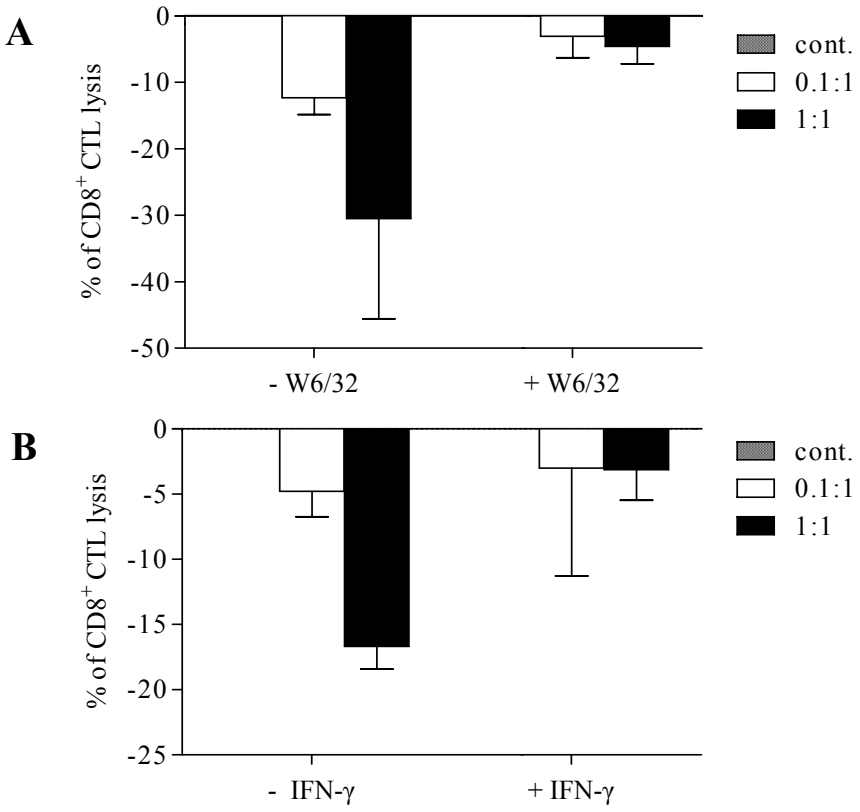

Fig. 13: MHC class I-restricted allogeneic CD8$^+$ CTL cytotoxicity of SDC and MDC pretreated with or without IFN-γ. (A) The relative lysis of SDC and MDC of CaSki preincubated with or without HLA-A, -B, -C Ag-specific mAb W6/32 at different E:T ratios. (B) The relative lysis of SDC and MDC of CaSki pretreated with or without IFN-γ at different E:T ratios. Representative for three independent donors. Mean values ± SD of three determinations.

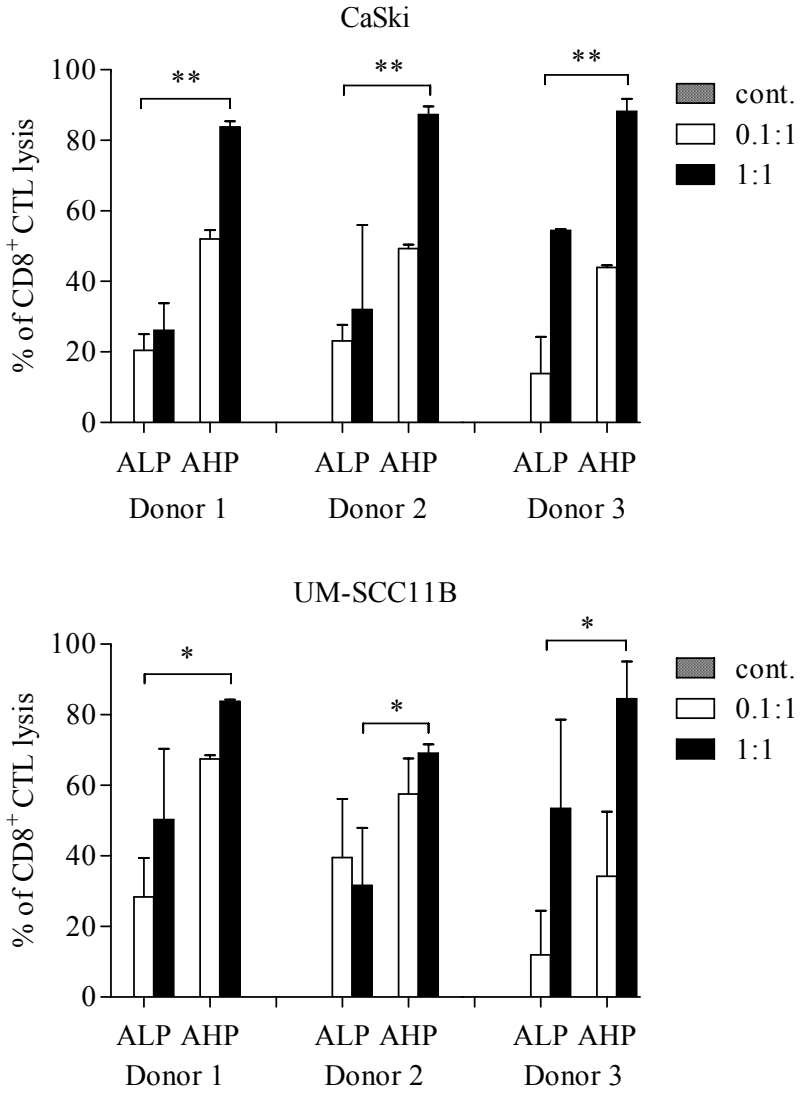

Fig. 14: Allogeneic CD8$^+$ CTL cytotoxicity against AHP and ALP of SDC. AHP were stronger killed than ALP for both CaSki ($P<0.01$) and UM-SCC11B ($P<0.05$) SDC in three independent donors. Mean values ± SD of three determinations. Significant differences: * $P<0.05$; ** $P<0.01$.

5. Discussion

5.1 Stemness Characteristics of SDC

Mounting evidence suggests that CSC can be expanded from permanent cancer cell lines including HNSCC [45, 50] and cervical carcinoma cell lines [48, 51] based on expression of the stem cell marker ALDH1, and this ALDH1 positive population may have the most potent tumor-initiating activity. In this study, we enriched cancer stem cell-like cells derived from spheroids grown from HNSCC and cervical cancer cell lines by the method described previously [17]. We analyzed their stemness characteristics by comparing ALDH1 expression and the level of stemness-related markers with the parental monolayer cell lines. Our results reveal that spheroid-derived cells express a higher level of ALDH1 and stemness-related markers than the monolayer-derived tumor cells. This demonstrates that spheroids subcultivated from cancer cell lines exhibit CSC characteristics and are therefore useful for CSC research.

5.2 Expression of Surface Markers on SDC and MDC

It is well documented that HNSCC [92] and cervical carcinoma [101] can downregulate the expression of MHC class I and antigen processing machinery components. Although MHC class I expression of brain tumor stem cells has been investigated and controversial data were reported [91, 99], the expression level on HNSCC and cervical carcinoma stem cells is still unclear. Our study shows that SDC and MDC derived from all 3 tumor cell lines investigated express MHC class I on the cell surface. The level of expression by CaSki SDC is reduced to approximately 50% of the level on MDC, but there is no difference detected on SDC vs MDC of cell lines UM-SCC11B and UD-2. More importantly, we found that the subpopulation of $ALDH1^{high}$ cells (AHP) from SDC of CaSki cells also expressed MHC class I about two times lower than $ALDH1^{low}$ cells (ALP). A recent study demonstrates that exposure to IFN-γ upregulates the cell surface expression of MHC class I on CD133-positive cells [99]. In our analysis, incubating SDC of 3 tumor cell lines with IFN-γ significantly increased the expression of MHC class I. This was also true for AHP derived of SDC. Moreover, CaSki cells were the most sensitive cell line reacting to IFN-γ, by upregulation of MHC class I, MHC class II, and CD54 expression on both SDC and MDC. Relative to MHC class I, MHC class II and CD54, other immune-related molecules like CD58, CD80 and CD86 were less susceptible.

5.3 $CD8^+$ CTL Cytotoxicity to SDC and MDC

In order to study cancer stem cell susceptibility to T cell lysis, we adapted the VITAL-FR assay to measure and to compare the sensitivity of the SDC and MDC population. VITAL-FR is an extension to the VITAL assay, a flow cytometry-based assay system assessing CTL frequency and function. 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 it is a sensitive and flexible flow cytometry-based in-vitro assay for clinically relevant specific CTL function [95]. By using PI staining and excluding positive cells from the gating during analysis of the cytometric results, we also take into account the freshly killed cells that have not yet undergone apoptosis. This could enhance the result of the VITAL-FR assay quantifying the decrease in gated target cells.

As increasing evidence supports the notion that CSC are responsible for cancer progression, therapy resistance, and relapse, it would be necessary to develop immunotherapy targeting CSC to inhibit tumor recurrence more effectively [4]. Moreover, it was demonstrated that vaccination with DCs loaded with glioblastoma multiform (GBM)-derived CSC could induce CTL reactive against CSC and could prolong survival in an animal model, and in human brain tumor patients as well[78]. Another group has shown that CD133-positive brain tumor stem cells (BTSC) are subject to immunologic recognition and elimination by peptide-specific $CD8^+$ CTL [91]. Even though these reports have examined the reaction between CSC and the innate or adaptive immune system, none of them take into account the potential different immunogenicity of CSC and the corresponding proliferated and “differentiated” bulk tumor cells. The crucial point of our study is that we observed that CaSki SDC appear to be more resistant to the recognition and destruction by MHC class I-restricted allogeneic $CD8^+$ CTL than the matched MDC. Importantly, this was determined in an assay quantifying the lysis of both populations in the same reaction. $CD8^+$ CTL are potent cytotoxic effector cells of the adaptive immune system that play a major role in host defence against tumors. Their activity requires MHC class I-restricted peptide epitope presentation of specific antigens and costimulatory signals. MHC class I at the APC surface can interact with the T cell receptor and induce $CD8^+$ CTL lysis of target cells. As a result, downregulated MHC class I level on CaSki SDC may hamper the reaction between SDC and $CD8^+$ CTL and lead to some resistance to lysis. However, UM-SCC11B SDC and MDC seem to have the same sensitivity to the killing by allogeneic $CD8^+$ CTL. This may be attributable to the different expression of MHC class I, CD54 or NK cell-activating ligands on

CaSki SDC and MDC but similar level on UM-SCC11B SDC and MDC.

Anhua Wu and his colleagues [99] described that CD133-positive cancer stem-like cells did not express MHC class I or NK cell-activating ligands, which rendered them resistant to immune surveillance. However, IFN-γ may partially restore their immunogenicity and potentiate their lysis by NK cells. In our assays, pretreatment with IFN-γ also rendered SDC and MDC more sensitive to allogeneic $CD8^+$ CTL lysis. This resulted in enhanced SDC lysis as compared to MDC. One might speculate that SDC are more sensitive to the restoration of immunogenicity by IFN-γ than MDC. Another important point supporting the potential enhancement of immunogenicity of cancer stem cells is that AHP of SDC were more susceptible to the lysis of allogeneic $CD8^+$ CTL than ALP despite a comparable level of MHC class I expression. More work or alternate methods are needed to explain this difference.

5.4 Perspective

In an autologous immune system - like a patient's intrinsic immune response - a T cell recognizes tumor antigen presented on the MHC molecules of APC and subsequently antigen-specific T-cells are activated. However, there was no autologous blood of these patients available who had donated the cell lines years ago. We circumvented the problem in this initial study by using allo-antigen specific T cell lines to evaluate the immunogenicity of CSC-derived of cell lines cultured as spheroids. Moreover, the allogeneic response overwrites the tumor antigen-specific response making this system an ideal tool for investigating the antigen-independent effect by e.g. adjuvants like IFN-γ. In our study, healthy donors' T cells recognized CD80-positive allogeneic tumor cells acting as artificial allogeneic APC, and expressing allogeneic MHC molecules used as artificial generic antigens. This resulted in allo-antigen-reactive T cells lines. These allo-antigen-reactive T cells could in turn kill the tumor cells and this allogeneic response is independent of specific tumor antigen. After having established the best methodology and combination with adjuvants, a future step could be to use autologous tumor and blood and investigate responses to specific CSC antigens.

Our allogeneic model system is well suited for investigations like ours. It is advantageous because more cell lines can be used to show generic applications, the immune response is presumably stronger since tumor-specific responses are often weak or suppressed, and a universal CSC-tumor antigen is not yet available. Moreover, our test system could be also useful to screen for the effects of different adjuvants or molecules to regulate the immune response. For further research, epitope-specific $CD8^+$ CTL (e.g. as a model antigen CMV pp65-specific $CD8^+$

CTL) or tumor antigen-specific $CD8^+$ CTL (e.g. HPV, ALDH1, p53-specific $CD8^+$ CTL) investigated by our VITAL-FR assay may reveal that CSC could be targeted by specific immunotherapy and could improve on our basic understanding to create vaccines or enhance future treatment approaches.

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8. Curriculum Vitae and Publications

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

Publications:

1. **Tian Liao**, Yunfei Lu. HER2 gene and breast cancer. China Practical Medical. 2007; 2(9): 89-91.
2. Qingyi Liu, Yunfei Lu, Fengyun Cong, **Tian Liao**. Density and area of lymph vessels in human gastric cancer and its relationship with lymph node metastasis. Acta Anatomica Sinica. 2008; 39(4): 602-606.
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11. Zhang G-Y, **Liao T**, Gao W-Y. Genetic Modifiers in pulmonary Fibrosis. N Engl J Med. 2011 Jul 14;365(2):178-179.

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9. Statement

I declare that the experiments described in the thesis were carried out by me.

Erklärung

„Ich, [Tian Liao], erkläre, dass ich die vorgelegte Dissertation mit dem Thema: [Immunogenicity of cervical and HNSCC-derived putative cancer stem-like cells] selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

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