Immunoregulatory Properties of ALDH1⁺ Cancer Stem-Like Cells Derived from Carcinoma Cell Lines of the Cervix Uteri

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Abstract

Increasing evidence suggests that cancer stem cells (CSCs) might preferentially initiate and sustain neoplastic growth and disease progression through immunoevasive and immunomodulatory functions. These observations will challenge the targeting of cells modulated by CSCs or CSC themselves by immunotherapy. Cervical cancer is the second most common cancer in women, and the fifth deadliest. However, the immunologic properties of cervical cancer stem cells have not yet been explored. Aldehyde dehydrogenase isoform 1 (ALDH1) was found as a surface marker in CSC of cervical cancer; In this study, we evaluated the effect on proliferation, activation, apoptosis and function of cervical cancer cell lines derived ALDH1⁺ cancer stem-like cells with ALDH1⁻ cells on pre-stimulated T cells in a two-chamber assay. In addition, we also compared their role in innate immunity with respect to the polarization of macrophages. We show that cervical cancer cell line derived ALDH1⁺ cells had a stronger suppressive effect on T cell proliferation, activation, cytokine production, and cytotoxic T cell effector functions than ALDH1⁻ cells. Moreover, we found ALDH1⁺ cells induced T cell polarization the Macrophage to an M2 phenotype. Consistently, we have found in 3 cervical cancer cell lines this strong immunosuppressive activity exerted by CSC. All T cell functions investigated were suppressed upon co-culture with ALDH1⁺ cells significantly stronger than by ALDH1⁻ cells. These data support the role of CSC in the complex interplay of the host’s immune system leading to a sustained growth of tumor eventually allowing the tumor to immune-escape. Conversely, these data also support future efforts to effectively target CSC.

Key words: Cancer stem cell; ALDH1; immunosuppression; tumor immunity; cell sorting.

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, prevention by screening and the absence of medical support. Despite recent advances in conventional treatments such as surgery or chemoradiation therapy, the prognosis for most patients with advanced cervical cancer remains poor (1). It is, therefore, desirable to develop a deeper understanding of the biology of this disease to adapt and complement current therapeutic strategies to increase their effectiveness.

CSCs represent a subpopulation of cells of malignant tumors that is characterized to be the cell origin of a tumor held responsible for tumorigenesis, tumor differentiation, tumor maintenance, metastasis,
and tumor relapse following therapy (2, 3). Increasing
evidence suggests that CSCs might preferentially ini-
tiate and sustain neoplastic growth and disease pro-
gression through immunoevasive and immunomod-
ulatory functions, which will challenge the immuno-
therapy targeting of these cells (4). Recent findings
that suggest a negative correlation between degrees of
host immunocompetence and rates of cancer devel-
opment raise the possibility that CSC may possess the
phenotypic and functional characteristics to evade the
host immuno-surveillance and immune-mediated
rejection of tumor cells in immunologically intact in-
dividuals (5).

ALDH1 is the isoform of aldehyde dehydrogen-
ase (ALDH) that expressed in humans is a cytosolic
detoxifying isoenzymethat oxidizes intracellular al-
dehydes, and contributes to the oxidation of retinol to
retinoic acid in early stem cell differentiation (6). Re-
cent studies have shown that ALDH1 is a specific
marker for identification of cervical CSCs and plays a
crucial role in maintaining the self-renewal properties
and tumorigenicity in cervical cancer-derived CSCs
(7, 8). Moreover, ALDH1 immunoexpression has been
found in tissue sample of cervical cancer and has
prognostic value for cervical cancer survival (9, 10).

In this current study, we used the Aldefluor a-
say and fluorescence-activated cell sorting (FACS)
analysis to isolate ALDH1+ and ALDH1- cells from
three human cervical cancer cell lines. We then com-
pared the immunologic properties of ALDH1+ cancer
stem-like cells with ALDH1- cells. Our findings re-
cognize specific CSC-induced immunosuppression
interactions in the tumor microenvironment that
should be acknowledged when designing immuno-
therapeutic approaches against cervical cancer. In-
dependently the data also suggest that therapeutic
regimen of cervical cancer should be effectively ta-
argeting CSC to increase the possibility of cure.

Material and methods

Cell lines and cell culture

Cervical cancer cell lines were obtained from the
American Type Culture Collection (American Type
Culture Collection, Rockville, MD, ATCC): HeLa
(HPV18), CaSki (HPV16) and MRIH-215(HPV45).
Cells were maintained in Dulbecco’s modified Eagle’s
medium (DMEM; Invitrogen, Grand Island, NY) 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% CO2, and 95% air at-
mosphere.

Human peripheral blood mononuclear cell
(PBMC) preparation and differentiation of
monocyte-derived macrophages

PBMCs were isolated from blood of healthy do-
nors by Ficoll-Hypaque Plus (GE Healthcare, Uppsala,
Sweden) density gradient centrifugation and cul-
tured in Quantum 263 medium (Biochrom), supple-
mented with 10 ng/ml EGF and 10 ng/ml bFGF (Bi-
ochrom), interleukin (IL)-2 and IL-7 (10 IU/ml; Im-
muTools, Friesoythe, Germany), and 1% Penicillin
and Streptomycin. Monocytes were cultured in
Quantum 263 medium supplemented with 20ng /
mlMacrophage colony-stimulating factor (M-CSF;
R&D Systems, Abingdon UK) in order to induce dif-
ferentiation into macrophage at 37°C.

Aldefluor Assay and Cell Sorting

To isolate an Aldefluor-stained cell population
with ALDH1 enzymatic activity, we used the Ald-
fluor assay kit (StemCell Technologies, Durham , NC,
USA), which is designed for optimal identification
and isolation of stem cells through specific interaction
with human ALDH1 (11). The experiments were un-
dertaken according to the manufacturer's instruction.
Cervical cancer cells were disaggregated into single
cells by Trypsin/EDTA digestion. Then, the si-
ngle-cell suspension was washed twice in PBS without
Ca2+/Mg2+ and suspended in 1 ml ALDEFLUOR a-
say buffer containing 5 µl ALDH substrate (BAAA, 1
µmol/ml per 1×10⁶ cells) and incubated for 40 min at
37°C in the dark. As a negative control, for each sa-
mple, an aliquot was treated with 5 µl diethylami-
nobenzaldehyde (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.

The cells were then incubated at 4°C for 15 min
in the dark. And then cells were resuspended in PBS
buffer at 1×10⁷ cells per ml and separated on an Aria
cell sorter (BD Biosciences). The sorted cells were a-
alyzed for expression of ALDH1 by FACS after 24
hours and after six days. The sorting gates were es-
tablished, using as negative controls the cells treated
with DEAB.

Clone formation assay

After FACS sorting, the ALDH1+ and ALDH1-
cells were inoculated to Ultra-low attachment 6-well
plates (Fisher Scientific, Loughborough, UK) at a
density of 1000 cells/ml in Quantum 263 medium
supplemented with 10% FBS. Cells were cultivated
under standard culture conditions for 2 weeks and
then the clone numbers were counted microscopically.
Co-culture of PBMC with tumor cells

For two-chamber assays, transwell plates divided by a microporous membrane with a pore diameter of 0.4 μm were used. Sorted ALDH1+ and ALDH1- cells were seeded in the lower chamber and PBMCs in the upper chamber of a 24 well Transwell plate (Corning, NY, USA) at a 1:5 ratio (cancer cell: PBMC). Thus, cell populations were physically separated by a semi-permeable membrane. PBMCs were co-cultured with sorted ALDH1+ or ALDH1- cells for 6 days and then subjected to further flow cytometry analysis.

Cell cycle analysis and Ki-67 staining

ALDH1+ or ALDH1- cells were harvested and resuspended in 70% ethanol by adding ethanol at −20°C dropwise while vortexing. The cells were stored at −20°C overnight then washed with PBS, centrifuged and 1 μg FITC-conjugated mouse anti-human Ki-67 (DAKO, Glostrup, Denmark) was added to the cell pellet and incubated for 20 min at room temperature. Finally, cells were resuspended in 1 ml of a cocktail containing propidium iodide (2 μg/ml) and RNase (100 μg/ml) (Fermentas Life Science, St. Leon-Rot, Germany) and stained for 30 min followed by flow cytometry.

CFSE-dilution proliferation assay

PBMCs (1 × 10⁶/ml) were suspended in PBS and labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 7 minutes at 37°C. The cells were washed 3 times in an equal volume of PBS and resuspended in 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 ALDH1+ or ALDH1- cells as mentioned above. Proliferation of PBMCs was measured on day 6 by flow cytometry on the basis of CFSE dilution.

Flow-cytometric (FACS) analysis

Monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (Percp), or allophycocyanin (APC) against human CD8, CD14, CD33, CD69, CD137, CD154, CD163 (all BD Pharmingen, CA, USA), were used to characterize PBMCs. Briefly, 5×10⁴ 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) software. The percentage of positive cells was determined by subtracting the percentage of fluorescent cells in the control from the percentage of cells positively stained with the appropriate antibody.

MHC-dextramer analysis

PBMCs (1 × 10⁶) from HLA-A2+ donors were stained with 10 μl dextramer complexes of CMVpp65-derived peptide NLVPMVATV/HLA-A*0201-APC (Immundex, Copenhagen, Denmark) for 10 min in the dark at room temperature according to the manufacturer instructions. 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.

Generation of CD8+ CMV-specific CTLs and cytotoxicity assay

Freshly isolated PBMCs from 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 × 10⁶ per ml. IL-2 and IL-7 (10 IU/ml) was added every 2-3 days. At 7-day intervals, cells were restimulated with peptide.

CD8+ CMV-specific CTL cytotoxicity assay

To investigate the CD8+ CMV-specific CTL cytotoxicity, a VITAL-FR assay was performed as previously described (12). An autologous B lymphoblastoid cell line (BLCL) was generated from each HLA-A2+ healthy donor by infection of PBMC with supernatant from the Epstein-Barr virus–producing cell line B95-8 (ATCC) + 1 ng/ml cyclosporin A and were maintained in RPMI 1640 medium containing 10% bovine serum. Activated CMV-specific CTL derived from HLA-A2-positive donors were used as effector cells and were co-cultured with ALDH1+ or ALDH1- cells as mentioned above. Proliferation of PBMCs was measured on day 6 by flow cytometry on the basis of CFSE dilution.
lated 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]×100= specific lysis (%).

**Luminex-based assay for cytokine quantification**

Human Cytokine/Chemokine Luminex bead immunoassay kit, Procartaplex™, 34 Plex from eBio-science was used to measure the levels of cytokines and chemokines in the supernatant of cervical cancer cells or co-cultures of activated PBMC and cervical cancer cells. The concentrations of the following soluble mediators were measured: Eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-related oncogene alpha (GRO-alpha), IFN-alpha, IFN-gamma, IL-1 beta, IL-1 alpha, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, interferon-induced protein (IP-10), monocyte chemotactrant protein (MCP)-1, human macrophage inflammatory protein alpha (MIP-1 alpha), MIP-1 beta, RANTES, stromal cell-derived factor 1 alpha (SDF1-alpha), TNF-alpha, TNF-beta. Each sample was measured in triplicate according to the manufacturer’s specifications. Standard curves for each analyte were generated by using the reference analyte concentration supplied by the manufacturer.

The measurement was performed using the Bio-Plex system in combination with Bio-Plex Manager software, version 4, using fiveparametric curve fitting (Bio-Rad Bioplex; Bio-Rad).

**Quantitative real-time PCR**

Total RNA was extracted by using TRIzol reagent (Invitrogen) and converted to cDNA by RT-PCR using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). 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. Primer sequences are listed in table 1.

**Western Blot Analysis**

Aliquots of protein extracts (20 µg) were prepared for Western blotting as described by Williams et al.(13). Nuclear protein (20 µg) was electrophoresed using 4%–20% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 5% milk in Tris buffered saline-Tween 20 and probed with antibodies to Sox2 (Santa Cruz Biotechnology), Oct4 (Abcam, Cambridge, MA), and Nanog (Abcam) and horseradish peroxidase-conjugated secondary antibody (Invitrogen and Abcam). The membrane was then visualized using an enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). Membranes were stained with Ponceau-S (Fisher Scientific) to determine equal loading and transfer.

**Statistical Analysis**

All statistics analyses were performed using the SPSS software for Windows (version 15) (SPSS, Chicago, IL). Student’s t-test was used to analyze statistical significance of differences of the experimental data.

**Results**

**Characterization of the stemness properties of the ALDH1+cells from cervical cancer cell Lines**

To investigate the mechanisms by which CSCs modulates host immune responses, we first undertook fluorescence-activated cell sorting to separate CSCs-enriched populations from cervical cancer cell lines (Fig. 1A). Then a clone formation assay was conducted to determine the clonogenic ability of ALDH1 sorted cells in vitro. After 14 days of culture, the colonies that formed were quantified macroscopically (Fig. 1B). As shown in Fig.1C, the ALDH1+ subpopulation in CaSki, HeLa and MRIH215 cell lines have a higher clone formation efficiency as compared to the ALDH1- subpopulation (*P < 0.05).

**Table 1. Primer sequences used for RT-PCR in immunosuppressive expression analysis.**

<table>
<thead>
<tr>
<th>Transcript name</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
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<tr>
<td>SOX2</td>
<td>GGGAAATGGGAGGGGTGCAAAAGAGG</td>
<td>TTTGCGTGAGTGTGGATGGGATTGGTG</td>
</tr>
<tr>
<td>Nanog</td>
<td>AATACCTCAGCCTCACCAGCATG</td>
<td>TGGCTCACACCATTCATCTTCTTC</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>GACAGGGGGAGGGGAGGCTAGG</td>
<td>CTCTCCCTCAACAACTGGCCCTCCAAAC</td>
</tr>
<tr>
<td>Arginase II</td>
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<td>IL-8</td>
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<td>TCCACAAACCCTCTGCA</td>
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<td>GGAAGGACGACCCAGCTGCCTC</td>
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<tr>
<td>β-actin</td>
<td>CCCCCAGGGCCACCAGGCC</td>
<td>GGCTGGGCGTTGGAGGT</td>
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Figure 1. Characterization of ALDH1+ and ALDH1− cells derived from cervical cancer cell lines. (A) FACS-sorting strategy for the isolation of ALDH1+ (upper panel) and ALDH1− (lower panel) cervical cancer cells. Representative picture of the cell line CaSki is shown. (B) ALDH1+ or ALDH1− single cells were cultured in 6-well ultra-low attachment plates. Three weeks after inoculation, ALDH1+ cells produced spheroid colonies, while ALDH1− cells did not. (C) Clone formation assay with ALDH1+sorted and cloned cells. Mean values ± SD of three determinations. Statistically significant differences are * P < 0.05. (D) Messenger RNA isolated from ALDH1+ and ALDH1− cells was quantified for expression of the indicated TF. The ratio of expression in ALDH1+ to ALDH1− cells is shown. Significant differences were * p<0.05. The mRNA level of stemness-related TF Nanog, Oct3/4, and Sox2 were increased remarkably in ALDH1+ cells. (E) The protein expression levels of Nanog, Oct3/4, and Sox2 in ALDH1+ and ALDH1− cells from three cervical cancer cell lines were analyzed by Western blot analysis. (F) Double staining with Ki-67 and propidium iodide for the measurement of proliferation and cellular DNA content. Single cells were gated on FL2-W and FL2-A. DNA content and Ki-67 expression was evaluated by flow cytometric analysis. (H) The percentage of quiescent cells in the ALDH1+ cells as compared to that in the ALDH1− cells. Mean values ± SD of three determinations. Significant differences are * p<0.05.

It was reported that Sox2, Oct3/4, and Nanog, which form a self-organized core of transcription factors that function to maintain pluripotency and self-renewal capacity in human embryonic (14, 15) and cancer stem cells (16). We then conducted quantitative real-time PCR and Western blot to compare the mRNA and protein expression levels of these transcription factors in the sorted ALDH1+ and ALDH1− cells from cervical cancer cell lines (Fig. 1D). Notably, the mRNA and protein levels of Sox2, Nanog, and Oct4 were found to be elevated in the ALDH1+ cells from all three cervical cancer cell lines (Fig. 1D and Fig.1E). There is particularly a 47-fold increase in Sox2 mRNA expression in the ALDH1+
cells from HeLa cells relative to the parental ALDH1- cells (Fig. 1D).

It has been suggested that the majority of anti-cancer drugs target actively dividing (cycling) cancer cell population, but leave quiescent CSCs intact, which may consequently lead to cancer metastasis and relapse that can appear many years after curative treatment of a primary tumor (17). To assess the proliferation rate of the ALDH1 sorted cells from the three cervical cancer cells, we performed a co-staining of DNA with propidium iodide and Ki-67, a well-known proliferation marker (18). As shown in Fig. 1F, the cells that reside in the G1/G0 peak and are negative for Ki-67 staining are considered to be the quiescent population (G0 phase). Importantly, ALDH1+ cells from CaSki, HeLa and MRIH215 all exhibit a significant increase in the proportion of cells residing in the stationary (non-proliferating) G0 phase, compared with their parental ALDH1- cells (Fig. 1G).

**Inhibition of T cell proliferation and function by ALDH1+ cells from cervical cancer cell Lines**

CSCs have been described to have a lower susceptibility for immunologic recognition (19). We therefore employed CFSE cell proliferation assay to investigate the influence of the cervical cancer derived ALDH1+ cells on T cell proliferation. As shown in Fig. 2, our results demonstrated that ALDH1+ cells from the three cervical cancer cell lines display significantly higher capacity to suppress the proliferation of activated T cells than ALDH1- cells (P<0.05). Consistent with these findings, co-culture with ALDH1+ cells from the three cervical cancer cell lines significantly decreases the expression levels of CD69, CD137, and CD154 in T cells and those of IFN-γ, IL-2, and TNF-α in CD4+T cells than ALDH1- cells (Fig. 3 and Fig.4).

![Figure 2. ALDH1+ and ALDH1- cells inhibit the proliferation of T cells. T cells from healthy donors were stained with CFSE and stimulated with anti-CD3/CD28 coated Dynal beads in the presence of ALDH1+ or ALDH1- cells. ALDH1+ and ALDH1- cells were plated underneath the 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 representative of 1 of 3 experiments of identical design. T cells stimulated with or without anti-CD3/CD28 bead in the absence of cancer cells for 6 days were used as positive control, or negative control, respectively (data not shown).](http://www.jcancer.org)
Figure 3. Suppression of the expression of activation markers by cervical CSCs. The expression of CD69, CD137 and CD154 on anti-CD3/anti-CD28-activated T cells from healthy donors co-cultured with cervical cancer ALDH1\(^+\) or ALDH1\(^-\) cells for 6 days. T cells stimulated with or without anti-CD3/CD28 beads in the absence of cancer cells for 6 days were used as positive control, or negative control, respectively. Representative FACS plots are shown in the lower panel. Statistical significance: * p<0.05; ** p<0.01.
Figure 4. Suppression of cytokine expression by cervical CSCs. The expression of IFN-γ, IL-2 and TNF-α in T cells from healthy donors co-cultured with cervical cancer ALDH1+orALDH1- cells of the three cell lines at a 5:1 ratio in a transwell assay. T cells stimulated with anti-CD3/anti-CD28 beads and concurrently co-cultured with the ALDH1+orALDH1- cells for 6 days. Representative FACs plots are shown in the lower panel. Statistical significance: * p<0.05; ** p<0.01.

Induction of M2 macrophages (cells) and myeloid derived suppressor cells

Because M2 macrophages are considered to be anti-inflammatory and immunosuppressive and MDSC are a heterogeneous population of immature cells with suppressive function (20), we next compared the differently polarized macrophage and MDSC in PBMCs by co-culture with ALDH1+or ALDH1- cells. CD14 is a specific monocyte/macrophage marker. CD33 is expressed on not terminally differentiated myeloid cells and CD163 is linked to macrophage anti-inflammatory functions (21). We used triple-color flow-cytometry to identify and quantify macrophages (CD14), their maturation status (CD33) and their polarization (M2; CD163). PBMCs were co-cultured with ALDH1+or ALDH1- cells for 6 days and then adherent cells were collected for further flow cytometry analysis. As shown in table 2, the proportions of both CD14+CD33+CD163+ immature M2 macrophage and CD68-CD163+ myeloid cells in monocytes from 2 healthy donors co-cultured with ALDH1+ cells were significantly higher than those with ALDH1- cells in all three cell lines.

Inhibition of CD8+ CMV-specific CTL function by ALDH1+ cells from cervical cancer cell Lines

To investigate the effect of cervical cancer derived ALDH1+ cells on the CD8+ CMV-specific CTL
functions, a VITAL-FR assay was performed as described in the Materials and Methods (12) (Fig.5A and Fig.5B). At lower E:T ratios of 0.3:1, 1:1 and 3:1, there was no significant difference in the CMV CTL cytotoxic lysis between control cultures and co-cultures with ALDH1+ or ALDH1- cells (Fig. 6A). However, CMV-specific CTL induces substantial lysis of BLCL when a E:T ratio of 10:1 was used (Fig. 6A). Although both ALDH1+ or ALDH1- cells derived from all three cervical cancer cell lines could suppress the ability of activated CMV pp65495-503 peptide-specific CTL to lyse pp65495-503-pulsed autologous BLCL, strikingly, ALDH1+ cells exhibit greater inhibitory activity on the cytotoxic functions of CD8+ CMV-specific CTL than ALDH1- cells (Fig. 6A).

Having observed the suppressive effects of cervical cancer cell lines derived ALDH1+ cells from on CD8+ CMV-specific CTL cytotoxic lysis, we then questioned whether or not the ALDH1+ cells from cervical cancer cell lines affect the percentage of CD8+ CMV-specific CTL in the PBMCs from one donor. As a control, there are no significant changes in the percentage of CD8+ CMV-specific CTL when they are co-cultured with ALDH1+ cells (day 3: 1.09%; day 6: 0.95%), compared with that in the presence of ALDH1- cells (day 3: 2.37%; day 6: 2.62%) (P<0.05) (Fig. 6B). Intriguingly, the percentage of CMV-specific CD8+ T cells significantly declines when they are co-cultured with ALDH1+ cells (day 3: 1.09%; day 6: 0.95%), compared with that in the presence of ALDH1- cells (day 3: 2.37%; day 6: 2.62%) (P<0.05) (Fig. 6B). These data suggest that CaSk ALDH1+ cells may induce T cell apoptosis, thereby leading to the decline in the number of T cells in PBMCs.

Figure 5. 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 10⁵ specific and 10³ 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.

Table 2: Characterization of monocyte differentiation in PBMC co-cultures with ALDH1+ and ALDH1- cervical cancer cells.

<table>
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<td>ALDH1+</td>
<td>3.75±2.51</td>
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<td>4.44±0.72</td>
<td>1.16±0.13*</td>
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<td>2.47±0.72</td>
<td>0.41±0.18</td>
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<td>ALDH1+</td>
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<td>ALDH1-</td>
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The results represent mean value±SD of three independent determinations.

*Represents statistically significant differences of monocytes co-cultured with ALDH1+ cervical cancer cellscompared to those co-cultured with ALDH1- cervical cancer cells.

Monocytes were cultured in the absence of cancer cells for 6 days used as control.
Figure 6. Functional suppression of CTL by cervical CSCs. The specific lysis of target cells by CMV-specific T cells from healthy donors in the presence of ALDH1⁺ or ALDH1⁻ cells of the three cervical cancer cell lines at a 5:1 ratio in a transwell assay is shown. CFSE-labeled autologous B-LCLs pulsed with CMV pp65495-503 peptide were defined as specific target cells, whereas Far Red-labeled autologous B-LCLs were defined as control target cells, respectively. Effector cells (E) were co-cultured with ALDH1⁺ or ALDH1⁻ cells from the three cell lines for 3 days. Then the two target cells (T) and effector cells (E) were incubated in the presence of ALDH1⁺ or ALDH1⁻ cells for another 3 days at different E:T ratios, respectively, and autologous CMV-specific CTL lysis was assessed by flow cytometry together. (A) The relative autologous CMV-specific CTL cytotoxic lysis of CMVpp65-pulsed B-LCL at different E:T ratios in the presence of ALDH1⁺ or ALDH1⁻ cells is shown. Mean values ± SD of three determinations. (B) The percentage of CMV-specific, dextramer reactive CD8⁺ T cells in the presence of ALDH1⁺ or ALDH1⁻ cells at a ratio of 5:1 on day 3 and day 6.
Increased expression levels of immunosuppressive cytokines and chemokines in ALDH1+ cells from cervical cancer cell lines

To explore the potential mechanisms by which how ALDH1+ cells from the CaSki and the MRIH215 cell lines modulate host immune responses, we then performed quantitative real-time PCR to compare the mRNA expression levels of cytokine, including Arginase II, IL-8, TGF-β, IDO, and MIC-1, in ALDH1+ cells and ALDH1- cells. We found that the mRNA levels of Arginase II, IL-8, TGF-β, and MIC-1 were significantly increased in the ALDH1+ cells in those two cell lines, compared with those in ALDH1- cells (P<0.05) (Fig. 7). However, the mRNA level of IDO was only remarkably elevated in CaSki cervical cancer cells (Fig. 7).

Next we employed a Luminex-based quantitative approach to measure the levels of secreted cytokines and chemokines in the supernatant of cervical cancer cells or co-cultures of activated PBMC. ALDH1+ cells from CaSki cell line secrete significantly higher levels of immunosuppressive cytokines, including IL-6, IL-1RA, IL-8, IP-10 and SDF-1a, into the culture medium than those of ALDH1- cells cultures (Fig. 8A). Similarly, the supernatant of the ALDH1+ cells from the MRIH215 cell line contains significantly higher levels of immunosuppressive cytokines, including IL-6, IL-1RA, GRO-alpha, IL-8, IP-10, MCP-1, SDF-1a, and RANTES, than that from the ALDH1- cells cultures (Fig. 8B). These findings demonstrate that cervical cancer cell lines derived ALDH1+ cells may secrete high levels of immunosuppressive cytokines such as IL-6, IL-1RA, IL-8, IP-10, and SDF-1a, thereby inhibiting host immune responses.

Cervical cancer cell lines derived ALDH1+ cells inhibit cytokine production by anti-CD3/anti-CD28- activated PBMCs

We next investigated whether or not ALDH1+ cells from the CaSki and the MRIH215 cell lines affect the cytokine production by activated PBMCs. As shown in Table 3, T cells stimulated with anti-CD3/CD28 beads for 3 days in the absence of cancer cells were used as a positive control. The ALDH1+ cells from CaSki and MRIH215 cell line significantly inhibit the production of IFN gamma, IL-2, IL-21, TNF-alpha, TNF-beta, MIP-1alpha, MIP-1 beta and GMCSF production by anti-CD3/anti-CD28-activated PBMCs from 2 donors, compared with ALDH1- cells (P<0.05).
ALDH1 expression, the ALDH1+ cells demonstrated when tumorigenic cell lines were fractionated by iterative properties of self-renewal and expression of responsible for self-renewal and pluripotency, was they cooperatively maintain the regulatory network tors of human embryonic stem cell pluripotency and Oct3/4, Sox2, and Nanog, which are the core regul a- defined as those cells that have self-renewal ability. In parental tumor (11).

e. ALDH1+ cells are able to maintain the tumorigenic cell fraction, are able to self-renew and to recapitulate the heterogeneity of the tumors (8). In breast cancer, for example, Ginestier et cancer, Chen et al showed that 10^5 ALDH1+cervical and other epithelial cancers (9, 22 -24). In cervical cancer, Chikamatsu et al. reported that cells with high ALDH activity con-

### Discussion

The immunologic features of cells with CSC-phenotype enriched from cervical cancer cell lines have not been defined previously, and to our knowledge, this is the first study to show that this subpopulation mediates many of the key features of immunosuppression. The ability of CSC to rebuild the tumor from a single cell could explain many of the differences that discriminate tumor cells from differentiated somatic cells like immortality, quiescence, invasion leading to metastasis, and recurrence after treatment. ALDH1 was recently shown to be a suitable marker to identify putative CSC of cervical cancer and other epithelial cancers (9, 22-24). In cervical cancer, Chen et al showed that 10^4 ALDH1+cervical SiHa cells in xenotransplanted mice resulted in all cases in the generation of visible tumors 2 weeks after injection, while 10^5 ALDH1- cells failed to generate tumors (8). In breast cancer, for example, Ginestier et al. reported that cells with high ALDH activity contain the tumorigenic cell fraction, are able to self-renew and to recapitulate the heterogeneity of the parental tumor (11).

Cancer and normal stem cells (SCs) share proliferative properties of self-renewal and expression of key transcription factors (TFs). In the present study, when tumorigenic cell lines were fractionated by ALDH1 expression, the ALDH1+ cells demonstrated the capability of spheroid colony formation under nonadherent conditions in serum-free media which is defined as those cells that have self-renewal ability. In addition, we demonstrated that the expression of Oct3/4, Sox2, and Nanog, which are the core regulators of human embryonic stem cell pluripotency and they cooperatively maintain the regulatory network responsible for self-renewal and pluripotency, was up-regulated in ALDH1+ cells derived from the three cervical cancer cell lines.

In general, normal stem cells and cancer stem cells are more quiescent than differentiated cells in terms of cell division (25); cells resting in G0 are therefore less accessible to cell cycle-dependent treatments. In our study, we also found that ALDH1+cells had a higher proportion of Ki-67 negative cells compared to the corresponding ALDH1- cells, suggesting that ALDH1+ cells are more quiescent than ALDH1 cells. As shown for other epithelial cancers, Chen et al found that spheroid derived cells from head and neck squamous cell carcinoma contain a higher proportion of quiescent cells than parental monolayer-derived cells (11). This demonstrates that ALDH1+ from cervical cancer cell lines exhibit CSC characteristics.

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. The immunosuppressive properties of CSCs had been confirmed in other tumor types. For example, Chikamatsu et al. 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 (26). Culture supernatants of CD44+ cells contained significantly higher levels of IL-8, G-CSF, and TGF-β than those of CD44- cell cultures. Similarly, Tomas et al. reported that allogeneic glioblastoma multiforme CSCs but not their paired FBS cultured non-CSC tumor lines could inhibit the mitogen-induced (PHA+ and Con A) proliferation of T cells from healthy donors (27). Our results demonstrated that ALDH1+ cells from cervical

| Table 3. Cytokine production by anti-CD3/anti-CD28-activated PBMCs from 2 donors co-cultured with ALDH1 sorted tumor cells. |
|-----------------|----------------|----------------|---------------|----------------|---------------|----------------|
|                 | IFN gamma | IL-2 | IL-21 | TNF-alpha | TNF-beta | MIP-1alpha | MIP-1beta | GMCSF |
| donor 1          |           |      |       |           |           |            |           |       |
| MRH215 ALDH1+   | 353.67±185.29 | 21.50±3.27 | 27.50±9.37 | 32.67±7.66 | 91.17±43.52 | 810.50±398.29 | 2159.05±374.16 | 19.55±2.65 |
| ALDH1-           | 2356.33±359.01** | 317.83±103.24** | 97.17±39.54** | 632.67±227.24** | 1421.17±421.33** | 3652.34±564.18** | 8297.45±2088.88** | 1198.55±412.51** |
| CaSki ALDH1+     | 1153.33±121.68 | 28.19±6.31 | 29.17±5.51 | 52.15±8.35 | 235.53±49.23 | 1312.17±487.82 | 4439.21±1367.52 | 17.21±1.39 |
| ALDH1-           | 3198.50±1125.95* | 639.12±232.54** | 167.25±59.37* | 919.35±429.74** | 2539.04±969.32** | 4712.39±1638.13** | 10697.19±369.57** | 1795.39±921.49** |
| Control          | 3298.25±1654.24 | 2387.57±852.35 | 85.37±8.29 | 1189.76±157.36 | 1796.38±382.02 | 11725.67±1187.36 | 17594.38±1398.57 | 1682.45±258.55 |
| donor 2          |           |      |       |           |           |            |           |       |
| MRH215 ALDH1+   | 1245.6±235.70 | 1598.04±275.24 | 91.7±4.98 | 526.37±81.45 | 1056.65±159.63 | 3496.17±413.32 | 3698.17±401.26 | 161.15±26.13 |
| ALDH1-           | 7365.42±379.83** | 1599.33±332.06 | 518.17±89.47** | 2457.33±433.23** | 5042.00±733.56** | 15208.70±398.76** | 15639.50±392.37** | 7959.14±1365.37** |
| CaSki ALDH1+     | 1367.67±539.75 | 2368.17±425.35 | 109.57±5.16 | 743.71±245.75 | 1959.38±315.74 | 6646.33±1251.93 | 6359.13±1168.93 | 1056.05±597.35 |
| ALDH1-           | 8658.71±3013.26** | 3595.07±573.15* | 509.05±268.15* | 4214.52±1795.37** | 5151.05±1693.43* | 17475.52±677.54** | 12371.76±1503.47** | 8539.08±4184.16* |
| Control          | 8922.83±847.18 | 2175.76±602.19 | 798.71±214.45 | 4219.33±826.36 | 6244.49±1366.62 | 12037.66±820.55 | 25686.38±183.66 | 9764.38±1725.59 |

The results represent mean value ± SD of three independent determinations.

*Represents statistically significant differences of T cells co-cultured with ALDH1+cervical cancer cells compared to those co-cultured with ALDH1 cervical cancer cells. **P < 0.05.
cancer cell lines consistently showed greater suppressive activity on the proliferation of T cells compared to ALDH1 cells.

Moreover, as for T cell activation, our results showed that ALDH1+/cells when compared with ALDH1 cells inhibited more strongly CD3/CD28 polyclonal activation and the expression of CD69, CD137 and CD154, which are considered T cell activation marker molecules. Further studies were conducted to investigate the inhibition of cytokine production of T cells by ALDH1+cells. Consistent with the observations above, ALDH1+ cells suppressed more strongly CD3/CD28 induced production of IFN-γ, IL-2, and TNF-α in pre-activated effector T cells. These findings indicated that ALDH1+ cells from the investigated cervical cancer cell lines had a more immunosuppressive effect on T cell proliferation, activation, and cytokine production than ALDH1 cell.

Monocytes and macrophages are essential for innate and adaptive host defense and play a central role in the inflammatory response (28). Macrophages are a heterogeneous population and are typically defined as being of a classically activated M1 macrophage phenotype or alternatively activated M2 macrophage phenotype (29). M1 macrophages are characterized by their ability to kill tumor cells. In contrast, M2 macrophages promote tumor cell growth and metastasis (30). Myeloid differentiation is often disturbed in cancer, leading to reduced frequencies of immunostimulatory dendritic cells and an over-representation of immunosuppressive immature myeloid cells, and macrophages (31). A few recent studies analyzing M2-type macrophages indicated that a dense M2 macrophage infiltration is associated with poor survival in different carcinomas (32). Moreover, de Vos van Steenwijk et al. reported that the numbers of CD163+ CD14- correlated to intraepithelial Foxp3+ lymphocytes and might reflect immunosuppressive DCs or DC-derived macrophages (21). In our reports we also demonstrated that the cervical CSCs contributed to the conversion of monocytes to an immunosuppressive immature M2 macrophage (CD14+CD33+CD163+) or MDSC (CD163+CD14+) phenotype.

VITAL-FR is an extension to the VITAL assay, a flow cytometry-based assay system assessing CTL frequency and function (12). The sensitivity and reproducibility of the VITAL-FR assay has been described before, proving it is a highly sensitive and flexible flow cytometry-based in-vitro assay for clinically relevant specific CTL function. Here, we adapted the VITAL assay to quantify and compare the efficacy of CMV-specific T cell-mediated target cell killing in the presence of ALDH1+ or ALDH1- cells. We found that CMV-specific CTL co-cultured with ALDH1+ cells showed stronger functional inhibition and a decrease in the frequency of CMV-specific CD8+ T cells than those co-cultured with ALDH1- cells. Consistent with this observation, ALDH1+ cells showed greater suppressor activity on the cytotoxic T cell effector functions of established CMV-specific T cells than ALDH1- cells. 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 prohibited CTL degranulation and cytolyis (33). 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.

The high tumorigenic and metastatic properties of CSCs could be based on their increased ability to produce cytokines and chemokines, which, through autocrine and paracrine mechanisms, stimulate proliferation of tumor cells and migration and proliferation of stromal cells to form a network of blood vessels that support tumor growth (34). We performed a comprehensive analysis of these factors in cervical cancer cell lines derived ALDH1+cells and ALDH1 cells. We found that ALDH1+ cells, in comparison with ALDH1- cells, produced significantly higher levels of proinflammatory cytokine IL-6 and chemokines GRO-alpha(CXCL1), IL-8 (CXCL8), MCP-1 (CCL2), SDF-1α (CXCR4), RANTES (CCL5), IP-10(CXCL10) which are potent stimulators of angiogenesis and tumor cell proliferation and play an important role in tumor progression and metastasis in a variety of human cancers, including cervical cancer(35, 36). Furthermore, ALDH1+ cells from cervical cancer cell lines showed higher levels of IL-1RA. IL-1RA is a naturally occurring antagonist for IL-1. Fujiwaki et al. showed that IL-1 RA is overexpressed in tissue and serum from patients with cervical carcinoma and in univariate analysis predicted poor disease-free survival (37). Thus, CSCs seem to produce increased levels of cytokines and chemokines, which modulate the tumor microenvironment that tumor cells exploit to their advantage as compared to non-CSCs.

Moreover, our study indicates that ALDH1+ cells exhibited a significantly higher inhibition of production of αCD3/αCD28 stimulated immune-cell derived cytokines and chemokines (IL-2, IL-21, TNF-β, IFN-γ and TNF-α, MIP-1α, MIP-1β, and GMSF) than ALDH1- cells. These cytokines and chemokines are
mainly produced by activated T cells, indicating a second mechanism of ALDH1+ cells mediated suppression of T cell-mediated immune responses. This production of immunosuppressive factors may represent another powerful strategy used by tumors to escape immune surveillance.

Conclusion

The ALDH1+ population within the CaSki, HeLa and MIRI-H-215 cervical cancer cell lines 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 by targeting CSC.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

References


