

Published in final edited form as:

Head Neck. 2011 February ; 33(2): 208–215. doi:10.1002/hed.21420.

IMMUNOREGULATORY PROPERTIES OF CD44+ CANCER STEM-LIKE CELLS IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Abstract

Background—CD44 was found as a surface marker in cancer stem cell (CSC) of squamous cell carcinoma of the head and neck (SCCHN); however, the immunologic properties of such CSCs have not yet been elucidated.

Methods—The immunologic properties of CD44+ cancer stem-like cells were compared with those of CD44– cells using flow cytometry and enzyme-linked immunosorbent assay.

Results—CD44+ cells exhibited weak HLA-A2 and class II expression. Interestingly, downregulation of transporter antigen processing (TAP)2 was found in CD44+ cells. The CD44+ cell population produced significantly higher levels of interleukin (IL)-8, granulocyte colony-stimulating factor (G-CSF), and transforming growth factor (TGF)- β than the CD44– cell population. Moreover, CD44+ cells have been shown to not only more strongly inhibit T-cell proliferation, but also to more efficiently inhibit regulatory T cells (Treg cells) and myeloid-derived suppressor cells (MDSC) as compared with CD44– cells. Additionally, CD44+ cells suppressed Th1 responses and enhanced regulatory T cell responses.

Conclusion—CSCs might have higher malignant potential with numerous escape strategies from immune attack.

Keywords

cancer stem cells; CD44; squamous cell carcinoma of the head and neck; immunosuppression; tumor immunity

It is well recognized that solid tumors are heterogeneous, composed of cells with different phenotypic characteristics, proliferative, differentiated, and/or with malignant potential. Recently, there has been overwhelming evidence that only a minority of cancer cells with stem cell properties, cancer stem cells (CSCs), are responsible for the maintenance and growth of the tumor. These CSC subpopulations show a capacity for highly tumorigenicity, self-renewal, and differentiation. To date, human CSCs have been identified and purified in a variety of malignancies, including those of the brain, breast, prostate, colon, pancreas, and head and neck.^{1–6} Moreover, several studies have demonstrated that drug or radiation treatment of tumor cells can enrich and maintain the CSC subpopulation in vitro and in

vivo,^{7–10} suggesting that CSCs are responsible for tumor regeneration after conventional cancer treatments. Thus, CSCs have high malignant potential; however, the immunologic properties of CSCs remain unclear.

In general, it has long been thought that antitumor immunity plays an important role in the protection against the development of malignancy. In earlier stages of cellular transformation, immunosurveillance can detect and eliminate tumor cells; however, with a developing tumor, tumor variants with reduced immunogenicity, and/or acquired various mechanisms to corrupt the host antitumor response and escape from the host immune system arise, survive and grow in the host.^{11,12} As a result, interactions between tumor cells and host immune cells in the tumor microenvironment create an immunosuppressive network in which CSCs might actively participate in inducing immunosuppressive ability and promoting evasion from immunosurveillance due to their malignant potential. Indeed, Levina et al¹³ have reported that lung CSCs selected by treatment with chemotherapeutic drugs produce higher levels of angiogenic and growth factors, such as vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-8, and granulocyte colony-stimulating factor (G-CSF), than a parental cell line. Moreover, CSCs expressed higher levels of cancer-associated antigens, including embryonic cancer antigens, which are often detected in malignantly transformed cells.¹³ On the other hand, Kawasaki et al¹⁴ found that CSCs derived from a prostate cancer cell line showed an increased expression of the glycoprotein CD200, which is involved in immunosuppression and immune tolerance.

More recently, we have demonstrated the identification, expansion, and characterization of CD44+ cancer stem-like cells in a squamous cell carcinoma of the head and neck (SCCHN) cell line.¹⁵ Under serum-free medium culture conditions, we enriched a subpopulation of CD44+ cells that possess marked capacity for forming tumor spheres, proliferation, migration, and invasion in vitro. Furthermore, the CD44+ cell population that had been purified using immunomagnetic beads was significantly more resistant to various chemotherapeutic agents than the CD44– cell population. In the present study, we compared the immunologic properties of CD44+ cancer stem-like cells with CD44– cells. Our findings provide new insights into our understanding of immunosuppressive mechanisms in the tumor microenvironment and novel therapeutic approaches against CSCs in SCCHN.

MATERIALS AND METHODS

Cell Line and Culture Conditions

A human cultured cancer cell line, Gun-1, was established from a squamous cell carcinoma of the hypopharynx.¹⁶ Gun-1 was cultured with Serum-Free Expansion medium (StemCell Technologies, Vancouver, Canada) supplemented with epidermal growth factor (EGF; Calbiochem, Darmstadt, Germany) and basic fibroblast growth factor (bFGF; Calbiochem; 20 ng/mL each), as previously described.¹⁵

Antibodies

The fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and allophycocyanin (APC)-conjugated anti-CD44 monoclonal antibodies (mAb) were purchased from BD Pharmingen (San Diego, CA). The HLA-A2-specific mAb MA2.1 and BB7.2 and the HLA class I-specific mAb W6/32 were kindly provided by Dr A. B. DeLeo (University of Pittsburgh Cancer Institute, Pittsburgh, PA). HLA-A24-specific mAb (anti-HLA-A23, A24 mAb, IgG2b) was purchased from One Lambda, (Canoga Park, CA). The low-molecular-weight protein (LMP)2-specific mAb SY-1, LMP7-specific mAb HB2, LMP10-specific mAb TO-6, TAP1-specific mAb NOB1, TAP2-specific mAb NOB2, tapasin-specific mAb TO-3, HLA-class II-specific mAb LGII-612.14, and β 2 microglobulin (β 2m)-specific mAb NAMB-1

were developed and characterized as described previously.^{17–20} Labeled mAbs for staining CD4+CD25+ regulatory T-cells (Treg cells; APC-conjugated anti-CD4 mAb, FITC-conjugated anti-CD25 mAb) and myeloid-derived suppressor cell (MDSCs; FITC-conjugated lineage cocktail containing anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56 mAb, PE-conjugated anti-HLA-DR mAb, and APC-conjugated anti-CD33 mAb) were purchased from BD Pharmingen. PE-conjugated anti-FOXP3 mAb was from eBioscience (San Diego, CA).

Flow Cytometry

Trypsinized cells were resuspended, incubated with mAb for 30 minutes at 4°C, washed twice with phosphate-buffered saline (PBS) containing 0.1% fetal bovine serum and 0.1% NaN₃, and fixed with 1% paraformaldehyde in PBS. Intracellular staining of tumor cells with antigen processing machinery (APM) component-specific mAbs was performed as previously described²¹ and permeabilization with 0.1% (w/v) saponin in PBS-bovine serum albumin buffer was used to visualize intracytoplasmic targets. FITC-conjugated goat anti-mouse monoclonal antibody (BD Pharmingen) was used as a secondary antibody. To determine the frequency of Treg cells in peripheral blood mononuclear cells (PBMCs), the harvested PBMCs were stained with CD4+CD25+ FOXP3+ using a FOXP3 staining buffer kit (eBioscience) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry (Beckton Dickinson, Franklin Lakes, NJ). Respective immunoglobulin G isotype-matched controls (BD Pharmingen) were used as negative controls.

Magnetic Cell Sorting

The cells were incubated with anti-CD44-biotin (BD Pharmingen) for 5 minutes at 4°C. After washing once, 20 µL anti-biotin microbeads (Miltenyi Biotec)/1 × 10⁷ cells were added for 15 minutes at 4°C. Subsequently, cells were washed once, resuspended, and applied to MACS separation columns (Miltenyi Biotec). Positive (CD44+) and negative (CD44-) fractions were resuspended in SFM with EGF and bFGF for further experiments, respectively.

Cytokine Enzyme-Linked Immunosorbent Assay

Cytokines in culture supernatants of CD44+ and CD44- cells were quantified with the ELISArrays (SABiosciences, Frederick, MD) according to the manufacturer's instructions. Briefly, the sorted cells were seeded at 5 × 10⁵ cell/mL in a 24-well plate, and culture supernatants were harvested after 48 hours.

Carboxyfluorescein Succinimidyl Ester-Based Suppression Assay

The responder PBMCs obtained from healthy donors were stained with 1.5 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probe/Invitrogen, Grand Island, NY), and cocultured with the sorted CD44+ or CD44- cells. Briefly, responder cells were stimulated with expander beads (Miltenyi Biotec) in AIM-V medium containing IL-2 (100 IU/mL) in 96-well plates (1 × 10⁵ cells/well). The sorted CD44+ or CD44- cells were added to responder cells at different ratios and cocultures were incubated for 6 days. Proliferation of T cells was measured by flow cytometry on the basis of CFSE dilution.

Induction of Regulatory T Cells and Myeloid Derived Suppressor Cells

Twenty-four-well Transwell chambers with a 0.4 µm porous membrane (Corning Costar, Lowell, MA) were used. Briefly, the sorted cells were plated underneath the Transwell chamber (2.5 × 10⁴ cells/well), and PBMCs (1 × 10⁵ cells) were added to the inner chamber. Cells were cultured in AIM-V medium for 6 days. After harvest, PBMCs were stained for

flow cytometry. Treg cells and MDSCs were defined as CD4⁺CD25⁺FOXP3⁺ and Lin[−]HLA-DR-CD33⁺ cells, respectively.²²

Cytokine Production by Phytohemagglutinin-Activated Peripheral Blood Mononuclear Cells Cocultured with Tumor Cells

PBMCs obtained from healthy donors were plated at 1×10^6 cells per well of 24-well plates in the presence of phytohemagglutinin (PHA; 10 µg/mL) in AIM-V medium and equipped with inner Transwell chambers. Sorted CD44⁺ or CD44[−] cells (2×10^5 cells) were placed in the Transwell insert. After 48 hours, supernatants were collected and levels of interferon (IFN)- γ , IL-2, and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA; Endogen, Pierce Biotechnology, Rockford, IL).

Statistical Analysis

The 2-tailed *t* test and the Scheffe test were used for statistical analysis of data. The *p* values <.05 were considered significant. Analyses were performed using Stata 9.0 (Stata Corp, College Station, TX).

RESULTS

HLA Molecules and Antigen Processing Machinery Component Expression in CD44⁺ and CD44[−] Cells

As reported previously,¹⁵ the CD44⁺ population in Gun-1 cultured in SFM containing EGF and bFGF was increased up to approximately 40%, and the expressions of HLA molecules and APM components were compared in CD44⁺ and CD44[−] cells by flow cytometry. As shown in Figure 1A, there was no difference in the expression of HLA class I, HLA-A24, and β 2-microglobulin molecules in these 2 subpopulations. Meanwhile, the Gun-1 cell line originally had allele-specific deletion of HLA-A2 molecules; however, CD44⁺ cells expressed HLA-A2 molecules. This finding was confirmed using 2 different HLA-A2-specific mAb, MA2.1, and BB7.2. Moreover, the expression level of HLA-A2 molecules on CD44⁺ cells was modest compared to that of PBMCs obtained from HLA-A2⁺ healthy donors (data not shown). On the other hand, unexpectedly, CD44⁺ cells, but not CD44[−] cells, expressed HLA class II molecules. These findings suggest that CD44⁺ cells may have different immunologic properties to CD44[−] cells. Among 6 APM components tested, the expressions of LMP-2, -7, -10, TAP-1, and Tapasin were similar in CD44⁺ and CD44[−] cells. In contrast, the TAP-2 molecule was downregulated in CD44⁺ cells compared to CD44[−] cells (Figure 1B).

Cytokine Production by CD44⁺ and CD44[−] Cells

The supernatants of sorted CD44⁺ and CD44[−] cells were analyzed for levels of cytokines. Supernatants of CD44⁺ and CD44[−] cell cultures were positive for IL-4, IL-6, IL-8, TNF- α , G-CSF, and transforming growth factor-beta (TGF- β) and negative for IL-2 and IL-10 (Table 1). The supernatant of CD44⁺ cell cultures had significantly higher levels of IL-8, GCSF, and TGF- β than those of CD44[−] cell cultures, whereas levels of cytokines, including IL-4, IL-6, and TNF- α in CD44⁺ cell cultures, were comparable in supernatants of CD44[−] cell cultures.

Inhibition of T-cell Proliferation

To assess the effect of CD44⁺ and CD44[−] cells on T-cell proliferation, the sorted CD44⁺ and CD44[−] cells were cocultured with CFSE-labeled T cells. After 6 days of coculture with anti-CD3/anti-CD28 stimulus, proliferation of T cells was measured using flow cytometry. In cocultures, both CD44⁺ and CD44[−] cells inhibited the proliferation of anti-CD3/anti-

CD28 stimulated T cells in a dose-dependent manner (Figure 2). Moreover, CD44⁺ cells showed greater suppressor activity than CD44⁻ cells.

Induction of Treg Cells and Myeloid Derived Suppressor Cells

Because several tumor-derived factors have been implicated in the development of immune-suppressive cells, we next compared the percentages of Treg cells and MDSC in PBMCs by coculture with sorted CD44⁺ or CD44⁻ cells. Representative dot plots of Treg cells (CD4⁺CD25⁺FOXP3⁺) and MDSC (Lin⁻HLA-DRCD33⁺) are shown in Figure 3A. As expected, cocultures in the presence of tumor cells showed increased proportions of Treg cells and MDSCs in the PBMCs. Moreover, the proportions of both Treg cells and MDSCs in PBMCs cocultured with CD44⁺ cells were significantly higher than those with CD44⁻ cells (Figure 3B).

Cytokine Production by Phytohemagglutinin-Activated Peripheral Blood Mononuclear Cells Cocultured with Tumor Cells

The supernatants of PHA-activated PBMCs cocultured with CD44⁺ or CD44⁻ cells were analyzed for levels of cytokines (Table 2). Both CD44⁺ and CD44⁻ cells showed significant inhibition of IFN- γ and IL-2 production by PHA-activated PBMCs. Furthermore, CD44⁺ cells significantly enhanced inhibition of IFN- γ production compared to CD44⁻ cells. Meanwhile, CD44⁺ but not CD44⁻ cells significantly enhanced IL-10 production by PHA-activated PBMCs. These findings suggest that CD44⁺ cells may suppress T helper type 1 (Th1) responses and induce regulatory T cell response more efficiently than CD44⁻ cells.

DISCUSSION

The primary objective of this study was to evaluate the immunologic properties of CD44⁺ cancer stem-like cells in the SCCHN cell line. CD44 was found as a surface marker in CSC of head and neck cancer, and several different cancers including breast,² prostate,³ and pancreas.⁵ Here, we were able to demonstrate a variety of unique immunologic functions of CD44⁺ cancer stem-like cells, as opposed to CD44⁻ cells. In general, tumor cells can escape immune responses by numerous mechanisms, including the production of immunosuppressive factors and low levels or a lack of expression of tumor antigens, major histocompatibility complex molecules, or co-stimulatory molecules. Indeed, allele-specific deletion of HLA-A2 molecules was observed in a parental cell line.¹⁶ Defective expression of HLA class I-tumor antigen-derived peptide complexes led to the escape from cytotoxic T lymphocyte recognition and destruction, whereas such tumor cells became more susceptible to natural killer cell-mediated lysis. Interestingly, CD44⁺ cells restored HLA-A2 expression; however, at very low levels compared to normal HLA-A2-positive PBMCs, suggesting that such CD44⁺ cells may not be recognized by either cytotoxic T lymphocytes or natural killer cells. Moreover, CD44⁺ cells exhibited a weak HLA class II expression on the cell surface, although CD44⁻ cells did not express HLA class II. In studies using embryonic stem cells and mesenchymal stem cells, the expressions of HLA antigens have been shown to depend on their levels of differentiation^{23–25}; therefore, CSCs and their differentiated derivatives, non-CSCs, may show different levels of HLA antigen expression. However, this issue needs further detailed study. In addition, downregulation of TAP2 was found in CD44⁺ cells. To date, defects in the expression and/or functions of APM components are found in various malignancies.^{26–29} These abnormalities may have a negative impact not only on the interactions of tumor cells with the immune system, but also on clinical outcomes. Ogino et al.²⁷ have reported that the expression of APM components correlated with the extent of CD8⁺ T-cell infiltration in laryngeal carcinoma. Moreover, LMP2 and HLA class I antigen downregulations were significantly associated with reduced patient survival.²⁷ Thus, our findings support that CSCs might be less immunogenic than non-CSCs.

Next, we investigated how CD44⁺ cells contribute to the downregulation of antitumor immunity in the tumor microenvironment. Tumor cells initially produce a variety of cytokines to induce immune tolerance and promote angiogenesis and carcinogenesis. In SCCHN, Young et al³⁰ indicated that both primary tumors and cell lines derived from tumor biopsies have been shown to secrete detectable amounts of the proinflammatory cytokines IL-1 α , IL-6, IL-8, and GM-CSF, and also inhibitory factors, such as TGF- β , PGE2, and IL-10. As expected, this cell line also produced IL-4, IL-6, IL-8, TNF- α , G-CSF, and TGF- β . It is interesting that CD44⁺ cells produced significantly higher levels of IL-8, G-CSF, and TGF- β than CD44⁻ cells. These cytokines are known to promote tumor cell proliferation, angiogenesis, invasion, and metastatic dissemination.^{31–33} Thus, CSCs seem to produce increased levels of cytokines, which modulate the tumor microenvironment that tumor cells exploit to their advantage as compared to non-CSCs. Alternatively, TGF- β is also a potent inducer of epithelial-mesenchymal transition (EMT), which is a key developmental program required for tumor invasion and metastasis. Recently, several studies have demonstrated that the induction of EMT in cancer cells is associated with the acquisition of stem-like properties,^{34–36} suggesting that EMT promotes the generation of CSCs. Accordingly, CSCs may maintain their stem-like properties themselves through autocrine and paracrine mechanisms.

Our data demonstrate that CD44⁺ cells show 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 MDSC than CD44⁻ cells. Moreover, CD44⁺ cells also suppressed Th1 responses and enhanced regulatory T cell responses. To date, several distinct cell subsets with immune suppressive activity, including CD4⁺ CD25⁺FOXP3⁺ naturally occurring Treg cells, IL-10-producing Tr1, and MDSC have been identified. Recent studies have demonstrated that these immune regulatory cells accumulate in the tumor microenvironment, promote tumor growth, and downregulate antitumor responses.^{11,37,38} Our findings strongly suggest that CD44⁺ cells possess preferential capacity to enhance immunosuppressive ability, leading to the dysfunction of effector T-cell populations. Thus, in the tumor microenvironment, CSCs might play a pivotal role in establishing an immunosuppressive network and subsequent facilitation to activate the immunosuppressive pathway.

Taken together, our data suggest that CSCs have higher malignant potential with numerous escape strategies from immune attack, including less immunogenicity, production of inhibitory cytokines, and the induction and recruitment of immune regulatory cells. Tumor escape from the host immune system has been a major problem in immunotherapy, and CSCs could be particularly refractory to current immunotherapy.³⁹ Accordingly, the development of novel immunotherapeutic strategies to efficiently overcome CSC-driven immune suppression might be urgently needed.

Acknowledgments

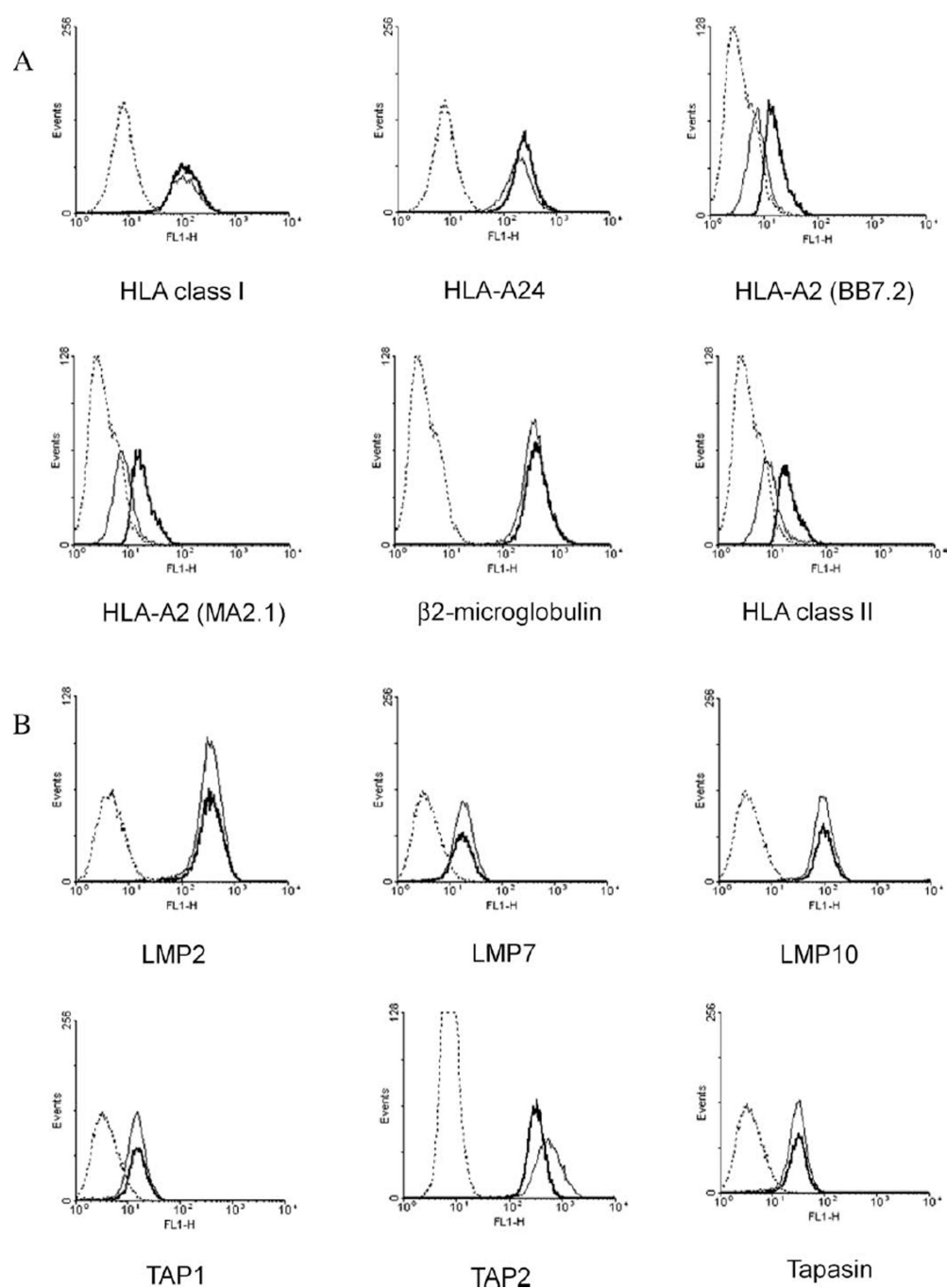
This work was supported in part by grants-in-aid (20592013 to K.C., 20592014 to K.M.) from the Ministry of Education, Cultures, Sports, Science, and Technology, Japan

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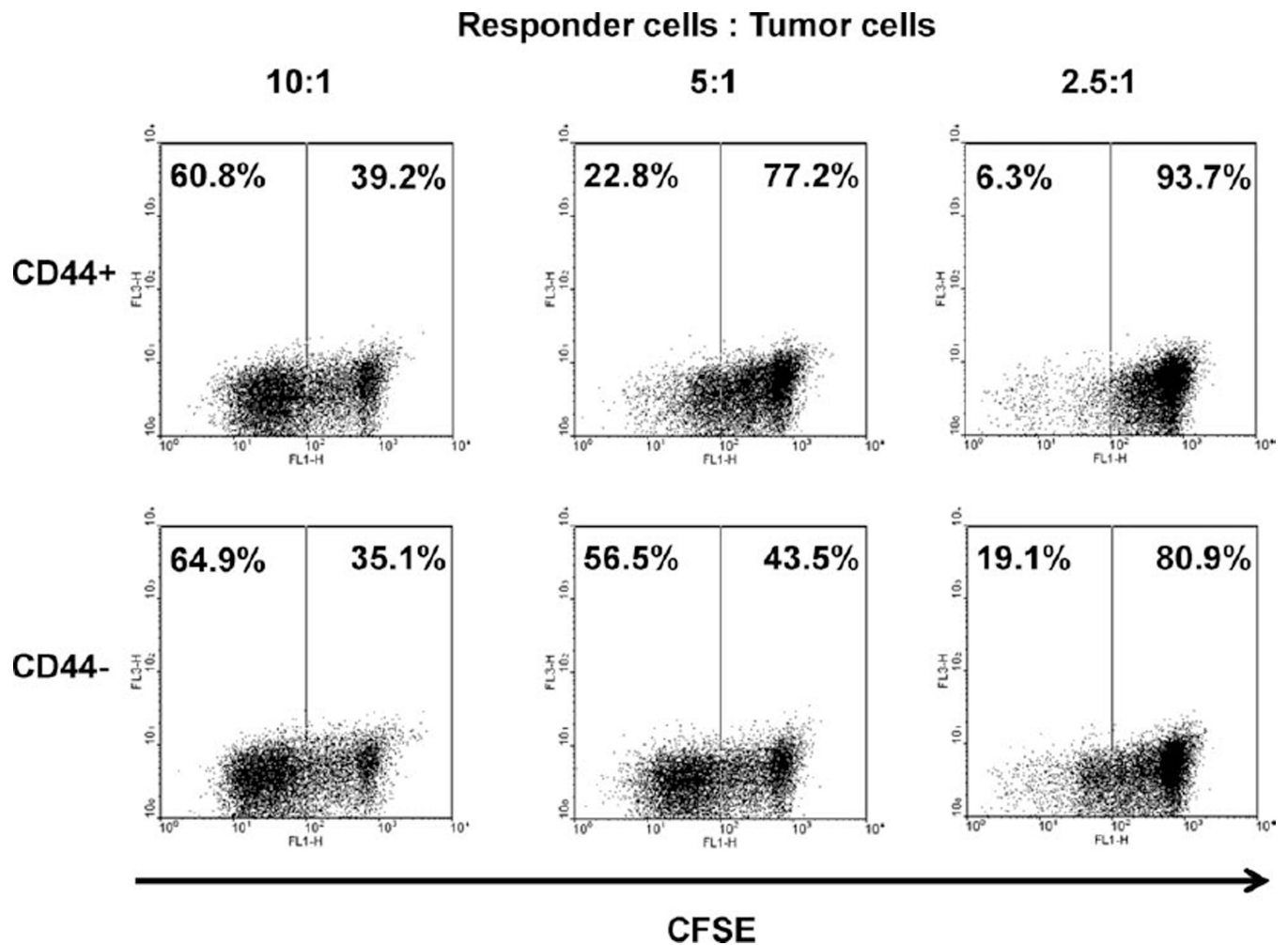
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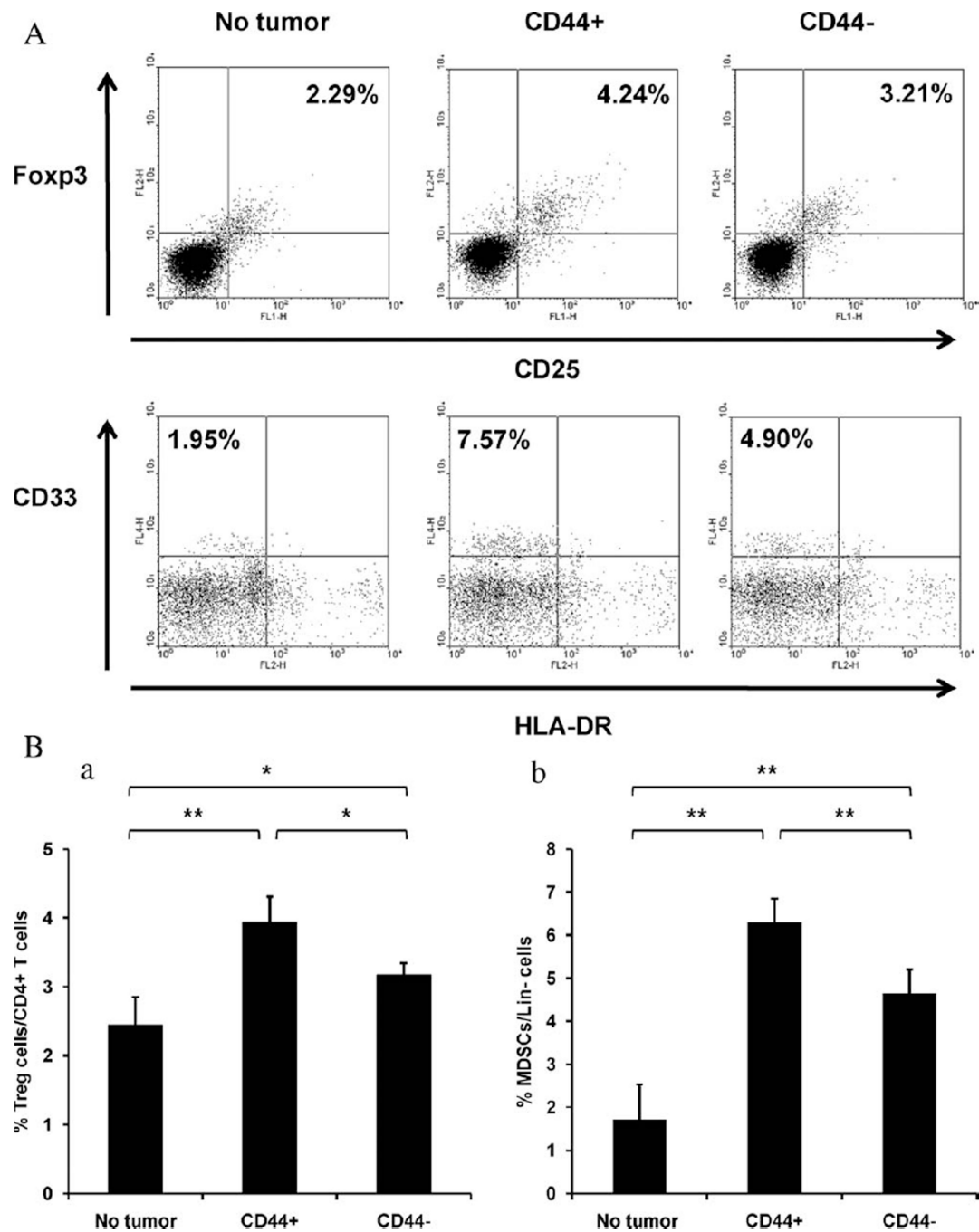
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**FIGURE 1.**

Flow cytometry analysis of CD44⁺ and CD44⁻ populations in the squamous cell carcinoma of the head and neck (SCCHN) cell line. **(A)** Expression of surface HLA-class I, HLA-A2, HLA-A24, β 2-microglobulin, and HLA class II molecules on the surface of tumor cells. **(B)** Expression of antigen processing machinery (APM) components (LMP2, LMP7, LMP10, TAP1, TAP2, and Tapasin) in tumor cells. On the histograms, the thin line represents expression on the CD44⁻ population, whereas the bold line represents expression on the CD44⁺ population. Representative results from 1 of 3 experiments are shown.

**FIGURE 2.**

Suppression activity of CD44+ and CD44- cells on T-cell proliferation. Sorted CD44+ and CD44- cells were cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells for 6 days with anti-CD3/anti-CD28 stimulus. Proliferation of T cells was measured by reduction of CFSE staining intensity using flow cytometry. Representative results from 1 of 5 experiments are shown.

**FIGURE 3.**

Frequency of regulatory T cells (Treg cells; CD4+CD25+FOXP3+) and myeloid derived suppressor cells (MDSC; Lin-HLADR-CD33+) in peripheral blood mononuclear cells (PBMCs) cocultured with sorted CD44+ and CD44- cells. Sorted cells were plated underneath the Transwell chamber (2.5×10^4 cells/well), PBMCs (1×10^5 cells) were added to the inner chamber, and then cultured for 6 days. Flow cytometry analysis was performed with gates set on CD4+ T-cells and Lin-cells, respectively. (A) Representative results from 1 of 3 experiments. (B) Mean values of the percentage of CD25+FOXP3+ cells in CD4+ cells

and HLA-DR-CD33+ cells in Lin-cells from 3 independent experiments. Asterisks indicate significant differences (* $p < .05$ and ** $p < .01$).

Table 1

Levels of cytokines measured in culture supernatant of CD44+ and CD44– cells.

Cytokines	Mean ± SD (pg/mL)		<i>p</i> value
	CD44+ cells	CD44– cells	
IL-2	Not detected	Not detected	–
IL-4	100.8 ± 32.6	85.2 ± 5.9	.459
IL-6	867.4 ± 326.6	1306.2 ± 401.9	.216
IL-8	3686.8 ± 500.1	2604.1 ± 380.9	.014
IL-10	Not detected	Not detected	–
TNF-α	68.3 ± 27.7	63.5 ± 15.2	.759
G-CSF	1073.5 ± 362.3	618.9 ± 232.0	.040
TGF-β	642.8 ± 129.6	195.7 ± 134.4	.007

Note: Sorted CD44+ and CD44– cells were seeded at 5×10^5 cell/mL in 24-well plates, and culture supernatants were harvested after 48 hours. The levels of cytokines in supernatants were measured by enzyme-linked immunosorbent assay (ELISA). The results are expressed as mean ± SD from 5 independent experiments.

Table 2

Cytokine production by PHA-activated PBMCs cocultured with tumor cells.

Cocultured with	Mean \pm SD (pg/mL)		
	IFN- γ	IL-2	IL-10
CD44+ cells	1003.8 \pm 57.7	60.8 \pm 4.3	603.4 \pm 38.6
CD44- cells	1205.1 \pm 30.1	56.4 \pm 2.9	533.2 \pm 42.1
No tumor cells	1269.3 \pm 24.5	95.6 \pm 6.4	506.1 \pm 9.4

* p < .05.

Note: Culture supernatants from PHA-activated PBMCs cocultured with tumor cells were harvested after 48 hours as described in Materials and Methods. The levels of cytokines in supernatants were measured by ELISA. The results are expressed as mean \pm SD from 4 independent experiments.