

Article

Differential Regulation of Soluble and Membrane 4-1BB and OX-40 Within the Peripheral Compartment of Cervical Cancer

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Abstract

Background: Members of the tumor necrosis factor receptor superfamily (TNFRSF), including 4-1BB (CD137) and OX-40 (CD134), play central roles in regulating cytotoxic lymphocyte activation and anti-tumor immunity. In addition to their membrane-bound forms, soluble receptor isoforms may modulate signaling by influencing ligand availability and receptor engagement. However, the peripheral regulation of soluble and membrane 4-1BB and OX-40 in cervical cancer remains incompletely defined. **Methods:** Plasma concentrations of soluble 4-1BB (s4-1BB) and soluble OX-40 (sOX-40) were quantified by ELISA in 20 treatment-naïve cervical cancer (CC) patients and 20 healthy controls (HCs). Membrane expression (m4-1BB and mOX-40) on circulating T cells and NK cells was evaluated by flow cytometry. Correlation and stage-stratified analyses were performed. **Results:** Plasma levels of s4-1BB and sOX-40 were significantly elevated in CC patients compared with HCs. Membrane 4-1BB expression was increased on both T cells and NK cells, while OX-40 expression was selectively elevated in NK cells, with no difference observed in T cells. A moderate positive correlation was identified between s4-1BB and T-cell membrane 4-1BB expression. Notably, membrane 4-1BB expression decreased in advanced-stage disease, whereas soluble concentrations remained unchanged. **Conclusions:** These findings demonstrate differential regulation of soluble and membrane 4-1BB and OX-40 in the peripheral compartment in cervical cancer, highlighting distinct regulatory layers within the TNFRSF axis.

Keywords: 4-1BB; OX-40; TNFRSF; soluble receptors; membrane expression; cervical cancer; immune modulation



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1. Introduction

Cervical cancer (CC) remains a major public health burden, particularly in low- and middle-income countries, where approximately 90% of cases occur and mortality rates are

up to 18-fold higher than in high-income regions [1]. In countries such as Mexico, despite sustained public health efforts and improvements in diagnostic strategies, cervical cancer remains the gynecological malignancy with the highest fatality rate [2].

This persistent mortality burden highlights the need to elucidate the biological mechanisms underlying cervical cancer progression. The host immune response, particularly T cell- and natural killer (NK) cell-mediated immunity, is a critical determinant of tumor development and disease outcome [3].

Within these cytotoxic populations, members of the tumor necrosis factor receptor superfamily (TNFRSF) have emerged as critical regulators of cancer progression. TNFRSF signaling exerts paradoxical effects within the tumor microenvironment. While certain members of this family promote proliferation, invasion, metastasis, and angiogenesis, others activate death domain-dependent pathways that trigger tumor cell apoptosis [4].

Among these receptors, 4-1BB and OX-40 have been identified as central mediators of antitumor immune responses [5]. Both receptors recruit TNF receptor-associated factors (TRAFs), adaptor proteins that connect TNFRSF to activation of NF- κ B and mitogen-activated protein kinases (MAPKs), including JNK, ERK, and p38. While 4-1BB binds TRAF1, TRAF2, and TRAF3, OX-40 recruits TRAF2, TRAF3, TRAF5, and TRAF6 [6].

Notably, 4-1BB (TNFRSF9, CD137) is a 27 kDa inducible costimulatory glycoprotein receptor encoded by the *TNFRSF9* gene on chromosome 1p36.22. It is a 255-amino-acid type I transmembrane protein containing an N-terminal signal peptide, an extracellular region with four cysteine rich domains (CRDs), a transmembrane segment, and a cytoplasmic signaling domain harboring a TRAF binding motif [7]. This receptor is expressed on activated T cells, monocytes, dendritic cells, and NK cells, while its ligand, 4-1BBL (TNFSF9), is primarily found on antigen presenting cells [8]. Engagement of 4-1BB by its ligand promotes antitumor immunity by enhancing immune cell survival, proliferation, and effector function, particularly within cytotoxic lymphocyte populations [9].

Similarly, OX-40 (TNFRSF4, CD134) is a 50 kDa type I transmembrane glycoprotein encoded by the *TNFRSF4* gene on chromosome 1p36.33. It is composed of 275 amino acids and contains an extracellular ligand binding domain formed by four cysteine rich domain repeats [10]. OX-40 is inducibly expressed on activated T and NK cells, as well as other lymphoid populations, while its ligand OX-40L is typically expressed on activated antigen presenting cells [11]. Interaction between OX-40 and its ligand promotes T and NK cell activation, enhances cytokine production, and increases cytotoxic activity against target cells. This pathway plays a key role in amplifying immune responses, particularly in the context of infection and cancer [12].

Immune surveillance can be compromised within the tumor microenvironment through different mechanisms, including the release of soluble TNFRSF molecules or by shedding receptor extracellular domains that sequester their ligands, effectively neutralizing costimulatory signaling and weakening antitumor immunity [13].

Given the central role of 4-1BB and OX-40 in regulating cytotoxic lymphocyte function and the ability of tumors to induce exhaustion and the modulation of TNFRSF signaling through soluble receptor forms, a better understanding of their expression patterns in cervical cancer is needed. This study aims to evaluate the membrane-bound expression of 4-1BB and OX-40 in T cells and NK cells, as well as to quantify their soluble forms, in order to characterize their potential contribution to immune regulation in cervical cancer.

2. Materials and Methods

2.1. Sample Collection

A total of 20 women with newly diagnosed treatment-naïve cervical cancer were enrolled in this study. The cohort included 10 patients with early-stage disease (stages

I–II) and 10 patients with advanced-stage disease (stages III–IV). Patients were recruited from the Antiguo Hospital Civil de Guadalajara Fray Antonio Alcalde and the Instituto Jalisciense de Cancerología. Additionally, a healthy control group consisting of 20 age-matched women with no history of cancer, infectious diseases, or autoimmune disorders was recruited.

All procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the institutional research, bioethics, and biosafety committees of both participating hospitals. Written informed consent was obtained from all participants prior to inclusion in the study.

Peripheral blood samples were collected in EDTA-coated tubes. Plasma was separated by centrifugation at 3500 rpm for 10 min and stored at -80°C until further analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Stemcell Technologies, Vancouver, BC, Canada; cat. no. 07851), and subsequently cryopreserved in liquid nitrogen until evaluation.

2.2. ELISA

Plasma concentrations of soluble 4-1BB (s4-1BB) and soluble OX-40 (sOX-40) were quantified using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. Soluble 4-1BB was measured using the Human TNFRSF9 ELISA Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA; cat. no. EHTNFRSF9), and soluble OX-40 was determined using the Human OX-40 ELISA Kit (Invitrogen, cat. no. BMS296).

Plasma samples were thawed and diluted prior to analysis. Standard curves were generated using recombinant standards provided with each kit, and blank wells were included as controls. All samples and standards were analyzed in duplicate. Absorbance was measured at 450 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland).

Concentrations of s4-1BB and sOX-40 were calculated by interpolation from the respective standard curves using a four-parameter logistic (4PL) regression model for s4-1BB and a five-parameter logistic (5PL) regression model for sOX-40. Final concentrations were calculated according to the corresponding dilution factor.

2.3. Flow Cytometry

Cryopreserved PBMCs were thawed and cell viability was assessed by trypan blue exclusion. Samples with viability greater than 95% were included for analysis. The surface expression of 4-1BB and OX-40 (m4-1BB and mOX-40) was evaluated on T cells (CD3^{+}) and NK cells ($\text{CD3}^{-}\text{CD56}^{+}$) by multiparametric flow cytometry.

Briefly, 5×10^5 cells per sample were incubated with the following fluorochrome-conjugated monoclonal antibodies: CD3-FITC (BioLegend, San Diego, CA, USA; cat. no. 300406), CD56-PE-Cy7 (BioLegend, San Diego, CA, USA; cat. no. 362510), 4-1BB-PE (BioLegend, San Diego, CA, USA; cat. no. 309804), and OX-40-APC-Fire 750 (BioLegend, San Diego, CA, USA; cat. no. 350032).

Data acquisition was performed using an Attune NxT Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA). Instrument compensation was established using compensation beads (Becton Dickinson, San Jose, CA, USA; cat. no. 552843). A total of 250,000 events within the lymphocyte gate were acquired for each sample.

Data were analyzed using Kaluza software version 2.1 (Beckman Coulter, Brea, CA, USA).

2.4. Statistical Analysis

Data distribution was assessed using the D'Agostino–Pearson normality test. For normally distributed data, comparisons between groups were performed using an unpaired Student's *t*-test. Non-normally distributed data were analyzed using the Mann–Whitney U test. Correlations were evaluated using Spearman's rank correlation test. A *p*-value < 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism version 10 (GraphPad Software, Boston, MA, USA).

3. Results

3.1. Plasma Levels of s4-1BB and sOX-40 Are Elevated in Cervical Cancer

To determine whether systemic levels of costimulatory receptors are altered in cervical cancer and given that soluble forms of these molecules can function as regulatory decoys by sequestering their respective ligands and modulating receptor–ligand interactions, plasma concentrations of soluble 4-1BB and OX-40 were quantified in healthy controls and patients with cervical cancer (Figure 1). Levels of s4-1BB were significantly increased in CC patients compared to HCs (HC: 212.3 pg/mL vs. CC: 329.6 pg/mL; *p* = 0.035). Similarly, circulating sOX-40 levels were significantly elevated in CC patients (HC: 0.405 vs. CC: 1.477; *p* = 0.017). These findings demonstrate differences in circulating soluble 4-1BB and OX-40 levels between healthy individuals and patients with cervical cancer.

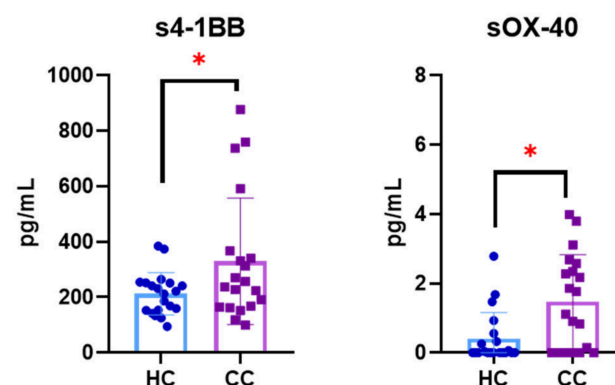


Figure 1. Plasma levels of soluble 4-1BB and OX-40 in cervical cancer patients. Plasma concentrations of s4-1BB and sOX-40 in HC and CC patients. Statistical significance was determined using an unpaired Student's *t*-test for s4-1BB and the Mann–Whitney U test for sOX-40. * *p* < 0.05.

3.2. Surface Expression of m4-1BB and mOX-40 in Circulating T and NK Cells from Cervical Cancer

Given that the functional relevance of costimulatory receptors is highly dependent on the cellular context, we next assessed the membrane-bound expression of 4-1BB and OX-40 on circulating T and NK cells (Figure 2). The gating strategy is provided in Supplementary Material (Figure S1).

The proportion of peripheral T cells expressing m4-1BB was significantly increased in CC patients compared with healthy controls, approximately doubling in frequency (HC: 2.56% vs. CC: 5.13%; *p* < 0.0001). A similar pattern was observed within the NK cell compartment, where the percentage of m4-1BB⁺ cells was markedly higher in CC patients (HC: 8.84% vs. CC: 16.86%; *p* = 0.0002).

In contrast, no difference was detected in the proportion of T cells expressing mOX-40 between groups (HC: 5.17% vs. CC: 5.21%; *p* = 0.981). However, NK cells from CC patients exhibited a significant increase in mOX-40 expression compared with controls (HC: 2.94% vs. CC: 4.88%; *p* = 0.017).

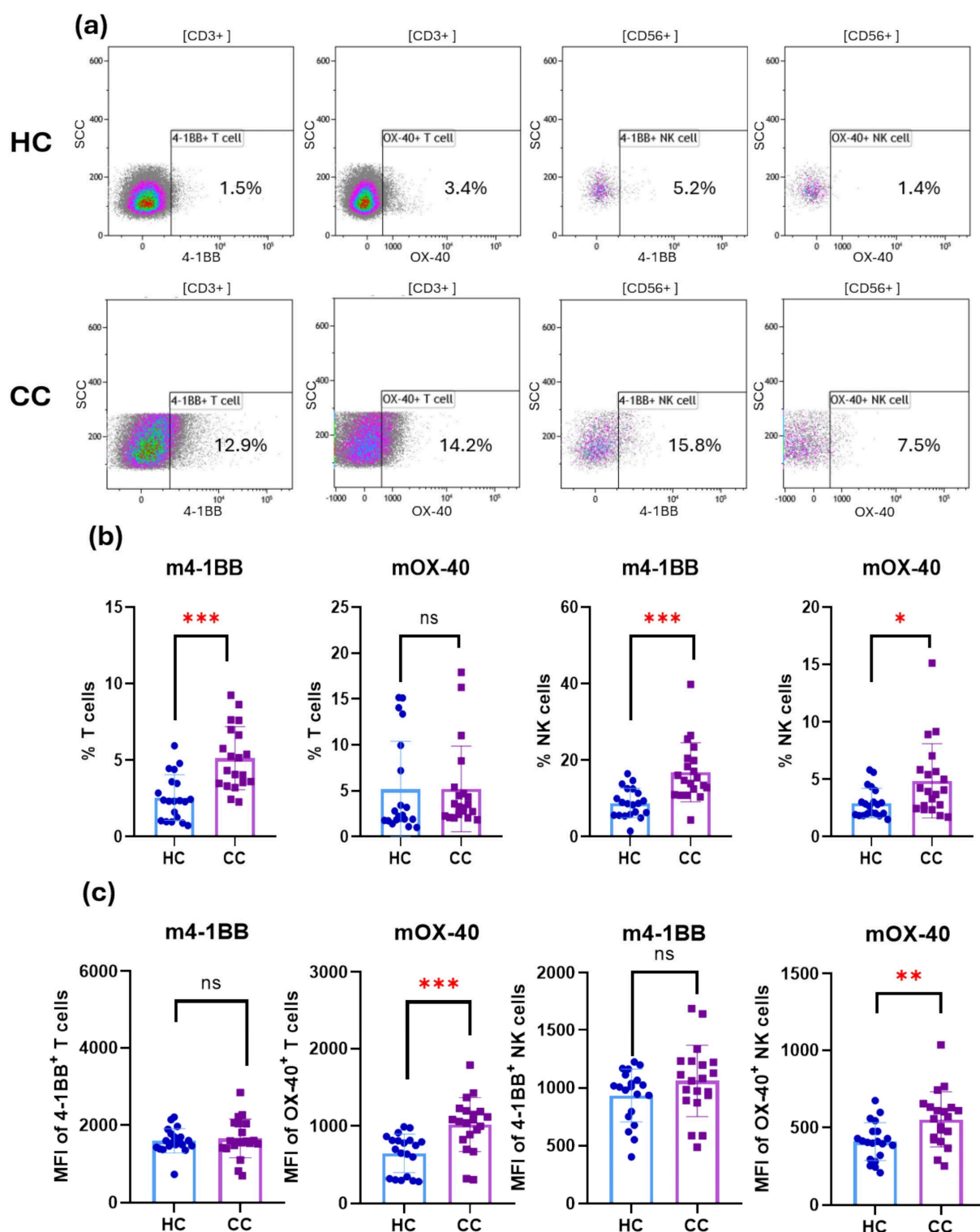


Figure 2. Membrane-bound 4-1BB and OX-40 expression on peripheral T and NK cells from healthy controls and cervical cancer patients. (a) Representative flow cytometry dot plots showing 4-1BB⁺ and OX-40⁺ T cells (CD3⁺) and NK cells (CD3⁻ CD56⁺) from a healthy control (upper panels) and a cervical cancer patient (lower panels). (b) Percentage of 4-1BB⁺ and OX-40⁺ T cells and NK cells in HC and CC subjects. (c) Median fluorescence intensity (MFI) of 4-1BB and OX-40, calculated exclusively within the corresponding 4-1BB⁺ or OX-40⁺ T-cell and NK-cell populations. Statistical significance was determined using an unpaired Student's *t*-test. * *p* < 0.05; ** *p* < 0.005; *** *p* < 0.0005; ns, not significant.

Median fluorescence intensity (MFI) was calculated exclusively within the gated 4-1BB⁺ and OX-40⁺ populations of circulating T and NK cells. No significant differences were observed in the MFI of 4-1BB⁺ T or NK cells between healthy controls and cervical cancer patients. In contrast, the MFI of OX-40⁺ cells was significantly increased in both T cells (HC: 647.9 vs. CC: 1020; $p = 0.0004$) and NK cells (HC: 410.8 vs. CC: 553.6; $p = 0.0054$) from CC patients. Together, these findings demonstrate a differential modulation of costimulatory receptor expression across circulating lymphocyte subsets in cervical cancer.

3.3. Association Between Soluble Receptor Levels and Cellular Expression

To determine whether circulating soluble receptor levels reflect membrane-bound expression on peripheral lymphocytes, Spearman correlation analyses were performed between plasma concentrations of soluble 4-1BB and OX-40 and the corresponding membrane expression levels (in median fluorescence intensity) on T and NK cells (Figure 3). A moderate positive correlation was observed between plasma s4-1BB concentrations and membrane 4-1BB expression on circulating T cells ($r = 0.5308$, $p = 0.0160$), indicating a significant linear association between systemic and cellular levels of this receptor.

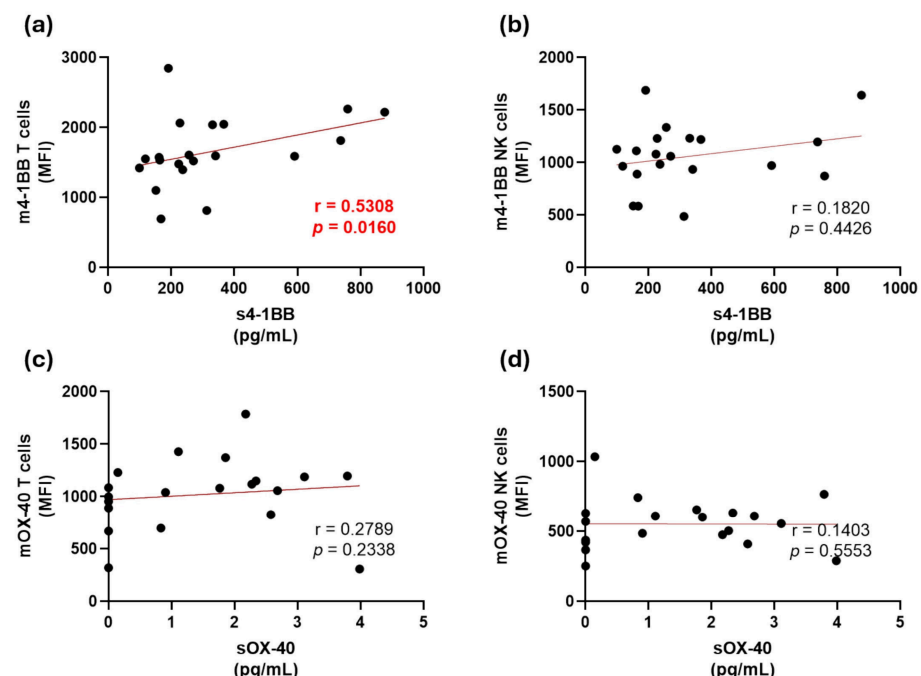


Figure 3. Correlation between soluble receptor levels and membrane expression on circulating lymphocytes. (a) Correlation between s4-1BB and membrane 4-1BB expression on T cells. (b) Correlation between s4-1BB and membrane 4-1BB expression on NK cells. (c) Correlation between plasma sOX-40 levels and membrane OX-40 expression on T cells. (d) Correlation between sOX-40 and membrane OX-40 expression on NK cells. Each dot represents an individual subject. Correlations were evaluated using Spearman's test. r and p values are indicated in the corresponding panels. A p -value < 0.05 was considered statistically significant.

In contrast, no significant correlations were detected between s4-1BB and membrane 4-1BB expression in NK cells. Likewise, plasma sOX-40 levels did not correlate with membrane OX-40 expression on either T or NK cells. These findings demonstrate a selective linear association between soluble and membrane 4-1BB within the T-cell compartment, whereas no corresponding relationship was observed for OX-40.

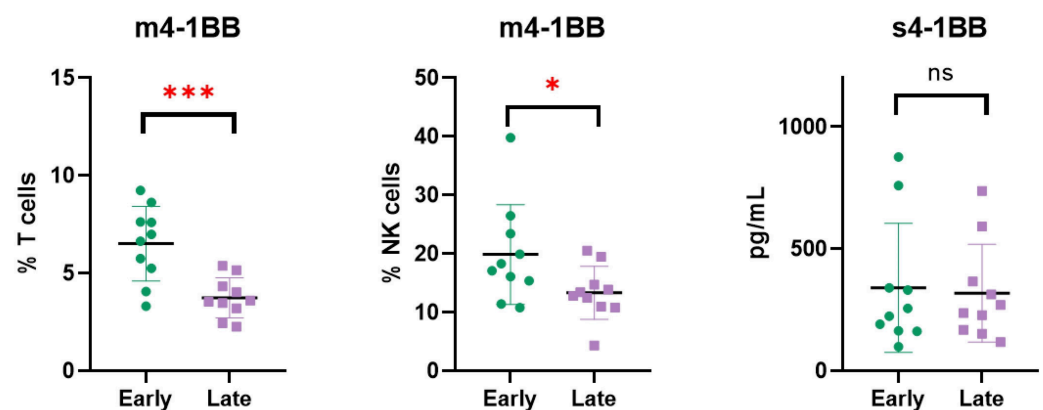
In addition, Spearman correlation analyses were performed to evaluate the association between the percentages of 4-1BB⁺ and OX-40⁺ T cells and NK cells and the plasma concentrations of their corresponding soluble forms. No statistically significant correlations

were identified between the frequency of receptor-positive cells and the levels of soluble 4-1BB or soluble OX-40 (Figure S2).

3.4. 4-1BB and OX-40 Expression with Disease Stage in Cervical Cancer

To determine whether alterations in the 4-1BB/OX-40 axis are associated with disease progression, cervical cancer patients were stratified into early (stages I–II) and advanced (stages III–IV) disease. Plasma concentrations of soluble receptors and membrane expression levels on circulating T and NK cells were compared between stage groups to evaluate stage-dependent differences (Figure 4).

(a)



(b)

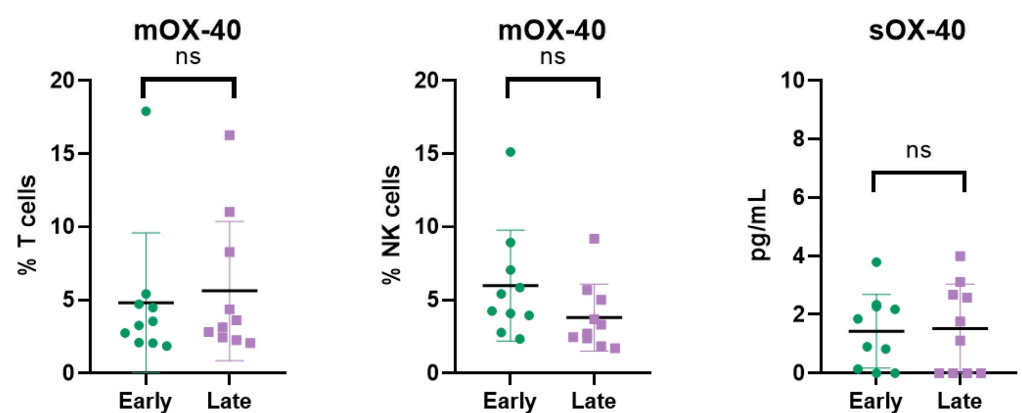


Figure 4. Stage-dependent analysis of membrane and soluble 4-1BB and OX-40 in cervical cancer patients. (a) Expression of m4-1BB on T and NK cells, as well as plasma s4-1BB levels in early and advanced stages. (b) Expression of mOX-40 on T and NK cells, as well as plasma sOX-40 levels in early and advanced. *: $p < 0.05$; ***: $p < 0.0005$; ns: no-significance.

A significant reduction in membrane 4-1BB expression was observed in advanced-stage patients. The proportion of circulating T cells expressing 4-1BB was markedly decreased in late-stage disease compared with early-stage patients (Early stage: 6.51% vs. Late stage: 3.74%; $p = 0.0008$). Similarly, within the NK cell compartment, the frequency of 4-1BB⁺ cells was significantly lower in advanced stages (Early stage: 19.89% vs. Late stage: 13.34%; $p = 0.0458$). In contrast, plasma s4-1BB concentrations did not differ between early and advanced stages (Early stage: 340.7 pg/mL vs. Late stage: 318.4 pg/mL; $p = 0.8334$), indicating that the stage-associated changes were restricted only to the membrane-bound form.

The OX-40 axis displayed a distinct pattern. In contrast with 4-1BB, no significant differences were detected in the percentage of OX-40⁺ T cells between stages (Early stage:

4.80% vs. Late stage: 5.62%; $p = 0.7053$). Likewise, NK cell expression of OX-40 did not significantly differ between early and advanced disease (Early stage: 5.97% vs. Late stage: 3.79%; $p = 0.1356$), although a numerical reduction was observed in late-stage patients. Plasma sOX-40 levels also remained unchanged across disease stages (Early stage: 1.43 vs. Late stage: 1.52; $p = 0.8856$).

To determine whether differences in lymphocyte composition could contribute to the interpretation of soluble CD137 and OX-40 levels, the proportions of circulating CD3⁺ T cells and CD3⁺CD56⁺ NK cells were compared between healthy controls and cervical cancer patients, as well as between early- and advanced-stage disease. Cervical cancer patients exhibited a modest reduction in the proportion of circulating CD3⁺ T cells compared with healthy controls, whereas NK-cell frequencies were comparable between groups. Importantly, no significant differences in the proportions of either CD3⁺ T cells or CD3⁺CD56⁺ NK cells were observed between early- and advanced-stage cervical cancer patients (Supplementary Figure S2), suggesting that the reduced membrane CD137 expression observed in advanced-stage disease is unlikely to be explained by differences in the relative abundance of these lymphocyte populations.

4. Discussion

Costimulatory receptors of the TNF receptor superfamily (TNFRSF) provide context-dependent signals that regulate lymphocyte activation, persistence, and effector function. Among them, 4-1BB and OX-40 are inducible receptors whose activation depends on receptor clustering and ligand engagement, ultimately converging on NF- κ B signaling to promote immune effector responses [14].

Here, we investigated the systemic and cellular distribution of 4-1BB and OX-40 in cervical cancer by measuring soluble receptor concentrations in plasma and membrane expression on circulating T and NK cells. This dual-compartment approach provides an integrated view of receptor availability, particularly for TNFRSF members, in which membrane expression and soluble isoforms together influence signaling output [15].

Although the cohort size was limited, the concordant findings across soluble and membrane compartments support the biological relevance of our observations. A major finding was the elevation of soluble 4-1BB and OX-40 in cervical cancer compared with healthy controls. Soluble immune receptors, generated through alternative splicing and/or proteolytic ectodomain shedding, are increasingly recognized as modulators of immune responses by influencing ligand availability and receptor–ligand interactions [16]. For TNFRSF members, these mechanisms may alter receptor clustering and signaling, even when circulating receptor concentrations are modest [17].

Mechanistically, the increase in s4-1BB is consistent with accumulating evidence that CD137 exists as a soluble receptor generated through alternative splicing and proteolytic shedding [18]. Although the mechanisms responsible for its release were not investigated here, metalloproteases such as ADAM10 and ADAM17 have been implicated in regulating CD137 availability at the cell surface. Interestingly, tumor cell lines from multiple cancer types including renal, lung, melanoma, and hepatocellular tumor cells can produce s4-1BB under hypoxic conditions, where it binds 4-1BBL and interferes with costimulatory signaling [19]. Accordingly, s4-1BB has emerged as a dynamic biomarker in immuno-oncology [20], supporting the concept that circulating s4-1BB is a regulated component of the CD137 signaling axis rather than merely a passive byproduct [21].

Similarly, sOX-40 is a soluble isoform detectable in human serum that has been associated with cancer progression [22]. Like other soluble TNFRSF members, sOX-40 may modulate OX-40/OX-40L signaling by altering ligand availability and receptor engagement. Consistent with this concept, structural and functional studies support the notion

that changes in receptor form (membrane versus soluble) can influence OX-40 signaling output [23].

At the cellular level, we observed increased frequencies of circulating m4-1BB⁺ T cells, m4-1BB⁺ NK cells, and mOX-40⁺ NK cells in cervical cancer. These findings are consistent with the inducible nature of TNFRSF costimulatory receptors following lymphocyte activation and with the role of 4-1BB signaling in supporting cytotoxic immune responses across both adaptive and innate compartments [24].

Unlike many solid tumors, cervical cancer develops in the context of persistent high-risk HPV infection, and the observed alterations in 4-1BB and OX-40 may therefore reflect chronic virus-driven immune stimulation in addition to tumor-associated immune dysregulation. Sustained expression of the viral oncoproteins E6 and E7 maintains continuous antigenic stimulation, promoting prolonged activation and remodeling of T- and NK-cell responses. Under these conditions, inducible costimulatory receptors such as 4-1BB and OX-40 may initially be upregulated, whereas persistent immune stimulation may subsequently alter their expression and signaling dynamics. Thus, the increased membrane and soluble receptor expression observed in this study may reflect the combined effects of persistent viral antigen exposure and tumor-associated immune remodeling rather than malignant transformation alone [25].

Increased 4-1BB expression has been reported across multiple malignancies. Elevated peripheral 4-1BB expression has been observed in patients with oropharyngeal and oral squamous cell carcinomas [26] and chronic lymphocytic leukemia, where NK cells exhibit significantly higher 4-1BB expression than healthy controls [27]. In cervical cancer, increased 4-1BB expression has been associated with an exhausted phenotype and reported in peripheral CD8⁺ and CD4⁺ T cells [28], as well as in NK cell subsets, including CD56^{dim} and CD56^{bright} populations [29]. Beyond the peripheral compartment, 4-1BB has been extensively characterized within the tumor microenvironment across several solid tumors, including colorectal cancer, ovarian cancer, non-small cell lung cancer, intrahepatic cholangiocarcinoma, glioblastoma, and hepatocellular carcinoma [30].

Regarding OX-40, most studies evaluating its expression in solid tumors have reported that increased infiltration of OX-40⁺ lymphocytes is associated with favorable clinical outcomes, supporting its relevance as a marker of effective anti-tumor immunity. In advanced gastric cancer, OX-40 expression on both CD4⁺ and CD8⁺ T cells has been associated with improved prognosis [31]. Likewise, OX-40⁺CD4⁺ tumor-infiltrating lymphocytes have been described in breast cancer, sarcoma, melanoma, and colorectal cancer, where they frequently correlate with improved survival [32]. Similarly, high OX-40 expression within tumor infiltrates has been linked to favorable clinical outcomes in non-small cell lung cancer [33]. In contrast, our previous studies in cervical cancer demonstrated increased OX-40 expression on both tumor-infiltrating and circulating T and NK cells exhibiting exhaustion-associated phenotypes, suggesting that the biological significance of OX-40 expression may be highly context-dependent [27,28].

When stratifying by disease stage, we observed a consistent reduction in the frequency of m4-1BB⁺ T cells and NK cells in advanced disease relative to early stages, whereas plasma s4-1BB concentrations remained unchanged. This divergence suggests that stage-related remodeling of the 4-1BB axis is more readily captured at the cell-surface level than by systemic soluble measurements. Because TNFRSF signaling is strongly influenced by receptor density and higher-order clustering, a reduction in the proportion of m4-1BB⁺ lymphocytes could have important functional implications for ligand-dependent activation, even when circulating soluble receptor levels remain stable [34].

Importantly, our findings are consistent with those reported by Zizzari et al., who identified circulating 4-1BB⁺ T cells as a surrogate marker of an effective anti-tumor im-

immune response and improved clinical outcome. In our cohort, membrane-bound 4-1BB⁺ T cells were most abundant in early-stage disease but declined significantly with disease progression. Rather than conflicting with the proposed role of 4-1BB⁺ T cells, this pattern may suggest that membrane 4-1BB expression dynamically reflects the evolving host immune response during cervical cancer progression. Early-stage disease may be characterized by a more robust anti-tumor immune response capable of sustaining 4-1BB expression, whereas advanced disease is associated with progressive immune dysregulation that may compromise the maintenance of activated 4-1BB⁺ T cells [35].

Importantly, this stage-related dissociation between membrane-bound and soluble receptors suggests that TNFRSF regulation is dynamic during cervical cancer progression. In early-stage disease, increased membrane expression together with elevated soluble levels may reflect enhanced immune activation and receptor shedding. In contrast, the persistence of elevated soluble receptors despite declining membrane expression in advanced disease suggests that soluble forms are not simply passive byproducts of surface abundance but may instead arise through distinct regulatory mechanisms, including sustained inflammatory signaling, alternative splicing, proteolytic shedding, or release from different immune cell populations. This uncoupling may therefore reflect progressive immune dysregulation and altered costimulatory signaling in advanced cervical cancer.

By contrast, the frequency of mOX-40⁺ T and NK cells, as well as plasma sOX-40 concentrations, remained unchanged across disease stages. Although mOX-40 showed a tendency to decrease in NK cells with disease progression, this was not accompanied by changes in soluble OX-40, suggesting that sOX-40 is not a reliable surrogate of membrane OX-40 expression. Consistent with this interpretation, plasma s4-1BB concentrations positively correlated with the frequency of m4-1BB⁺ T cells, whereas no significant correlations were observed between soluble and membrane OX-40. These findings indicate that, despite belonging to the same TNFRSF family, the membrane and soluble forms of OX-40 are regulated differently from those of 4-1BB. Similarly, evidence from other cancers indicates that elevated circulating sOX-40 may have clinical associations without necessarily mirroring receptor expression on PBMCs, underscoring that soluble and membrane OX-40 are not obligatorily coupled [36].

From a translational perspective, these findings may be relevant for therapeutic strategies targeting 4-1BB and OX-40. Because agonistic TNFRSF antibodies depend on receptor clustering for optimal activity [37], circulating soluble receptors could influence ligand availability or interfere with therapeutic agonists in the extracellular space. Although this possibility remains to be investigated for s4-1BB and sOX-40 in cervical cancer, circulating s4-1BB has already been incorporated as a pharmacodynamic biomarker in clinical immunotherapy studies, supporting its utility as a measurable component of the 4-1BB signaling axis during immune modulation [38].

While stage-associated changes were more readily detected at the membrane level, the persistent elevation (with respect to healthy controls) of sOX-40 and s4-1BB despite declining percentages of m4-1BB positive NK and T cells suggests that the soluble and membrane compartments are regulated independently. Although the mechanisms underlying this dissociation were not investigated, differential proteolytic shedding, alternative splicing, or contributions from distinct immune cell populations may explain the sustained presence of soluble receptors. Future mechanistic studies will be required to determine how these processes regulate the biological activity of soluble 4-1BB and OX-40 during cervical cancer progression.

This study has several limitations. The cohort size was modest, and analyses were confined to peripheral blood without parallel evaluation of tumor-infiltrating lymphocytes or functional assays assessing responsiveness to 4-1BBL and OX-40L. Consequently,

the functional significance of the altered receptor distribution remains to be determined. Notably, membrane 4-1BB expression declined toward healthy donor levels in advanced disease while circulating s4-1BB remained elevated, suggesting that soluble receptors may influence ligand availability and costimulatory signaling independently of surface receptor density. Although soluble TNFRSF members have been proposed to sequester ligands and modulate pathway activity, we did not directly evaluate whether plasma enriched in s4-1BB or sOX-40 attenuates ligand- or agonist-induced signaling.

Additionally, although previous work from our group has characterized exhaustion-associated phenotypes in cervical cancer, the flow cytometry panel used in the present study was not designed to distinguish functional T-cell subsets. Consequently, we could not determine whether the 4-1BB⁺ and OX-40⁺ T-cell populations comprised activated, suppressive (e.g., regulatory T cells), or exhausted T cells. This limitation is particularly relevant because regulatory T cells are known to express both OX-40 and 4-1BB and may contribute to immunoregulatory responses [39]. Future studies incorporating multiparametric immunophenotyping, including markers for CD4⁺, CD8⁺, FOXP3⁺, and T-cell activation or exhaustion, will be necessary to better define the cellular sources of 4-1BB and OX-40 expression in cervical cancer. In addition, functional assays evaluating the effects of plasma with high soluble receptor concentrations on costimulatory responses would help clarify the biological significance of the membrane-soluble dissociation observed in this study.

5. Conclusions

In summary, this study demonstrates that the 4-1BB/OX-40 axis undergoes compartment-specific modulation in cervical cancer, characterized by increased circulating soluble forms and differential membrane expression patterns. While membrane 4-1BB expression was elevated in early-stage patients, we observed a significant stage-dependent decline in advanced disease. Crucially, soluble levels of both 4-1BB and OX-40 remained persistently high regardless of stage. This “uncoupling” finding suggests that soluble and membrane-bound TNFRSF receptors constitute distinct but interconnected regulatory layers.

The pattern observed between systemic soluble concentrations and surface receptor availability suggests that multiple mechanisms—including alternative splicing, proteolytic shedding, receptor internalization, and ligand sequestration—may differentially shape each compartment. It is possible that the high systemic concentration of these soluble forms contributes to a “colder” immune environment by acting as a molecular sponge. By sequestering costimulatory ligands, these soluble decoy receptors effectively preempt T and NK cell reinvigoration, even when therapeutic agonists are present. Furthermore, our results challenge the assumption that soluble receptors are less informative than membrane density; instead, persistent soluble levels in the face of declining membrane expression may serve as a superior systemic monitor for immune exhaustion and receptor turnover. Collectively, these findings support a model where the dynamic remodeling of receptor distribution, rather than uniform expression, dictates the immune landscape during cervical cancer progression.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/receptors5030028/s1>, **Figure S1.** Flow cytometry gating strategy. Singlets were identified using FSC-H versus FSC-A, and lymphocytes were then gated using SSC-A versus FSC-A. CD3⁺ cells were identified as T cells, whereas CD3[−]CD56⁺ cells were identified as NK cells. **Figure S2.** T-cell and NK-cell compartments. (a) Percentages of T and NK cells among PBMCs from HD and CC patients. (b) Percentages of T cells and NK cells among PBMCs from patients with early- and late-stage CC.

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