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ORIGINAL ARTICLE

Tumor-infiltrating lymphocytes contain a higher proportion of FOXP3$^+$ T lymphocytes in cervical cancer

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Background/Purpose: The subpopulations and functions of tumor-infiltrating lymphocytes (TILs) from cervical cancer (CC) are altered. Dysfunction of TIL could be partially because of the inhibition by regulatory T (T_{reg}) cells. *FOXP3* is the control gene for the T_{reg} cells.
Methods: We investigated the distribution of TILs and FOXP3$^+$ cells in CC ($n = 10$) and cervical intraepithelial neoplasia ($n = 8$) tissues. Double-immunofluorescence and confocal-based image quantitative microscopic analysis were used to calculate the number of cluster of differentiation (CD)4$^+$CD25$^+$FOXP3$^+$ T_{reg} cells around the tumor cells.
Results: The CD4$^+$CD25$^+$FOXP3$^+$ phenotype of T_{reg} cells was accumulated around the tumor cells. CC contains a significantly higher proportion of the FOXP3$^+$ T cells than in cervical intraepithelial neoplasia ($p < 0.001$). Moreover, CC with lymph node metastasis has a higher proportion of the FOXP3$^+$ T cells than that without lymph node metastasis ($p < 0.05$).
Conclusion: The increased accumulation of T_{reg} cells suggests that T_{reg} cells are important in the immunopathogenesis of CC.

Introduction

Immunological tolerance to self-antigens is a tightly regulated process. The immune tolerance is maintained in the periphery through a variety of mechanisms, including a population of regulatory T (T_{reg}) cells that actively suppresses the function of autoreactive T cells. These T_{reg} cells can be identified by their expression of cluster of differentiation (CD)4, the interleukin (IL)-2 receptor alpha chain (CD25), and the forkhead family transcription factor FOXP3. They have the ability to inhibit the development of autoimmunity when transferred into the appropriate host. Recent studies have shown that the forkhead/winged-helix protein FOXP3 is expressed predominantly in T_{reg}, and is both necessary and sufficient for their development and function.[1] The transcription factor FOXP3 is not only a key

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intracellular marker, but is also a crucial developmental and functional factor for $CD4^+CD25^+$ T_{reg} cells.[2–4] During the past few years, several phenotypically distinct T_{reg} cell populations have been suggested, and the classic ones are thymus-derived $CD4^+CD25^+FOXP3^+$ T cells, T_{reg} cells.[5] Recently, Woo et al[6] observed increased numbers of T_{reg} cells in patients with non-small-cell lung cancer and ovarian cancer compared with normal blood donors. A higher frequency of the T_{reg} cells in peripheral blood has been reported in patients with various cancers, including breast cancer,[7] gastric cancer,[8] and hepatocellular carcinoma.[9]

Cervical cancer (CC) is the second most common cancer among women worldwide, with 400,000 new cases diagnosed each year. Infection by human papillomavirus is a major risk factor for the development of invasive cervical carcinoma and its precursor cervical intraepithelial neoplasia (CIN). CIN I reflects active human papillomavirus replication and is rarely precancerous, most often resolving spontaneously. In contrast, CIN II and III represent potential cancer precursors; more than 12% of CIN III lesions will progress to cancer, if left untreated.[10] The cytotoxic potential of freshly isolated tumor-infiltrating lymphocytes (TILs) is usually not expressed.[11] Our recent study illustrated two possible pathways of cancer immune escape: down-regulation of perforin and direct blockade of cytotoxicity to CC through the engagement of inhibitory natural killer (NK) receptors (CD94/NKG2A) with their ligand.[12] Furthermore, we found that the cancer-derived IL-15 and/or transforming growth factor (TGF)-β could be the cause of the up-regulation of CD94/NKG2A in TILs, which does not occur among normal tissue-infiltrating or naive T lymphocytes.

$CD8^+$ T cells and NK cells use the perforin and granzyme pathways to kill the infected cells and the tumor cells. Activated human T_{reg} cells express granzyme A and kill T cells and antigen-presenting cells through perforin.[13] FOXP3 is considered as a T-cell marker and is more specific than other markers, such as glucocorticoid-induced tumor necrosis factor receptor (GITR) and Cytotoxic T-Lymphocyte Antigen-4 or the co-expression of CD4 and CD25.[14] Transfection of FOXP3 gene will convert naive $CD4^+CD25^-$ T cells toward a regulatory phenotype in both mice and humans, and therefore, this molecule represents a functionally important marker of this T_{reg}-cell population.[2,4,15] These observations suggest that the FOXP3 is presently the most definitive marker of the T_{reg} cells, and its detection could allow a better understanding of the mechanisms through which T_{reg} cells are involved in autoimmune and infectious diseases, as well as transplantation and tumor immunity. In this study, we focused on the $FOXP3^+$ cells and their role in CC progression.

Materials and methods

Paraffin blocks of human CC ($n = 10$) and CIN ($n = 8$) tissues were obtained from the Tissue Bank of National Taiwan University Hospital. The procedure was approved by the institutional review board of National Taiwan University Hospital.

Immunohistochemistry on paraffin sections was performed using the pressure-cooking method of antigen retrieval (20 minutes, citrate buffer, pH 6.5). The slides were cooled and transferred to phosphate-buffered saline. Before staining, endogenous peroxidase was blocked. The slides were incubated overnight at 4°C with the primary antibody and then washed three times with phosphate-buffered saline. Immunodetection was performed with biotinylated antimouse secondary antibodies (10 minutes), followed by peroxidase-labeled streptavidin (DAKO, Glostrup, Denmark) (10 minutes) and visualized using diaminobenzidine chromogen as the substrate (DAKO).

For immunofluorescence staining, tissues were stained with polyclonal rabbit anti-human-CD3 (DAKO, ready-to-use, Glostrup, Denmark), mouse anti-human FOXP3 monoclonal antibody (1:20 dilution, 236A/E7; Abcam, Cambridge, UK), mouse anti-human CD4 monoclonal antibody (1:20 dilution, Ab-8; Lab vision, Suffolk, UK), mouse anti-human CD25 monoclonal antibody (1:50 dilution, 4C9; Novocastra, Newcastle, UK), and mouse anti-human CD8 monoclonal antibody (C8/144B; DAKO; ready-to-use), followed by Alexa Fluor 488-conjugated goat antimouse IgG1, Alexa Fluor 594-conjugated goat antimouse IgG2b, and Alexa Fluor 488-conjugated donkey antigoat IgG (all 2 μg/mL, Molecular Probes, Eugene, OR, USA). Sections were then examined using a confocal laser scanning microscope LSM 510 (Carl Zeiss, Jena, Germany). An immersion-oil Plan-Apochromat 63×/1.4 objective was used. Double fluorescence for green and red channels was imaged using the excitation of argon–krypton laser at the wavelengths of 488 nm and 543 nm. Positive cells were quantified by ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA) and counted in 20 high power fields using confocal microscopy. Quantification was performed on the digitalized image of systematic randomized selected fields by two independent observers.

Statistical analysis was performed using the SPSS software program for Windows (version 12.0; SPSS, Chicago, IL, USA). The percentage of $FOXP3^+/CD3^+$ cells was compared by Mann-Whitney *U* test between CIN and CC. Then the Kruskal-Wallis test was applied to compare the percentage of $FOXP3^+/CD3^+$ cells among four groups (CINI/II, CIN III, CC with lymph node metastasis, and CC without lymph node metastasis). If the Kruskal-Wallis test is positive ($p < 0.05$) then Mann-Whitney test was used for pairwise comparison of subgroups. A p value <0.05 was considered as significantly different.

Results

Immunohistochemistry showed that in these CC specimens, most of the CD4, CD8, and FOXP3-expressing cells were located around the tumor sites (Fig. 1A). This result indicates that TILs cannot migrate into tumor cells, and remain outside the tumor cells instead. In CIN, the infiltration of some $CD4^+$ and $CD8^+$ cells into epithelial cells was observed. However, few FOXP3-expressing cells had infiltrated into epithelial cells (Fig. 1B).

In individuals with malignant CC ($n = 10$), we identified a substantial population of $CD4^+CD25^+$ T cells by immunofluorescence staining in paraffin-embedded tissue sections. Consistent with the above observation of the $FOXP3^+$ cells, $CD4^+CD25^+$ T cells in the tumor environment indicated that $CD4^+CD25^+$ cells preferentially accumulate around the

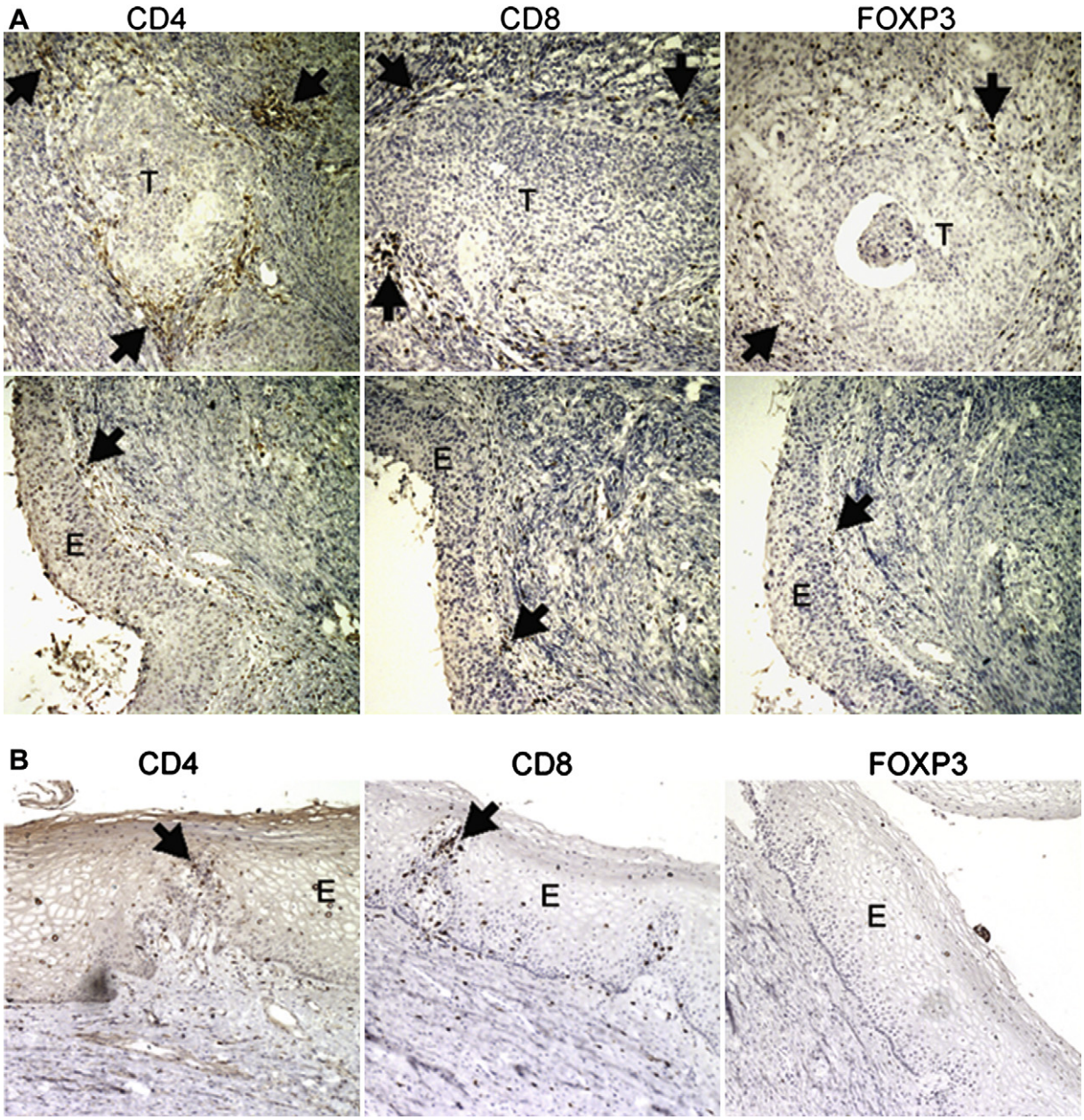

Figure 1. Paraffin-embedded tissue sections were stained with antibodies to human CD4, CD8, and FOXP3 from patients with (A) cervical cancer and (B) cervical intraepithelial neoplasia. Infiltrating cells of positive staining are indicated by black arrows. Original magnification, 100×. E = epithelial cells; T = tumor cells.

tumor cells, not inside the tumor (Fig. 2A). By using double-staining analysis, we showed that the $FOXP3^+$ cells expressed CD4 and a large amount of CD25. As shown in Fig. 2B, over 95% of the FOXP3-expressing cells were $CD4^+$ T cells, and up to 75% of the FOXP3-expressing cells were $CD25^+FOXP3^+$. This result indicates that most of the $FOXP3^+$ cells are $CD4^+CD25^+FOXP3^+$ cells. In summary, we showed that the $CD4^+CD25^+FOXP3^+$ phenotype of tumor-associated T_{reg} cells had accumulated in the tumor environment, especially around the tumor cells.

In immunofluorescence staining analysis, there were $FOXP3^+CD25^+$ cells in close contact with the $CD8^+$ T cells. $FOXP3^+$ T cells in the tumor mass were in proximity to the infiltrating $CD8^+$ T cells, suggesting that physical contact between $CD4^+CD25^+$ T cells and $CD8^+$ cytotoxic T cells might mediate regulatory functions. Therefore, cell-cell contact mediated suppression might be necessary for the $CD4^+CD25^+$ T_{reg} cells. Close contact between $FOXP3^+CD25^+$ cells and $CD8^+$ T cells suggests that tumor-associated $FOXP3^+$ T cells might be involved in the dysfunction of the $CD8^+$ cytotoxic T cells in tumors through cell-cell contact mediated suppression.

We found that CC contained a significantly higher proportion of the $FOXP3^+$ T cells than in CIN ($p < 0.001$, Fig. 3A). Furthermore, we classified the specimens into four groups: CIN I/II, CIN III, CC without lymph node metastasis, and CC with lymph node metastasis. The percentage of the $FOXP3^+CD3^+$ T cells in the $CD3^+$ T cells was higher in

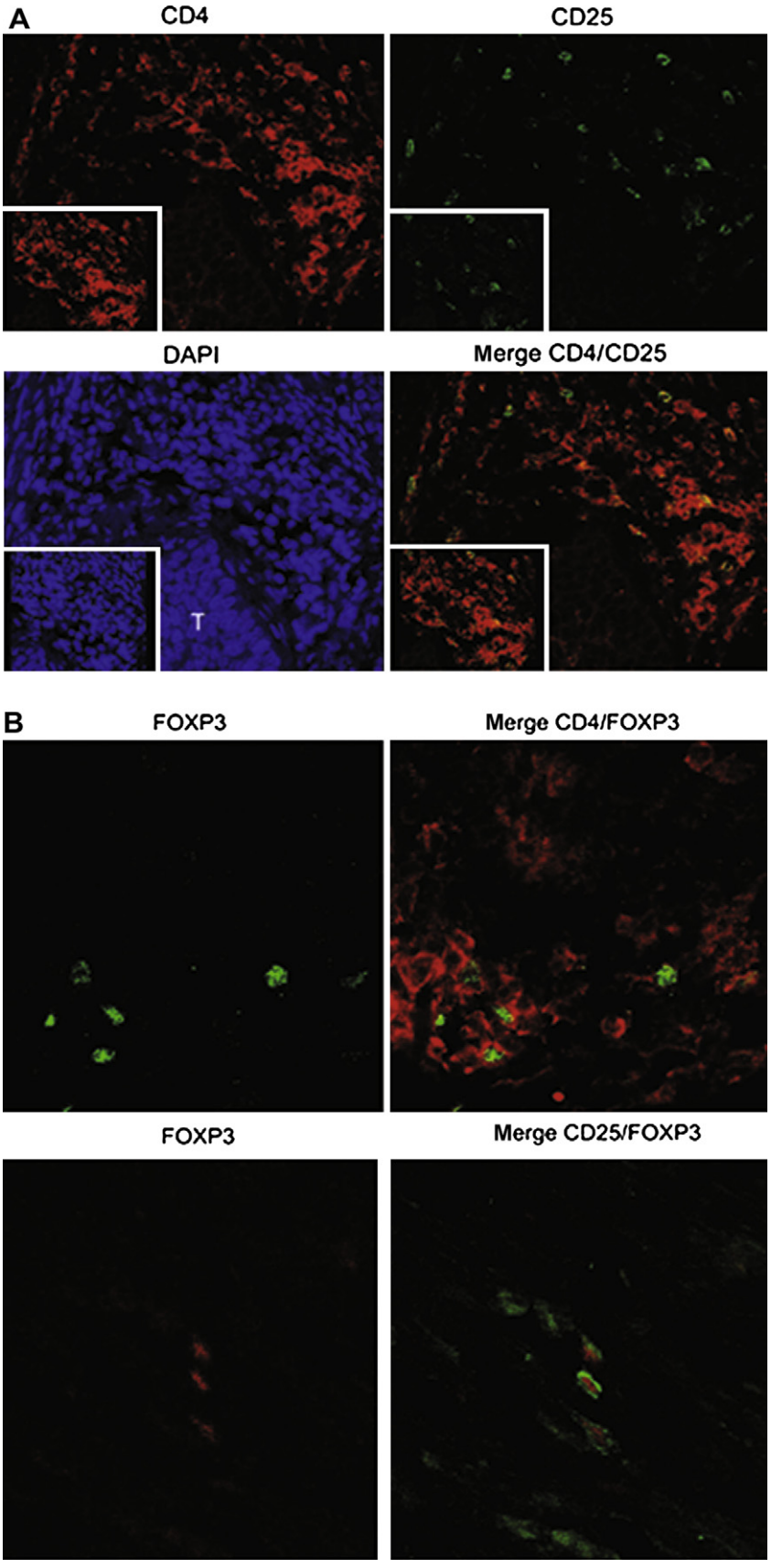

Figure 2. (A) $CD4^{+}CD25^{+}$ cells preferentially accumulate around the tumor cells. Cervical cancer tissue sections were analyzed by immunofluorescence multicolor staining and examined by confocal microscopy. The nucleus was stained by DAPI. (B) $FOXP3^{+}$ T cells in a cervical tumor mass. Tissues were stained with antibodies to human FOXP3, human CD25 and CD4. All of the $FOXP3^{+}$ T cells express CD4, and most strongly express CD25 ($n = 3$ for each). The box showed original magnification, 630×. DAPI = 4′-6-diamidine-2-phenyl indole; T = tumor cells.

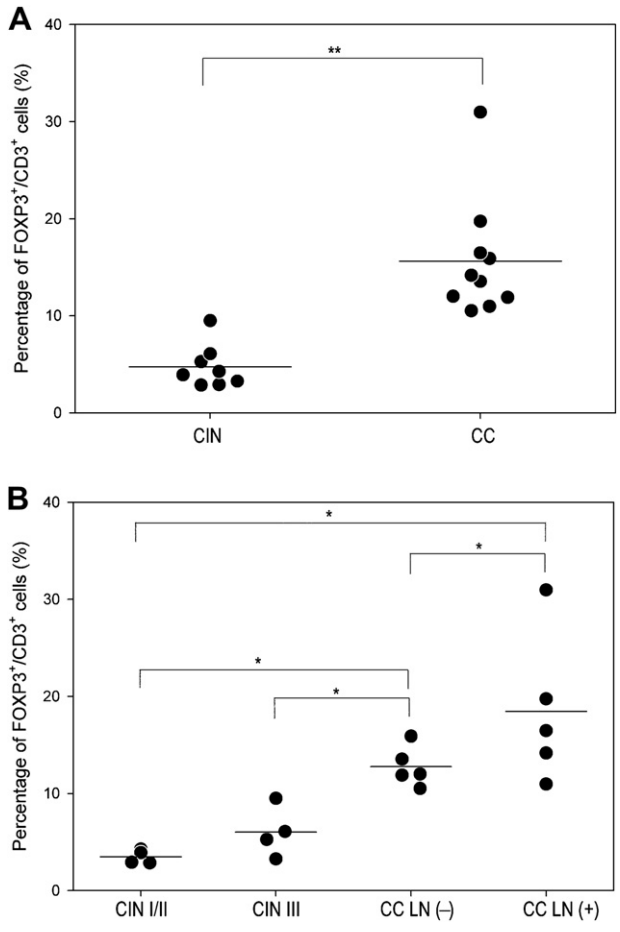

Figure 3. (A) Cervical cancer tissues contain a higher percentage of FOXP3$^+$ cells in the T cell population than in cervical intraepithelial neoplasia. (B) In later stages of cervical cancer, patients have a higher proportion of the FOXP3$^+$ T cell infiltration. Representative samples are shown. $*p < 0.05$, $**p < 0.001$ by Mann-Whitney *U* test; CC LN(−) = cervical cancer patients without lymph node metastasis; CC LN(+) = cervical cancer patients with lymph node metastasis; CIN = cervical intraepithelial neoplasm.

advanced stages. For example, CC without lymph node metastasis had more FOXP3$^+$ T cells than CIN III or CIN I/II (both $p = 0.014$, Fig. 3B). Similarly, CC with lymph node metastasis had more FOXP3$^+$ T cells than in CIN III or CIN I/II (both $p = 0.014$). Furthermore, CC with lymph node metastasis had more FOXP3$^+$ T cells than in CC without lymph node metastasis ($p = 0.045$).

Discussion

T cells or CD3$^+$ T lymphocytes belong to a group of white blood cells known as lymphocytes, and play a central role in cell-mediated immunity. T_{reg} cells, particularly CD4$^+$CD25$^+$ T_{reg} cells, are key mediators of peripheral tolerance.[16] Engendering strong antitumor immunity may thus involve breaking T_{reg}-mediated peripheral tolerance to tumor-associated antigens. Consistent with this concept, experimental depletion of T_{reg} cells in mice with tumors improves immune-mediated tumor clearance and enhances the response to immune-based therapy.[17,18] Recently, a study used systemic application of agonistic anti-GITR antibodies, and they demonstrated inactivated T_{reg} cells, increased intratumoral invasion of the CD8$^+$ T cells and complete tumor eradication in 70% of treated animals.[19]

Humans with cancer have increased number of peripherally circulating tumor T_{reg} cells.[6,7] Curiel et al[20] showed that the T_{reg} cells in human ovarian cancers express intracellular Cytotoxic T-Lymphocyte Antigen-4, GITR and FOXP3, inhibit tumor-assciated antigens-specific immunity, and contribute to tumor growth.[20] This demonstrated that the human T_{reg} cells have an important immunopathological role in human cancer by suppressing endogenous tumor-assciated antigens-specific T cell immunity. Furthermore, this study further linked this immunopathological role to clinical outcomes, by demonstrating that there is an inverse correlation between the tumor T_{reg} cell content and patient survival.

Consistent with the observation in human ovarian cancer,[20] our data show that this phenomenon can also be shown in CC. We found a significant trend toward a higher accumulation of the T_{reg} cells around the cervical tumor mass in advanced tumor stages. Therefore, T_{reg} cells seem to exist preferentially around the tumor mass. This implies that T_{reg} cell-mediated immunosuppression is a crucial tumor "immunoediting" mechanism and the main obstacle of successful tumor immunotherapy.

An important question is "Where do these T_{reg} cells come from?" By focusing on the tumor microenvironment—the active battlefront between tumors and the host immune system—the sources of T_{reg} cells in tumors can be summarized as follows: trafficking, differentiation, expansion, and conversion. Natural T_{reg} cells differentiate in the thymus and migrate to peripheral tissue. The mouse thymus produces T_{reg} cells as a separate lineage that expresses FOXP3. Because tumor-associated T_{reg} cells express FOXP3 mRNA and protein, it is possible that these cells traffic to tumors from the thymus, bone marrow, lymph nodes, and peripheral blood under the influence of tumor microenvironmental chemokine ligand 22.[20]

T_{reg} cells can be induced and differentiated in the periphery such as in the tumor microenvironment. Tumor environmental factors, such as vascular endothelial growth factor, IL-10, and TGF-β, suppress dendritic cell (DC) differentiation and function resulting in immature and/or partially differentiated DCs. Tumors convert DCs into TGF-β-expressing immature myeloid DCs that are capable of promoting T_{reg}-cell proliferation.[21] On the other hand, normal mature DCs stimulate the *in vivo* expansion of self-antigen-specific T_{reg} cells in mice.[22,23] As a result, thymus-derived T_{reg} cells in the tumor microenvironment might clonally expand the following stimulation by tumor-associated DCs.

Based on our previous studies, CC cells express TGF-β to drive the tumor-encountered T cells toward Th2/Tc2 polarity.[24] TGF-β is abundantly expressed in most, if not all, cancer cells, but is not or only very weakly expressed in normal cervical epithelial or stromal cells.[12] Therefore, we consider that T_{reg} accumulation in CC is due to TGF-β-mediated conversion from the CD4$^+$CD25$^-$ T cells. Furthermore, a recent study also showed that, using mice with a reporter introduced into the endogenous *Foxp3* locus, IL-6, an acute phase protein induced during inflammation, completely inhibits the generation of FOXP3$^+$ T_{reg} cells

induced by TGF-β.[25] This inhibition of T_{reg} development by different cytokines demonstrates that the development of the FOXP3$^+$ T_{reg} cells may be affected by multiple cytokines.

Our previous data, using flow cytometry and kinetic cytotoxicity assays, demonstrated retrained CD8$^+$ T lymphocyte cytotoxicity.[12] A recent report showed that the location of immune cells within human colorectal tumors could predict clinical outcome.[26] Therefore, the relative microenvironmental positions among these immune-modulating and CC cells need further verification. In the present study, we showed that TILs within tumor sites are located around the tumor cells. The possible reason to explain this distribution is tumor immune privilege. One of the many mechanisms adopted by tumors to destroy immune surveillance is to "counterattack" TILs. Tumors might establish an immune-privileged site to protect themselves from the immune response. Numerous studies have demonstrated FasL expression in many diverse types of human cancer, where it may contribute to tumor immune privilege.[27] Two independent studies found that the tumor-expressed FasL was significantly associated with a local decrease in the number of *in situ* TILs in human esophageal cancers.[28,29] There was a consistent local decrease in the number of TILs in FasL$^+$ tumor islands relative to matched FasL$^-$ tumor islands within every tumor examined. The local decrease in TILs was concomitant with a consistent increase in apoptosis among TILs.[28]

The accumulation of T_{reg} cells in tumors predicts a marked severity in cervical dysplasia, providing the "smoking gun" that links the T_{reg} cells and the immunopathogenesis of human cancer. Taken together, these data might be helpful for developing immune-boosting strategies based on ridding the cancer patient of this cell population.

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