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Glucocorticoid-induced tumor necrosis factor receptor expression in patients with cervical human papillomavirus infection

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ABSTRACT

Introduction: The progression of human papillomavirus (HPV) infection in the anogenital tract has been associated with the involvement of cells with regulatory properties. Evidence has shown that glucocorticoid-induced tumor necrosis factor receptor (GITR) is an important surface molecule for the characterization of these cells and proposes that GITR ligand may constitute a rational treatment for many cancer types. We aimed to detect the presence of GITR and CD25 in cervical stroma cells with and without pathological changes or HPV infection to better understand the immune response in the infected tissue microenvironment. **Methods:** We subjected 49 paraffin-embedded cervical tissue samples to HPV DNA detection and histopathological analysis, and subsequently immunohistochemistry to detect GITR and CD25 in lymphocytes. **Results:** We observed that 76.9% of all samples with high GITR expression were HPV-positive regardless of histopathological findings. High GITR expression (77.8%) was predominant in samples with ≥1,000 RLU/PCB. Of the HPV-positive samples negative for intraepithelial lesion and malignancy, 62.5% had high GITR expression. High GITR expression was observed in both carcinoma and high-grade squamous intraepithelial lesion (HSIL) samples (p = 0.16). CD25 was present in great quantities in all samples. **Conclusions:** The predominance of high GITR expression in samples with high viral load that were classified as HSIL and carcinoma suggests that $GITR^+$ cells can exhibit regulatory properties and may contribute to the progression of HPV-induced cervical neoplasia, emphasizing the importance of GITR as a potential target for immune therapy of cervical cancer and as a disease evolution biomarker.

Keywords: Human papillomavirus. Immune response. Immunohistochemistry.

INTRODUCTION

Human papillomavirus (HPV) infects the basal and parabasal cells of squamous epithelium in the female anogenital tract, and HPV types 16, 18, 31, 33, and 45 in particular are believed to put patients at high risk for the development of high-grade cervical intraepithelial neoplasia (CIN) and cervical carcinoma[1].

Infection progression has been associated with several factors, including the persistence of HPV, the presence of high-risk oncogenic HPV types, high viral load, integration of viral DNA, and E6 and E7 viral oncoprotein activity[1]. Evidence shows that regulatory T cells (T_{reg}) are also involved in the progression to cervical neoplasia in HPV-infected patients[2-5]. HPV-specific $CD4^+$ regulatory cells isolated from lymph node biopsies of patients with cervical carcinoma were found to suppress proliferation and cytokine (interferon-γ, interleukin [IL]-2) production by responder T cells[5].

T_{reg} cells play a crucial role in modulating the elimination of pathogens and tumor antigens and perform their function through immunosuppressive cytokine production and immunosuppression induction mediated by cell-to-cell contact, having the ability to suppress the activation, proliferation, and effector function of different cell types contributing to the immune response[6,7]. T_{reg} cells are subdivided into several subpopulations, one being the natural T_{reg} cells ($CD4^+CD25^+T_{reg}$), which numerically represent the largest group of cells with suppressor activity[8].

Studies show that T_{reg} cells are activated with greater sensitivity than naïve effector T cells after antigenic stimulation, which has been attributed mainly to their *semi-activated* state that is thought to be due to the increased expression of CD25 (α-chain of the IL-2 receptor), glucocorticoid-induced tumor necrosis factor receptor (GITR) markers, and others[9-11].

The detection of T_{reg} cells has been challenging owing to the lack of exclusive surface molecules for these cells. Studies have shown that the presence of transcription factor

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FOXP3 (*forkhead box p3)* is highly specific and that its transduction into naïve T cells increases the molecular expression associated with T_{reg} cells, such as that of CD25 and GITR[12,13].

Evidence shows that another characteristic surface molecule of cells with regulatory properties, T_{reg} cells in particular, is the GITR[14] — a tumor necrosis factor receptor superfamily member — which is predominantly expressed in $CD25^+$ $CD4^+$ T_{reg} cells and plays an important role in the regulation of mucosal immune responses[15-19]. Recent studies have demonstrated that *in vivo* GITR ligation using an agonist anti-GITR monoclonal antibody, DTA-1, can augment anti-tumor T-cell responses by modulating T_{reg} cells, which makes targeting GITR a potential immunotherapeutic approach to cancer treatment[20-22].

Given the findings that indicate the involvement of cells with regulatory properties, especially T_{reg} cells, in the progression of cervical malignant lesions[3,4,23,24], this study aimed to detect both CD25 and GITR markers in lymphocytes of cervical stroma to better understand the immune response in the microenvironment of HPV infection, which may shed light on novel therapeutic interventions against intraepithelial neoplasia and cervical cancer of viral etiology, and perhaps also make GITR a possible candidate biomarker for disease evolution.

METHODS

Samples

Forty-nine patient cervical samples embedded in paraffin and selected on a non-probabilistic form by convenience sampling from 2000 to 2002 in the Cancer Prevention Center of Campo Grande, Mato Grosso do Sul, Brazil, were used. These samples previously underwent a Hybrid Capture II reaction (Digene, Gaithersburg, MD, USA) to quantify the viral load for group B - high oncogenic risk types that were classified into scores from 0 to 3: 0 (HPV-negative samples); 1 (1 to < 100U of light released for probe; relative light units/positive control to group B (RLU/PCB); 2 (100 to <1,000 RLU/PCB); and 3 (≥1,000 RLU/PCB). On the basis of histopathological analysis, the samples were classified as low-grade squamous intraepithelial lesions (LSIL) (CIN I); high-grade squamous intraepithelial lesions; (HSIL) (CIN II, III); carcinoma, and negative for intraepithelial lesion and malignancy (NILM).

Immunohistochemistry of CD25 and GITR

The Immunohistochemistry (IHC) reaction was developed using antigen retrieval with wet heat and 0.05M ethylenediaminetetraacetic acid (EDTA) pH 8.0 for the detection of CD25 marker[25] and 10mM Tris and 1mM EDTA for the GITR marker. The primary antibodies used included anti-human IL-2R (eBioscience®, San Diego, CA, USA; clone B-B10/cod.BMS134) and anti-GITR (R&D systems®, Minneapolis, MN, USA; goat IgG/cod.AF689).

The detection system was a Universal LSAB + Kit/HRP (Dako®, Carpinteria, CA, USA), and diaminobenzidine (Dako®) was used as a chromogen. Counterstaining was performed in hematoxylin, and the slides were observed under common optical microscopy with 10× and 40× objective lenses. Samples showing brown staining on characteristic cells were considered positive. Human tonsil tissue was used as the external control of the reaction over which the primary antibody (positive control) and phosphate buffer pH 7.4 containing 1% albumin (negative control) was applied.

Quantitative analysis

According to the presence of immunomarked cells, the histological sections were classified in low (small quantities) and high (large quantities) scores. The slides were analyzed by 2 independent observers, previously calibrated ($\kappa = 0.98$), and the final result of discordant cases was obtained by common analysis to produce a consensus.

Statistical analysis

Analysis of the frequencies among the histopathological findings and viral load according to GITR expression intensity were compared using Fisher's exact test.

Ethical considerations

This study was approved by the Research Ethics Committee of the Universidade Federal de Mato Grosso do Sul, protocol number 975, July 31, 2007.

RESULTS

We observed a predominance of GITR in large quantities (7/9; 77.8%) in the samples with ≥1,000 RLU/PCB (viral load 3), although increases in viral load did not have a statistical correlation with high GITR expression (p=0.40). Regardless of histopathological findings, among all samples with high GITR expression, 76.9% (20/26) were HPV-positive (viral load, 1-3). Among the NILM samples, 40% (8/20) were HPV-positive (viral load, 1-3) and 62.5% (5/8) of these showed high GITR expression, while among NILM-HPV negative samples (viral load, 0), only 33.3% (4/12) showed high GITR expression **(Table 1)**.

A frequency analysis of the histopathological findings according to GITR expression intensity is shown in **Table 2**. High GITR expression was predominant in the carcinoma and HSIL samples (p = 0.16) **(Figure 1)**.

All samples showed intense staining for CD25 regardless of the result of viral load or histopathological findings **(Figure 2)**.

DISCUSSION

In the present study, we observed that among the high GITR expression samples 76.9% were HPV-positive and 23.1% were HPV-negative. The expression of this marker was predominant in samples with high viral load as well as high-grade lesions and carcinoma.

A number of surface and secreted molecules have been associated with T_{reg}, and GITR has been recognized as $CD4^+$ T_{reg} markers in mice and humans[22,26,27]. In this context, it is of interest that $GITR^+$ T_{reg} cells might be involved in the failure of

TABLE 1 - Distribution of histopathological findings according to viral load and GITR expression (n=49).

	NILM		LSIL		HSIL		CA		
	GITR		GITR		GITR		GITR		
Viral load	low	high	low	high	low	high	low	high	Total
0	8	4	0	0	0	1	0	1	14
1	1	2	5	2	3	4	1	1	19
2	1	1	0	0	2	2	0	1	7
3	1	2	1	1	0	1	0	3	9
Total	11	9	6	3	5	8	1	6	49

GITR: glucocorticoid-induced tumor necrosis factor receptor; NILM: negative for intraepithelial lesion and malignancy; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion; CA: carcinoma. Viral load - 0 (negative); 1 (1 to < 100 RLU/PCB); 2 (100 to < 1,000 RLU/PCB); 3 (≥ 1,000 RLU/PCB); RLU/PCB: relative light unit/positive controls to group B; GITR - low: small quantities of immunomarked cells; GITR - high: large quantities of immunomarked cells.

TABLE 2 - Frequence of histopathological findings according to the intensity of GITR expression.

	Histopathological (n/%)							
	NILM		LSIL		HSIL		CA	
GITR	n	%	n	%	n	%	n	%
low	11	55.0	6	66.7	5	38.5	1	14.3
high	9	45.0	3	33.3	8	61.5	6	85.7
Total	20	100.0	9	100.0	13	100.0	7	100.0

GITR: glucocorticoid-induced tumor necrosis factor receptor; NILM: negative for intraepithelial lesion and malignancy; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion; CA: carcinoma. GITR - low: small quantities of immunomarked cells; GITR - high: large quantities of immunomarked cells. (p=0.16).

the immune system to control the development of HPV-induced cancer. Studies have demonstrated increased frequencies and suppressive activity of T_{reg} cells in patients with high-grade lesions and cervical cancer. In addition, compared to colorectal cancer, skin melanoma, and bronchial carcinoma, HPV-derived CIN lesions and cervical carcinomas have higher numbers of T_{reg} cells[23,24].

One study that investigated the influence of tumor-infiltrating T_{reg} cells on tumor-specific T cell responses found that T_{reg} cells in patients with liver cancer upregulated GITR expression compared with T_{reg} cells in tumor-free liver tissue and blood[28]. Another study identified increased numbers of T_{reg} $GITR^+$ cells in tumor-positive lymph nodes compared with tumor-negative nodes in the same patient[29]. Both studies propose that GITR ligand could be a promising treatment for cancer and that GITR and GITR ligand are good candidates for disease evolution biomarkers.

Studies investigating the natural history of HPV infection have shown that viral clearance may vary from 4-16 months according to the virus' oncogenicity[30-32]. However, it has been observed that persistent infection with a higher likelihood of progression to high-grade lesions and invasive carcinoma can occur in the face of an ineffective immune response. In this context and considering that HPV infection is restricted to epithelial cells, the importance of the local immune response is highlighted, making the components present in the microenvironment crucial for lesion development or regression[33-36]. The role of GITR has been unclear until now, emphasizing the importance of the present study to clarify the immune response in the cervical microenvironment.

The presence of high GITR and CD25 expression levels found in HPV-derived CIN lesions and cervical carcinomas indicates that these cells may play an important role in the downregulation of immune responses[37]. A strong association between these markers and T_{reg} cells was demonstrated in a study that found GITR expression in only those cells that also expressed CD4 and CD25, and most of them co-expressed FOXP3[38]. The association of GITR and CD25 with negatively regulated T helper-activated lymphocytes has been demonstrated in experiment with C57BL/6 $GITR^{+/+}$ mice (wild type), which showed decreased IL-2 expression compared to C57BL/6 $GITR^{-/-}$[39].

The relevance of cells expressing the studied markers in immune response suppression is emphasized by studies that evaluate in vitro regulatory activity through cytokine expression by $CD4^+$ T cells, $CD4^+CD25^+GITR^+$ cells, and $CD4^+CD25^-GITR^+$ cells. These studies showed that the first produced cytokines that activated the immune response and the last 2 increased immunosuppressive cytokine levels[15,17].

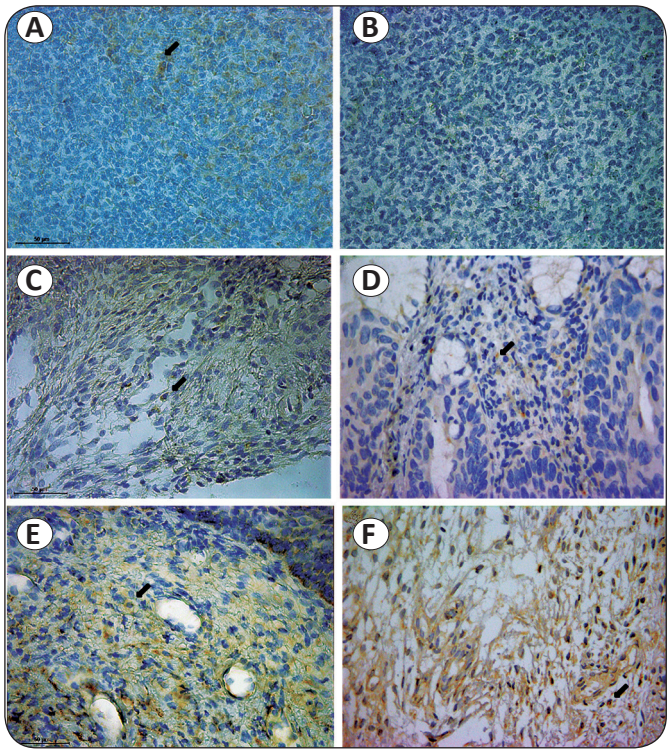

FIGURE 1 - GITR expression in HPV positive cervical lesions. a: positive control human tonsil stained with anti-human GITR antibody; b: negative control human tonsil - omitting primary antibody, showed no staining; c: CIN II and d: NILM, with GITR expressing cells in small quantities; e: CIN III and f: CIN I, with GITR expressing cells in large quantities. All figures are presented in the same magnification (400X). Black arrows indicate positive-staining cells with anti-human GITR antibody. GITR: glucocorticoid-induced tumor necrosis factor receptor; HPV: human papillomavirus; CIN: cervical intraepithelial neoplasia.

It is unclear whether increased frequencies of regulatory cells are a cause or consequence of high viral load and chronic infection[2,40,41]. The predominant expression of GITR in samples with high viral load and classified as HSIL and carcinoma in this study suggest that GITR$^+$ cells can exhibit regulatory properties. The lack of a correlation between GITR and viral load or GITR and histopathological findings can be explained by the small sample size. Additional studies are required to confirm these observations.

Further longitudinal studies are required to assess the true association between HPV persistence and immunoregulatory cell involvement in lesion progression and the development of neoplasia. Studies have demonstrated increased frequencies and suppressive activity of T_{reg} cells in HPV-infected patients with cervical cancer and its precursor lesions (CIN) and suggest that T_{reg} cells may be a potential marker of cervical disease persistence. One longitudinal analysis of T_{reg} cell frequencies showed a modest decline 1 year after curative surgery or chemoradiation[3,4].

Finally, on the basis of the finding that GITR configures a surface molecule characteristic of cells with a regulatory profile, our results suggest that GITR$^+$ cells may play a role in the development of a favorable microenvironment for the progression of HPV-induced cervical neoplasia that omits proper activation of the immune response for antigen elimination. Additional studies have been made by the same group including the characterization of FOXP3$^+$/CD25$^+$, CD4$^+$/transforming growth factor-β$^+$ and IL-10 - secreting cells in HPV-infected samples by using IHC to help elucidate the role of T_{reg} cells in cervical intraepithelial neoplasia (CIN) and cervical cancer (manuscript in preparation).

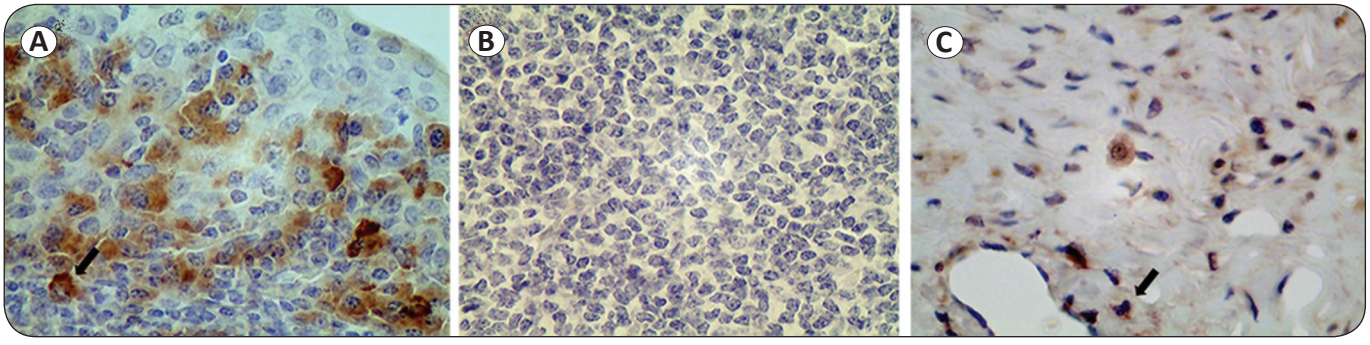

FIGURE 2 - CD25 expression in HPV positive cervical lesions. A: Positive control human tonsil stained with anti-human IL-2R antibody; B: negative control human tonsil - omitting primary antibody, showed no staining; C: CIN II, with CD25 expressing cells in large quantities. All figures are presented in the same magnification (400X). Black arrows indicate positive-staining cells with anti-human IL-2R antibody. HPV: human papillomavirus; IL-2R: IL-2 receptor; CD25: α-chain of the IL-2 receptor.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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