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Decreased levels of alternative co-stimulatory receptors OX40 and 4-1BB characterise T cells from head and neck cancer patients

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ABSTRACT

Background and aim: Head and neck cancers (HNC) are aggressive tumours. Tumour-specific T cells are frequently identified in patients with cancer, although they fail to control tumour progression. A family of proteins called co-stimulatory receptors regulate the function of T cells and may account for T cell dysfunction in cancer. Our aim was to characterise co-stimulatory receptors on T cells in HNC patients to identify novel targets for immunotherapy.
Methods: Peripheral blood mononuclear cells were isolated from HNC patients and healthy controls and the expression of co-stimulatory (OX40, 4-1BB, ICOS) and co-inhibitory (CTLA-4, PD1) receptors was analysed on $CD4^+$ and $CD8^+$ T cells using flow cytometry.
Results: We found that the levels of co-stimulatory receptors OX40 and 4-1BB were significantly lower on $CD4^+$ T cells from HNC patients. This was more pronounced in locally advanced tumours (T3/T4) compared to early carcinomas (T1/T2). PD-1 levels were higher on $CD8^+$ T cells in HNC patients compared to controls. Human papilloma virus (HPV)-specific $CD8^+$ T cells appeared to be more affected than Influenza-specific T cells.
Conclusions: Our results indicate that expression of co-stimulatory receptors on T cells from HNC patients is imbalanced with a preponderance of inhibitory signals, and reduction of stimulatory signals, especially in advanced disease. Restoring this balance could improve T cell therapy outcomes in HNC.

Introduction

Head and neck squamous cell carcinomas (HNC) are a significant cause of morbidity worldwide (Grandis et al. 2004). Advanced stage HNC (stages 3 and 4) is usually treated with a combination of surgery, radiotherapy and chemotherapy. However survival rates remain poor and frequent adverse effects associate with the available current treatment modalities. This raises the need for development of novel therapeutic tools.

Cancers may elicit anti-tumour immune responses by virtue of tumour specific antigens, which interact with the immune system of the host. Indeed, tumour antigen specific T cells are frequently identified in patients with cancer, although they often fail to control tumour progression (Van den Eynde and van der Bruggen 1997). Understanding the reasons underlying this failure can open up new avenues of T cell immunotherapy in cancer. Of note, tumour associated antigens have been identified in head and neck tumours (Venuti et al. 2004). The oncogenic human papilloma virus (HPV) (subtypes 16 and 18) has been associated with 60–70% of oro-pharyngeal cancers, a common HNC (Venuti et al. 2004). Of relevance to our work, circulating T cells specific for HPV with anti-tumour activity have also been identified in patients with oro-pharyngeal cancers (Albers et al. 2005).

The survival and activation of T cells following antigen encounter depends on the balance between co-stimulatory and co-inhibitory receptors (Acuto and Michel 2003). Members of the CD28 co-stimulatory receptor family on T cells interact with members of the B7 family on antigen presenting cells such as dendritic cells and macrophages, and the signals thus transduced regulate the response of T cells to antigens (Sharpe and Freeman 2002). Some of these receptors (CD28, ICOS, OX40 and 4-1BB) provide positive (co-stimulatory) signals and promote T cell activation, while negative (co-inhibitory) signals are generated by receptors like CTLA-4 and PD-1 that limit the T cell response (Alegre et al. 2001; Dong et al.

Abbreviations: HNC, head and neck cancer; HPV, human papilloma virus.

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2001; Watts 2005; Sharpe et al. 2007). Alterations in co-stimulatory pathways in antigen specific T cells in cancers may well account for the failure of T cells to control tumour progression.

Our aim was to characterise the expression of co-stimulatory (ICOS, OX40, 4-1BB) and co-inhibitory (PD-1 and CTLA-4) receptors on $CD4^+$ and $CD8^+$ T cell subsets as well as on tumour HPV antigen-specific T cells in patients with HNC. In this study we demonstrate for the first time that both $CD4^+$ and $CD8^+$ T cells in HNC have altered expression of co-stimulatory and co-inhibitory receptors. In particular, we show that these alterations are more profound in the HPV tumour antigen-specific T cell population.

Materials and methods

Study population

Blood samples were obtained from 18 patients with newly diagnosed HNC cancers before any treatment and 12 healthy controls. The demographic details of patients and controls are provided in Supplementary Tables 1 and 2. Patients with acute or chronic infections or co-existing inflammatory disorders (i.e. autoimmune diseases, diabetes, renal failure and coronary artery disease) were excluded from the study. The study was approved by the local research ethics committee and informed consent was obtained from all study subjects.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient using Histopaque (Sigma-Aldrich). The cells were then cultured in RPMI 1640 (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM L-glutamine, 25 U/ml IL-2 (Roche) and 5% heat inactivated pooled human serum (BioWhittaker). For T cell activation, cells were stimulated with 1 μg/ml immobilised anti-CD3 and anti-CD28 antibodies (eBioscience) for 4 days in the presence of 50 IU/ml human recombinant IL-2 (Roche).

Phenotypic analysis

$CD4^+$ and $CD8^+$ T cell subsets were identified by staining with APC-labelled anti-CD4 and FITC-labelled anti-CD8 monoclonal antibodies (BD Biosciences). The expression of co-stimulatory receptors on these two subsets of T cells was evaluated using flow cytometry and monoclonal antibodies specific for ICOS, OX40, 4-1BB, CTLA-4 and PD-1 (BD Biosciences). Isotype-matched control antibodies were used to assess non-specific staining. The samples were acquired on a FACSCalibur (BD Biosciences) flow cytometer and data analysis was performed using FlowJo software version 7 (Tree Star).

Analysis of antigen-specific $CD8^+$ T cells

Oro-pharyngeal cancer patients were tested for expression of HLA-A2 using FITC-labelled anti-HLA-A2 monoclonal antibodies (BD Biosciences) and flow cytometry. PBMCs from $HLA\text{-}A2^+$ patients were incubated with APC-labelled HLA-A2/HPV (YMLDLQPET) or HLA-A2/Influenza M1 (GILGFVFTL) iTAg MHC tetramers (Beckman Coulter) as per the manufacturer's protocol. Then cells were stained with monoclonal antibodies to CD8 and co-stimulatory receptors (OX40, 4-1BB, PD-1 and CTLA-4) to characterise the expression co-stimulatory receptors on HPV-specific and Flu-specific $CD8^+$ T cells. Two million events were collected during flow cytometric analysis on a FACSCalibur machine (BD Biosciences) and data analysis was performed using FlowJo software (Tree Star).

Detection of cytokines

The interleukin-7 (IL-7) and IL-15 ELISA kits were purchased from R&D Systems, while the IL-21 kit was from eBioscience. The quantification of cytokines was performed according to the manufacturer's instructions. Blood was allowed to coagulate for at least 30 min at room temperature, and then centrifuged at $1500 \times g$ for 10 min and the serum was stored at a temperature below $-20\,^{\circ}C$ until analysis.

Statistical analysis

Data were compared using the two tailed t test for unpaired samples with unequal variance. Probability values (p) of less than 0.05 were considered statistically significant. Statistical analysis was performed using the GraphPad Prism software version 5.02.

Results

$CD4^+$ T cells from HNC patients express lower levels of the co-stimulatory receptors OX40 and 4-1BB compared to control subjects

Co-stimulatory receptors have crucial roles in the optimal activation and function of T cells. We investigated the status of these receptors in $CD4^+$ T cells from HNC patients. As only CD28 is expressed constitutively on resting T cells (Acuto and Michel 2003), while others are induced following activation (OX40, 4-1BB, ICOS, CTLA-4, PD-1) (Alegre et al. 2001; Sharpe and Freeman 2002), we analysed the expression of these receptors on $CD4^+$ T cells both in the resting state and after activation with antibodies against CD3 and CD28. As expected, resting $CD4^+$ T cells expressed low levels of co-stimulatory receptors (Fig. 1A). Following activation, the expression of OX40, 4-1BB, ICOS and CTLA-4 was dramatically upregulated on $CD4^+$ T cells (Fig. 1B). Comparison of co-stimulatory receptor expression in HNC patients and control subjects revealed that $CD4^+$ T cells from HNC had significantly lower levels of OX40 and 4-1BB (Fig. 1C). A similar trend was observed for another co-stimulatory receptor ICOS. Of note, the co-inhibitory receptor CTLA-4 was present in similar levels on $CD4^+$ T cells from HNC patients and controls (Fig. 1C).

Decreased levels of OX40 and 4-1BB on $CD4^+$ T cells from HNC patients are a characteristic of advanced tumours

Next, we analysed whether the decrease in OX40 and 4-1BB was correlated with the clinical stage of the disease. For this purpose, we classified the HNC patient group according to the tumour stage (early, T1/T2; advanced, T3/T4) and nodal stage (without nodal involvement, N0; nodal involvement, N+). We found a progressive decrease in the expression of co-stimulatory receptors OX40 and 4-1BB on $CD4^+$ T cells with increasing tumour stage (Fig. 2A). Indeed, only $CD4^+$ T cells from HNC patients with advanced tumours (T3/T4) showed a significant decrease in OX40 and 4-1BB (Fig. 2A). Comparison of HNC patients with or without nodal involvement did not reveal any difference in the levels of co-stimulatory receptors between the two groups (Fig. 2B), suggesting that OX40 and 4-1BB downregulation correlates with an advanced tumour stage but not with the nodal status. As HPV16 has a high prevalence in HNC, we also investigated the relationship between the HPV (p16) status and alterations in co-stimulatory receptors. No significant differences were found in the expression of OX40 or 4-1BB on $CD4^+$