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Decreased functional response to Toll like receptor ligands in patients with oral cancer

B. Paleja [a], A. Anand [a], D. Chaukar [b], A. D'Cruz [b], S. Chiplunkar [a,*]

[a] Chiplunkar Lab, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Navi Mumbai, India
[b] Department of Surgery, Tata Memorial Centre, Parel, Mumbai, India

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ABSTRACT

Patients with oral cancer (OC) show dysregulation of variety of anti tumor immune responses. To assess the role of Toll like receptor (TLR) signaling in peripheral blood lymphocytes (PBL) from OC patients, we analyzed the expression of TLR2, TLR3, TLR4 and TLR9 on various lymphocyte subsets. Results revealed an increased expression of TLRs on unconventional T cells (like γδ T cells, NKT cells and $CD4^+CD8^+$ T cells) as compared to conventional αβ T cells. Functional studies using TLR ligands (CpG, Poly I:C, LPS and Pam3CSK4) showed defects in the TLR mediated signaling in PBLs of OC patients. Proliferation of OC PBLs in response to stimulation with TLR ligands was significantly decreased. TLR ligand induced IFN-γ production by PBLs from OC patients were low as compared to HI. Stimulation with TLR ligands upregulated the levels of activation markers (CD25 and CD69) on PBLs from HI but not from OC patients. TLR ligands CpG, Poly I:C, LPS and Pam3CSK4 significantly augmented the tumor directed cytotoxic response of PBLs from HI but not from OC patients. Our data suggests that impairment of TLR function on PBLs may be another strategy adopted by tumor cells to dampen tumor directed immune responses.

1. Introduction

Cancer of the oral cavity is a serious and growing problem in many parts of the globe. Despite the several advancements in therapeutic regimens the 5-year survival rate for oral cancer has not improved significantly and remains about 50% [1]. Oral squamous cell carcinoma represents more than 90% of all oral cancers (OC) [2]. Major risk factors for oral cancer include smoking, tobacco products, alcohol use and HPV infections. India has a higher incidence compared to developed countries due to the habits of tobacco chewing and bidi smoking. In India there are 75,000–80,000 new cases of oral cancer each year and the incidence rates of cancers of the oral cavity in both males and females in all urban cancer registries are among the highest in the world [3].

Earlier reports from our lab and others have shown a variety of immunosuppressive mechanisms in patients with oral cancer. These include presence of tumor derived inhibitory factors (like PGE2, IDO), tumor induced apoptosis of T cells, release of inhibitory cytokines (like IL-10, TGF-β), high frequencies of suppressive T cell populations or degradation of CD3-ζ, key T cell signaling molecule [4–10]. These molecular mechanisms explain tumor specific immunosuppression which are not restricted to the immediate tumor environment but present more generalized immunosuppression observed with tumor progression.

The innate immune system represents the first line of defense against invading pathogens or altered or transformed cells. Toll like receptors (TLRs) are one of the important members of a group of receptors called pattern recognition receptors (PRR) that are involved in recognition of pathogen associated molecular patterns (PAMPs) [11]. TLRs recognize conserved structures from microorganisms of viral, bacterial or fungal origin. TLRs also recognize a variety of endogenous ligands like heat shock proteins, fibrinogen, hyaluronic acid, etc. Binding of the ligand to TLR results in activation of various downstream signaling molecules. The adaptor molecule myeloid differentiation protein 88 (MyD88) is utilized for signaling through all the TLRs except TLR3, which signals through the recruitment of Toll/IL-1R domain containing adaptor-inducing IFN-β (TRIF). TLR engagement results in the activation of a variety of transcription factors including NF-κB and induction of a various responses like cytokine production, proliferation and expression of costimulatory molecules [12].

Toll like receptors have been extensively studied on the myeloid cells of the innate immune system, such as macrophages and dendritic cells and have traditionally been considered to indirectly

Abbreviations: PGE2, prostaglandin E2; IDO, indole 2,3-dioxygenase; Pam3, Pam3CSK4; PIC/Poly I:C, polyinosinic:polycytidylic acid; LPS, lipopolysaccharide; CpG ODN, CpG oligodeoxynucleotide Type A.

* Corresponding author. Address: Chiplunkar Lab, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Chairperson, Academic & Training Program, Kharghar, Navi Mumbai, Maharashtra 410210, India.
E-mail address: schiplunkar@actrec.gov.in (S. Chiplunkar).

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control T cell responses through the innate immune system. However recent reports have shown that T cells themselves express TLRs and TLR signaling contributes directly to T cell mediated immune responses [13]. An effective anti-tumor immune response involves the co-ordinated action of several components of the immune system. There are no reports on expression of Toll like receptors on lymphocytes of patients with oral cancer and their functional significance in anti-tumor immunity.

To elucidate the role of TLR in oral cancer patients, we investigated the expression pattern of TLR2, TLR3, TLR4 and TLR9 on subsets of peripheral blood lymphocytes (PBLs) obtained from healthy volunteers and oral cancer patients. We demonstrate that oral cancer patients show altered expression of TLR on PBLs. Moreover, compared to HI functional responses of lymphocytes to TLR ligands are significantly impaired in OC patients. This study suggests that dysfunctional TLR signaling pathway may contribute to the impaired immune responses observed in oral cancer patients.

2. Materials and methods

2.1. Study group

Heparinized blood was collected from patients with squamous cell carcinoma of the oral cavity (Stage II-III) and as controls, from healthy individuals (age and sex matched). Peripheral blood was collected from OC patients at the time of diagnosis, before start of any treatment. The lymphocyte absolute counts in healthy donors ranged from 1.24 to 3.6 $\times$ 10^9/L (Mean 2.46 $\times$ 10^9/L ± 0.6). In oral cancer patients the lymphocyte absolute counts ranged from 1.2 to 3.10 $\times$ 10^9/L (Mean 2.09 $\times$ 10^9/L ± 0.65). The study was approved by the Institutional Ethics Committee and written informed consent was obtained from the patients and healthy individuals prior to collection of blood samples.

2.2. Cell proliferation and cytokine secretion

PBLs were isolated from heparinized venous blood from oral cancer patients and healthy donors by Ficoll–Hypaque (FH, Sigma, USA) density gradient centrifugation. Isolated PBLs were cultured in RPMI 1640 (Invitrogen Life – Technologies, NY) medium supplemented with 10% Fetal Calf Serum (FCS; Invitrogen Life Technologies, Grand Island, NY), antibiotics (Penicillin, Streptomycin, gentamycin, mycostatin) and L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2. Cells were incubated at a concentration of 1 $\times$ 10^5 - cells/well in a 96 well round bottom plate in the presence of either 1 μg/ml Pam3CSK (Calbiochem), 10 μg/ml Poly I:C (Sigma), 10 μg/ml LPS (Sigma) or 2 μg/ml CpG ODN A (Invivogen) in the presence and absence of rIL-2 (1 IU/ml), only PBLs and PBLs incubated with rIL-2 served as controls. Proliferation was measured by uptake of ^{3}H Thymidine during the last 18 h of a 3-day culture period. Cells were harvested and radioactivity was measured in a scintillation counter.

PBLs were stimulated with TLR ligands as described above. After 24 h culture supernatant were collected and cytokines secreted (IL-6, IL-10, TNF and IFN-γ) was determined using cytometric bead array (BD Pharmingen, USA) as per manufacturer's instructions. Samples were acquired on BD FACSAria flow cytometer and analyzed using FCAP Array software.

2.3. Flow cytometry

Expression of cell surface antigens on different lymphocyte subsets were determined by multi-color cytometry. Following

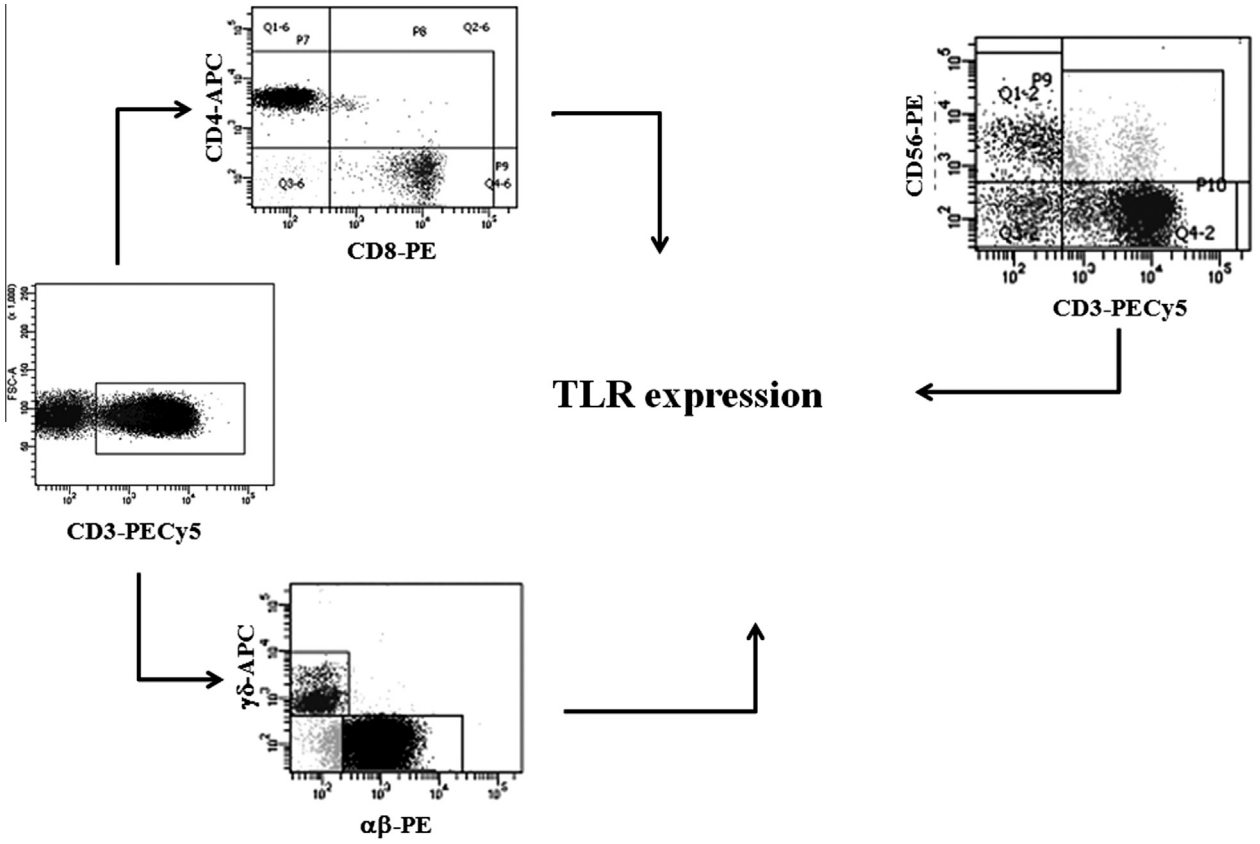

Fig. 1. Gating strategy employed to identify different lymphocyte subsets. Representative dot plot analysis of flow cytometry illustrating the gating strategy employed to identify different lymphocyte populations in peripheral blood.