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Limiting Dilution Analysis of Proliferating and Cytotoxic Lymphocytes in the Peripheral Blood and Tumours of Oral Cancer Patients

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Frequencies of proliferating and cytotoxic lymphocytes from the peripheral blood and tumour tissue of oral cancer patients and healthy individuals were monitored using limiting dilution analysis. Significantly lower precursor frequencies of proliferating lymphocytes were observed in the peripheral blood and tumour tissue of oral cancer patients. A high frequency of natural killer (NK) cells but low cytotoxic T lymphocytes (CTL) was observed in the peripheral blood compartment of oral cancer patients as compared to healthy individuals. A marked reduction in both NK and CTL frequencies in the tumour tissue compared to the peripheral blood was observed. In the tumour tissues, increased percentages of activated $CD4^+$ lymphocytes as compared to $CD8^+$ lymphocytes were observed. Our results suggest that impaired proliferative and cytotoxic potential of tumour infiltrating lymphocytes may play an important role in the escape of tumour cells from the immune system.

Key words: oral cancer, limiting dilution analysis, proliferating T lymphocytes, cytotoxic T lymphocytes, natural killer cells, tumour infiltrating lymphocytes, dual colour flow cytometry

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INTRODUCTION

Oral cancer is a predominant malignancy in India and has been associated with the use of tobacco and alcohol [1]. Deficiencies in both T and B cell responses have been reported in patients with oral cancer [2, 3]. The degree of immunodeficiency is variable and depressed immune responses have been reported at loco-regional and systemic levels in these patients [4].

Squamous cell carcinomas of the oral cavity are generally well infiltrated by mononuclear lymphocytes. Increased numbers of tumour infiltrating lymphocytes (TIL) around and in the tumour have been associated with improved prognosis and prolonged survival [5]. Histological and immunohistological techniques have been used to obtain information on the location, distribution and phenotype of the TILs [5]. These studies, however, do not address the crucial question of the state of activation or function of the lymphocytes in the local tumour microenvironment.

In order to gain a better insight into the functional repertoire of lymphocytes in the peripheral blood compartment and tumour tissues of oral cancer patients, the microcultures were phenotyped using dual colour flow cytometry to verify the involvement of T cell subsets (CD4, CD8) and natural killer (NK) cells that may contribute to their lytic potential.

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MATERIALS AND METHODS

Patients and controls

Blood samples were obtained from 10 untreated patients with primary tumours of oral cavity sites involving the tongue, buccal mucosa or alveolus. Tumour tissues were obtained from these patients immediately after surgery. All the patients had clinically and histologically proven squamous cell carcinoma stage II ($n = 2$), III ($n = 1$) and IV ($n = 7$), according to the TNM system of UICC (3rd Edition). Blood samples were also collected from 10 healthy individuals (aged from 25 to 50 years) who served as controls.

Separation of peripheral blood T lymphocytes (PBL-T) and TIL

Peripheral blood lymphocytes were obtained from heparinised venous blood by Ficoll–Hypaque (FH, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) gradient centrifugation and suspended in complete RPMI-1640 medium (Gibco, Grand Island, New York, U.S.A.) supplemented with 10% inactivated human AB blood group serum,

penicillin (100 IU/ml), streptomycin (50 μg/ml), gentamycin (40 μg/ml), mycostatin (5 μg/ml), L-glutamine (2 mM) and 2-mercaptoethanol (5×10^{-5} M). T cells were enriched from peripheral blood lymphocytes using nylon wool columns as described by Julius *et al.* [6].

For separation of TIL, tumour tissue was collected in sterile RPMI-1640 medium containing antibiotics and processed as described earlier [7]. In brief, tumour tissues were minced finely in medium and stirred gently with 0.5 mg/ml collagenase type IV (Sigma), 0.2 mg/ml deoxyribonuclease type I (Sigma) and 5 U/ml hyaluronidase type I-S (Sigma) for 2 h at 37°C. Single cell suspension containing tumour cells and TIL was loaded on a 75–100% FH gradient. After centrifugation, TIL at the interface of 75% and 100% FH were collected and washed twice with plain RPMI medium. The yield of TIL ranged from 0.5 to 2×10^6 cells per tumour tissue. The viability of TIL was >90% when stained with erythrosin B. The presence of T cells (>90%) in TIL was confirmed by immunoperoxidase staining of cytospin smears of TIL using anti-CD3 monoclonal antibody (MAb).

Microcultures of TIL and PBL-T

A limiting dilution microculture assay described by Moretta *et al.* [8], with minor modifications, was used. The lymphocytes were seeded in 96-well round bottomed microtitre plates (Nunc, Denmark) at 0.75, 1.5, 3, 6, 12, 25, 50 and 100 cells/well as 24 replicates of each dilution with irradiated (4000 R) allogeneic PBL (2×10^4) as feeder cells in a final volume of 200 μl of complete RPMI-AB medium. At the onset of culture, phytohaemagglutinin-M (PHA, 1% v/v Gibco) was added. After 48 h, 50 u/ml recombinant interleukin-2 (rIl-2, a gift from Hoffmann La Roche, Switzerland) was added. Microcultures were supplemented weekly with irradiated feeder cells and rIL-2. A set of cultures containing irradiated feeder cells, PHA and rIL-2 served as controls. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

The cultures were scored microscopically for growth at regular intervals. The microcultures established from peripheral blood were analysed for proliferation and cytotoxicity after 18 days whereas those from tumours were analysed after 21 days. These time points for the assays were decided after confluency of growth was observed in cultures containing the highest cell number plated.

Assays for proliferation and cytotoxicity

For assessing proliferation, the cultures were pulsed with 0.5 μCi/well of tritiated thymidine (^{3}H-TdR; Board of Radiation and Isotope Technology, India, specific activity 6–9 mCi/mMole). The incorporated radioactivity was measured after 18 h by liquid scintillation counting. Cultures in which ^{3}H-TdR uptake exceeded the mean uptake detected in control cultures (irradiated feeder cells cultured with PHA and rIL-2) by greater than 3 standard deviations (SD) were defined as positive.

Cytotoxic activities of individual microcultures were tested in a 4 h ^{51}chromium (^{51}Cr) release assay. Each microculture was split into two different 100 μl aliquots and tested for cytotoxicity against ^{51}Cr (Amersham International Bucks, U.K.; specific activity 250–500 mCi/mg) labelled targets (5×10^3 cells/well). To assess cytotoxic T lymphocyte (CTL) activity, lectin-dependent cellular cytotoxicity (LDCC) using concanavalin A (20 μg/ml; Sigma) with Raji cells (B-lymphoblastoid cell line) was carried out [9]. Natural killer cell activity was determined using NK sensitive K562 cells (erythroleukaemic cell line) as targets. Percent specific lysis was calculated using the following formula:

$$\% \text{ specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100.$$

Cultures in which the percentage specific release exceeded by greater than 3 S.D. the mean percentage specific release detected in control cultures (irradiated feeder cells cultured with PHA and rIL-2), were defined as positive.

Determination of frequency of proliferative and cytotoxic T-lymphocyte precursors

Minimal estimates of frequencies of proliferating and cytotoxic T-lymphocyte precursors (PTL-p and CTL-p, respectively) were obtained by the minimum chi square method from Poisson's distribution relationship between the responding cell number and logarithm of the percentage of non-responding cultures as described by Taswell [10].

Statistical analysis

Differences in frequencies of PTL-p and CTL-p observed between patients and healthy individuals were analysed by Student's *t* test.

Phenotypical analysis of the microcultures

Microcultures set up for estimating the precursor frequencies of proliferating and cytotoxic cells were also analysed for phenotypes of lymphocytes using single and dual colour flow cytometry after 18 days for PBL microcultures and after 21 days for TIL microcultures. Cells (5×10^5) were suspended in 100 μl of phosphate buffered saline (0.01 M, pH 7.5) containing 1% fetal calf serum (Gibco) and incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labelled MAbs against CD3, CD4, CD8, CD56 and HLA-DR markers (Dako) for 30 min at 4°C. Single and dual colour flow cytometric analysis was carried out on an EPICS 753 (Coulter Electronics, U.S.A.) flow cytometer. Cells stained with mouse isotypes (all subtypes) conjugated to FITC and PE served as negative controls.

RESULTS

Precursor frequencies of proliferating T cells

The frequencies of proliferating and cytotoxic lymphocyte precursors in the peripheral blood of oral cancer patients and healthy individuals and in oral tumours were estimated by a limiting dilution microculture technique. As seen in Fig. 1, mean precursor frequencies of proliferating lymphocytes from peripheral blood (1/14) and tumour tissue (1/23) of oral cancer patients were significantly reduced compared to those observed in the peripheral blood of healthy individuals (1/8). Oral tumours showed a markedly impaired clonogenicity of infiltrating lymphocytes, the frequencies of PTL-p ranged from 1/34 to 1/17. In peripheral blood of oral cancer patients the frequency ranges were 1/32 to 1/7 which were lower than PTL-p frequency ranges observed in

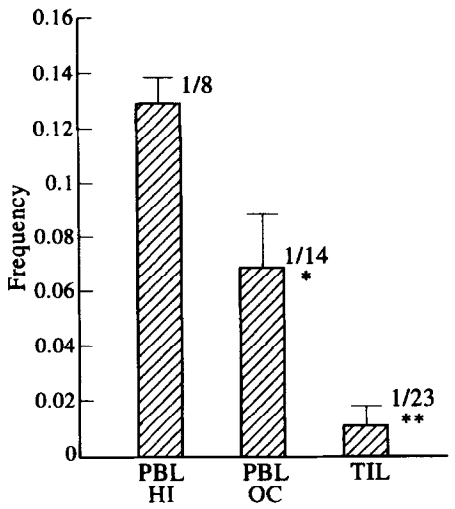

Fig. 1. PTL-p frequencies in PBL and TIL of oral cancer patients (OC, $n = 10$) and healthy individuals (HI, $n = 10$). Statistical significance between PTL-p frequencies of PBL of healthy individuals and oral cancer patients [*, $P < 0.05$] and PBL and TIL of oral cancer patients [, $P < 0.05$].**

healthy individuals (1/9 to 1/6). All data fitted the single-hit Poisson model with P values of >0.05 in regression analysis.

Precursor frequencies of cytotoxic lymphocytes

Two different assays, namely LDCC and NK, were employed to assess the cytotoxic potential of the microcultures, LDCC assay allows detection of cytolytic potential of lymphocytes irrespective of antigen specificity of effector cells and acts by triggering the lytic machinery essentially via the TCR [9]. Frequency of NK like effectors was significantly higher in the peripheral blood of oral cancer patients (1/7) compared to healthy individuals (1/29). In the tumour tissues, the frequencies of NK cells were markedly low (1/63), indicating local immunosuppression at the tumour site (Fig. 2).

Comparison of CTL-p frequencies demonstrated a lower frequency (1/42) of cytotoxic lymphocytes in the peripheral blood of oral cancer patients compared to healthy individuals (1/14). Although no statistically significant differences were observed in the frequencies of cytotoxic lymphocytes at the tumour site and in the peripheral blood compartment of oral cancer patients, the CTL-p frequency ranges at the tumour site were lower (1/134 to 1/79) compared to higher ranges (1/88 to 1/8) observed in the peripheral blood of oral cancer patients (Fig. 3).

Phenotypical analysis of cultured lymphocytes from peripheral blood and tumour tissue

As significant differences were observed in the frequencies of cytotoxic lymphocytes at the tumour site and in the peripheral blood compartment of oral cancer patients, single and dual colour flow cytometric analysis was performed to investigate subsets of lymphocytes involved in mediating cytotoxicity. Microcultures of five oral tumours were analysed and representative data are presented in Fig. 4. A dominant $CD3^+$ lymphocyte population (93%) was observed in the microcultures established from peripheral blood and tumour tissues of oral cancer patients. Compared to the peripheral blood compartment which showed 17% and 21% of $CD4^+$ and $CD8^+$ lymphocytes, respectively, higher percentages of $CD4^+$ (68%) and $CD8^+$ (51%) lymphocytes were observed in the tumour tissue. Interestingly the percentage of $CD56^+$ lymphocytes was significantly higher at the tumour site (87%) than in the peripheral blood (11%). Dual colour flow cytometric analysis of the microcultures was performed with anti-CD4 MAb or anti-CD8 MAb conjugated to PE and anti-HLA-DR MAb conjugated to FITC. As shown in Fig. 4, the total percentages of lymphocytes in the peripheral blood expressing CD4 and HLA-DR were 17% and 87%, respectively. The percentage of lymphocytes exhibiting a dual expression of $CD4^+$ $HLA\text{-}DR^+$ phenotype was 15%. Similarly the

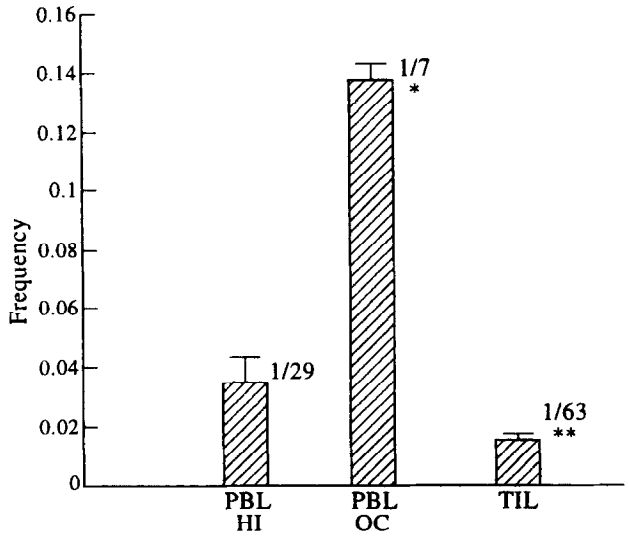

Fig. 2. NK cell frequencies in PBL and TIL of oral cancer patients (OC, $n = 10$) and from healthy individuals (HI, $n = 10$). Statistical significance between precursor frequencies of PBL of healthy individuals and oral cancer patients [*, $P < 0.05$] and PBL and TIL of oral cancer patients [, $P < 0.01$]**

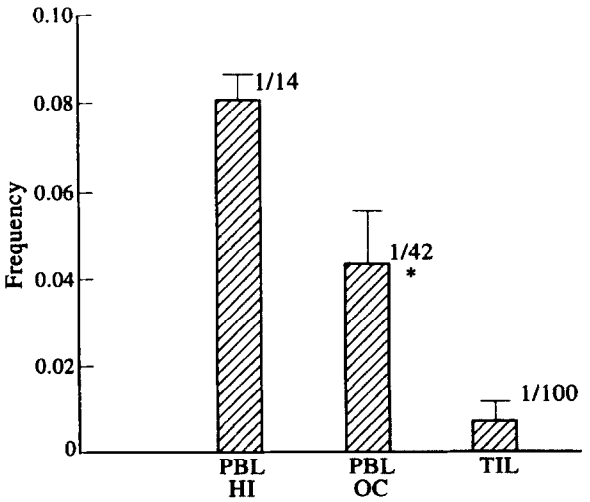

Fig. 3. CTL-p frequencies in PBL and TIL of oral cancer patients (OC, $n = 10$) and from healthy individuals (HI, $n = 10$). Statistical significance between precursor frequencies of PBL of healthy individuals and oral cancer patients [*, $P < 0.05$]

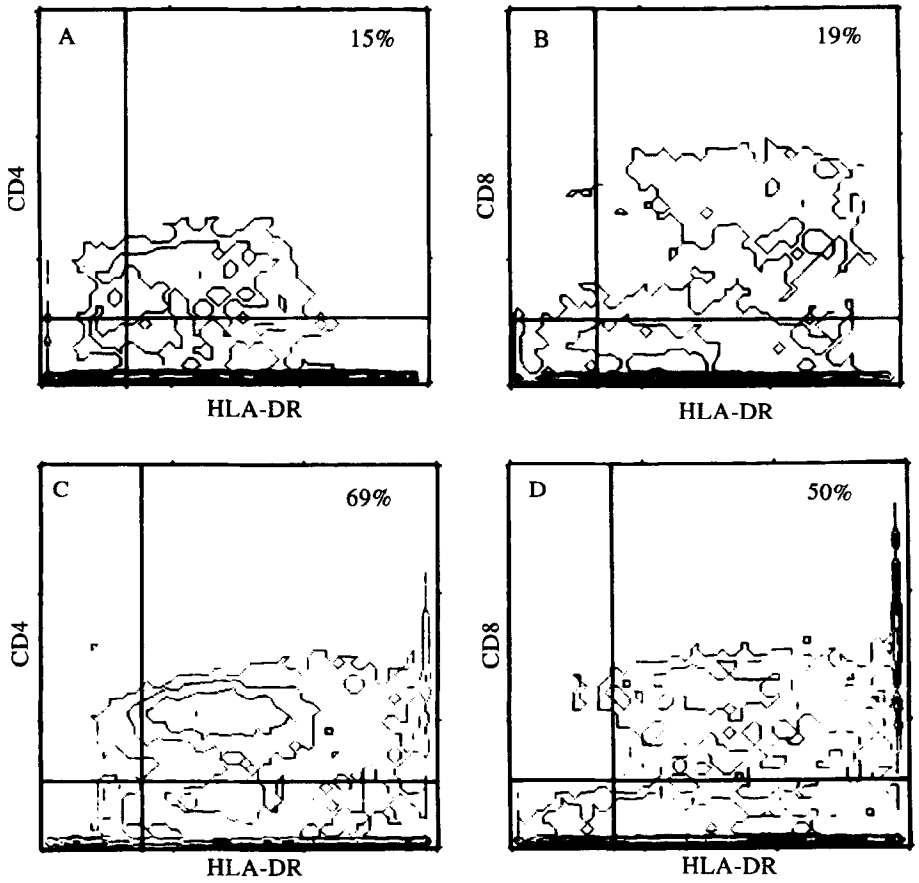

Fig. 4. Dual colour flow cytometric analysis of lymphocytes from peripheral blood (A, B) and tumour tissue (C, D) of oral cancer patients. MAbs used were PE-conjugated anti-CD4 and anti-CD8 and FITC conjugated anti-HLA-DR. Numbers in figures represent the percentages of cells showing dual markers.

total percentage of $CD8^+$ lymphocytes was 21% while the percentage of $HLA\text{-}DR^+$ lymphocytes was 81%. $CD8^+$ $HLA\text{-}DR^+$ expression was seen on 19% of lymphocytes. Tumour infiltrating lymphocytes consisted of 70% and 99% of $CD4^+$ and $HLA\text{-}DR^+$ cells, respectively, with 69% of these cells exhibiting a dual expression $CD4^+$ $HLA\text{-}DR^+$ phenotype. The total percentage of $CD8^+$ lymphocytes in TILs was 51% and $HLA\text{-}DR^+$ was 99%. $CD8^+$ $HLA\text{-}DR^+$ phenotype was observed on 50% of the TILs (Fig. 4).

Microcultures established from peripheral blood of healthy individuals were similarly phenotyped. The cultures exhibited dominant $CD3^+$ phenotype (90%) with 56% and 23% of $CD4^+$ and $CD8^+$ lymphocytes, respectively. The percentage of $CD56^+$ lymphocytes was 16%. Dual colour flow cytometric analysis revealed that lymphocytes expressing $CD4^+$ and $HLA\text{-}DR^+$ were 64% and 90%, respectively. Out of these, 60% $HLA\text{-}DR^+$ lymphocytes also expressed CD4. Lymphocytes expressing $CD8^+$ and $HLA\text{-}DR^+$ were 27% and 83%, respectively. The percentage of lymphocytes exhibiting a dual expression of CD8 and HLA-DR phenotype was 26%.

DISCUSSION

In the present investigation, we have used the limiting dilution microculture technique to quantitate the pool size of proliferating and cytotoxic (NK, CTL) lymphocyte precursors in the peripheral blood and tumour tissues of oral cancer patients. This method allows extensive clonal proliferation of virtually all T cells [8].

Our studies demonstrated that frequencies of proliferating T cells in tumour tissues and peripheral blood of oral cancer patients were significantly lower than in healthy individuals. The impaired clonogenic potential of precursor lymphocytes in the tumour milieu and in circulation of oral cancer patients could be due to the presence of intrinsic inhibitory factors. Many tumours release inhibitory molecules which downregulate proliferative and anti-tumour activities of immune cells found in the tumour microenvironment [11, 12]. A T-lymphocyte suppressor factor produced by human glioblastoma cells has been identified to be TGF-β [13]. Vitolo *et al.* [14], using *in situ* hybridisation with radiolabelled cDNA antisense probes demonstrated the presence of TGF-β message in mononuclear cells present in immediate proximity of squamous cell carcinoma of head and neck (SCCHN). In certain solid tumours TGF-β producing mononuclear cells may be responsible for local immunosuppression [15]. Decreased PTL-p frequencies of TILs in oral tumours observed by us have also been reported for other solid tumours such as breast, oesophagus, colon, lung and nasopharyngeal carcinomas [16–18].

It has been reported that TILs possess a significant cytotoxic potential despite their reduced proliferative capacity [16, 19]. We have analysed precursor frequencies of lymphocytes mediating NK and CTL like activities in tumor tissues and peripheral blood of oral cancer patients. TIL

exhibited a low frequency of non-MHC restricted NK like effectors compared to the peripheral blood. Snydermann *et al.* [5] reported that NK cell activity varied considerably in peripheral blood of patients with SCCHN. Percentages of NK cells were consistently lower in TIL and lymph node lymphocytes compared to PBL in these patients.

In the present investigations, we have used LDCC to assess the cytolytic potential of lymphocytes. LDCC can be employed to analyse the cytotoxic potential of MHC restricted CTL in the absence of appropriate antigen specific target cells [9], i.e. autologous tumour cells. In our studies, a decreased LDCC activity was observed both in the peripheral blood and tumour tissues of oral cancer patients indicating that the ability of lymphocytes to mediate cytotoxicity was significantly low in these patients.

In order to understand the involvement of lymphocyte subsets in mediating these cytotoxic functions, we immunophenotyped the microcultures using single or dual colour flow cytometry. We observed more $CD56^+$ cells in the microcultures established from TIL suggesting preferential accumulation of lymphocytes with the potential to mediate non-MHC restricted cytotoxicity at the tumour site. However, our frequency analysis data demonstrated a lower frequency of NK like effectors, despite high percentages observed by flow cytometry. As suggested by Moy *et al.* [20] and Keong *et al.* [21], the NK activity may be suppressed at the tumour site. Although CD56 expression is characteristic of lymphoid effectors that kill target cells in MHC unrestricted fashion, not all CD56 cells are typical NK cells. Fuchshuber and Lotzova [22] characterised the oncolytic efficacy of NK cell subsets generated from a highly NK enriched population in long term IL-2 cultures. They observed that the $CD16^+/CD56^+$ NK cell subset was superior in its cytotoxic activity against tumour targets compared to the $CD16^-/CD56^+$ subset. Their studies suggested that NK cell subsets are not equally oncolytic and that oncolytic effects may also be tumour dependent. It remains to be investigated if TIL of oral tumours contain higher percentages of $CD16^-/CD56^+$ lymphocytes which may contribute to their low cytotoxic function despite increased expression of CD56. Our study thus demonstrates a functional difference in lymphocytes isolated from the tumour site and peripheral blood of oral cancer patients. An increase in $CD4^+$ compared to $CD8^+$ lymphocytes was observed in the tumour tissue. Both $CD4^+$ and $CD8^+$ lymphocytes were in an activated state ($HLA\text{-}DR^+$) in the peripheral blood and tumour tissue, percentages of activated $CD4^+$ lymphocytes being higher at the tumour site. It therefore appears that both $CD4^+$ and $CD8^+$ cells significantly contribute to the cytotoxic activity observed in oral cancer patients. A predominance of $CD4^+$ clones obtained by limiting dilution was also reported for other tumours [19, 23]. Although the role of $CD4^+$ lymphocytes in the destruction of tumour cells is still not clear, $CD4^+$ TIL clones which can interact with MHC-class I on tumour cells have been reported from patients with solid tumours [24, 25]. It thus appears that the impaired proliferative and cytotoxic potential of TIL might play an important role in the escape of tumour cells from the immune system. The possible role of tumour cells in inducing reduced proliferative and cytotoxic potential of TILs needs to be explored further which would have important implications in designing immunotherapeutic strategies.

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