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Research paper

Isolation of viable and functional T-cells from human palatine tonsils

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ABSTRACT

Increasing clinical evidence indicates that removal of the palatine tonsils enhances the risk for adults to suffer from severe illnesses. Together with recent experimental findings pointing to the presence of immunologically competent immune cells these findings illustrate that adult palatine tonsils likely play an appreciable role in the host immune response. T-cells are abundant in the palatine tonsil and are a pivotal entity of the adaptive immune response. However, investigation of T-cells from tonsils has been widely neglected and largely restricted to immune phenotyping. Accordingly, methodological literature describing the experimental preparation and isolation of T-cells from tonsils is scarce and has rarely been complemented with rigorous tests of T-cell functionality. We report here on a comparative investigation of three isolation protocols composed of permutations of different tissue grinding approaches, density gradient centrifugation and automated magnetic collection of CD4/CD8 T-cells. Importantly we put a strong emphasis on assessing the impact of the preparative procedures on the functionality of T-cells at the level of viability and functional response to T-cell receptor (TCR) ligation. The reported, optimized preparation protocols allow for the rapid isolation of highly viable, functional T-cells within 2.5 h and represent a useful, affordable approach for the analysis of tonsillar T-cells.

1. Introduction

Due to the localization in the oropharynx directly at the entrance to the upper respiratory and gastrointestinal tracts the palatine tonsils are the most easily accessible secondary lymphoid tissue in humans. They represent the foremost obstacle protecting the body against exogenous pathogens (Nave et al., 2001). Palatine tonsils consist of a surface of stratified squamous crypt epithelium, follicular germinal centers with a mantle zone and an interfollicular area (van Kempen et al., 2000). Tonsillectomy is routinely performed to treat chronic and recurrent acute tonsillitis, defined as two or more tonsillitis episodes within one year requiring antibiosis (Sada-Ovalle et al., 2012). However, an increasing body of evidence shows that the removal of the palatine tonsil is associated with increased risk in adults to develop multiple sclerosis (Lunny et al., 2013), acute myocardial infarction (Janszky et al., 2011), Hodgkin's lymphoma (Vestergaard et al., 2010) or a deep neck infection (Wang et al., 2015). These findings strongly indicate that palatine tonsils play a more prominent role in immune defense than anticipated.

T-cells are present in high numbers in palatine tonsils and are mainly found in the extra-follicular spaces (Nave et al., 2001). In contrast to other secondary lymphoid organs, palatine tonsils do not only provide a niche for antigen/pathogen recognition by naïve T-cells but also foster the maturation of immature precursors (Sada-Ovalle et al., 2012). Given the easy accessibility and the large numbers of resident T-cells palatine tonsils are an attractive and commonly used source for lymphoid cells (Vidal-Rubio et al., 2001; Sada-Ovalle et al., 2012). However, probably due to the chronically underestimated functional importance of palatine tonsils there is only very few published records on methodological procedures for the isolation of tonsillar T-cells. Moreover, to our knowledge, the consequences of purification procedures on the functionality of T-cells have not been comprehensively addressed so far.

The aim of this study was to compare different isolation approaches for the enrichment of tonsillar T-cells. For that purpose we implemented and characterized three different approaches of T-cell purification from adult palatine tonsil tissue obtained by tonsillectomy from patients with chronic tonsillitis. Importantly, we placed particular attention on monitoring the repercussion of purification steps on the functionality of the T-cells, i.e. by assessing their response to TCR ligation at the level of activation marker upregulation and clonal expansion. Our findings illustrate that all three approaches achieve a high yield and purity of T-cell fractions (>90% $CD3^+$) without detectable T-cell

Abbreviations: Ab, antibody; CD, cluster of differentiation; CFSE, 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester; FCS, fetal calf serum; TCR, T-cell receptor.

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activation as assessed by T-cell activation marker surface expression. However one procedure stood up in terms of higher T-cell viability and optimal response to TCR stimulation.

2. Materials and methods

2.1. Patients and surgical method of palatine tonsil extraction

We examined palatine tonsils from 3 adult patients with chronic tonsillitis, 1 female and 2 males, between 20 and 34 years old. All three patients underwent a bilateral tonsillectomy. The surgical interventions were performed at the Department of Otorhinolaryngology, Jena University Hospital, Germany. Written consent for tonsil utilization was obtained from all patients as approved by the ethical review committee from the medical faculty of the Friedrich-Schiller-University Jena (No. 3972-01/14).

2.2. Materials and reagents

Human MicroBeads CD4, CD8 and C-tubes were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany); 70 μM cell strainer from BD Pharmingen™ (Franklin Lakes, USA); RPMI 1640 medium and PBS from Biochrome AG (Berlin, Germany); Ficoll Histopaque®-1077, DMEM, Collagenase type IV and DNAse type I from Sigma-Aldrich (St. Louis, USA); Falcons from Greiner Bio One GmbH (Frickenhausen, Germany); 24-well plates from Corning Incorporated (Corning, USA); Streptavidin from Dianova (Hamburg, Germany); FCS from Biowest LLC (Kansas City, USA); EDTA from Applichem (Darmstadt, Germany); BSA, Penicillin and Streptomycin from PAA Laboratories GmbH (Pasching, Austria); CFSE from Enzo Lifescience Inc. (Lörrach, Germany); and Sytox® AADvanced™ from Life Technologies Corporation (Carlsbad, USA).

2.3. Antibodies

Antibodies for flow cytometry analysis: anti-human CD3-FITC (clone MEM-57), anti-human CD4-PE (clone MEM-57), anti-human CD8-APC (clone MEM-31) and anti-human CD25-FITC (clone TB-30) were purchased from ImmunoTools GmbH (Friesoythe, Germany); anti-human CD69-APC (clone FN50) was from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Antibodies for T-cell stimulation: anti-human CD3 Biotin (clone OKT3), anti-human CD28 purified (clone CD28.2) and anti-human CD3 purified (clone OKT3) were purchased from eBioscience Inc. (San Diego, USA).

2.4. Palatine tonsil tissue preparation and T-cell purification

In the present study three different strategies for the isolation of $CD4^+$ and $CD8^+$ T-cells from freshly removed tonsils were performed and compared (Fig. 1). Immediately after surgery, the palatine tonsils were stored in PBS/0.5% BSA/2 mM EDTA on ice. To enable a side-by-side comparison of all three preparation protocols described below, each tonsil was manually dissociated and split into three parts, one for each approach (see Fig. 1 for experimental flowchart).

Approach A: Manual dissociation: The tonsillar tissue was placed on a 70 μM cell strainer and carefully squashed with a plunger. The homogenate was re-suspended in PBS/0.5% BSA/2 mM EDTA at room temperature and centrifuged for 10 min/500 *g*/4 °C. The pellet was washed once with warm PBS/0.5% BSA/2 mM EDTA followed by a cell-count determination. The cell suspension was used for magnetic purification as described below.

Approach B: Tonsils were manually dissociated and pressed through a 70 μM cell strainer as in approach A. Following the washing with PBS/0.5% BSA/2 mM EDTA the tissue pellet was re-suspended in 10 ml RPMI-medium. The cell suspension was carefully layered on top of a 15 ml Ficoll-Histopaque®-1077 cushion in a 50 ml conical tube and spun for 20 min at 400 g at room temperature in a swinging-bucket rotor without brake. The mononuclear cells in the interphase were carefully transferred into a new tube and washed with PBS/0.5% BSA/2 mM EDTA. The obtained cell suspension was processed for automated magnetic selection as described below.

Approach C: Tonsils were manually dissociated as before and prepared for automated homogenization with the gentle-MACS device. To this end the grinded tissue was suspended in 8 ml pre-warmed PBS/0.5% BSA/2 mM EDTA supplemented with 0.5 mg/ml collagenase type IV and 0.02 mg/ml DNAse type I in a C-tube (Miltenyi Biotec GmbH). The tissue sample was placed into the gentleMACS™ Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and homogenized using the pre-installed program C, followed by incubation for 15 min at 37 °C and a second homogenization round with program C. The homogenate was placed on a 70 μM cell strainer and carefully squashed with a plunger and taken up in 8 ml DMEM. After centrifugation for 10 min at 500 g in the cold, the pellet was washed once with PBS/0.5% BSA/2 mM EDTA and resuspended in 10 ml DMEM. The cells were layered carefully on a Ficoll-Histopaque®-1077 cushion and centrifuged for 20 min and 400 *g* at room temperature as for approach B. The mononuclear cells in the interphase were transferred into a new tube and washed once. The obtained single cell suspension was processed for magnetic purification as follows.

Single cell suspensions generated by all three protocols were subjected to a final automated positive selection of CD4/CD8 T-cells in the autoMACS Pro Separator device using the Human MicroBeads CD4, CD8 kit (Miltenyi Biotec GmbH). The number of cells in the single cell suspensions was determined and separation was performed following the manufacturer's instructions. The purity of the final $CD4^+/CD8^+$ cell preparations was determined via flow cytometry.

2.5. T-cell cultivation and TCR stimulation

Human T-cells were cultured in RPMI 1640 medium supplemented with penicillin and streptomycin and 3% (for 18 h stimulation) or 10% (for 6 d stimulation) heat-inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2. TCR stimulation with soluble monoclonal antibodies was accomplished by the addition of 1.7 μg each of biotinylated anti-CD3ε and anti-CD28 antibodies (Abs) Abs per 0.5×10^6 T-cells. CD3ε and CD28 Abs were mixed before administration for dual stimulation. Abs were further cross-linked by the addition of 5 μg/ml streptavidin. For the surface immobilized stimulation cell culture plates were coated with 5 μg/ml anti-CD3ε diluted in PBS by rocking the plates on an orbital shaker 2 h, at 37 °C, followed by washing with PBS prior to seeding of the T-cells in the presence of 1.7 μg soluble anti-CD28 per 0.5×10^6 T-cells.

2.6. Flow cytometric analysis of T-cell activation

Immediately after magnetic isolation 0.5×10^6 cells per sample were analyzed by flow cytometry. Cells were washed once with PBS/1% BSA and stained with appropriate human antibody panels (see Section 2.3). All samples were co-stained with the life/dead dye Sytox® AADvanced™ to eliminate dead cells from the analysis. Cell mixtures were incubated at 4 °C for 30 min, and cells were washed once with PBS/1% BSA and processed for flow cytometry. For the analysis of TCR-induced responses purified $CD4^+/CD8^+$ T-cells were challenged with various TCR/co-stimulatory molecule Ab combinations for the indicated periods of time as described in Section 2.5. Reactions were stopped by washing the T-cells with ice-cold PBS/1% BSA followed by staining at 4 °C for 30 min with anti-human FITC-CD25 or APC-CD69 in combination with Sytox® AADvanced™ for live/dead cell discrimination. Cells were washed with PBS/1% BSA and used for flow cytometry. Flow cytometry data were acquired using a FACSCalibur (BD Pharmingen™, Franklin Lakes, USA) and analyzed with FlowJo software (TreeStar Inc., Ashland, USA).