Histol Histopathol (2021) 36: 1273-1283

**http://www.hh.um.es**

Histology and Histopathology
*From Cell Biology to Tissue Engineering*

**ORIGINAL ARTICLE** **Open Access**

# Epithelial component and intraepithelial lymphocytes of conjunctiva-associated lymphoid tissue in healthy children

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**Summary.** Conjunctiva-associated lymphoid tissue (CALT) plays a key role in protecting the eye surface by initiating and regulating immune responses. The aim of this study was to investigate in healthy children the proportion of intraepithelial lymphocytes (IELs), the degree of viability and/or apoptosis and cell proliferation in three different topographic areas of the conjunctiva. Superior tarsal, superior bulbar, and inferior tarsal-bulbarfornix conjunctival cells were collected by brush cytology (BC) from 24 healthy paediatric subjects (13 boys and 11 girls, mean age 6±2 years) who were to undergo strabismus correction surgery under general anaesthesia. Subsequently, these cells were analysed phenotypically and functionally by flow cytometry (FC). Flow cytometry analysis showed that not all the cells obtained by BC were of the epithelial lineage, but that there was a population of CD45+ cells (IELs) regularly present in the conjunctiva of healthy children. These IELs were mostly T-lymphocytes (CD3+) and B-lymphocytes (CD19+), with higher levels of T-lymphocytes (CD3+) in the upper areas than in the inferior tarsal-bulbar-fornix, where the highest levels of B-lymphocytes (CD19+) were found. In the apoptosis assay, two groups of cell populations were differentiated by cell size and complexity (cytoplasmic granularity), with more complex cells predominating in the upper areas of the conjunctiva and less complex cells being more abundant in the inferior tarsal-bulbar-fornix. Finally, the proliferative capacity of the conjunctival epithelium was significantly higher in the upper tarsal zone than in the rest of the zones analysed. These results suggest that the epithelial component and the IELs of CALT are also regularly present in the conjunctiva of the healthy child, varying in phenotype, viability and cell proliferation according to the different conjunctival regions analysed, which could lead us to believe that each conjunctival zone plays a different, specific role in the regulation of the immune response at the ocular level.

**Key words:** Conjunctiva, Epithelium, Brush cytology, IELs, Immunophenotype, Apoptosis, Cell cycle, Healthy children

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DOI: 10.14670/HH-18-385

## Introduction

Mucosal-associated lymphoid tissue (MALT) is a highly specialised compartment of the immune system that protects the surfaces of all mucosal tissues, which are part of the systemic immune system, although with some independence. It is the body's first line of defence against the invasion of both new and previously exposed pathogens (Tomasi, 1994; Hein, 1999; Mowat, 2003). The cellular components of MALT include mainly T-lymphocytes, B-lymphocytes, IgA-producing plasma cells, antigen-presenting cells, macrophages and occasionally mast cells and eosinophils in the interfollicular region. The existence of a MALT system in adult humans has been widely studied, but its function in children is less well-understood. MALT may be particularly important in children to control pathogen

**Abbreviations.** MALT, Mucosal-associated lymphoid tissue; CALT, Conjunctiva-associated lymphoid tissue; HEV, High endothelial venules; IELs, Intraepithelial lymphocytes; LPL, Lamina propria lymphocytes; BC, Brush cytology; DMEM, Dulbecco's Modified Eagle Medium; FC, Flow cytometry; FS, Forward scatter; SS, Side scatter; MPI, Mean proliferation index

invasion when long-term protective adaptive immunity is not yet fully developed (Ghazarian et al., 2017).

At the ocular level, this MALT is represented by the eye-associated lymphoid tissue which acts as an anatomical and functional unit protecting in a coordinated way the ocular surface and its attachments, and which includes conjunctiva-associated lymphoid tissue (CALT), and also lacrimal drainage-associated and lacrimal gland-associated lymphoid tissues (Franklin and Remus, 1984; Knop and Knop, 2000, 2005a,b). This tissue is connected by the recirculation of lymphocytes, through highly specialised vessels (high endothelial venules, HEV) (Haynes et al., 1999), to other distant regions of the immune system, indicating that these systems are integrated.

The conjunctiva is a transparent mucous membrane that covers the inner side of the eyelids (palpebral conjunctiva), the anterior sclera (bulbar conjunctiva), and forms a retraction between both sections of the conjunctiva (fornix). Histologically, the conjunctiva is similar to other mucous membranes, consisting of a stratified, unkeratinised epithelium of two to six layers of epithelial cells interspersed with goblet cells, and a lamina or stroma formed by fibrovascular connective tissue containing lymphatic vessels and nerves. It contains a wide range of immune cells distributed throughout the conjunctiva, belonging to the relatively non-specific innate immune system and the specific adaptive immunity, which keep the ocular surface integrated, known as CALT. The thickness of the conjunctival epithelium is not the same in all topographic regions of the conjunctiva, i.e. it is thinner in the palpebral part and increases in thickness gradually toward the limbus.

CALT is formed by organised lymphoid tissue, composed of a variable number of lymphoid follicles where B-lymphocytes predominate, accompanied by diffusely dispersed T-lymphocytes in the periphery, and by diffuse lymphoid tissue, mainly composed of T-lymphocytes arbitrarily distributed among the epithelium (intraepithelial lymphocytes, IELs) and among the underlying connective tissue (*lamina propria* lymphocytes, LPL), accompanied by plasma cells producing IgA (Franklin and Remus, 1984; Knop and Knop, 2000, 2005a,b). Organised lymphoid tissue represents the afferent arm or immune system induction site of the ocular mucosa, and diffuse lymphoid tissue probably represents the efferent arm or effector site (Knop and Knop, 2000). Therefore, as in the gut, the eye contains its own local lymphoid tissues organised as CALT, which serves as an inducer site and as an effector site of the immune responses and is thus involved in initiating and regulating the local immune response, maintaining immune tolerance, and contributing to maintaining eye surface homeostasis.

Among the components of CALT, IELs play the main role in the immune regulation of the eye surface. IELs are resident lymphoid cells that intercede between the epithelial cells of the conjunctiva. They are mobile and constantly patrol the space between epithelial cells above the basement membrane (Mcdonald et al., 2018), produce cytokines and exert a mainly cytotoxic effect both under normal conditions and in disease and are important regulators of tissue homeostasis in mucosal tissues (Stern et al., 2013). Despite their shared properties and location, IELs encompass a surprising diversity of lineages (Mcdonald et al., 2018).

Although the role of CALT has been classically associated with inflammatory processes, it became evident a few years ago that this tissue, under normal conditions, plays a fundamental role in maintaining the immunological integrity of the healthy human conjunctiva (Knop and Knop, 2005b; Reinoso et al., 2012; Agnifili et al., 2014). It has also been shown that the density of the IELS varies depending on the conjunctival area observed, as reflected in a study by Knop and Knop (2000). Most of the IELs in the human conjunctiva are T-lymphocytes (CD3+) with a clear predominance of cytotoxic T-lymphocytes (CD3+ CD8+), over helper T-lymphocytes (CD3+CD4+); however, these authors also point out that there is an inverse distribution in the lymphocytes of the lamina propria. In addition, Reinoso et al. (2012) showed that IELs in normal adult human conjunctiva reside mainly in the tarsal conjunctiva, gradually decreasing towards the fornix and bulbar conjunctiva.

These same authors showed that most of the IELs in the human conjunctiva of healthy adults are T-lymphocytes, followed by B-lymphocytes and NK-lymphocytes, although in different proportions depending on the anatomical area of the conjunctiva. They showed that each zone of the normal human conjunctiva presents a different profile in terms of phenotype, viability and cell proliferation (Reinoso et al., 2012). Although IELs play a crucial role in the defence mechanisms of the ocular surface, it is not entirely clear whether its distribution evolves with age.

The aim of this study was to characterize the immune cell component in paediatric normal conjunctiva. So far we describe the distribution of IELs, their viability and/or apoptosis degree and the cell proliferation within three different topographic areas of the conjunctiva in healthy children.

## Materials and methods

### *Subjects*

This study was approved by both the institutional review board of the IOBA-University of Valladolid and University Clinic Hospital of Valladolid and followed the tenets of the Declaration of Helsinki. Twenty-four healthy children (11 females and 13 males, mean ± standard deviation age 6±2 years) agreed to participate in this study (in this case, their legal representatives) after the procedures were fully explained and signed informed consent was obtained. Brush cytology (BC) samples were obtained from the superior tarsal and

bulbar and inferior tarsal-bulbar-fornix conjunctiva before correction of strabismus or botulinum toxin injection surgery under general anaesthesia in paediatric patients. The decision to select pre-surgical patients was due to the fact that undergoing an invasive procedure scheduled for their surgical pathology saved the patient from a further uncomfortable episode in obtaining the samples. The fact that the samples were collected once the patient was under general anaesthesia moments before the intervention facilitated the collection of the samples in these paediatric patients. Finally, the fact that the samples were taken just before the surgery was done to interfere as little as possible with the scheduled surgery, since we obtained the samples while the surgeons were preparing for the operation.

In order to determine their suitability for the proposed study, all participants underwent a previous ophthalmological evaluation at the time of the pre-operative visit scheduled by their surgeon. At the end of their routine visit, and if they met the criteria for inclusion and exclusion, which were basically absence of systemic or local disease, absence of inflammatory or allergic diseases, no topical or systemic medication that might interfere with our results, we proceeded to explain to the parents the nature of the research to assess their acceptance to participate in the study, the protocols were explained before signing the informed consent, which was signed by the parents or legal representatives of the minors.

On the day of the participant's scheduled surgery, after general anaesthesia, samples were taken from the conjunctiva of the eye to be operated on (if both eyes were to be operated on, the sample was taken from the one being operated on first, so as to interfere as little as possible with the scheduled operation), immediately after which the surgeon proceeded with the surgery in the usual manner.

### *Conjunctival brushing*

Minimally invasive collection of conjunctival cells was performed by BC (Martínez-Osorio et al., 2009; Reinoso et al., 2011, 2012), in the superior tarsal, superior bulbar and inferior tarsal-bulbar-fornix conjunctiva after selecting an area of clinically absent follicles under the operating microscope (Fig. 1). Once the patient was under general anaesthesia and after instilling topical anaesthesia (0.04% oxybuprocaine), conjunctival cells were taken by gently rotating the brush (Cytobrush® plus GT, Medscand Medical, Sweden) over each of the three areas mentioned, independently and consecutively. Conjunctival cells were always collected by the same clinician (co-author MTCS). Cells were then detached from the brush by gentle rotation for 30 s in an Eppendorf tube containing 1.2 ml of a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham F12 medium (DMEM/F-12) supplemented with 10% foetal bovine serum (Gibco-Invitrogen, Inchinnan, UK). BC was then performed three more times (with a new brush each time) at the same location and the samples were subsequently detached in the same tube prior to being transported to the laboratory for flow cytometry analysis, which was done less than 30 min after sampling.

### *Flow cytometry (FC) analysis*

Immunophenotype, viability, and proliferative capacity of the BC-recovered cells were analyzed by flow cytometry. First, each cell suspension was equally divided into four different tubes of 300 $\mu$l. Each tube was then washed with 2 ml of Cell Wash Solution (BD Biosciences, San Jose, CA), centrifuged at 500×g for 5 min, and analyzed for a panel of specific immune cell markers. After removal of the supernatant, the remaining cells were processed for three different analyses: cell lineage (Tubes 1 and 2), viability (Tube 3), and cell cycle (Tube 4). For each assay, at least 5,000 conjunctival cells were analyzed. The flow cytometry analysis was performed with a Cytomics FC 500 Cytometer (Beckman-Coulter, Fullerton, CA) using 488 nm excitation with an argon-ion laser for Fluorescein Isothiocyanate (FITC), Phycoerythrin (RD1), Phycoerythrin-Texas Red-X (ECD), Propidium Iodide,


**Fig. 1.** Brush cytology (BC) from superior tarsal **(A)** and bulbar **(B)** and inferior tarsal-bulbar-fornix conjunctiva **(C)** in a representative healthy child.

Phycoerythrin-Cyanine 5, and Phycoerythrin-Cyanine 7. The data collected were analyzed using the Cytomics RXP software program (Beckman-Coulter). Controls included crossreactivity of the fluorescence signals of each channel, as well as isotype-matched unspecific monoclonal antibodies used as negative controls. Appropriate flow cytometry gates were applied to analyze the cell phenotype, apoptotic stage, and cell cycle.

Cell lineage analysis

The phenotype of the BC-recovered conjunctival cells was determined by staining them with two panels of fluorochrome conjugated mouse anti-human monoclonal antibodies as described previously (Reinoso et al., 2011, 2012). In the first panel, we used the following monoclonal antibodies: anti-CD3 Phycoerythrin-Cyanine 5 (clone UCHT1), anti- CD4 RD1 (clone SFCI12T4D11), anti-CD8 ECD (clone SFCI21Thy2D), and anti-CD45 FITC (clone B3821F4A). All antibodies used in this panel were purchased from Beckman-Coulter. We used the following monoclonal antibodies in the second panel: anti-CD3 FITC (clone 33-2A3) from Immunostep (Salamanca, Spain), anti-CD16 Phycoerythrin (clone B73.1), and anti-CD56 Phycoerythrin (clone NCAM 16.2) from Caltag Laboratories (Burlingame, CA), and anti-CD19 Phycoerythrin-Cyanine 5 (clone J4.119) and anti-CD45 Phycoerythrin-Cyanine 7 (clone J33) from Beckman-Coulter. Cell suspensions were incubated in the dark with these antibodies at room temperature for 15 min. They were then incubated in the dark with 1 ml of FACS Lysing solution (BD Biosciences) at room temperature for 15 min to lyse any residual red cells under gently hypotonic conditions and also to preserve epithelial cells and leukocytes. Afterwards, the cells were gently agitated, and flow cytometry analysis performed.

Cell viability analysis

The percentages of live, early and late apoptotic, and dead BC-recovered conjunctival cells were determined using an Annexin V-FITC and Propidium Iodide commercial kit (Beckman-Coulter) as formerly described (Martínez-Osorio et al., 2009; Reinoso et al., 2011, 2012). Following the manufacturer's protocols, cell suspensions were washed with cold PBS (2 ml) and centrifuged at 500×g for 5 min. The supernatant was removed and the cells were resuspended with 100 µl of annexin binding buffer at 4°C. Cells were then incubated at 4°C in the dark with 1 µl of annexin V-FITC (25 µg/ml) and 5 µl of PI (250 µg/ml) for 10 min. Finally, 400 µl of binding buffer at 4°C was added, gently agitated, and flow cytometry analysis performed. Annexin V is an anticoagulant that binds to negatively charged phosphatidylserine. Phosphatidylserine is an internal plasma membrane phospholipid that is exposed on the outer layer of the plasma membrane in the early apoptotic cascade, before the cell becomes permeable to PI. Therefore, vital and early apoptotic cells with intact cell membranes showed no fluorescent signal for PI. Early apoptotic cells are only stained with Annexin V, and double staining with Annexin V and Propidium Iodide detected late apoptosis. Necrotic cells are only stained by Propidium Iodide, and double negative cells were viable cells.

Cell cycle analysis

The DNA content of the BC-recovered conjunctival cells was determined by staining them with Propidium Iodide after cell permeabilization (Coulter DNA Prep Reagents Kit; Beckman-Coulter) as described previously (Martínez-Osorio et al., 2009; Reinoso et al., 2011, 2012). To study the cell cycle, the remaining 300 µl of the BC samples was used to determine DNA content. After the cell suspension was centrifuged at 500×g for 5 min, the supernatant was removed and cells were permeabilized in 50 µl of DNA-Prep LPR at RT. DNA-Prep Stain™ containing PI and RNA-ase III-A (1 ml) was added, gently agitated, and incubated for 30 min at RT in the dark, according to the manufacturer's instructions. The preparations were stored in the dark before the analysis. A $G_0/G_1$ doublet discrimination using pulse height vs. area (Wersto et al., 2001) was performed before cell cycle phase analysis. The mean proliferation index (MPI) was calculated as the ratio of $(S+G_2/M)/(S+G_2/M + G_0/G_1)$, where S was the percentage of cells in the S phase, $G_2/M$ the percentage of $4n$ cells in $G_2$ and M phases, and $G_0/G_1$ the percentage of $2n$ cells in $G_0$ and $G_1$ phases.

*Statistical analysis*

Statistical analysis was performed by a licensed statistician (co-author IF) using R version 3.4.0 statistical software (R Core Team, 2017). The percentage of events obtained by flow cytometry analysis was expressed as mean ± SD. $P<0.05$ was the criterion of significance for all statistical tests.

An ANOVA with three repeated measurements was used to evaluate the differences between zones with the intra-subject factor: zone. To check the normality of the residues, the Shapiro-Wilk test was used and to check the sphericity hypothesis; the Mauchly test, using the Greenhouse-Geisser epsilon correction, was used in case of hypothesis rejection. To carry out the adjustment of the models, the package car of R was used to evaluate the differences between areas. Once a significant p-value was found with the global contrast, a Student's t-test was used for two related samples applying the Bonferroni correction or the non-parametric alternative, the Friedman test, in case it was not possible to assume the normality of the dependent variable. To compare groups two to two, the symmetry hypothesis for repeated measurements was contrasted, using a test based on

permutations in the package coin of R.

## Results

### *Flow cytometry*

Different ratios of IEL subtypes detected within distinct conjunctival zones

Based on the forward scatter (FS) vs. side scatter (SS) dot plots of the flow cytometry data, most (>75%) of the BC-recovered conjunctival cells were of epithelial origin. However, by using positive CD45 staining and a low SS signal, which detects cellular complexity or cytoplasmic granularity, a population of IELs was also detected (Fig. 2A). There were significantly fewer $CD45^+$ IELs in superior bulbar conjunctiva (9.87±16.01%) compared to the superior tarsal and inferior tarsal-bulbar-fornix regions (10.82±6.3%, $p<0.05$ and 21.88±20.2%, $p<0.001$, respectively; Fig. 3A). Immunophenotypic analysis of IELs revealed that most of these lymphocytes were $CD3^+$ T cells, followed by $CD19^+$ B lymphocytes and residual $CD3^-CD16^+/CD56^+$ NK lymphocytes (Fig. 2B,C). The superior tarsal conjunctiva had significantly ($p<0.05$) more $CD3^+$ T cells (65.92±17.77%) than the inferior tarsal-bulbar-fornix (55.76±15.68%). Although the lymphocyte infiltration was high, and there were more B lymphocytes in the inferior fornix, the small sample size did not allow us to reach statistically significant differences of this increase over the superior conjunctival areas. The superior bulbar conjunctiva had significantly ($p<0.05$) more $CD16^+/CD56^+$ NK lymphocytes (2.57±2.68%) than the superior tarsal conjunctiva (0.97±0.88%; Fig. 3A).

When $CD3^+$ T cell subtypes were analysed (Fig. 2D-E), no significant differences were found in the percentage of $CD4^+$ T cells and $CD8^+$ T cells between the three regions. Consequently, the CD4/CD8 ratio was similar for the three regions. The CD3/CD19 ratio was also similar for the three regions (Fig. 3B).

Variation in viability between cell types in different conjunctival regions

In the three conjunctival zones, two distinct cell populations based upon cell size (FS scale) and complexity (SS scale) were differentiated in the apoptosis assay. Most of the BC-recovered cells were larger, more complex, with heterogeneous complexity

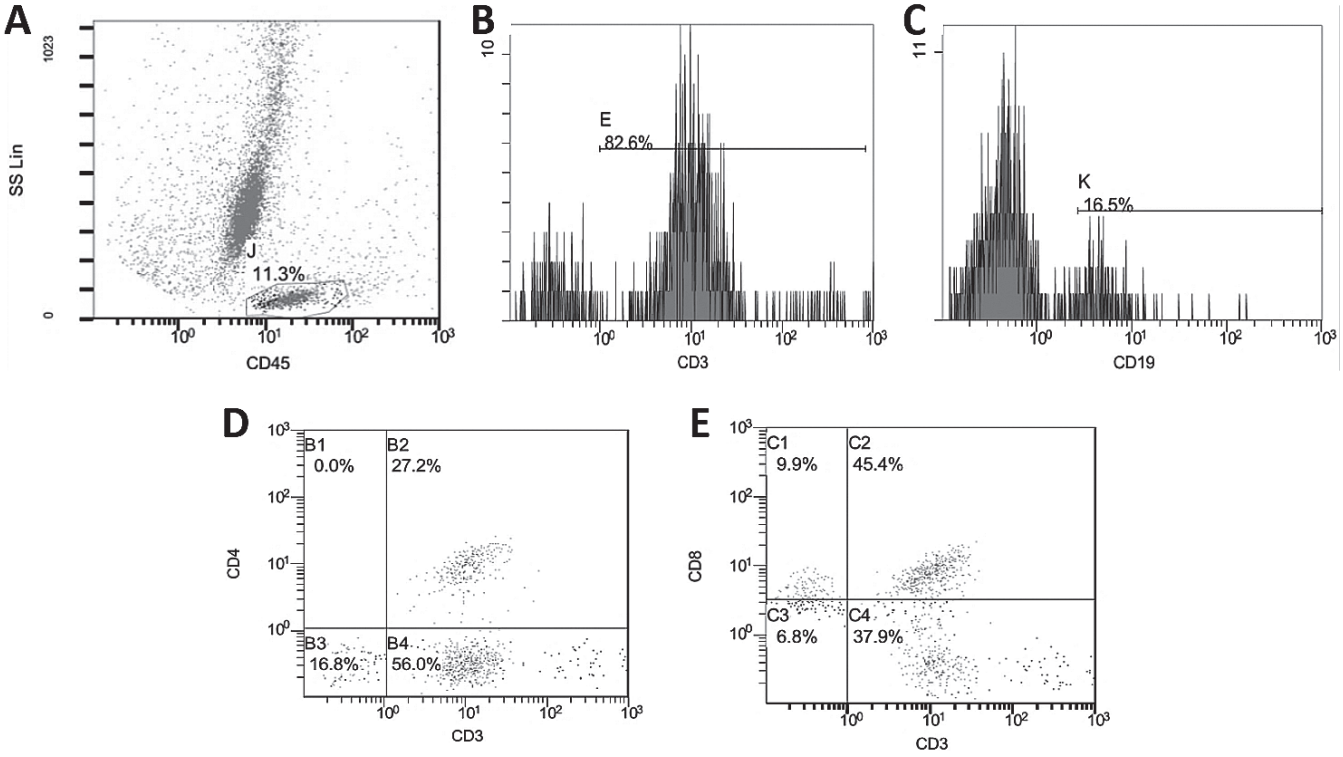


**Fig. 2.** Immunophenotypic analysis from a representative healthy child. **A.** Side scatter (SS) vs. CD45 dot plot showed the lymphocyte population (CD45+ and low cellular complexity, SS) that was gated for analysis (gate J). **B.** Fluorescence histogram of CD3-PC5 gated on CD45+ cells showed a CD3+ T-lymphocyte population (segment E). **C.** Fluorescence histogram of CD19-PC5 gated on CD45+ cells showed a CD19+ B-lymphocyte population (segment K). **D.** CD3 vs. CD4 dot plot gated on CD3+ cells showed a CD3+CD4+ T-helper lymphocyte population (region B2). **E.** CD3 vs. CD8 dot plot gated on CD3+ cells showed a CD3+CD8+ T-cytotoxic lymphocyte population (region C2). PC5, phycoerythrin-cyanine 5.

and had poor viability. The remainder was composed of smaller, less complex cells, with higher viability (Fig. 4). The smallest proportion of the more complex cells, 37.79±12.94%, was present in the inferior tarsal-bulbar-fornix region ($p<0.001$; Fig. 5A). Moreover, this area had the lowest percentage of late apoptotic and dead cells ($p<0.001$) and the highest viability ($p<0.01$) compared to the other conjunctival areas.

On the contrary, the percentage of less complex cells was significantly higher in the inferior tarsal-bulbar-fornix region (59.44±15.96%) than in the upper tarsal and bulbar conjunctiva (42.86±15.29%, $p<0.01$ and 31.35± 22.74%, $p<0.001$, respectively; Fig. 5B). In this smaller and less complex population, cell viability was

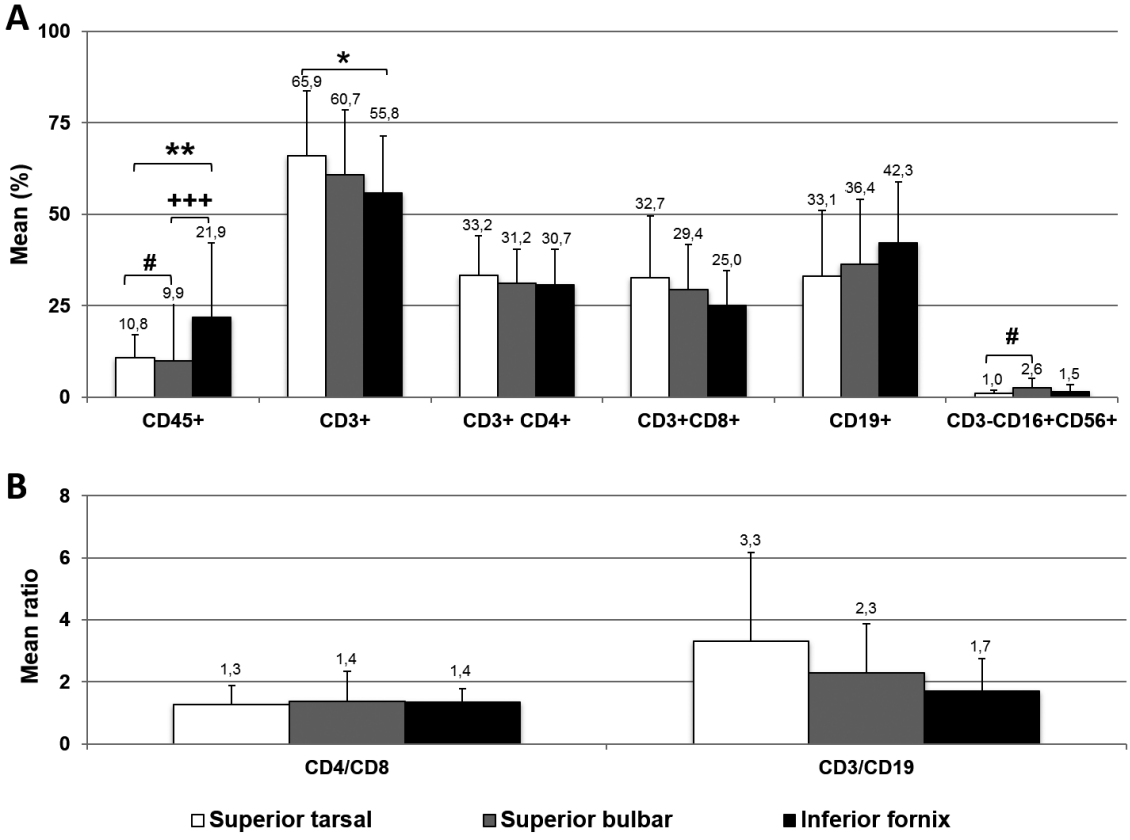


**Fig. 3.** Immunophenotypic analysis of intraepithelial lymphocytes (IELs) recovered by brush cytology (BC) from superior tarsal and bulbar and inferior tarsal-bulbar-fornix conjunctiva. **A.** Mean percentage of cells bearing CD45, CD3, CD4, CD8, CD19, CD16/56, or combinations thereof. **B.** Mean ratios CD4/CD8 and CD3/CD19. +++$p<$ 0.001 bulbar vs. fornix; *$p<0.05$, **$p<0.01$ tarsal vs. fornix; #$p<$ 0.05 tarsal vs. bulbar.

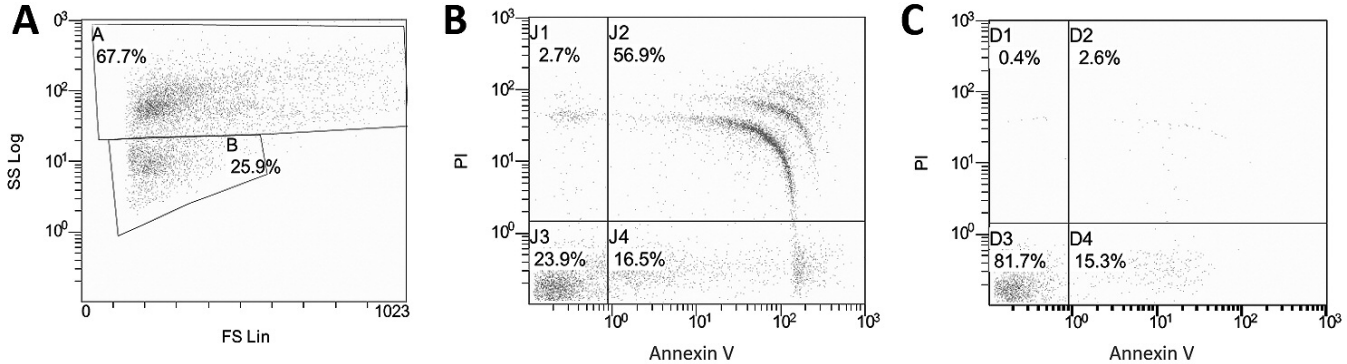


**Fig. 4.** Flow cytometry (FC) dot plots of viability cell analysis of conjunctival cells recovered by brush cytology (BC) from a representative healthy child. **A.** Side scatter (SS) vs. forward scatter (FS) dot plot of conjunctival cells showed two different populations: larger, more complex cells (gate A) and smaller, less complex cells (gate B). **B.** Dot plot of annexin V-FITC vs. PI gated on the larger and more complex cells showed a high level of early (region J4) and late (region J2) apoptotic cells. **C.** Dot plot of annexin V-FITC vs. PI gated on the smaller and less complex cells showed high viability (region D3). FITC, fluorescein isothiocyanate; PI, propidium iodide.

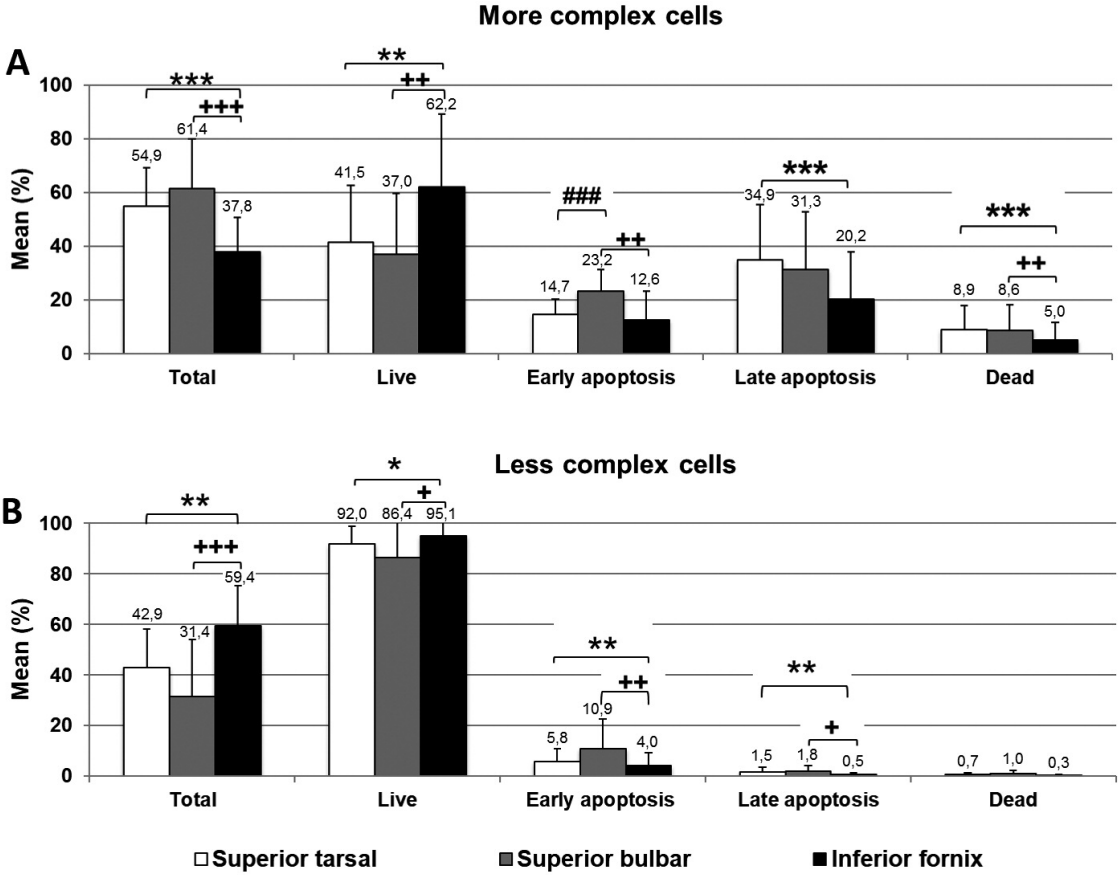


**Fig. 5.** Cell viability analysis in conjunctival cells obtained by brush cytology (BC) from superior tarsal and bulbar and inferior tarsal-bulbar-fornix conjunctiva. Mean percentage of total, live, early and late apoptotic; and dead cells in the larger **(A)**, more complex population and the smaller **(B)**, less complex population. +p< 0.05, ++p< 0.01, +++p<0.001 bulbar vs. fornix; *p<0.05, **p<0.01, ***p<0.001 tarsal vs. fornix, ###p<0.001 tarsal vs. bulbar.

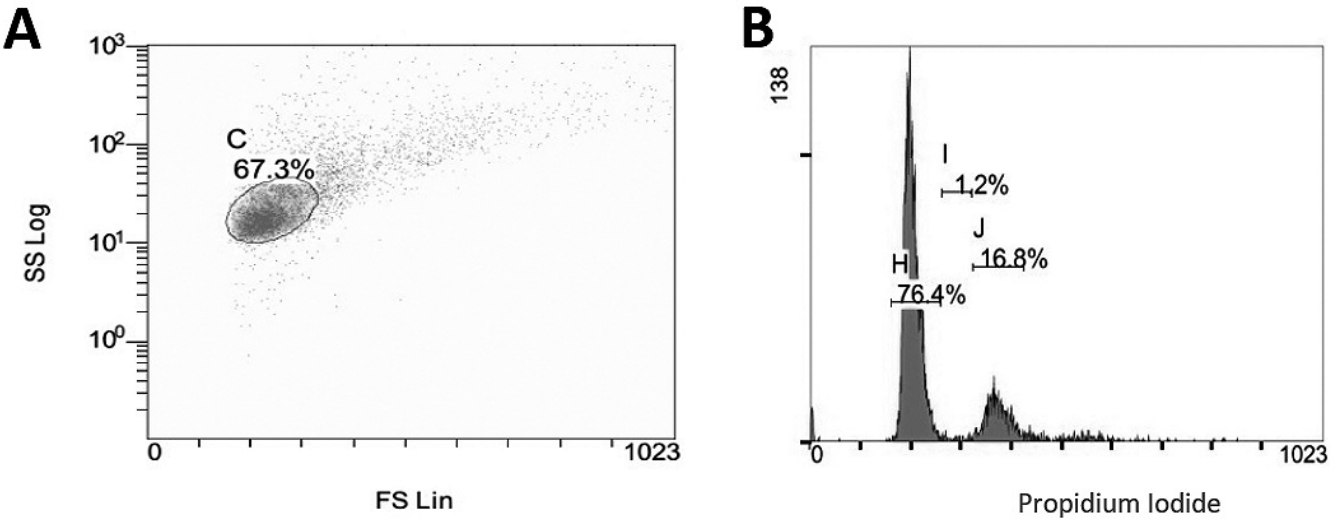


**Fig. 6.** Flow cytometry (FC) dot plots of cell cycle analysis of conjunctival cells recovered by brush cytology (BC) from a representative healthy child. **A.** Side scatter (SS) vs. forward scatter (FS) dot plot of conjunctival cells shows that only cells of homogeneous size and complexity (gate C: 67.3% of cell sample) were analyzed for DNA content. **B.** Linear fluorescence histogram of propidium iodide (PI) gated on the homogeneous population shows different phases of the cell cycle (H, 2n cells in G0 and G1 phases; I, cell population in the S phase; J, 4n cells in G2 and M phases).

significantly higher in the inferior tarsal-bulbar-fornix region, 95.14±6.09% than in the upper tarsal and bulbar conjunctiva (92.03±6.68% and 86.35±14.18%, respectively; p<0.01, Fig. 5B). Consequently, these superior areas had the highest levels of cells in early and late apoptosis and dead cells.

Comparison of cell cycle phases among different conjunctival zones

In the inferior tarsal-bulbar-fornix area, 78.76±9.38% of the cells were in the $G_0/G_1$ phases of the cell cycle (resting or pre-cycling), which was significantly (p<0.05) higher than in the superior tarsal area (70.21±8.37%; Fig. 3). On the other hand, the percentage of superior tarsal conjunctiva cells in G2/M phases, 16.83±6.8%, was significantly higher than in the other topographical zones (p<0.01) (Fig. 6). The MPI of the superior tarsal conjunctiva (21.52±7.69) was also significantly higher than in the superior bulbar and inferior tarsal-bulbar-fornix conjunctiva (10.82±6.44%, p<0.01 and 10.82±5.91%, p<0.05, respectively; Fig. 7).

## Discussion

It is clear that the presence of CALT plays a key role in initiating immune responses related to the ocular surface, as it is present not only when an inflammatory process takes place, but also regularly in all healthy subjects (Knop and Knop, 2000; Reinoso et al., 2012; Agnifili et al., 2014), participating in the secretion of immune mediators as well as in the regulation of the local immune response. Our study focuses on the IELs population present in the conjunctival epithelium of a cohort of healthy children. To our knowledge, all published studies on IELs in humans have been conducted in the adult population, and there are no studies in the literature that include subjects of paediatric age. Therefore, to the best of our knowledge, this is the first study in the characterisation of this tissue in this age range. Moreover, it is necessary to emphasise that such samples are very difficult to obtain, paediatric cases are even harder to recruit and to work with and cellularity is always low in conjunctival BC. For all these reasons, we truly believe that this study has a special relevance and importance.

In recent years, several studies have shown that BC (Martínez-Osorio et al., 2009; Reinoso et al., 2011, 2012) is a technique capable of collecting a great quantity of cells directly in suspension, with the consequent advantages when it comes to their subsequent characterisation. These authors have also demonstrated that this technique is capable of isolating more viable cell populations than with other techniques such as conjunctival impression cytology (Brignole-Baudouin et al., 2004; Calonge et al., 2004), and maintaining the morphology of the recovered cells intact (Fujihara et al., 1997a; Yagmur et al., 1997). The combination of BC together with FC analysis is a useful tool for determining the presence of cell infiltrates and IELs in the human conjunctival epithelium and investigating the physiopathology of ocular surface diseases (Fujihara et al., 1997b; Reinoso et al., 2011).

Immunophenotypic analysis showed that most of the cells recovered from the conjunctiva of healthy children are of the epithelial lineage, as had already been demonstrated in previous studies (Soukiasian et al., 1992; Fujihara et al., 1997a; Tsubota et al., 1999; Martínez-Osorio et al., 2009). Our results also revealed the existence of a considerable population of CD45+ cells, i.e. IELs, interspersed between the different epithelial layers of the conjunctiva in the healthy child,

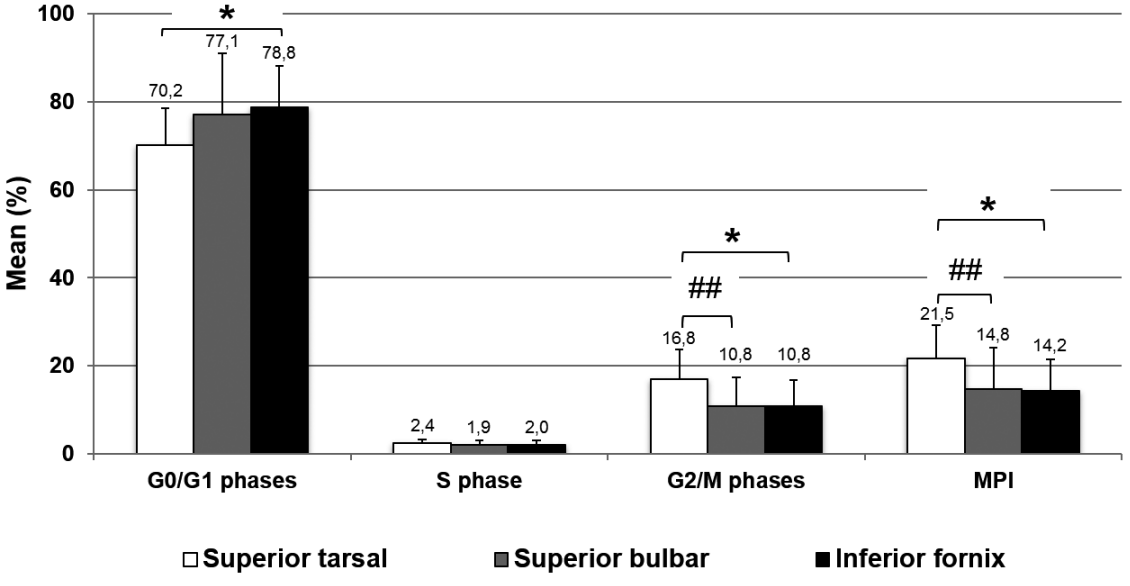


**Fig. 7.** Cell cycle analysis in conjunctival cells obtained by brush cytology (BC) from superior tarsal and bulbar and inferior fornix conjunctiva. *p<0.05 tarsal vs fornix, ##p<0.01 tarsal vs bulbar. MPI, mean proliferation index.

as had also been described in previous work in the adult population (Reinoso et al., 2012) and in animals (Steven and Gebert, 2009; Giuliano and Finn, 2011; Siebelmann et al., 2013; Crespo-Moral et al., 2020). However, in the present study, prominently higher levels of lymphocyte infiltration were observed than those found in the healthy adult population (Reinoso et al., 2012) and those found in patients with dry eye syndrome (Reinoso et al., 2011) in the three regions analysed, mainly in the inferior tarsal-bulbar-fornix.

One of the observations made by Reinoso et al. (2012) was that the high levels of IELs found in the lower fornix could probably be due to anatomical reasons, since with the rotational movements of the brush in the inferior fornix zone, cells from several zones can be dragged at once, which does not allow for the collection of cells exclusive to this zone but would also include cells from the lower tarsal and bulbar conjunctiva, which could lead to a falsely high and/or more variable cell count. In addition, there is a possibility that, as this is the thinnest epithelium, there may occasionally be bleeding and blood cells with a consequent increase in B cells from this area. This aspect might be increased in the paediatric population due to its smaller dimensions. However, it is necessary to emphasise that in this study we observed significantly higher levels of IELs as compared to other age groups in the three analysed topographic areas of the healthy child's conjunctiva under normal conditions, requiring further study along with more patients to confirm these observations.

Our results also showed that most of the IELs residing in the conjunctival epithelium of the healthy child are predominantly T lymphocytes, followed by B lymphocytes and, in smaller percentages, NK lymphocytes. These results are consistent with what has been previously published, which indicates the predominance of T cells over B cells (Knop and Knop, 2000; Reinoso et al., 2012). In addition, in this study, we observed a much greater infiltration of B cells than those described in previous publications, mainly in the inferior fornix area. Taking into account that the thickness of the conjunctival epithelium is not the same in all the topographic regions of the conjunctiva, these findings might suggest that, in this area, as it is thinner, it is possible that conjunctival brushing may occasionally cross the basal membrane of the epithelium and reach the lamina propria with a consequent increase in B-lymphoid cells from lymphoid follicles (Reinoso et al., 2012). This possibility, together with the fact that this harvesting area could be contaminated with blood has forced us to stop using this region for ulterior studies using this technology.

With regard to the subtypes of T-lymphocytes (CD3+), we did not observe significant differences either in helper T-lymphocytes (CD3+CD4+) or cytotoxic T-lymphocytes (CD3+CD8+) in the three areas analysed. These results suggest that, in the healthy child, there may be a crucial balance of CD4+ and CD8+ cells under normal conditions, that would represent a physiological immune balance. We can say that these findings differ from the results of previous studies (Dua et al., 1994; Knop and Knop, 2000; Reinoso et al., 2012; Agnifili et al., 2014), where a predominance of cytotoxic T-lymphocytes (CD3+CD8+) versus cooperating T-lymphocytes (CD3+CD4+) was observed in the conjunctival epithelium in adults. On the other hand, inflammatory disorders of the eye surface, dysregulation of conjunctival mucosal immunity and loss of immune tolerance are associated with an increase in the CD4+/CD8+ ratio in the human conjunctival epithelium.

With regard to the analysis of cell viability, it should be remembered that this parameter gives us information on the cellular turnover of a tissue. For this to occur safely, a physiological mechanism of programmed cell death called apoptosis is essential, so it plays a crucial role both in the maintenance of cell homeostasis and in the regulation of the immune response (Potestio et al., 1998; Gupta, 2005). Our results corroborated what had been described in previous studies (Stern et al., 2005; Reinoso et al., 2011, 2012), i.e. the existence of two cell populations totally differentiated by their size and cytoplasmic complexity: a major population composed of large, complex, low viability cells that we call 'more complex cells' and, on the other hand, another minor population composed of small, less complex, but higher viability cells that we call 'less complex cells'. An aspect to highlight in our study are the differences found between the upper areas and the inferior fornix. We observed that, in the superior conjunctiva, both upper tarsal and upper bulbar, we found a higher percentage of complex cells that were generally less viable, in contrast to what was observed in the inferior fornix, where we found a higher percentage of simple cells that were more viable. These results could indicate that, in this topographic area of the healthy child's conjunctiva, i.e. the inferior fornix, there might be more proliferating cells, which is expressed as a low percentage of cells in apoptosis and a higher number of living cells, which may be associated with the fact that, in this area the cells might be less differentiated (smaller size and less granularity), or as previously proposed, this harvesting area might include cells from peripheral blood.

These results are similar to previous studies where the expression of pro-apoptotic proteins in the superficial epithelial layers (the 'more complex cell' population) and apoptosis inhibiting factors in the more basal layers of the conjunctival epithelium (the 'less complex cell' population) are evident (Tan et al., 2000a; Giebel et al., 2005). In light of these results, it could be interpreted that the group of 'more complex cells' might correspond to the cells located in the most superficial layers of the conjunctiva, while the group of 'less complex cells' might correspond to the cells that come from deeper or basal layers of the conjunctiva and would include IELs that are probably less differentiated.

With regard to the cell cycle, in this study, we measured by flow cytometry the amount of DNA in the

cells obtained by brush cytology, and consequently the stage of the cell cycle, which can be used as a marker of the proliferative state (Thompson et al., 1991; Tan et al., 2000b). Our results show that most conjunctival cells obtained from healthy children were in the growth or pre-cycle stages ($G_0/G_1$), followed by those with proliferative capacity (S and G2/M stages), as described in previous studies in the adult population (Reinoso et al., 2012).

In our study, it was evident that the inferior fornix had the highest levels of cells in G0/G1 phase and a low rate of proliferation (followed closely by the upper bulbar area), suggesting that the inferior fornix area might be the predominant site for the location of the healthy child's conjunctival epithelial stem cells, which are likely to be slowly cycling stem cells. These results are similar to those observed by Pellegrini et al. (1999), which suggest that, generally, stem cells cycle slowly in vivo, thus remaining in the $G_0$ phase of the cell cycle in certain situations but show high proliferative capacity in vitro.

These results are consistent with other studies in which conjunctival stem cells were found in the basal layers of the conjunctival epithelium of the fornix (Wei et al., 1995; Pauklin et al., 2011; Stewart et al., 2015). Similar results regarding their location were also obtained in some animals (Wei et al., 1993; Lavker et al., 1998). We might say that the region of the inferior fornix would be the preferred area for the conjunctival stem cells of the healthy child, as this area provides greater physical protection. However, there are other studies in adults that suggest the presence of epithelial stem cells in the bulbar conjunctiva (Pellegrini et al., 1999; Nagasaki and Zhao, 2005; Vascotto and Griffith, 2006; Reinoso et al., 2012). Thus, taking into account previous studies, it may be that the possible locations of the epithelial stem cells of the healthy conjunctiva are preferentially distributed in the inferior fornix and bulbar conjunctiva (Pellegrini et al., 1999), or it could be that their location varies according to age. We emphasise that these reference studies did not study individuals of paediatric age, so these differences might be due to the age of the subjects. One limitation in this study was how extremely difficult it was to recruit subjects in this age range, given the particular anatomical location of the IELs that is not easily accessible for clinical and instrumental examinations.

In conclusion, this study demonstrates the existence of an infiltrating IEL population, mostly T and B lymphocytes, drawing a gradient along the conjunctival epithelium of healthy children. This epithelium holds two cell lineages that are completely different in terms of their viability, size, and cell complexity. The IELs present in each topographical zone from healthy children conjunctiva have a unique profile of immunophenotype and viability that might be related to a differentiated regional functionality. The upper tarsal region has the highest percentage of T CD8+ lymphocytes, while the inferior fornix has the greatest infiltration of predominant follicular B lymphocytes. Also, the inferior fornix had the highest percentage of living cells with the lowest proliferation rate. With respect to the upper bulbar zone, it presented the lowest degree of lymphocyte infiltration.

Adequate knowledge of the immune system of the ocular mucosa in healthy individuals, in particular knowledge of IELs in children, will help to better understand the pathophysiology of ocular surface diseases, whether they are of infectious, allergic or autoimmune aetiology, and is thus crucial to the development of new diagnostic and therapeutic strategies.

*Conflict of interest.* No conflicts of interest, financial or otherwise, are declared by the authors.

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Accepted October 26, 2021