

A comparative study of the characteristics of intraepithelial and lamina propria lymphocytes of the human nasal mucosa

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The phenotypes and receptors of the intraepithelial lymphocytes which are a major component of the mucosal immune system are of considerable interest. Intraepithelial lymphocytes, especially those of the intestines, are well documented. The nasal intraepithelial lymphocytes probably play a key role in the immune defense of the entire respiratory tract. However, documentation of these lymphocytes is limited to only a few nondescriptive studies by immunohistological methods. In our previous paper, we examined the subsets of the nasal intraepithelial lymphocytes by two-color flow cytometry after culture with monoclonal antibody against CD3 and PMA. The mucosal immune system, which comprises the lymphoid tissue associated with the mucosal surface, consists of lymphocyte populations from both the epithelium and the lamina propria. In this paper (a sequel to our previous study), we have carried out a comparative study of the intraepithelial lymphocytes and lamina propria lymphocytes of the human nasal mucosa.

In the 42 patients (with allergic rhinitis/infectious hypertrophic rhinitis) whom we examined, T lymphocytes were predominant in the epithelium as well as the lamina propria. B lymphocytes were detected only in the lamina propria. CD8⁺ T cells (suppressor/cytotoxic) and CD4[−]8[−] (double-negative) (DN) comprised the major population of the intraepithelial lymphocytes. The CD4⁺/CD8⁺ ratio was 0.5 ± 0.1 . In contrast, the lamina propria showed a predominance of CD4⁺ T cells (helper/inducer), with a CD4⁺/CD8⁺ ratio of 1.3 ± 0.2 . CD3⁺4[−]8[−] (double-negative T cells) were comparable in number in the epithelium and the lamina propria. CD8⁺ T cells of the epithelium were predominantly cytotoxic T cells, and CD4⁺ T cells were predominantly helper T cells. In the lamina propria, CD8⁺ T cells were predominantly of the suppressor type.

In the analysis of the T-cell receptors of the epithelium and the lamina propria, there was a predominance of the $\alpha\beta$ receptor-bearing T cells in both. However, in the double-negative T-cell population of the epithelium, $\gamma\delta$ heterodimer predominated over $\alpha\beta$.

In order to verify the influence of culture conditions on the percentages of the individual subsets of lymphocytes, we examined the peripheral blood lymphocytes by flow cytometry before and after culture under the same conditions. We found no significant change in the different subsets caused by culture except a slight reduction in the percentage of suppressor T cells as well as a significant increase in activated T cells. We therefore wish to emphasize that the nasal intraepithelial lymphocytes are heterogenous in phenotype, and are dominated by double-negative cells and CD8⁺ T cells. Moreover, the nasal lamina propria lymphocytes are also heterogenous in phenotype, and differ in proportion from the intraepithelial lymphocytes. What is of utmost interest is the preferential location of the $\gamma\delta$ receptor-bearing T cells in the epithelium and their relative absence in the lamina propria.

R. Pawankar, M. Okuda

Department of Otorhinolaryngology, Nippon Medical School, Bunkyo-ku, Tokyo, Japan

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Dr Minora Okuda
Department of Otolaryngology
Nippon Medical School
Bunkyo-ku, Tokyo 113
Japan

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Clinical aspects

Under conditions of natural exposure to even extremely low doses of environmental pollutants, allergens, on contact with T lymphocytes, stimulate a cascade of cellular and humoral reactions. Although a variety of cells such as eosinophils, mast cells, and neutrophils, and a host of biologically active, soluble mediators can contribute to an inflammatory response, only lymphocytes interact with the foreign body in a highly specific manner. The predominance of cytotoxic T cells and helper T cells in the epithelium is significant, as they are the first to come into contact with foreign bodies and initiate an immune reaction. The presence of a significant population of B cells in the lamina propria can be explained, as they play a vital role in antibody synthesis. The suppressor type of CD8+ T cells in the lamina propria probably play a secondary role by downregulating the actions of the helper T cells. It is significant that the epithelium, as well as the lamina propria, has a heterogeneous population of lymphocytes occupying different proportions in each tissue, with a preferential location for the $\gamma\delta$ receptor-bearing, double-negative T cells in the nasal epithelium, as reported in other epithelial sites. Further studies on the functional significance of these lymphocytes are necessary to elucidate their precise role in the nasal mucosa.

Since the nasal epithelium is the outermost barrier with which foreign bodies first come into contact, the cells residing in and migrating into it play a major role in the first line of defense, not only of the upper respiratory tract but also of the entire respiratory system. Although a variety of cells are known to populate the nasal epithelium, lymphocytes are predominant (16, 23, 24), as in the intestines (4) and lower respiratory tract (6).

Recently, there have been many studies of intestinal intraepithelial lymphocytes (IEL) (1, 9, 10–12, 17, 18, 20). However, there have been few studies of nasal intraepithelial lymphocytes (nIEL) in man (limited to CD4+, CD8+, and B cells) by immunocytochemistry; these are, in addition, controversial (2, 5, 7, 13, 14, 19, 22, 24). In our previous paper, we reported in detail, for the first time, the heterogeneity of the nIEL and their receptors (15). As already mentioned in our previous paper, the shortcomings of immunohistologic methods influenced our resort to flow cytometric analysis, which is now widely preferred over conventional immunohistologic methods.

Moreover, the few comparative studies of the phenotypes and receptors of the human nasal intraepithelial lymphocytes and human nasal lamina propria lymphocytes (nLPL) have compared only the CD4+, CD8+, and B-cell populations (2, 13, 22).

Since there is no documentation on detailed comparative analysis of nIEL and nLPL, our present study is a detailed comparative analysis of the lymphocyte subsets and receptors of nIEL and nLPL; these are as follows: cytotoxic T (Tc), suppressor T (Ts), helper T (Th), inducer T (Ti), helper/inducer T (Thi), suppressor/inducer T (Tsi), activated helper/

inducer T (aTh/i), activated suppressor/cytotoxic T (aTs/c), $\alpha\beta$ receptor-bearing ($\alpha\beta$ T), $\gamma\delta$ receptor-bearing T ($\gamma\delta$ T), natural killer (NK), and killer cell (K).

Material and methods

Twenty patients with house dust allergy, with a male to female ratio of 3:2 and an average age of 21.8 years, and 22 patients with nonallergic rhinitis (infectious hypertrophic rhinitis), with a male to female ratio of 7:4 and an average age of 39.5 years, were selected after careful screening by history, clinical evaluation, X-ray examination of sinuses, and allergy tests, for the intraepithelial lymphocyte study. Thirteen of 20 patients (examined for nIEL) with house dust allergy, with a male to female ratio of 12:1 and an average age of 18.7 years, and 18 of 22 patients with nonallergic rhinitis (infectious hypertrophic rhinitis), with a male to female ratio of 2:1 and an average age of 25.7 years, were employed for the study of lamina propria lymphocytes. The patients with allergic rhinitis included in our study had no associated symptoms of asthma or atopic eczema. We could not include normal persons in our study because of inability, for ethical reasons, to obtain sufficient tissue by conchotomy.

Under aseptic conditions, biopsies of the nasal turbinate were collected from patients undergoing conchotomy in PBS (phosphate-buffered saline (pH 7.2) (Wako-Junyaku, Japan) with 5% FCS (Gibco, UK) and 100 μ g/ml penicillin-streptomycin with 25 μ g/ml amphotericin B (Gibco, UK). The mucosal tissue, measuring about 2 $\times$ 3 cm in size, was then washed in PBS (containing 5% FCS and antibiotics/antimycotic).

For intraepithelial lymphocytes. The epithelial layer was scraped gently with a sterile surgical curette measuring 2.5×3.5 mm. The nasal scrapings thus obtained (approximately 30–40) were dissociated by gentle magnetic stirring for 10 min in PBS with 1 mmol dithioerythritol (Sigma, USA), as Guy-Grand et al. (4) have described. The cell suspension was then centrifuged at 400 g for 10 min at 4°C and the cell pellet washed in PBS (containing 5% FCS and antibiotics/antimycotic). Then the cells were counted and resuspended in culture medium RPMI 1640 (Gibco, UK), containing NaHCO_3 , 25 mmol HEPES, supplemented with 1 ng/ml PMA (phorbol-12 myristate, 13 acetate) (Sigma, USA), 1% antibiotics/antimycotic (Gibco, UK) and 20 ng/ml monoclonal antibody (mAb) against CD3 (OKT3) (Ortho Diagnostics, USA), in a 24-well culture plate at $1 \times 10^4/\text{ml}$, and incubated for 72 h at 37°C , 5% CO_2 and 100% humidity. After incubation for 72 h, the cells were harvested, the cell types were identified by May-Grunwald Giemsa staining, and the viable lymphocytes counted by Trypan blue dye exclusion test (8). The cells were adjusted to a concentration of $1 \times 10^6/\text{ml}$ and stained with FITC (fluorescent isothiocyanate) and PE (phycoerythrin)-labeled monoclonal antibodies (details of cell types, surface antigens, monoclonal antibodies, and sources are given in Table 1), and the lymphocyte subtypes were analyzed by single- and two-color flow cytometry (Epics C, Coulter, USA), as described in Okuda & Pawankar (15). The forward and side scatter gates were set to exclude cell debris, dead cells, and cells other than lymphocytes.

For the lamina propria lymphocytes. The mucosal layer, after removal of the epithelium, was incised

and the cells expressed by gentle squeezing over a nylon mesh; the remaining tissue was minced into minute pieces and subjected to magnetic stirring for 60 min in Ca- and Mg-free Hank's balanced salt solution, HBSS (Gibco, UK), containing 25 mmol HEPES and 0.01% collagenase (Sigma, USA). The cell suspension was then passed through nylon mesh, and the cells were centrifuged at 400 g for 10 min. Next they were washed, resuspended, and incubated in culture medium for 72 h, before being counted, stained, and analyzed by flow cytometry in the same manner as nIEL.

Peripheral blood lymphocytes. Mononuclear cells from the peripheral blood of 10 normal volunteers were purified by Ficoll-Hypaque density gradient (3), Lymphoprep (Daiichi Kagaku, Japan), and analyzed by flow cytometry before and after culture under the same culture conditions to confirm whether culture conditions induced any change in the percentages of the individual subsets of lymphocytes.

Statistics. The results are expressed as the mean $\pm$ SD. The significance of the difference was determined by Wilcoxon's test. A *P* value of less than 0.05 was considered to be significant. A comparison was made between the epithelial and the lamina propria lymphocytes.

Results

Intraepithelial lymphocyte subsets and T-cell receptors

CD3+ cells (total T cells) ($78.1 \pm 8.4\%$) comprised most of the intraepithelial lymphocyte population. The number of B cells was almost nil. CD8+ T cells (suppressor/cytotoxic) predominated over

Table 1. Cell types, surface antigens, monoclonal antibodies, and sources used in this study

Cell types	Surface antigens	mAbs	Sources
Pan T	(CD3+)	Leu 4	BD
Pan T	(CD3+)	OKT3	Ortho
Pan B	(CD20+)	Leu 16	BD
Helper/inducer T cell (Th/i)	(CD4+)	Leu 3a	BD
Helper T cell (Th)	(CD4+) (CD45RA-)	Leu 3a/2H ₄	BD/Coulter
Helper inducer T cell (Thi)	(CD4+) (CD29+)	Leu 3a/4B ₄	BD/Coulter
Suppressor inducer T cell (Tsi)	(CD4+) (CD45RA+)	Leu 3a/2H ₄	BD/Coulter
Suppressor/cytotoxic T cell (Ts/c)	(CD8+)	Leu 2a	BD
Suppressor T cell (Ts)	(CD8+) (CD11+)	Leu 2a/Mo1	BD/Coulter
Cytotoxic T cell (Tc)	(CD8+) (CD11-)	Leu 2a/Mo1	BD/Coulter
Activated T cell (helper/inducer) aT(h/i)	(CD4+) (HLA-DR+)	Leu 3a/HLA-DR	BD
Activated T cell (suppressor/cytotoxic) aT(s/c)	(CD8+)(HLA-DR+)	Leu 2a/HLA-DR	BD
$\alpha\beta$ receptor-bearing T cell ($\alpha\beta$ T)	(CD8+/CD4+) ($\alpha\beta$ TCR)	Leu 2a/Leu 3a/TCR-1	BD
$\gamma\delta$ receptor-bearing T cell ($\gamma\delta$ T)	(CD8+/CD4+) ($\gamma\delta$ TCR)	Leu 2a/Leu 3a/TCR δ 1	BD
Natural killer cell (NK)	(CD16+) (CD57-)	Leu 11/Leu 7	BD
Killer cell (K)	(CD8+) (CD57+)	Leu 2a/Leu 7	BD
Double-negative T cell (DN)	(CD3+) (CD4-CD8-)	Leu 4/Leu 3a/Leu 2a	BD

BD: Becton Dickinson.

mAbs: monoclonal antibodies.

Table 2. T-cell subsets of the cultured nasal intraepithelial lymphocytes

Subset	Subpopulation
CD8+ (35.9±14.1%) (n=42)	Ts (43.7±28.9%) (n=13)
	Tc (56.2±28.7%) (n=13)
CD4+ (19.1±10.1%) (n=42)	Th (60.1±30.8%) (n=13)
	Thi (21.9±7.6%) (n=10)
	Ti (39.8±30.8%) (n=13)
	Tsi (17.9±15.7%) (n=10)
CD4-8- (45.0±15.7%) (n=42)	CD3+4-8- (22.7±6.5%) (n=10)
CD4+8+ nil (n=4)	
CD4+/CD8+ 0.5±0.1 (n=42)	

* $P < 0.05$; ** $P < 0.01$.

CD4+ cells (helper/inducer). However, CD4-8- (double-negative) cells were the most numerous. CD3+4-8- (DN) (double-negative T) cells comprised approximately 50% of the total double-negative cells, the remainder being CD3-4-8- cells. Double-positive T cells were not detected. The average ratio of CD4+/CD8+ cells was 0.5 ± 0.1 (Table 2).

Of the CD8+ T cells, CD11- (Tc) cells predominated over CD11+ (Ts) cells. In the CD4+ T-cell population, CD4+2H4- (Th) cells predominated over CD4+2H4+ (Ti) cells. Tsi and Thi cells were comparable in values. Activated helper/inducer T cells and activated suppressor/cytotoxic T cells were also comparable. CD16+Leu7- (NK) ($3.3 \pm 2.6\%$) and CD8+Leu7+ (K) ($7.5 \pm 6.8\%$) cells were few. Because of the limited number of lymphocytes in the nasal epithelium, we could not examine all the subsets in some specimens.

Table 3. $\alpha\beta$ and $\gamma\delta$ TCR in cultured nasal intraepithelial lymphocytes

T-cell subset	Ratio $\alpha\beta/\gamma\delta$ (n=26)	Percentages in total cells	
		$\alpha\beta$	$\gamma\delta$
Total T	2.1±2.5		
CD4+	3.4±3.0	8.1±5.9%	2.8±2.3%
CD8+	2.6±2.0	10.1±7.8%	4.1±3.4%
CD4-8-	0.6±0.9	3.7±3.1%	5.1±3.6%

* $P < 0.05$; ** $P < 0.01$.

Table 4. T-cell subsets of the cultured nasal lamina propria lymphocytes

Subset	Subpopulation
CD8+ (23.9±6.9%) (n=26)	Ts (69.7±14.1%) (n=16)
	Tc (30.2±14.4%) (n=16)
CD4+ (31.1±6.1%) (n=26)	Th (45.1±23.5%) (n=16)
	Thi (28.5±13.1%) (n=10)
	Ti (54.9±23.5%) (n=16)
	Tsi (27.1±13.5%) (n=10)
CD3+4-8- (23.8±4.2%) (n=10)	
CD4+8+ nil (n=4)	
CD4+/CD8+ 1.3±0.2 (n=42)	

* $P < 0.05$; ** $P < 0.01$.

In the study of the intraepithelial T-cell receptors, $\alpha\beta$ T predominated over $\gamma\delta$ T. However, $\gamma\delta$ T predominated over $\alpha\beta$ T in the double-negative T-cell population (Table 3). Our results are well in accordance with our previous results.

Lamina propria lymphocyte subsets and T-cell receptors

CD3+ (T cells) comprised the major population of the lamina propria lymphocytes. B cells were found to comprise about $19.6 \pm 13.5\%$ of the total lymphocytes. The average ratio of T:B was 3.5. CD4+ T cells were predominant. The average ratio of CD4+/CD8+ was 1.3 ± 0.2 . CD3-4-8- cells and double-positive cells were not detected (Table 4).

CD8+ T cells were predominantly CD11+ (Ts) cells, and in the CD4+ T-cell population CD4+2H4+ cells (Ti) predominated over CD4+2H4- (Th). Thi and Tsi cells were almost equal in values. Activated suppressor/cytotoxic T cells and activated helper/inducer T cells were also comparable in number. NK cells ($6.4 \pm 4.4\%$) and K cells ($6.9 \pm 4.4\%$)

Table 5. $\alpha\beta$ and $\gamma\delta$ TCR in cultured nasal lamina propria lymphocytes

T-cell subset	Ratio $\alpha\beta/\gamma\delta$ (n=11)	Percentages in total cells $\alpha\beta$
Total T	8.5±3.1	
CD4+		13.9±6.3%
CD8+		8.9±3.1%
CD4-8-		8.9±5.4%

Table 6. Changes in the population of peripheral blood lymphocytes after culture

CD4+ cell	0.94±0.16↔
CD8+ cell	1.36±0.19↔
CD4- CD8- cell	0.72±0.38↔
CD4+/CD8+	0.69±0.10↔
Ts	0.57±0.25↓
Tc	1.08±0.05↔
Th	0.69±0.21↔
Ti	1.32±0.40↔
Thi	1.21±0.55↔
Tsi	1.28±6.72↔
act Th/i	6.59±3.91↑
act Ts/c	4.86±0.43↑
NK	0.75±0.95↔
K	1.03±0.74↔
αβ CD8+ cell	1.03±0.74↔
αβ CD4+ cell	0.92±0.46↔
Total αβT	1.11±0.28↔
Total γδT	1.14±0.71↔

↑ increased; ↓ decreased; ↔ no change.

The figures are expressed as the ratio of after to before culture in 10 pairs ($\bar{x} \pm \text{SD}$).

were few. The CD4+, CD8+, and CD3+4-8- subtypes of T lymphocytes of the lamina propria were mainly associated with the αβ heterodimer, total γδ being less than 4% (Table 5).

We analyzed the peripheral blood lymphocytes before and after culture under the same culture conditions, and estimated the change as elucidated by the ratio of after culture to before culture. Although there was a marked increase in the activated T cells and a slight reduction in the percentage of suppressor T cells, there was no significant change in the percentages of the other subsets (Table 6), as also stated by Wim et al. (21). In a comparison of the T-cell subsets of the epithelium and the lamina propria (Fig. 1), CD8+ T cells were predominant in the epithelium, and CD4+ T cells were predominant in the lamina propria. Of the CD8+ T cells, cytotoxic T was predominant in the epithelium, in contrast to the lamina propria, in which the suppressor subtype was predominant. Most of the CD4+ T cells of the epithelium were helper T cells (Fig. 2).

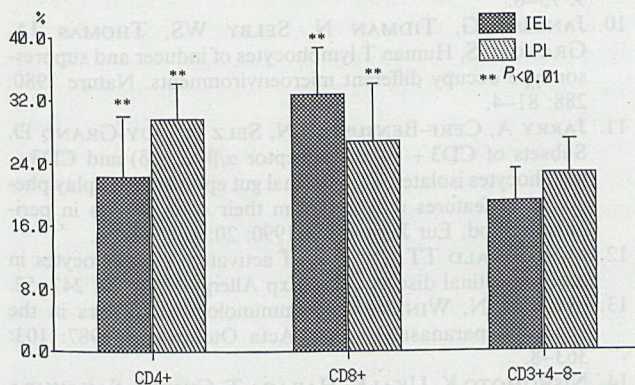


Fig. 1. Comparison of percentage of T-cell subsets of intraepithelial lymphocytes and lamina propria lymphocytes.

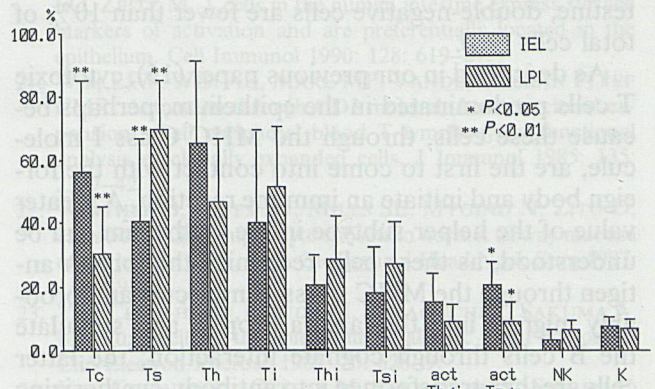


Fig. 2. Comparison of percentages of T-cell subpopulation of intraepithelial lymphocytes and lamina propria lymphocytes.

Activated CD4+ T cells and activated CD8+ T cells, which showed an increase caused by culture, were comparable in values and formed a minority of the total T-lymphocyte population. αβT was predominant in both epithelium and lamina propria, but γδT was preferentially located in the epithelium.

Discussion

The mucosal immune system, mainly consisting of the T-cell system, protects the mucosal tissues from external allergens and pathogens. It is significant that the regional immune system at such mucosal barriers is known to comprise a heterogeneous population of lymphocytes which are distinct, phenotypically and functionally, from the peripheral blood lymphocytes and other lymphoid organs. The advent of monoclonal antibodies and flow cytometry allows us to understand the regional immunity at a mucosal surface.

Our previous paper, which was the first report of its kind, dealt with the phenotypes and receptors of the human nasal epithelium (15). In this paper, we have studied the phenotypes and receptors of the lymphocytes that occupy the microenvironment of the epithelium as well as the lamina propria. T lymphocytes were found to be predominant in both epithelium and lamina propria. B lymphocytes were not detected in the epithelium, but comprised about 20% of the lymphocyte population of the lamina propria. The predominance of CD8+ (suppressor/cytotoxic) T cells in the epithelium is in accordance with studies (2, 10, 14-16, 24) in the nasal mucosa, and studies (1, 10, 11, 17, 19, 20) in the gut epithelium, but it contrasts with the findings of studies (13, 22) in the nasal mucosa. A high percentage of double-negative cells in the nasal epithelium is in accordance with our previous findings (15) as well as with findings in the intestines in certain diseases such as coeliac disease (12, 18), although in normal in-

testine, double-negative cells are fewer than 10% of total cells (20).

As described in our previous paper (15), cytotoxic T cells predominated in the epithelium, perhaps because these cells, through the MHC Class I molecule, are the first to come into contact with the foreign body and initiate an immune reaction. A greater value of the helper subtype in the epithelium can be understood, as these cells recognize the foreign antigen through the MHC Class II molecule and probably migrate into the lamina propria and stimulate the B cells through cognate interaction; the latter cells are then transformed into antibody-synthesizing plasma cells. In addition, on antigenic stimulation, the helper T cells release potentially active lymphokines which not only coordinate the immune function, but also alter the local physiology.

In contrast to the epithelium, CD4+ T cells predominate in the lamina propria, as found in studies (2, 13, 22) in nLPL and other studies (17, 20) in the intestinal lamina propria. B lymphocytes, which are known to play a major role in antibody synthesis, were found to occupy a significant part of the total lymphocyte population of the lamina propria. The nLPL population, predominant in suppressor T cells, seems to play a secondary role in the immune response by downregulating the actions of helper T cells (13) as well as controlling the synthesis of secretory IgA.

Comparative studies of the individual subsets of T lymphocytes in the epithelium and lamina propria in allergic as compared with nonallergic (infectious) rhinitis will be reported separately. We have no parallel comparison of nIEL except our previous paper (15) and comparisons drawn with iIEL (1, 9, 10–12, 17, 18, 20). The epithelium was characterized by a predominance of T lymphocytes, among which CD8+ T cells were predominant. A high percentage of double-negative cells, as seen in coeliac disease (12, 18), can be accounted for, since we have examined the nasal mucosa of patients with allergic and nonallergic (infectious) rhinitis and not normal nasal mucosa. The function of these double-negative T cells (usually associated with the $\gamma\delta$ heterodimer) is not yet well defined; they may play a significant role in the immune defense of the upper respiratory tract.

The preferential location of $\gamma\delta$ receptor-bearing cells in the epithelium and their relative absence in the lamina propria, as in the intestines (20), can be explained by the proposal of Janeway et al. (9) that $\gamma\delta$ TCRs mediate an immunologic surveillance of the epithelium by monitoring the integrity of the cell layers that separate the internal from the external milieu, and that $\alpha\beta$ TCRs are responsible for the surveillance of the internal milieu. The homing of the $\gamma\delta$ receptor-bearing cells in the nasal epithelium, like that of the iIEL (9, 11, 18, 20), which increases in

disorders such as coeliac disease (1, 20), suggests a highly specific role in these anatomic sites. Further studies of the repertoire of the $\gamma\delta$ TCRs and the lymphokines released by them are necessary for a complete understanding of their biologic role in the human nasal mucosa.

We wish to emphasize that the lymphocytes of the human nasal mucosa (both nIEL and nLPL) are heterogenous in phenotype and receptors, and together comprise the regional immunity of the upper respiratory tract. The integrity of this immune system and its role in immune defense rely entirely on the resident and migrating lymphocytes.

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