

# Intestinal Lamina Propria TcR $\gamma\delta$ <sup>+</sup> Lymphocytes Selectively Express IL-10 and IL-17

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## ■ Abstract

*Background:* The characteristics and roles of gut lymphocytes have been only partly elucidated, in particular with regard to activation patterns.

*Objectives:* To characterize lymphocytes from various parts of the gut and examine their activation pattern as a network.

*Methods:* Lymphocytes were isolated from the epithelium, the lamina propria, Peyer's patches, mesenteric lymph nodes, the spleen, and peripheral blood of naïve mice. They were then characterized for T cell phenotype, T cell receptors (TCRs), activation markers, and cytokine production.

*Results:* The results showed a gradient of cells with an increasing proportion of TcR $\gamma\delta$ <sup>+</sup>, CD8 $\alpha\alpha$ <sup>+</sup> cells towards the gut lumen, with the highest number found in intraepithelial lymphocytes. These cells, together with lamina propria lymphocytes (LPLs) were also characterized by a memory-like phenotype (CD25<sup>-</sup> CD45RB<sup>low</sup> and CD44<sup>high</sup>) and CD69 expression. CD8<sup>+</sup> TcR $\gamma\delta$ <sup>+</sup> LPLs produced IL-10 and IL-17, while TcR $\alpha\beta$ <sup>+</sup> LPLs were FoxP3 positive.

*Conclusions:* Gut lymphocytes express various receptors and cytokines according to their location. These specific features suggest a differential function for gut lymphocytes depending on their location.

**Key words:** TcR $\gamma\delta$ <sup>+</sup> lymphocytes. IL-17. IL-10.

## ■ Resumen

*Antecedentes:* Las características y funciones de los linfocitos intestinales se han explicado solo en parte, en concreto en lo que respecta a los patrones de activación.

*Objetivos:* Caracterizar como una red los linfocitos de distintas partes del intestino y estudiar su patrón de activación.

*Métodos:* Se aislaron linfocitos del epitelio, la lámina propia, las placas de Peyer, los ganglios linfáticos mesentéricos, el bazo y la sangre periférica de ratones no sometidos previamente a experimentación. Estos linfocitos se caracterizaron posteriormente en cuanto a fenotipo de células T, receptores de células T (TCR), marcadores de activación y producción de citocinas.

*Resultados:* Los resultados mostraron un gradiente de células con una proporción creciente de células CD8<sup>+</sup> TCR<sup>+</sup> hacia la luz intestinal, observándose el número más elevado en los linfocitos intraepiteliales. Estas células, junto con los linfocitos de la lámina propia (LLP), también se caracterizaron en cuanto a fenotipo de memoria (CD25<sup>-</sup> CD45RB<sup>bajo</sup> y CD44<sup>alto</sup>) y expresión de CD69. Los LLP CD8<sup>+</sup> TCR<sup>+</sup> produjeron IL-10 e IL-17, mientras que los LLP TCR<sup>+</sup> fueron positivos para FoxP3.

*Conclusiones:* Los linfocitos intestinales expresan distintos receptores y citocinas en función de su ubicación. Estas características específicas indican una función diferencial de los linfocitos intestinales según su ubicación.

**Palabras clave:** Linfocitos TCR $\gamma\delta$ <sup>+</sup>, IL-17. IL-10.

## Introduction

In higher vertebrates the gut is populated by a unique immunologic network, and in mice, almost 50% of all T cells are

located in the small intestine [1]. The gut is also characterized by a specific physical barrier, the gut mucosa, which separates a milieu loaded with microbial and food antigens from the sterile environment of the submucosa. In relation to this

barrier, and exposure or nonexposure to high loads of antigens, the intestinal immune system can be divided into 2 different compartments. In the outer compartment, T cells are scattered across the extensive surface of the intestinal wall. They are resident either in the lamina propria (LP), where they are referred to as lamina propria lymphocytes (LPLs), or directly between the epithelial cells, where they are referred to as intraepithelial lymphocytes (IELs). In the inner compartment, T cells are located in gut-associated lymphoid tissue (GALT) with an organization similar to that of conventional lymphoid structures. The GALT includes Peyer's patches (PPs), a series of domes scattered along the internal side of the ileum, and mesenteric lymph nodes (MLNs).

Constant antigen stimulation favors the expansion of specific T cell populations to the detriment of other populations and modifies the distribution of T cell phenotypes during an organism's lifetime [2,3]. These mechanisms balancing unresponsiveness and inflammation suggest the presence of specific T cell populations with specific functions in the different intestinal compartments. Previous studies have isolated and characterized cells from different parts of the gut [4-6]. The lack of responsiveness of these cells, and of IELS in particular, suggests that they might play a role in the homeostasis of the gut immune system, possibly by active mechanisms of oral tolerance. If the natural homeostasis is broken, the intestinal immune system, and more specifically the inner compartment, will respond with an inflammatory response to the pathogen in order to protect the host. However, despite their large numbers, intestinal T cells are not well characterized and studies integrating the various T cell phenotypes in the intestinal compartment with regard to activation markers and expression of cytokines are scarce and describe only 1 or 2 localizations but not the gut immune system as a whole [7-9]. In addition, the immune system of the gut might respond specifically to various mice strains. A large number of studies on food tolerance/allergy are currently using mouse models with the C3H/HeJ or the C3H/HeOuJ strains [10-13]. Both are genetically very similar but unlike C3H/HeOuJ mice, C3H/HeJ mice are resistant to endotoxin.

The aim of the present study was to characterize as a network the phenotypes and activation patterns of T cells isolated from various locations of the gut in young naïve C3H/HeOuJ mice, in particular with regard to T cell receptor (TcR)  $\alpha\beta$  and TcR $\gamma\delta$  expression as well as activation markers (CD25, CD44, CD45RB, CD69), homing molecules, (CCR9 and  $\alpha_{\text{IEL}}\beta_7$ ) and cytokine profile after *in vitro* activation (interferon [IFN] $\gamma$ , interleukin [IL]-4, IL-10, and IL-17) and FoxP3 expression. We used the endotoxin-sensitive mouse strain (C3H/HeOuJ) to characterize T cell phenotypes in the intestinal compartment because a normal response to endotoxins from the commensal flora of the gut is essential for immunological studies of gut lymphocytes.

## Materials and Methods

### Mice

Female C3H/HeOuJ mice purchased from Charles River (L'Arbresle, France) were housed at the School of Medicine

of the University of Geneva, Switzerland. The animals were between 4 and 5 weeks of age and were fed with standard mice pellets and conventional water. All the experiments were approved by the Animal Studies Ethics Committee of the University and performed in accordance with their guidelines.

### Antibodies and Reagents

Anti-TcR $\beta$  (H57-97), anti-TcR $\delta$  (GL3), anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 $\alpha$  (53-6.7), anti-CD8 $\beta$  (H35-17.2), anti-CD25 (3C7), anti-CD45RB (16A), anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-CD103 (M290), anti- $\gamma$ IFN (XMG1.2), anti-IL-4 (BVD4-1D11), anti-IL-10 (JES5-16E3) and anti-IL-17 (TC11-18H10) were acquired from BD Bioscience Europe (Erembodegem, Belgium). Anti-CCR9 (242503) was from R&D (Abdington, United Kingdom), anti-FoxP3 from eBioscience (San Diego, California, USA), and 7-amino-actinomycin D (7-AAD) from Sigma (Buchs, Switzerland).

RPMI 1640 medium (cRPMI) was supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 mM L-glutamine, 100  $\mu$ g/mL gentamicin, 15 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES), and 10% fetal calf serum (FCS) (all from Sigma, Buchs, Switzerland). HBSS (Sigma, Buchs, Switzerland) (cHBSS) was supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 mM L-glutamine, 100  $\mu$ g/mL gentamicin, 15 mM HEPES, 2 mM NaHCO<sub>3</sub>, and 10% FCS.

### Isolation of Lymphocytes

PP lymphocytes, LPLs, and IELS were isolated using modified methods previously described [14]. Briefly, fat was removed, the PPs were mechanically excised and kept on ice until cell isolation (see below), and the gut was extensively flushed with cHBSS. Intestinal pieces were opened longitudinally and cut into 5-mm pieces. The tissue was incubated in calcium- and magnesium-free cHBSS containing 2 mM EDTA and 1 mM dithiothreitol (Sigma) for 30 minutes at 37°C with magnetic stirring. It was then vigorously vortexed and filtered through a 70- $\mu$ m nylon filter. Intestinal IELS were obtained by filtration of the supernatant through a nylon wool column. To isolate LPLs, the remaining tissue was washed 3 times with cRPMI, and intestinal pieces were subsequently incubated with magnetic stirring for 30 minutes at 37°C in cRPMI supplemented with 100 U/mL collagenase D (Roche, Mannheim, Germany). The cells were separated from tissue debris by purification through a 70- $\mu$ m nylon filter. This step was repeated once.

PPs were incubated in calcium- and magnesium-free HBSS containing 2 mM EDTA and 1 mM dithiothreitol for 30 minutes at 37°C with magnetic stirring; they were then crushed and filtered.

Spleen and MLNs were harvested, crushed, and filtered to obtain a cell suspension. Each cell population was washed twice and lymphocytes were enriched by discontinuous 33%/40% Percoll (Bioscience, Uppsala, Sweden) on a lympholyte M gradient (Cederlane, Hornby, Canada) for 20 minutes at 600  $\times$  g at room temperature. Lymphocytes were harvested on the Percoll 40% lympholyte M interface.

To isolate peripheral blood mononuclear cells (PBMCs), the mice were bled before sacrifice and lymphocytes were isolated on a lympholyte M gradient.

All organs were isolated and analyzed during the same experiment. A preliminary experiment showed that spleen cell (SC) pre-incubation with EDTA, DTT, or collagenase using the same protocols as those used for intestinal T cell isolation did not modify T cell phenotypes (data not shown).

### Flow Cytometry

Surface marker analysis was performed using freshly isolated cells. Dead cells were excluded by 7-AAD and further analyses were performed by gating on CD3<sup>+</sup> cells. For intracellular cytokine and FoxP3 analysis, freshly isolated cells were activated with PMA (50 ng/mL/ionomycin (1 µg/mL) in cRPMI in the presence of GolgiPlug (BD Pharmingen, Franklin, New Jersey, USA) (1/1000), for 4 hours at 37°C in 0.5% CO<sub>2</sub>. The cells were fixed and permeabilized with Cytotfix/Cytoperm (BD Pharmingen) for 20 minutes on ice followed by 2 wash steps with Perm/Wash buffer (BD Pharmingen).

For staining, the cells were incubated for 20 minutes on ice

with purified rat IgG (Sigma, Buchs, Switzerland) to block Fc receptors. They were then incubated with the corresponding concentration of directly coupled antibodies for 30 minutes on ice.

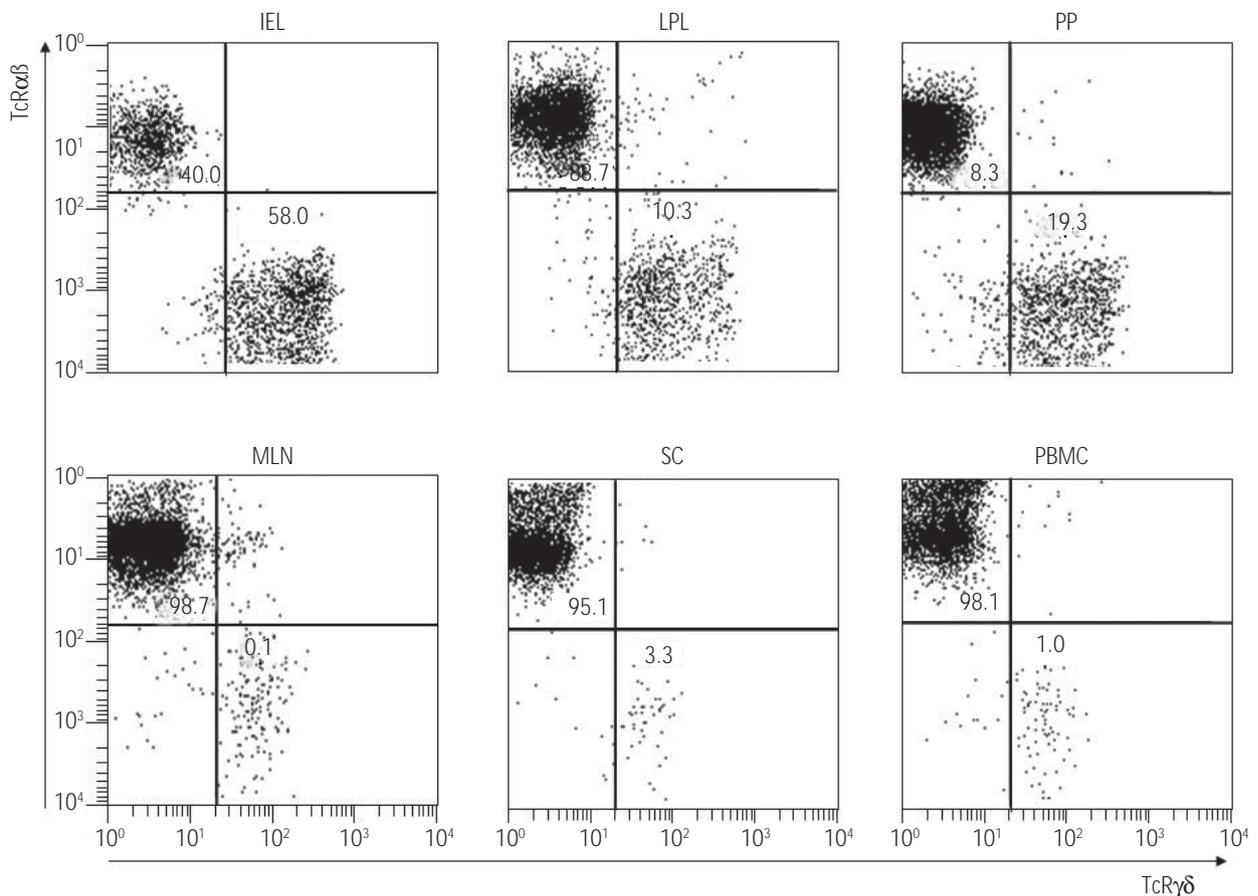
Multiparameter analyses were performed on a flow cytometer (FACSCalibur; BD Bioscience) equipped with the CellQuest software (BD Bioscience).

### Statistical Analysis

Data were expressed as means±SEM. Experiments were repeated 3 times with pooled cells from at least 3 mice per experiment in order to obtain a sufficient number of cells for each analysis.

## Results

The frequency of TcRγδ<sup>+</sup> and CD8<sup>+</sup> T cells was increased in the outer compartment of the intestine. T cell subpopulations



**Figure 1.** TcRαβ and TcRγδ expression on T cells. Isolation of intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patch (PP) cells, mesenteric lymph node (MLN) cells, spleen cells (SCs) and peripheral blood mononuclear cells (PBMCs) was performed at the same time, as described in the Materials and Methods section. Viable T cells were analyzed by gating on 7-AAD<sup>-</sup> and CD3<sup>+</sup> cells. Numbers represent the percentages of cells in each quadrant. Results from a representative experiment of 3, with cells pooled from at least 3 mice, are shown.

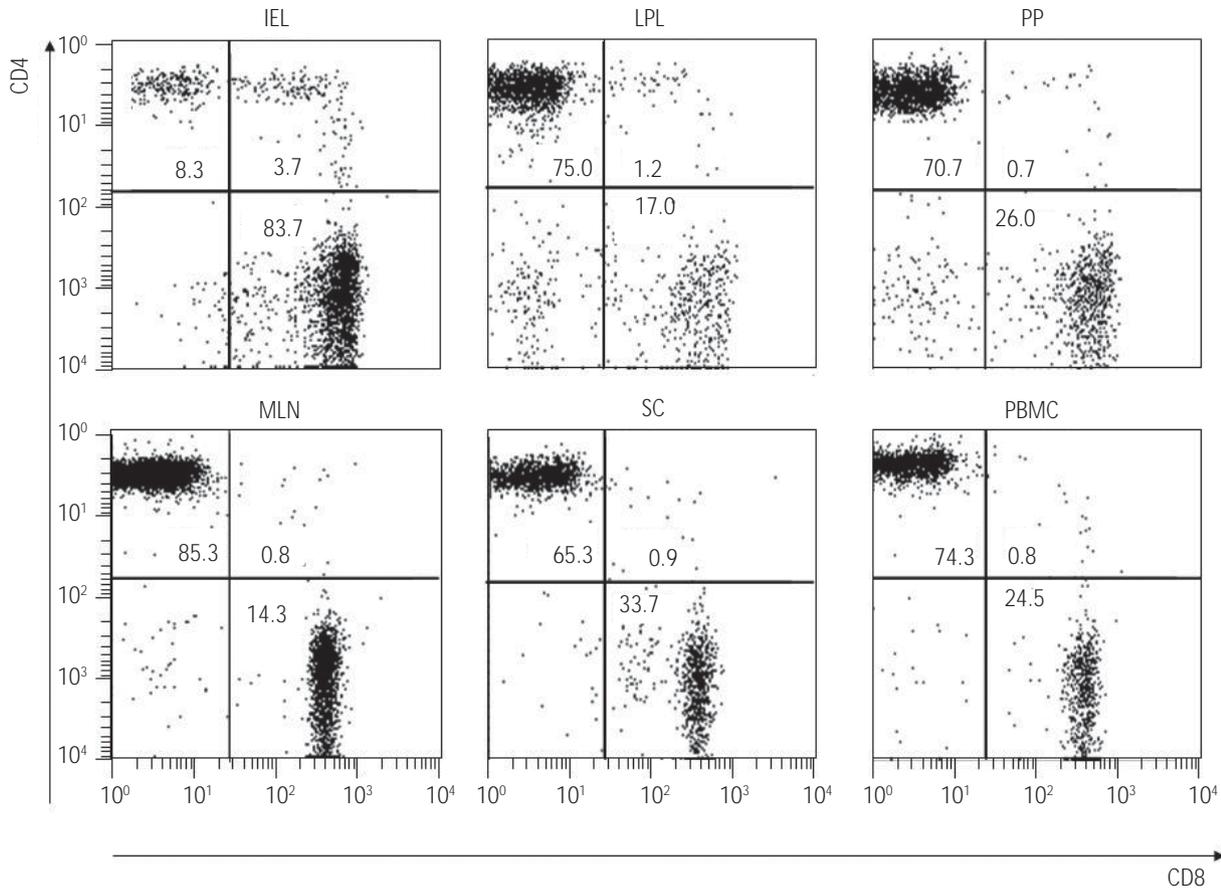


Figure 2. TCD4 and CD8 $\alpha$  expression in T cells from intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patch (PP) cells, mesenteric lymph node (MLN) cells, spleen cells (SCs) and peripheral blood mononuclear cells (PBMCs). Experimental conditions were identical to those described in Figure 1.

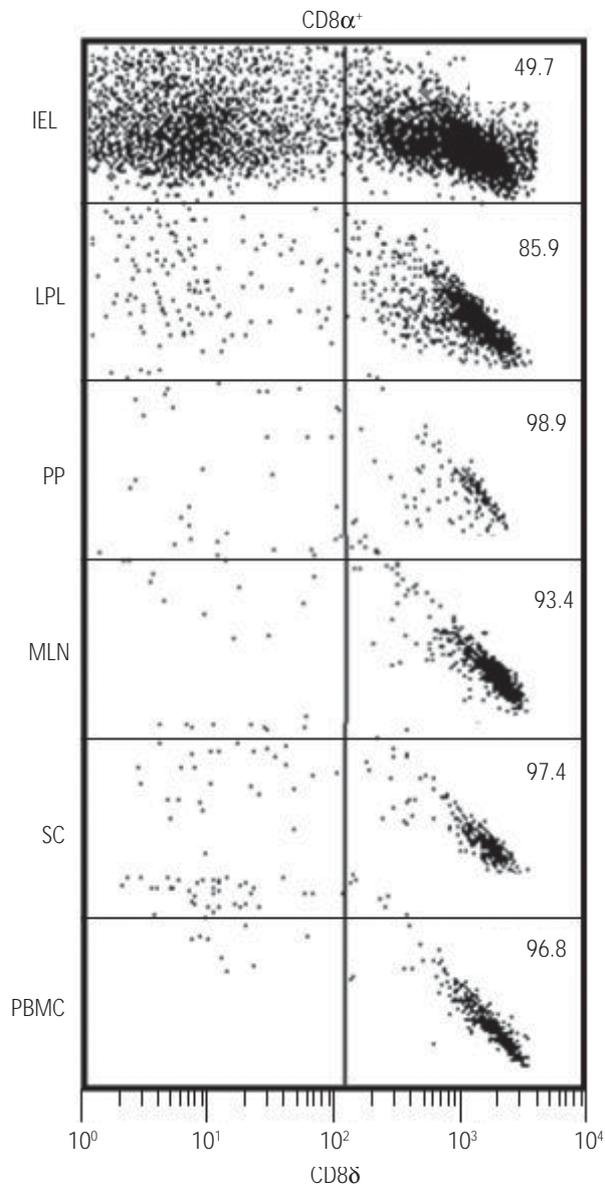
from the outer intestinal compartment (IELs and LPLs), the inner intestinal compartment (PP and MLN cells), and the systemic compartment (SCs and PBMCs) were quantified by flow cytometry. Figure 1 shows that in the inner compartment, MLN T cells expressed identical TcR receptors as those expressed in the systemic compartment (spleen and blood), with a majority of TcR $\alpha\beta$ <sup>+</sup> cells (98.7%, 95.1%, and 98.1%, respectively). In contrast, in PPs and the outer intestinal compartments, the expression of TcR $\alpha\beta$  decreased gradually, with 78.3% on PP cells, 88.7% on LPLs, and 40.0% on IELs. TcR TcR $\gamma\delta$ <sup>+</sup> expression in contrast was 19.3% on PPs, 10.3% on LPLs, and 58.0% on IELs.

Expression of CD4 and CD8 receptors was similarly specific to the location of the cells. Figure 2 shows a predominance of CD4<sup>+</sup> T cells in PBMCs, the spleen, MLNs, PPs, and the LP (65.3%-85.3%); the percentage of CD8<sup>+</sup> T cells, in contrast, ranged from 14.3% to 33.7%. In the epithelium, most T cells (83.7%) expressed the CD8 antigen, while only a small fraction (8.3%) expressed CD4. We also identified a third population of T cells, which expressed on their surface both CD4 and CD8

receptors (double positive), and were present in 3.7% of IELs but in less than 1% of cells in the systemic compartment.

#### *Fifty Percent of CD8<sup>+</sup> IELs Are Unconventional CD8 $\alpha\alpha$ <sup>+</sup> Cells*

It has been reported that CD8<sup>+</sup> T cells may express the homodimer  $\alpha\alpha$  chains instead of the heterodimer  $\alpha\beta$  chains on their CD8 molecule [15]. To define the frequency of CD8 $\alpha\alpha$ <sup>+</sup> cells in the various compartments analyzed, we gated on CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> cells and measured the frequency of CD8 $\beta\beta$ <sup>+</sup> cells (Figure 3). In the systemic and intestinal inner compartment, almost all the CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> cells were also CD8 $\beta\beta$ <sup>+</sup> cells (93.4% in MLNs and 98.9% in PPs). These results are consistent with the expression of the conventional  $\alpha\beta$  heterodimer phenotype of these CD8 molecules. In contrast, a large number of CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells in the epithelium were negative for CD8 $\beta$ , suggesting that these cells express the CD8 $\alpha\alpha$  phenotype. These CD8 $\alpha\alpha$ <sup>+</sup> T cells represented 50.3% of all the CD8<sup>+</sup> cells in the IELs and were also present in the LP as a minor subpopulation (14.1%).



**Figure 3.** CD8 $\beta$  expression in CD8 $\alpha^+$  T cells from intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patch (PP) cells, mesenteric lymph node (MLN) cells, spleen cells (SCs) and peripheral blood mononuclear cells (PBMCs). Cells were analyzed by gating on 7-AAD $^-$  and CD3 $^+$  and CD8 $\alpha^+$  cells. Numbers represent the percentage of CD8 $\alpha^+$  cells also positive for CD8 $\beta^+$  cells (CD8 $\alpha/\beta$  double positive cells). Results from a representative experiment of 3, with cells pooled from at least 3 mice, are shown.

### *TcR $\gamma\delta$ T Cells in the Mucosal Compartment Are CD8 $\alpha\alpha^+$ Cells*

In order to analyze the expression of TcR chains in CD4 and CD8 subpopulations, we gated on CD3 $^+$ CD4 $^+$ , CD3 $^+$ CD8 $\alpha^+$ , and CD3 $^+$ CD8 $\beta^+$  cells and measured the frequency of TcR $\alpha\beta$  and TcR $\delta\delta$ .

Figure 4A shows that TcR $\alpha\beta$  was expressed on the surface of almost all CD4 $^+$  cells (93.5%-99.6%) in the various compartments analyzed. TcR $\gamma\delta$ , in contrast, was expressed only on a minority of cells (0.1%-1.1%) (Figure 4B). For CD8 $\alpha^+$  and CD8 $\beta^+$  cells, TcR $\alpha\beta$  was also preferentially expressed in all the tissues analyzed, except in IELs where CD8 $\alpha^+$  cells were associated with TcR $\alpha\beta$  (66%) and TcR $\delta\delta$  (29.6%). CD8 $\beta^+$  cells, in contrast, expressed TcR $\alpha\beta$ .

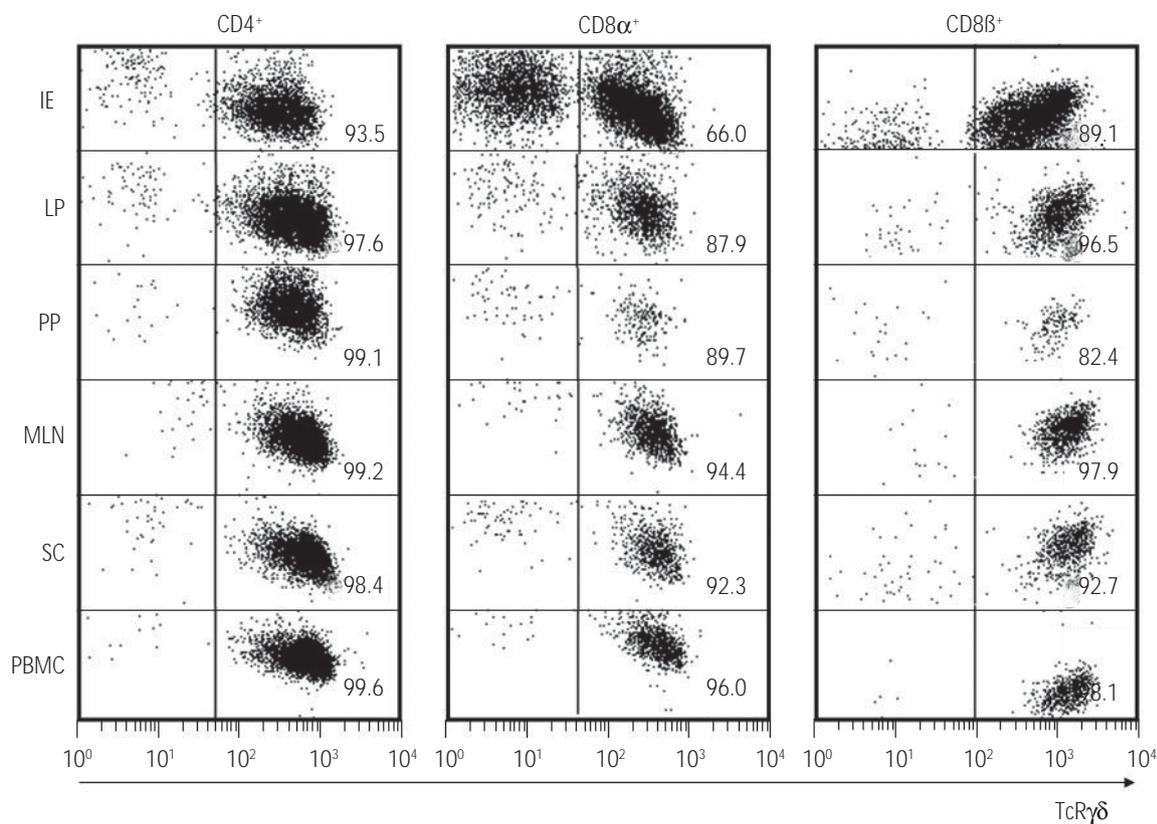
Next, we analyzed exclusively TcR $\gamma\delta^+$  and TcR $\alpha\beta^+$  cell populations in IELs (Figure 5). We clearly saw that TcR $\gamma\delta^+$  IELs were almost exclusively CD8 $\beta^+$  cells while TcR $\gamma\delta^+$  IELs were predominantly CD8 $\alpha\beta^+$  cells. Due to the low frequency of TcR $\gamma\delta^+$  cells in the other compartments (particularly in PBMCs, SCs, and MLNs), we were unable to gate directly on TcR $\gamma\delta^+$  cells to measure the frequency of CD8 $\alpha$  and CD8 $\beta$  chains. However, Figure 4B shows that the frequency of TcR $\gamma\delta$  in PBMCs, SCs, and MLNs was identical on CD8 $\alpha$  and CD8 $\beta$  gating, suggesting a preferential expression of the heterodimer CD8 $\alpha\alpha$  in TcR $\gamma\delta^+$  cells. In contrast, in the LP and PPs, the frequency of TcR $\gamma\delta^+$  cells was higher for CD8 $\beta^+$  cells than for CD8 $\alpha^+$  cells, with a preferential expression of the homodimer CD8 $\alpha\alpha$  on TcR $\gamma\delta^+$  cells.

### *IELs Express a Unique Memory-like Phenotype*

Due to their particular location, T cells in the intestinal compartment are more exposed to environmental pressure. To investigate the influence of the environment on T cell activation, we measured the expression of surface molecules characteristic of T cell subpopulation activation/memory in young naïve mice. We focused on the expression of major activation markers (CD25, CD69, CD45RB, and CD44) that are specific to intestinal homing ( $\alpha$ IELB7, CCR9) in CD4 $^+$  and CD8 $\alpha^+$  T cells.

In the PBMCs, SCs, and MLNs we found a majority of T cells that were negative for CD25 (Table 1) and CD69 (Figure 6B). In both subpopulations, the cells were CD44 $^{\text{low}}$  (56.6%-63.9%) and to a lesser extent CD44 $^{\text{high}}$  (22.7%-34.0%) (Figure 6A). These data demonstrate the presence of 2 T cell populations: one with CD25 $^-$ , CD69 $^-$ , and CD44 $^{\text{low}}$ , a phenotype characteristic of naïve T cells, and another with CD25 $^-$  CD69 $^-$  and CD44 $^{\text{high}}$ , a phenotype characteristic of memory T cells. The first phenotype was predominantly expressed in CD4 $^+$  cells whereas the second was predominantly expressed in CD8 $^+$  cells.

In contrast to PBMCs, SCs, and MLN cells, a predominant number of IELs and LPLs, (both CD4 $^+$  and CD8 $^+$ ) showed a memory-like T cell phenotype: CD25 $^-$  CD45RB $^{\text{low}}$  and CD44 $^{\text{high}}$  and also expressed CD69, which is a marker of early activation in lymphocytes. Interestingly, a small percentage of CD4 $^+$  IELs (41.0%), and LPLs (7.5%) were positive for  $\alpha$ IELB7 (Figure 6C), whereas almost all the CD8 $^+$  IELs (95.1%) expressed this integrin. Moreover, CD4 $^+$  T cells from

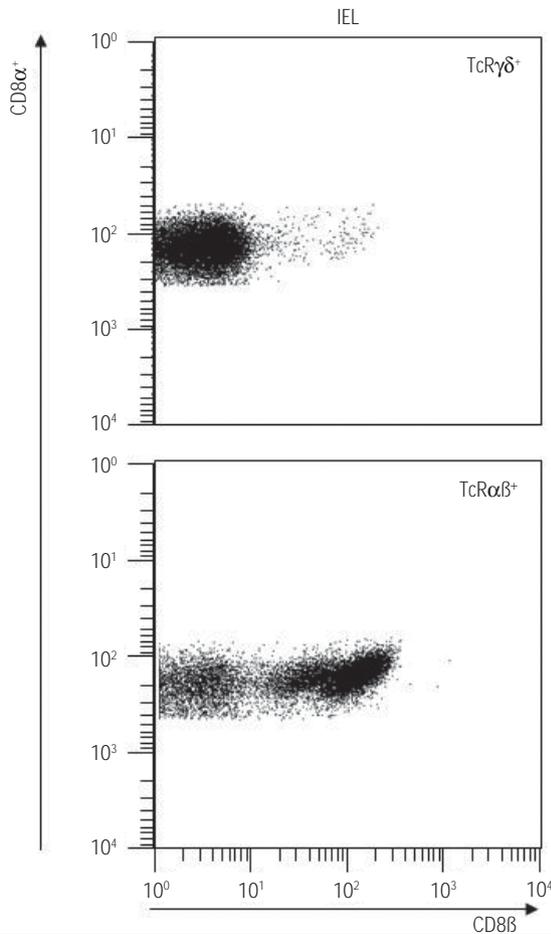


**Figure 4.** TcR $\alpha\beta$  (A) and TcR $\gamma\delta$  (B) expression analysis of CD4 $^+$ , CD8 $\alpha^+$ , and CD8 $\beta^+$  T cell subpopulations from intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patch (PP) cells, mesenteric lymph node (MLN) cells, spleen cells (SCs) and peripheral blood mononuclear cells (PBMCs). Cells were analyzed by gating on 7-AAD $^-$  and CD3 $^+$  cells and then on CD4 $^+$ , CD8 $\alpha^+$ , or CD8 $\beta^+$  cells. Results represent the percentage of TcR $\alpha\beta$  positive cells for each condition. Numbers represent the percentages of positive cells. Results from a representative experiment of 3, with cells pooled from at least 3 mice, are shown.

**Table 1.** Phenotypes of CD4 $^+$  and CD8 $^+$  Subpopulations Isolated From Systemic and Intestinal Compartments

	CD25, %		CD45RB, %		CCR9, %	
	CD4 $^+$	CD8 $\alpha^+$	CD4 $^+$	CD8 $\alpha^+$	CD4 $^+$	CD8 $\alpha^+$
IELs	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	2.6 $\pm$ 1.0	23.3 $\pm$ 8.8	77.2 $\pm$ 8.6	80.8 $\pm$ 5.3
LPLs	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	2.4 $\pm$ 1.3	22.3 $\pm$ 4.8	79.2 $\pm$ 7.3	66.3 $\pm$ 12.1
PP cells	0.1 $\pm$ 0.1	0.6 $\pm$ 0.5	10.2 $\pm$ 2.8	88.5 $\pm$ 11.3	22.1 $\pm$ 2.4	71.0 $\pm$ 12.4
MLN cells	0.3 $\pm$ 0.5	0.2 $\pm$ 0.3	14.8 $\pm$ 3.6	89.9 $\pm$ 7.6	22.0 $\pm$ 4.1	72.6 $\pm$ 13.4
SCs	0.2 $\pm$ 0.3	0.9 $\pm$ 0.5	30.2 $\pm$ 5.1	90.6 $\pm$ 11.3	43.4 $\pm$ 3.2	92.8 $\pm$ 6.2
PBMCs	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	17.7 $\pm$ 3.7	90.2 $\pm$ 5.0	10.1 $\pm$ 5.5	41.0 $\pm$ 10.9

Abbreviations: IELs, intraepithelial lymphocytes; LPLs, lamina propria lymphocytes; MLN, mesenteric lymph node; PP, Peyer's patch; SCs, spleen cells.



**Figure 5.** CD8 $\beta$  isotype expression in CD8 $^+$  intraepithelial lymphocytes. Cells were analyzed by gating on 7-AAD $^-$  and CD8 $^+$  cells and then on TcR $\gamma\delta^+$  or TcR $\alpha\beta^+$  cells. Results represent the dot-plot of gated cells, numbers represent the percentages of positive cells. Results from a representative experiment of 3, with cells pooled from at least 3 mice, are shown.

the outer compartment (IELs and LPLs) expressed more CCR9 on their surface (77.2%-79.2%) (Table 1) than did systemic T cells (10.1%-43.4%). In contrast, a large percentage of CD8 $^+$  cells from all compartments expressed CCR9 (66.3%-92.8%). The exception was PBMCs, in which only 41.0% of cells expressed CCR9 $^+$ .

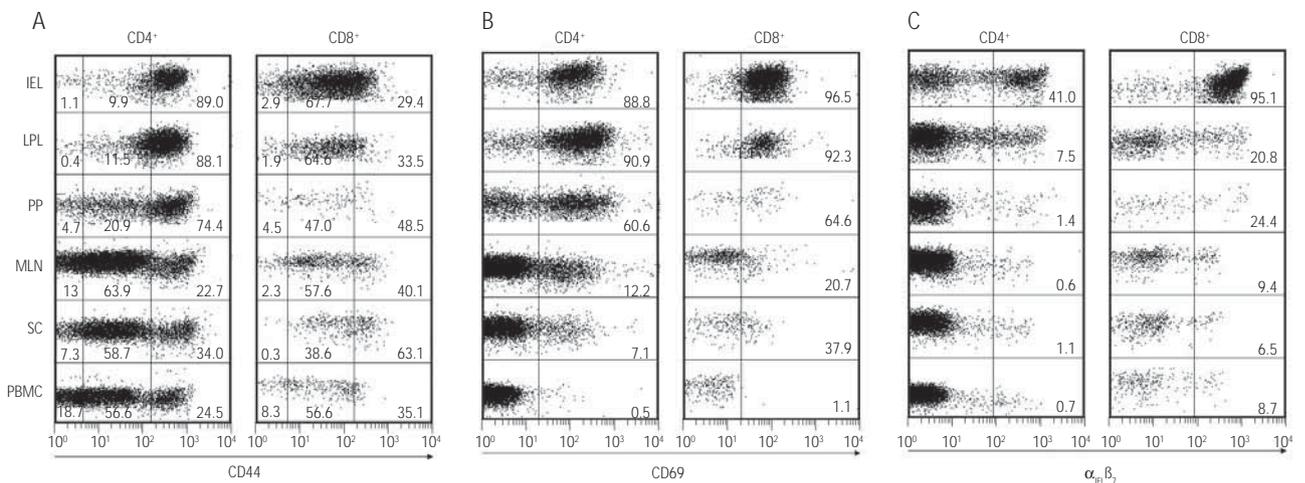
In the PPs we observed a mixed situation, with an increased percentage of classical naive CD25-CD45RB $^{\text{high}}$ CD44 $^{\text{low}}$  T cells compared to the outer compartment. Interestingly, the frequency of CD69 $^+$  cells was lower for CD4 $^+$  (60.6%) and CD8 $^+$  (64.6%) cells. All CD8 $^+$  IELs expressed  $\alpha_{\text{IEL}}\beta_7$ , but this was true for only low proportions of CD8 $^+$  LPLs and PP cells. Only half of CD4 $^+$  IELs and very few LPLs (7.4%) expressed  $\alpha_{\text{IEL}}\beta_7$ .

*Th17 Regulatory T Cells and Other T Regulatory Phenotypes Co-Exist in the Outer Compartment of the Intestine*

We also used flow cytometry to analyze the intracellular expression of major cytokines that modulate allergic inflammation after in vitro PMA/ionomycin activation. The following cytokines were measured; IFN $\gamma$  for type 1 helper (T $_{\text{H}}$ 1) cells, IL-4 for T $_{\text{H}}$ 2 cells, IL-17 for T $_{\text{H}}$ 17 cells, and IL-10. We also measured the expression of intranuclear FoxP3 factor to identify regulatory T (Treg) cells (Figure 7, Table 2).

We did not detect IL-4 after activation in any of the cells analyzed, and we detected only a few IFN $\gamma$ -producing T cells in PBMCs, SCs, and MLN cells (data not shown). These IFN $\gamma$ -producing T cells preferentially expressed CD8 $^+$ , and they were detected in PBMCs, SCs, and MLN cells.

Interestingly, when we gated on TcR subsets, we detected a large population of TcR $\alpha\beta^+$  LPLs producing IL-10 and/or IL-17 after 4 hours of in vitro stimulation (Figure 7AC). FoxP3 expression was found in CD4 $^+$ , but not in CD8 $^+$  or TcR $\gamma\delta^+$  cells (Figure 7B). CD4 and TcR $\alpha\beta$  FoxP3 $^+$  cells



**Figure 6.** CD44 (A) CD69 (B), and  $\alpha_{\text{IEL}}\beta_7$  (C) expression on T cell subpopulations from intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patch (PP) cells, mesenteric lymph node (MLN) cells, spleen cells (SCs) and peripheral blood mononuclear cells (PBMCs). Cells were analyzed by gating on 7-AAD $^-$  and CD3 $^+$  cells and then on CD4 $^+$  or CD8 $\alpha^+$  cells. Results represent the percentages of positive cells for CD44, CD69, and  $\alpha_{\text{IEL}}\beta_7$ . Results from a representative experiment of 3, with cells pooled from at least 3 mice, are shown.

Table 2. Phenotype of CD4<sup>+</sup> and CD8<sup>+</sup> Subpopulations Isolated From the Systemic or Intestinal Compartments

	IL-10, %		IL-17, %		FoxP3, %	
	CD4 <sup>+</sup>	CD8 $\alpha$ <sup>+</sup>	CD4 <sup>+</sup>	CD8 $\alpha$ <sup>+</sup>	CD4 <sup>+</sup>	CD8 $\alpha$ <sup>+</sup>
IELs	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	1.4 $\pm$ 0.4	0.1 $\pm$ 0.1	1.4 $\pm$ 0.3	0.7 $\pm$ 0.3
LPLs	0.1 $\pm$ 0.1	2.3 $\pm$ 0.9	2.3 $\pm$ 0.6	1.7 $\pm$ 0.3	11.2 $\pm$ 0.9	1.4 $\pm$ 0.4
PP cells	1.4 $\pm$ 0.2	1.4 $\pm$ 0.7	1.3 $\pm$ 0.1	0.6 $\pm$ 0.3	6.2 $\pm$ 0.8	2.0 $\pm$ 0.4
MLN cells	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	3.8 $\pm$ 0.4	1.0 $\pm$ 0.4
SCs	0.5 $\pm$ 0.3	0.7 $\pm$ 0.5	0.6 $\pm$ 0.3	0.1 $\pm$ 0.1	8.5 $\pm$ 0.7	0.5 $\pm$ 0.2
PBMCs	0.2 $\pm$ 0.1	0.7 $\pm$ 0.4	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1	5.4 $\pm$ 0.6	2.6 $\pm$ 0.7

Abbreviations: IELs, intraepithelial lymphocytes; IL, interleukin; LPLs, lamina propria lymphocytes; MLN, mesenteric lymph node; PP, Peyer's patch; SCs, spleen cells.

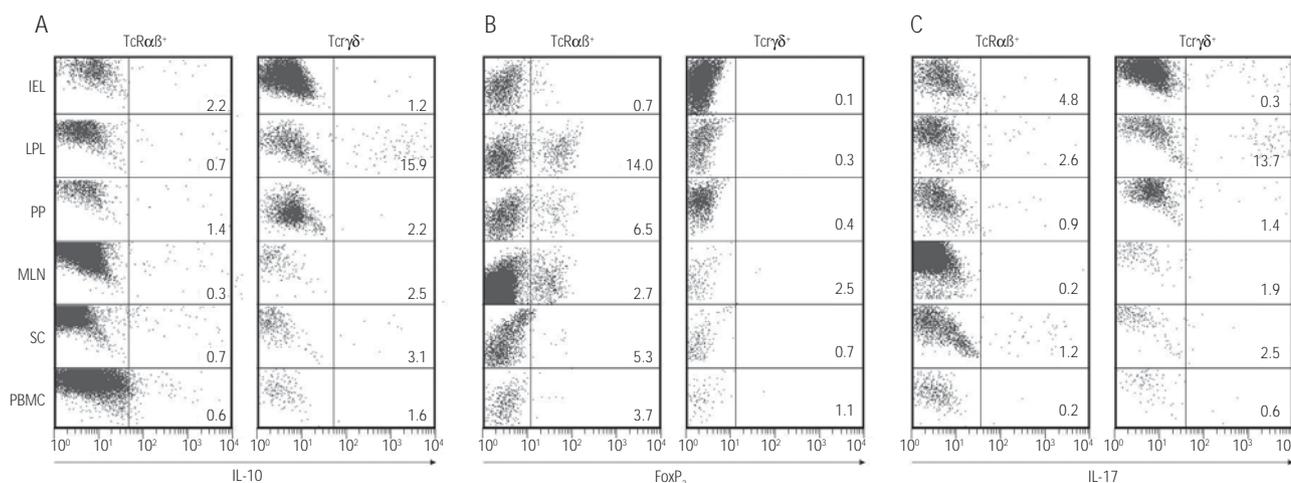


Figure 7. Interleukin (IL) 10 (A) FoxP3 (B) and IL-17 (C) production in T cell subpopulations from intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patch (PP) cells, mesenteric lymph node (MLN) cells, spleen cells (SCs) and peripheral blood mononuclear cells (PBMCs). Cells were activated for 4 hours with PMA/ionomycin, cytokines were measured after intracellular staining by flow cytometry. Cells from each compartment were gated on TcR $\alpha\beta$  or TcR $\gamma\delta$  cells and then analyzed for IL-10, FoxP<sub>3</sub>, and IL-17. Results from a representative experiment of 3, with cells pooled from at least 3 mice, are shown.

were found in all of the compartments analyzed except in the IELs. Interestingly, FoxP3 was highly expressed in CD4<sup>+</sup> TcR $\alpha\beta$ <sup>+</sup> LPLs (14.0% $\pm$ 0.7) compared to cells in the systemic compartment (PBMCs, 3.7%, SCs, 5.3%).

## Discussion

The analysis of T cell phenotypes from various locations of the gut, as well as from the spleen and peripheral blood has shown a specific phenotype for IELs and LPLs, characterized

by a predominance of TCR $\gamma\delta$ <sup>+</sup> CD8 $\alpha$ <sup>+</sup> IELs. IELs were also found to specifically express the activation markers CD44, CD69, and CCR9. In addition, CD8 $\alpha$ <sup>+</sup> T cells located in the LP were found to secrete IL-10 and IL-17, suggesting a T<sub>H</sub>17 phenotype, while CD4<sup>+</sup> LPLs were FoxP3 positive, suggesting a regulatory function.

The mucosal immune system of the gut is divided into 2 main compartments, each with a specific immune function: an inductive site, constituted by organized GALT comprising PPs, MLNs, the appendix, and isolated lymphoid follicles; and an effector site constituted by a lymphocyte population

scattered among 2 large intestinal structures: the LP and the epithelium [16]. In the inductive site, antigens are sampled and can activate T cells, while in the effector site, lymphocytes mainly contribute to inflammation and regulation by various pathways. This structural concept was initially based on the distribution of lymphocytes within inductive sites, contributing to an activation of B cells and leading to secretory immunoglobulin A production [16]. Subsequent studies have linked specific T cells to various sites in the gut and at least in part revealed specific functions for each population [17,18]. In addition, an intriguing subpopulation of CD8<sup>+</sup> TCR $\gamma\delta\alpha\alpha$ <sup>+</sup> cells has been described in the gut epithelium [19]. These cells are mostly anergic, and most probably play a role in gut homeostasis. The study reported here aimed to define the characteristics of T cells from various locations of the gut in order to gain a better understanding of the various players involved in immune homeostasis in naïve mice. In agreement with previous data, we showed that PBMCs, SCs, and MLN cells bear a majority of T cells expressing TcR $\alpha\beta$ , with up to two-thirds of CD4<sup>+</sup> cells. In these compartments, TcR $\gamma\delta$ <sup>+</sup> cells accounted for under 5% of the total number of T cells. Up to two-thirds of T cells in PPs and the LP expressed CD4 and the frequency of TcR $\gamma\delta$ <sup>+</sup> cells was between 10% and 20%. IELs were characterized by a predominance of CD8<sup>+</sup> cells with large numbers of CD8 $\alpha\alpha$  T cells. In addition, half of the cells expressed TcR $\gamma\delta$ . This peculiar T cell distribution suggests the presence of 2 major intestinal immune compartments: an inner compartment including the PPs and LP, located just below the epithelial barrier, and an outer compartment comprising the T cells located in the epithelium. Similar characteristics were noted for MLN cells and PBMCs (i.e. the peripheric system). The outer intestinal compartment was characterized by a large frequency of TcR $\gamma\delta$ <sup>+</sup> and CD8<sup>+</sup> T cells.

Previous studies have shown that the distribution of TcR $\alpha\beta$ <sup>+</sup>, TcR $\gamma\delta$ <sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells in the small intestine epithelium is relatively constant between mice from different strains and of the same age [20]. CD8 $\alpha\alpha$ <sup>+</sup> cells are not normally detected in the spleen or lymph nodes but are constitutively expressed on the majority of T cells residing within the epithelium of the small intestine [21]. Moreover, CD8 $\alpha\alpha$ <sup>+</sup> IELs are predominant in the mucosal T cell population of young mice, as we found in our study. In older animals, they are gradually replaced by conventional CD8 $\alpha\beta$ <sup>+</sup> T cells [22].

Co-expression of CD8 $\alpha\alpha$  and TcR diminishes or abolishes T cell activation by TcR. This was suggested by experiments where the constitutive expression of CD8 $\alpha\alpha$  in IELs permanently increased the minimal signal level required for restimulation of antigen-experienced T cells and thus downregulated the magnitude of the immune response [23]. In vitro experiments showed that the interaction of CD8 $\alpha\alpha$ <sup>+</sup> T cells with the thymic leukemia antigen on epithelial cells promotes the survival of activated T cells [24]. In the intestinal compartment, this marker is constitutively expressed by epithelial cells, prolonging their survival [25]. All these observations suggest that they act as memory precursors during primary activation [26].

TcR $\gamma\delta$ <sup>+</sup> cells are the first T cells to colonize the epithelium during embryogenesis and have a thymic and extrathymic origin. This epithelial T cell population is characterized by

limited gene rearrangement and is activated by a nonclassical MHC pathway, suggesting that the number of ligands may be limited [27]. Intestinal TcR $\gamma\delta$ <sup>+</sup> cells are implicated in the regulation of intestinal homeostasis and local inflammatory responses, and participate in epithelium maintenance after damage [28].

Our data analyzed in the light of previous publications show that the outer compartment is colonized by a large fraction of unique T cells which differ from peripheral T cells by their peculiar phenotype characteristic of cells known to have a low response to the antigenic stimulation of the gut lumen. In addition, we found that naïve IELs expressed a natural activation pattern with expression of CD44 and CD69.

The inner compartment (the LP and PPs) is colonized by 2 major T cell populations. First, CD4<sup>+</sup> T cells expressing TcR $\gamma\delta$  and T cells with similar characteristics to those seen in the epithelial compartment, and second, CD4<sup>+</sup> TcR $\alpha\beta$ <sup>+</sup> T cells similar in their memory-like phenotype to those isolated from the peripheral compartment. This suggests that these cells were already activated and resident in these compartments. The presence of TcR $\gamma\delta$ <sup>+</sup> cells in the inner compartment could be due to contamination of epithelial cells during the isolation protocol, but cells originating from such a contamination would not express CD8 $\alpha\alpha$ . A more likely explanation would be the migration of TcR $\gamma\delta$ <sup>+</sup> cells between intestinal compartments.

We found a similar pattern of classical T cell phenotypes in MLN cells as well as in blood-borne T cells. The MLN cells did not express an activation pattern, suggesting that they exert a similar function to cells located in the peripheral compartment (eg, PBMCs). These data suggest that cells colonizing the MLN originate from and recirculate to the peripheral compartment and are not previously activated intestinal cells such as those found in the inner intestinal compartment.

It is suspected that TcR $\gamma\delta$ <sup>+</sup> cells play an immunoregulatory role in the gut and in particular in the homeostasis of the immune response of TcR $\alpha\beta$ <sup>+</sup> cells. This has been demonstrated in TcR $\delta$ -deficient mice infected by the parasite *Eimeria*, in which the severity of infection was similar to that in control mice. However, increased inflammation was also observed in the TcR $\delta$ -deficient mice, suggesting that TcR $\gamma\delta$  cells regulate TcR $\alpha\beta$  T cell-mediated response in the epithelium [29]. Consequently, we were interested in characterizing intracellular cytokine secretion by flow cytometry and analyzing the presence of the Tregs marker, FoxP3, in naïve TcR $\gamma\delta$ <sup>+</sup> cells and TcR $\alpha\beta$ <sup>+</sup> cells after activation. Interestingly we observed a differential phenotypic response to PMA/ionomycin-induced activation of these markers, with TcR $\gamma\delta$ <sup>+</sup> LPLs expressing IL-10 and IL-17, and TcR $\alpha\beta$ <sup>+</sup> LPLs expressing FoxP3, but without upregulation of CD25. Further experiments showed that IL-10- and IL-17-expressing cells were CD8 $\alpha\alpha$ <sup>+</sup>, while FoxP3<sup>+</sup> cells were CD4<sup>+</sup>. IELs and LPLs did not secrete any other of the cytokines analyzed (IL-4 or IFN $\gamma$ ). These results clearly show that similar types of cells might behave differently according to their localization, with only TcR $\gamma\delta$ <sup>+</sup> LPLs showing response in our experiments. Our results are in agreement with previous reports that TcR $\gamma\delta$ <sup>+</sup> cells might be a major source of IL-17. Indeed, such cells have been found to secrete IL-17 in the lung in *Mycobacterium tuberculosis* infection [30] and in *Escherichia coli*-infected mice [31]. Splenic lymphocytes

from naïve mice have also been found to be major producers of IL-17 after PMA/ionomycin stimulation [32]. The role of IL-10 has been extensively demonstrated in the regulation of inflammation in the gut, as in IL-10-deficient mice showing features of inflammatory bowel disease cured by exogenous supplementation of IL-10 [33]. TcR $\gamma\delta^+$  IELs have been shown to be essential for self-tolerance in experimental diabetes [34], while TcR $\gamma\delta^+$  IELs have been found to secrete large amounts of IL-10 [35].

Considering our results and the current knowledge regarding the various types of T cells resident in the gut, we hypothesize that there is a gradient of CD8 $^+$ , TcR $\gamma\delta^+$ , and TcR $\alpha\alpha^+$  cells from the epithelium through the inner compartment of the gut towards the systemic immune system, and an opposite gradient of CD4 $^+$  and TCR $\alpha\beta^+$  cells. In addition, there is a differential pattern of activated cells in naïve mice with regard to activation molecules and cytokine secretion, suggesting that gut homeostasis is dependent on a fine balance of T cells based on their location as well as on TcR $\gamma\delta$  or TcR $\alpha\beta$  expression.

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