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Lymphoid cells in afferent and efferent intestinal lymph: lymphocyte subpopulations and cell migration

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SUMMARY

Gut wall emigrating cells have been characterized in the intestinal lymph. The intestinal lymph duct was cannulated in 6-month-old minipigs. Under non-restraining conditions the efferent lymph from the mesenteric lymph nodes was collected in seven normal animals. Lymph coming directly from the gut (afferent lymph) was also collected in 18 pigs after resection of the mesenteric lymph node chains 3 months previously. The intestinal lymph flow was similar in both groups (around 18 ml/h). The lymphoid cell yield was $1{\cdot}2 \pm 1{\cdot}0 \times 10^6$/h in control animals, while in mesenteric lymph node resected pigs it was around 20 times higher ($26{\cdot}2 \pm 17{\cdot}6 \times 10^6$/h). In the gut-derived lymph $76{\cdot}5 \pm 8{\cdot}8\%$ T lymphocytes were observed ($CD4^+$, $48{\cdot}1 \pm 15{\cdot}5\%$; $CD8^+$, $53{\cdot}6 \pm 12{\cdot}7\%$). The percentage of immunoglobulin-positive cells was lower (IgM^+, $10{\cdot}1 \pm 4{\cdot}5$; IgA^+, $1{\cdot}7 \pm 1{\cdot}1$). In 14 mesenteric lymph node resected pigs a mean of $5{\cdot}6 \pm 3{\cdot}1 \times 10^8$ lymphocytes from the gut lymph were labelled *in vitro* with a fluorescent dye and retransfused. The labelling index of fluorescent cells in the intestinal lymph increased rapidly and remained at a high level until 44 h after cell transfusion. A four-to-ten times lower labelling index was found in the spleen, various lymph nodes and Peyer's patches. Most of the recovered lymphocytes were T cells. This model provides access to the cell pool leaving the gut wall, thus allowing an examination of its role in the gastrointestinal tract and other mucosal-lined organs.

Keywords intestinal lymphatics lymphocyte migration pig lymphocyte subsets

INTRODUCTION

The intestinal immune system has the capacity to protect the organism against the invasion of many luminal antigens. The cooperation of the gut immune system's afferent branch, the organized lymphoid tissue (Peyer's patches (PP), isolated follicles and appendix), with the efferent branch, the lymphocyte populations in the lamina propria (LP) and the epithelium of the gut wall, is crucial for an effective immune response (for review see [1,2]). Migrating lymphoid cells connect the different parts of the mucosal immune system. Several studies have concentrated on the cell immigration to the PP and LP or on the cells leaving the gut wall. Using the frozen section assay it was observed that mucosa-derived lymphocytes preferentially migrate back to gut-associated lymphoid tissues [3,4]. There is evidence that high endothelial venule-like vessels mediate the immigration of lymphocytes to the LP of the gut [5]. The migration of stimulated or newly formed lymphocytes derived from the PP or the mesenteric lymph nodes has been examined using ^{3}H-thymidine as a label for cells synthesizing DNA. It is difficult to study subpopulations of the migrating blasts because a combination of autoradiography and immunocytology is required. Some of these cells are B blasts, becoming IgA-producing plasma cells for the secretory immunity in the LP (for review, see, for example [6]), but a marked population of T blasts was also observed [7]. Other techniques are necessary to examine gut wall emigrant cells. All cells in the gut wall can be labelled *in situ* by perfusing the tissue with a fluorescent dye using an extracorporal perfusion system or by injecting small doses of the dye directly into the tissue [8,9]. The phenotype and migration kinetics of the lymphoid cells leaving the labelled area can be studied easily using these methods. In sheep and rats these cells are mainly B lymphocytes and they migrate to all lymphoid organs within the body [9,10]. The experimental approaches described above do not, however, provide information about the high numbers of cells migrating through the gut wall. Although small lymphocytes represent the largest portion of cells leaving the intestinal wall, little is known about their function, their phenotype or their migratory behaviour [11].

The first aim of the present study was to characterize all lymphoid cells emigrating via lymphatics from the gut wall and from the mesenteric lymph nodes. The experiments were designed to be carried out over a long period with the animals awake and under non-restraining conditions, to minimize the effects of stress. Another aim was to examine the migration

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properties of lymphocytes that had emigrated from the intestinal afferent lymph. The experiments can be used as a basis for future characterization of the influence of intestinal antigens on the recirculation of lymphocytes through the compartments of the mucosal immune system. The dysregulation of lymphocyte recirculation through the gut wall may be a major pathophysiological process in mucosal diseases. Such a study can only be done in an animal model. The pig was used because it has several advantages: (i) it is an omnivorous animal; (ii) MoAbs are available for the different lymphocyte subsets [12]; (iii) although it has two types of PP, the development of both these PP does not show such a distinct difference as observed in sheep [13,14]; (iv) it is large enough for a selective cannulation of the major intestinal lymph duct to be performed and for the lymph to be collected without using metabolism cages; (v) as the lymph coming from the mesenteric lymph nodes is normally poor in lymphocytes [15,16], it is certain that after mesenteric lymphadenectomy almost exclusively afferent intestinal lymphocytes are recovered from the intestinal lymph duct.

MATERIALS AND METHODS

Animals and surgical techniques

In these experiments, 25 female minipigs of the Göttingen breed were used. Seven animals served as controls. To obtain gut-derived lymph in 18 minipigs the mesenteric lymph nodes (mLN) were removed as described previously [16]. In brief, at an age of 3 months the animals were laparotomized under i.v. thiobarbiturate anaesthesia (Trapanal; Byk Gulden, Konstanz, Germany). The lymph nodes of the right and left side of the mesentery were gently removed, and the blood supply of the mLN was interrupted by electrocauterization. The peritoneum was closed again with fine resorbable sutures (Dexon; Braun-Dexon GmbH, Spangenberg, Germany). The incision was closed using resorbable sutures and standard techniques. The animals recovered quickly, showing no clinical signs and no differences in weight gain in comparison with their non-operated group mates. At around 6 months of age (animal weight $21{\cdot}5 \pm 7{\cdot}2$ kg) the intestinal lymph duct cannulation was performed. The animals were starved for 40 h, but were given water *ad libitum*. Around 12 h before the operation the animals received a small amount of food mixed with vegetable oil. The duct could then be detected more easily during the operation because the lymph was white due to the resorbed oil [17]. Under i.v. thiobarbiturate anaesthesia a vena sectio of the external jugular vein was established. After a midline laparotomy the parietal peritoneum was opened below the pancreas near the confluence of the left renal vein and the posterior vena cava. To the left of the vena cava the intestinal lymph duct was found within the fatty connective tissue, running from the dorsal side of the pancreas caudally to the cysterna chyli. A special cannula (clear vinyl tube, diameter 1·6 mm (Dural Plastics and Engineering, Dural, Australia) in a silastic leading tube (Silastic; Dow Corning GmbH, Meerbusch, Germany)) was put into the duct and fixed there with fine sutures (5/0 Dexon; Braun-Dexon GmbH, Spangenberg, Germany). The cannula was exteriorized through an incision in the right abdominal wall. The abdominal wall was closed by consecutively suturing the different layers, and the cannula was fixed to the skin. The end of the cannula was put into a collecting flask that had been fixed with the help of a bag on the right flank of the animal. A special screw cap with a fitting for the cannula was used to prevent spillage or contamination of the lymph. After the cannulation the animals were kept in a normal box under non-restraining conditions. They had free access to water and food and were mostly in good condition.

Collection and examination of lymph samples

After implanting the cannula a first lymph sample was collected during the operation. Then the lymph was collected in 250-ml flasks containing 5 ml of sterile RPMI 1640 (Seromed Biochrom KG, Berlin, Germany) supplemented with antibiotics (6000 I.E. penicillin, 6 mg streptomycin, 75 μg amphotericin, 3 mg gentamycin, Seromed) and 1500 I.E. heparin to prevent clotting (Liquemin N 25 000; Roche, Grenzach-Wyhlen, Germany). The lymph bottle was changed at 8.00 a.m. and 7.00 p.m. The lymph volume was determined and the cells were washed twice (centrifugation 400 ***g***, 10 min, resuspension in RPMI 1640). After the second washing the cells were resuspended in a defined volume of RPMI 1640. The numbers of nucleated cells, lymphocytes and erythrocytes were determined using a haemocytometer and a phase contrast microscope at $\times$ 500 magnification. Differential counts to determine the proportion of lymphoid and non-lymphoid cells were carried out on Giemsa-stained cytocentrifuge preparations (Heraeus Sepatech, Osterrode, Germany).

Lymphocyte migration study

The afferent intestinal lymph collected in the sampling periods from 8 to 44 h after the operation was used for the cell migration study in 14 mesenteric lymph node resected pigs (mean weight $25{\cdot}3 \pm 5{\cdot}6$ kg). The cells were kept under sterile conditions. The washed cell suspensions were resuspended in standard RPMI 1640 and pooled. They were incubated at a concentration of 1×10^8 nucleated cells/ml with 50 μg FITC (Isomer I; Sigma, Munich, Germany) at 37°C for 15 min [18]. After washing, the number of nucleated cells and lymphocytes was determined in the suspension and $5{\cdot}6 \pm 3{\cdot}1 \times 10^8$ lymphoid cells were retransfused to the animals via the i.v. cannula.

Cell suspensions of intestinal lymph and lymphoid organs

The lymph was collected for around 90 h. The lymphocyte migration experiments finished either 20 h or 44 h after retransfusion of the *in vitro* labelled cells. The animals were killed by an overdose of Trapanal. A blood sample was taken and the lymphocytes were separated by Ficoll–Isopaque (Ficoll Density 1·077, Seromed) centrifugation [19]. Cell suspensions of the spleen, mesenteric lymph nodes, inguinal lymph nodes, bronchial lymph nodes and jejunal and ileal PP were prepared by gentle mincing and washing with RPMI 1640 (400 ***g***, 10 min) [19].

Immunocytological staining and flow cytometry

Indirect immunocytological staining was used to determine the lymphocyte subpopulations in the lymph samples [19]. In a microtitre plate for each subset a volume of $1{\cdot}5 \times 10^6$ cells from the suspensions was incubated for 30 min at 4°C with the different MoAbs against pig lymphocytes (CD2, CD4, CD8, IgM and IgA [12]). As second antibody a PE-labelled goat anti-mouse, diluted 1:50 (Dianova GmbH, Hamburg, Germany) was used. The cells were analysed with a flow cytometer (FACScan, Becton Dickinson, Erembodegem, Belgium). The

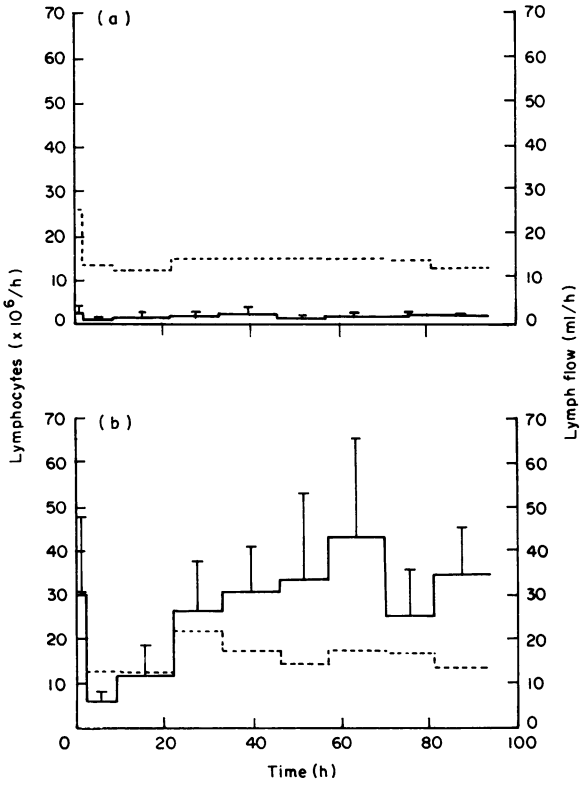

Fig. 1. Lymph flow/h and hourly output of lymphocytes from the intestinal lymph duct in (a) control minipigs and (b) minipigs after resection of the mesenteric lymph nodes (mLN). ——, Lymphocytes/h; ....., lymph flow/h.

lymphocytes were gated based on their forward and side scatter properties, and the cells positive for the specific antibody determined in the red fluorescence channel. For the cell migration study, in the lymph samples and the lymphoid organ suspensions the proportion of $FITC^+$ lymphoid cells was determined in the green fluorescence channel. To take account of the varying numbers of retransfused cells ($1{\cdot}7$–$13{\cdot}6 \times 10^8$ labelled cells) the labelling indices determined were recalculated based on a mean of $5{\cdot}6 \times 10^8$ retransfused cells (index/injected cell dose $\times 5{\cdot}6$) to facilitate the comparison between animals.

RESULTS

After the duct cannulation the pigs showed no clinical problems. During the whole collection period it was not necessary to restrain the pigs, even when the lymph collection bottle was changed. The effect of the successful reanastomosis of afferent and efferent lymphatics after removal of the mLN was tested by subserosal injection of Berlin blue dye at the end of the experiment. Only in mLN resected animals could the dye be observed in the duct cannula within 5 min after injection. The lymph flow in control pigs was $15{\cdot}3 \pm 5{\cdot}1$ ml/h and in mLN-resected animals $18{\cdot}1 \pm 9{\cdot}5$ ml/h (Fig. 1a, b). Some experiments failed due to obstruction of the cannula by coagulation (animals not included). In all animals of the present study a permanent lymph flow was observed during the whole experiment. The duration of the experiments ranged from 65 h to 94 h ($83{\cdot}4 \pm 12{\cdot}5$ h). In the lymph of both animal groups a marked number of erythrocytes was observed, ranging between 1 and

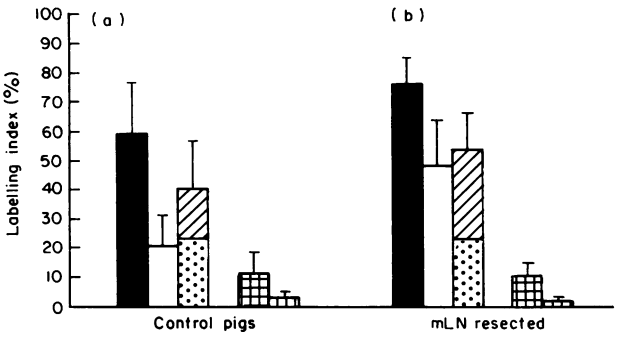

Fig. 2. Lymphocyte subsets in intestinal lymph in (a) control minipigs ($n=6$) and (b) minipigs after mesenteric lymph node (mLN) resection ($n=8$). The results are given as mean ± s.d. of all lymph samples collected from the intestinal lymph duct in all minipigs of each group. ■, $CD2^+$; □, $CD4^+$; ⊠, $CD8^+$ bright; ▩, $CD8^+$ dull; ⊞, IgM^+; ▥, IgA^+.

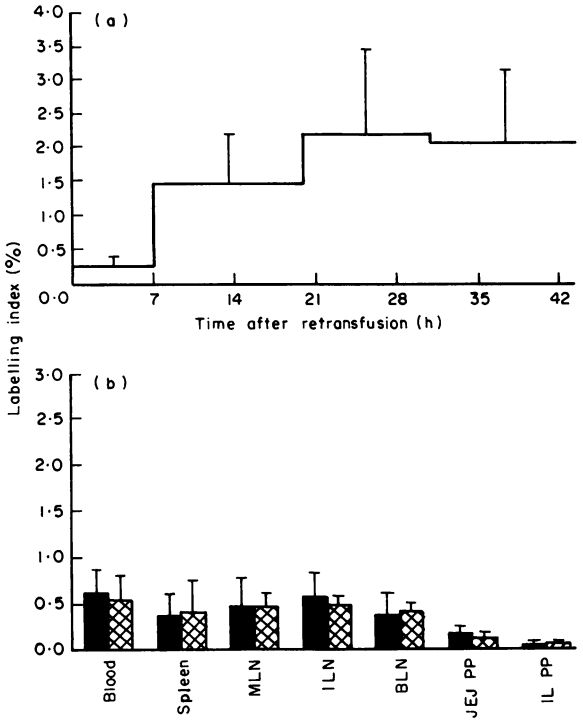

Fig. 3. $FITC^+$ lymphocytes after *in vitro* staining and retransfusion in (a) intestinal lymph and (b) various lymphoid organs up to 20 h ($n=7$) or 44 h ($n=6$) after cell retransfusion. mLN, Mesenteric lymph nodes of the large intestine; ILN, inguinal lymph node; BLN, bronchial lymph nodes; JEJ PP/IL PP, Peyer's patches of the jejunum or ileum. ⊠, 20 h; ■, 44 h after cell transfusion.

165×10^6/h in the first 55 h of the experiments. The erythrocyte yield in the lymph in some of the mLN-resected animals reached up to 870×10^6/h. The average lymphocyte yield was $1{\cdot}20 \pm 1{\cdot}0 \times 10^6$/h in control pigs, but in mLN-resected pigs it was around 20 times higher ($26{\cdot}2 \pm 17{\cdot}6 \times 10^6$/h; Fig. 1a, b). In all animals a decrease in the lymphocyte yield but not in the lymph flow was observed in the second and third sample. Among the lymphoid cells there were 1% plasmoblasts in

Table. 1. Migration of gut-derived lymphocytes: lymphocyte subsets of injected cells and of recovered FITC$^+$ cells.

	Injected cells			Recovered FITC$^+$ cells in gut lymph			Number of analysed
Pig no.	CD2$^+$	IgM$^+$	IgA$^+$	CD2$^+$	IgM$^+$	IgA$^+$	lymph samples
1	74·5	12·5	2·6	75·4	1·2	3·4	4
2	69·0	11·0	2·4	77·0	1·6	2·4	2
3	51·0	2·9	1·4	66·2	0·2	1·1	1
4	55·8	4·8	0·7	87·1	0·6	2·1	2
5	63·0	3·8	0·6	74·0	1·1	3·6	4

Results obtained in five minipigs by flow cytometry. For the immunocytological subset staining a PE-labelled second antibody was used (red fluorescence). After gating on FITC$^+$ cells (green fluorescence channel) the subset distribution of these cells was analysed in the red fluorescence channel of the flow cytometer.

control animals *versus* 4% in mLN-resected pigs. In the latter animals a marked proportion of mitotic figures was seen, showing the high stimulation of the afferent lymph. Neutrophils were also observed, their hourly output being comparable in both animal groups ($1{\cdot}4$–$7{\cdot}8 \times 10^6$/h). There was an initial increase in neutrophils/h, but their absolute numbers decreased after the second day of the experiment. In the afferent lymph veiled cells were sometimes observed. However, it was difficult to ascertain their morphology on the routine Giemsa-stained cytospots.

Lymphocyte subsets

The analysis of lymphocyte subsets in the intestinal lymph samples revealed interesting results. The pattern of the cell composition of afferent and efferent lymphocytes was comparable (Fig. 2). Most of the cells were CD2$^+$ T cells (control, $59{\cdot}2 \pm 17{\cdot}2$%; mLN-resected, $76{\cdot}5 \pm 8{\cdot}8$%). CD4$^+$ lymphocytes were outnumbered by CD8$^+$ cells. However, the staining was dull on some of the CD8$^+$ cells (CD8^{bright}/CD8^{dull}, control pigs 22·9%/17·1%; mLN-resected 22·6%/30·9%). The sum of CD4$^+$ and CD8$^+$ (bright + dull) cells equalled the T cell number in control pigs (Fig. 2), though it was higher in mLN-resected pigs (CD2$^+$, 76·5%; sum of CD4$^+$ and all CD8$^+$, 101·7%). These calculations provide evidence of a CD4$^+$CD8$^+$ double-positive subset in the mLN-resected animals.

Cell recirculation experiments

Eight hours after cell injection the labelling index in the lymph was around 0·25%. It increased up to 1·5% within the next 12 h and remained at that high level for up to 44 h (Fig. 3a). The index in the blood, spleen and mesenteric lymph nodes ranged from 0·4% to 0·6%. In the jejunal and ileal PP the index was only 10% of that in spleen and mLN (Fig. 3b). However, only 5% of the injected cell dose at most was recovered in the afferent lymph within the observation period of 44 h.

In some experiments the lymphocyte subsets of cells positive for FITC could be determined using the two-colour detection capabilities of the flow cytometer. In these experiments a gate was set on all FITC$^+$ lymphocytes. In comparison with the injected cell suspension, the labelled gut emigrants were mainly T cells (Table 1). The relative number of IgM$^+$ cells was reduced to 10% of the IgM$^+$ cell number in the inoculum. The IgA$^+$ cell number was comparable. In the mLN and the blood few if any FITC$^+$IgA$^+$ or FITC$^+$IgM$^+$ double-positive cells were found (0–1·5%).

DISCUSSION

The reanastomosis of mesenteric afferent and efferent lymphatics which occurs naturally after lymph node resection provides access to the cells coming directly from the gut wall. In pigs, successful collection of the gut lymph can easily be ensured due to the high lymphocyte numbers after mLN resection in comparison with the low cell number leaving the mLN via lymphatics in control pigs [15,16]. In sheep and rats [20,21], a few lymph nodes left *in situ* can lead to a mixture of both cell populations, because there is a comparably high number of lymphocytes in both lymph portions.

In this study the lymph flow and the lymphocyte yields in control and resected animals were comparable to those reported in earlier studies [15–17]. It is not known why pigs have such a high amount of erythrocytes, even in the lymph leaving the lymph node. Cannulation studies in pig lymphatics were often unsuccessful because of cannula obstruction due to rapid clotting [15]. Although the lymph flow was constant in the first period of the experiment, the lymphocyte yield decreased. This may be due to reduced peristalsis after the operation. The initial rise in the number of neutrophils was obviously caused by surgical trauma.

In mLN-resected animals the afferent gut lymph contained a marked number of plasmoblasts. Together with the mitotic figures only observed in this experimental group, this reflects that the cells leaving the gut are stimulated by antigen contact. Calculations revealed the presence of CD4 CD8 double-positive lymphocytes among gut-emigrant cells. This observation was not surprising, because double-positive lymphocytes have previously been observed in different lymphoid organs and the blood in pigs [22]. The appearance of this subset is additional evidence of the stimulation of the cells leaving the gut. After *in vitro* stimulation of pig lymphocytes an increased proportion of CD4$^+$CD8$^+$ lymphoblasts was detected [22]. In this double-positive population Saalmüller *et al.* [22] detected only a low expression of CD8. Therefore, it can be concluded from the

present experiments that the $CD8^{dull+}$ gut-emigrant cells are positive for CD4 too.

It is interesting that the major portion of T cells in the pig's intestinal lymph were $CD8^+$. This is different from sheep, where gut emigrants are mainly $CD4^+$ [11]. In rats it has been reported that the major portion of T cells emigrating from the *in situ*-labelled PP are $CD8^+$ [9]. However, the present results are in accordance with other results in pigs, where the $CD8^+$ cells outnumbered the $CD4^+$ subset in lymphoid organs and in the blood [19,23]. Among all cells leaving the gut the immunoglobulin-positive cells represented only a small portion, as has been reported in sheep [11,24]. The small amount of immunoglobulin-positive cells in gut lymph in comparison with the high percentage of immunoglobulin-positive cells in PP emigrants [9,10] is evidence that there has to be another source of preferentially T cells—possibly the LP—that contributes to the gut-emigrating lymphoid cells.

A further interesting aspect of this study is the recirculation pattern of the gut-derived lymphocytes in mLN-resected animals. The highest relative number of $FITC^+$ cells was found in the afferent lymph. The fact that many labelled lymphocytes have been in other compartments of the pig's body and start their emigration from the gut as much as 40 h after the cell transfer raises the question of preferential or merely random migratory patterns of gut-derived lymphocytes. The composition of the recovered cells showed that high numbers of T cells are recirculating through the gut wall. Recently, a large proportion of ^{3}H-thymidine-labelled specific T lymphoblasts were found in the villus LP of the gut wall within the first hours after injection in rats [25]. Later they could not be found there. A very interesting fact is the relative disappearance of the IgM^+ cells from the lymph. It could be expected that the immunoglobulin-positive gut emigrants would settle in the different lymph node groups to further mature. However, in mesenteric lymph nodes draining the large intestine that had not been resected during the first operation, the labelling index of $FITC^+IgM^+$ or of $FITC^+IgA^+$ cells was extremely low. Further experiments should concentrate on cell immigration to the LP of the gut wall to elucidate where the IgM^+ cells go and whether they undergo an isotype switch to IgA^+ plasmoblasts.

The pig model presented here can be used for further characterization of the cell traffic between the different parts of the gut immune system. This traffic is necessary for the development of lung immunity after oral vaccination [26]. For further understanding of the regulation of an intestinal immune reaction, it has to be clarified whether the gut emigrants mainly come from the PP or from the LP of the mucosa. Antigenic influences on the large pool of T cells recirculating through the compartments of the intestinal mucosa have to be analysed. There is evidence that these T cells are necessary for the specific homing of lymphoid cells of the B lineage to the LP [27].

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