Proliferating intestinal γ/δ T cells recirculate rapidly and are a major source of the γ/δ T cell pool in the peripheral blood

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The proliferation, recirculation and repertoire of gut-derived γ/δ T cells were studied in pigs *in vivo*. Proliferating γ/δ T cells (detected by BrdU labeling) are present in all intestinal compartments. In the gut lymph ~0.5% of all γ/δ T cells were proliferating. These gut-derived BrdU$^+$ γ/δ T cells re-enter the intestinal tissues, and re-appear in the intestinal lymph far more often than other cells: about 22% of i.v.-injected BrdU$^+$ γ/δ T cells were recovered again from the intestinal lymph within 72 h (compare with BrdU$^+$ B cells 2%, and other BrdU$^+$ T cells 10%). The contribution of the gut to the migrating γ/δ T cell pool in the blood became obvious: the proportion of BrdU$^+$ γ/δ T cells was three-times larger in control *versus* cannulated pigs. In 9-month-old pigs, clonally expanded T cells were identified in the intestine by complementarity-determining region 3 spectratyping of TCR-δ transcripts. Such expansions were not visible in the blood or intestinal lymph. The distribution of γ/δ T cells within the intestinal tract is likely to depend to a large degree on the proliferation and the migratory properties of these cells which are different to those of α/β T cells and B lymphocytes.

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1 Introduction

The functional role of γ/δ T cells is a matter of debate, but their antigen specificity, *e.g.* for food components [1], and their location along the mucosal surfaces are evidence of their role in the maintenance of the mucosal barriers [2]. The frequency of γ/δ T cells in various tissues — especially as intraepithelial lymphocytes (IEL) in the intestinal wall — and in the blood shows a wide range in different species [3]. In humans, the TCR-δ repertoire undergoes major changes with age and becomes oligoclonal in the adult intestine [4]. The thymic or intestinal origin and the migration of γ/δ T cells has still to be elucidated [5, 6]. The expression of CD8αα is thought to be a putative indicator for the extra-thymic origin of a γ/δ T cell subset [7]. Very little is known about the recirculation of γ/δ T cells through the compartments of the intestine such as the epithelium, lamina propria and the Peyer's patches (PP) and through other organs of the body [8].

To understand the contribution of the intestine to the thymus-independent development of γ/δ T cells, studies in parabiotic mice were previously performed, and it was demonstrated that the frequency of mixing of γ/δ T cells between the animals in parabiosis is low in the epithelium of the gut. A higher exchange of cells was observed in the lamina propria, whereas in the PP as well as in lymph nodes there was an exchange of the cells by 50%. Based on these experiments the hypothesis was put forward that γ/δ IEL develop *in situ*, and it was assumed that lamina propria γ/δ T cells are also to a marked extent derived from progenitors in the epithelium or the lamina propria itself [9].

Although the exchange of cell populations between the parabiotic animals was demonstrated in that study, the samples examined reflect only the static situation at the time of sampling. No migratory properties of defined subsets are available. Thus, it may well be true that the IEL remaining in the tissue during the observation period had entered this site earlier and had a long half-life in the epithelium, as observed by Penney et al. [10]. The rele-

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Abbreviations: CDR3: Complementarity-determining region 3 **IEL:** Intraepithelial lymphocyte **PP:** Peyer's patches

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vance of the recirculation and half-life in the tissue of γ/δ T cells for inducing immunity may be elucidated by the fact that several weeks after a vaccination against SIV in monkeys, a marked number of γ/δ T cells was seen within the lamina propria of the large intestine [11].

In our study we made an attempt to follow the traffic of γ/δ T cells through the compartments of the intestine in adult minipigs. This animal model eliminates the disadvantage of single observations at the end of an experiment and we were able to focus on migrating lymphocytes. With our model it is possible to collect directly those lymphoid cells emigrating from the intestinal wall in unrestrained animals for up to 2 weeks. Using this model we have already demonstrated that a large number of migrating T cells — proliferating and non-proliferating — emigrate into the intestinal lymph and that many lymph-derived IgA$^+$ plasma cell precursors accumulate in the intestinal lamina propria *in vivo* [12, 13]. In addition, a new concept for intestinal lymphocyte proliferation and migration was suggested [14].

Our experiments aimed to prove the hypothesis that lymphocyte recirculation is a crucial event for the distribution of proliferating gut γ/δ T cells that have their origin in the gut or might represent proliferating memory cells. Several important observations were made: (1) γ/δ T cells proliferate in the different compartments of the intestine; (2) γ/δ T cells originating from the intestine have a unique recirculation pathway — they quickly migrate back to the gut and exit rapidly again via intestinal lymph; (3) proliferating γ/δ T cells from the intestine are the major source of proliferating γ/δ T cells in the peripheral blood; (4) complementarity-determining region 3 (CDR3) spectratyping demonstrates that the subsets of intestinal γ/δ T cells are clonally expanded.

2 Results

2.1 γ/δ T cell recirculation study

Intestinal lymph contains a marked proportion of several γ/δ T cell subsets (see Table 1). The migratory pathways of these γ/δ T lymphocytes taken from intestinal lymph were analyzed in a cell recirculation study. The following lymphocytes populations were studied: all lymphocytes, γ/δ T cells, and proliferating γ/δ T cells. In four pigs, the intestinal lymph was collected over a period of 56 h. All lymphocytes were pooled and labeled *in vitro* using FITC. In addition, within this pool the cells in the cell cycle were marked by incubation with BrdU. A total of 2.80±0.64×10^9 FITC$^+$ cells were retransfused. This cell population contained 1.22±1.08% BrdU$^+$ γ/δ T cells (~34.2×10^6 in absolute numbers).

Table 1. Percentage of porcine CD2$^-$ γ/δ cell subsets among T cells in lymph, various organs and the blood of pigs (group A, *n*=4)

Source	Mean ± SEM %
Intestinal lymph[a)]	6.6±2.3
CD8αβ$^+$ γ/δ T cells	1.1±0.6
CD8αα$^+$ γ/δ T cells	4.8±1.8
CD8$^-$ γ/δ T cells	1.3±0.6
Jejunal PP	2.2±0.3
Ileal PP	1.6±1.1
Jejunal lamina propria	5.3±0.8
Ileal lamina propria	5.4±1.4
IEL	3.1±0.3
Mesenteric lymph nodes	2.2±0.4
Spleen	6.2±4.0
Blood	28.9±6.6

[a)] Within the lymph CD2$^-$ γ/δ T cell population, the indicated proportions expressing different CD8 molecules are shown underneath.

The appearance of all retransfused FITC$^+$ cells was studied in the lymph, using flow cytometry (Fig. 1A). The labeling index of FITC$^+$ cells in the lymph reached the highest level 36 h after retransfusion and remained constant until the end of the observation period (Fig. 1A). The total γ/δ cell population showed a comparable pattern of re-appearance, albeit on a lower level, resulting in 1.5% γ/δ T cells among all emigrating cells in the lymph. This level remained stable for the next 3 days (Fig. 1B). The retransfused BrdU$^+$ gut-derived cells initially had the same kinetics as all FITC$^+$ γ/δ cells; however, after reaching a peak level between 24 and 36 h, the number of emigrating BrdU$^+$ γ/δ T cells decreased (Fig. 1B).

2.2 Quantification of the migrating BrdU$^+$ γ/δ T cells and their depletion in the blood pool after lymph-duct cannulation

It was possible to quantify the recovered pools of BrdU$^+$ γ/δ T cells in absolute numbers in intestinal lymph, based on the number of injected and recovered BrdU$^+$ γ/δ T cells. This quantification demonstrated the unique migratory pathway of the γ/δ T cells through the intestine: ~22% of the BrdU$^+$ γ/δ T cells were recovered, whereas the recovery of BrdU$^+$ IgA$^+$ lymphocytes (~2 %), and BrdU$^+$ CD3 T cells (~10 %) was much lower (Fig. 2A).

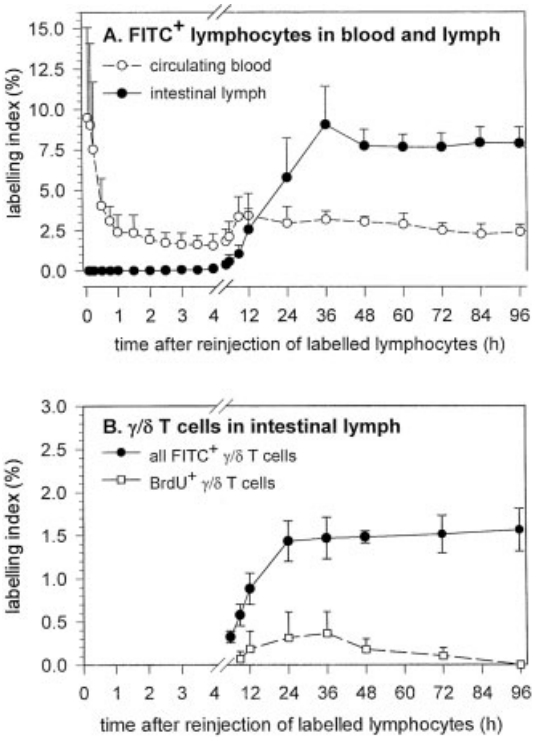

Fig. 1. Recirculation study after retransfusion of lymphocytes taken from gut lymph and labeled *in vitro* with FITC / BrdU. (A) The percentage of all FITC$^+$ recirculating cells was determined in the blood and intestinal lymph (mean ± standard deviation shown). (B) The proportion of FITC-labeled and of BrdU-labeled γ/δ T cells is different: after a peak at 36 h after cell transfer, the proportion of BrdU$^+$ γ/δ T cells decreases until 96 h (mean ± standard deviation shown).

A different approach was chosen to study the BrdU$^+$ γ/δ T cells further, and a depletion of this subset in the blood was observed. A group of four pigs (group B) with the intestinal lymph duct cannulated received a single dose of BrdU i.v. to label all cells in the S-phase of the cell cycle. Another group of three non-cannulated pigs served as controls. In both groups, blood samples were taken every 12 h to study the proportion of BrdU$^+$ γ/δ T cells. There was a marked reduction of the BrdU$^+$ γ/δ T cells in the blood in pigs with a lymph cannula running (Fig. 2B). These pigs had about 0.5% of this cell subset in the circulation whereas in control pigs the proportion of BrdU$^+$ γ/δ T cells was three-times higher. In absolute numbers the blood pool of BrdU$^+$ γ/δ T cells in control animals is 156×10^6 lymphocytes. The depletion of BrdU$^+$ γ/δ T cells shows a typical migration of this subset: the cells recirculate through the compartments of the intestine in a more efficient way than other subsets do.

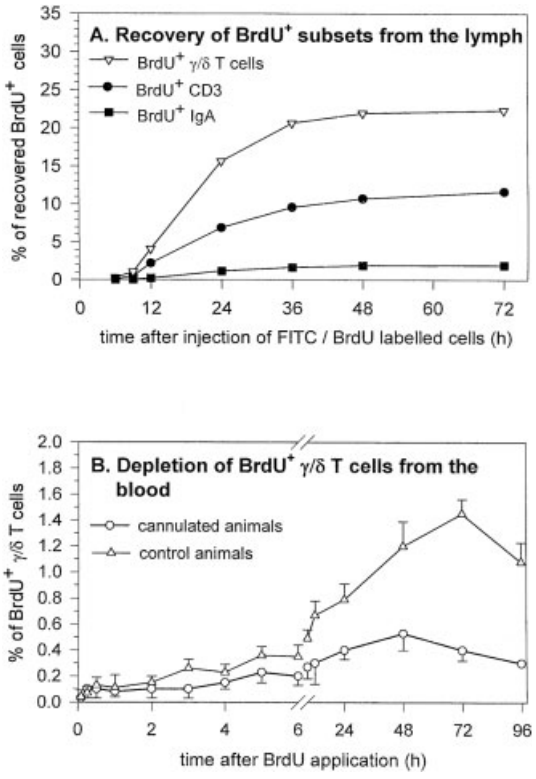

Fig. 2. (A) Recovery of BrdU$^+$ lymphocyte subsets from the intestinal lymph. Based on the number of injected cells and the number of BrdU$^+$ labeled cells recovered from the lymph, the proportion of re-appearing cells in the lymph was calculated. (B) Comparison of control (n=3) and intestinal lymph duct-cannulated (n=4) animals: the proportion of BrdU$^+$ γ/δ T cells in the peripheral blood was determined in the animals of both experimental groups (mean ± standard deviation shown).

2.3 Distribution and proliferation of intestinal γ/δ T cells

To detect the location of BrdU$^+$ γ/δ T cells, histological analyses were performed (groups C and D). One hour after an i.v. dose of BrdU, only single proliferating γ/δ T cells were observed in the epithelium, the lamina propria or the interfollicular area of the PP; it should be noted that the cells were only occasionally observed. Two weeks after a single BrdU pulse by i.v. injection, about 0.5 BrdU$^+$ γ/δ T cells per mm^2 were detected in the interfollicular area of the PP and in the lamina propria (Fig. 3). Four weeks after a BrdU pulse the frequency of cells was even higher in the interfollicular area than in the lamina propria. These observations obviously reflect the migration of the BrdU$^+$ γ/δ T cells through the traffic area of the gut immune system, as few if any BrdU$^+$ γ/δ T cells were present in the follicles of the PP.

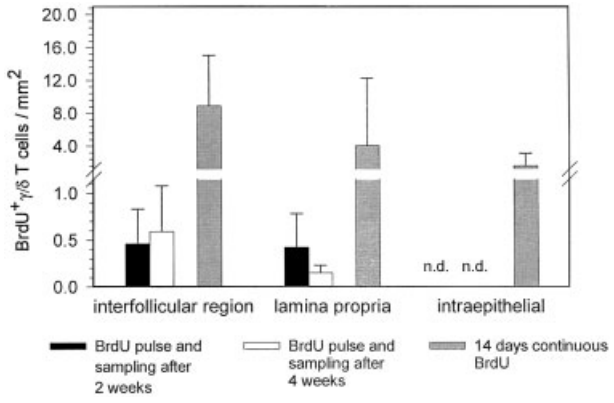

Fig. 3. BrdU$^+$ γ/δ T cells in the interfollicular region, the lamina propria and the epithelium. Pigs were studied 2 or 4 weeks after an i.v. BrdU pulse or after continuous labeling during a period of 14 days (BrdU injection every 8 h). The frequency of proliferating cells was lower among IEL, therefore BrdU$^+$ γ/δ T cells in this compartment were only determined after the 14-day continuous labeling. The frequency of cells is given per mm^2 of tissue area; for the IEL the frequency of cells was determined in the epithelium overlying the tissue area (mean ± standard deviation shown). n.d., not determined.

A continuous i.v. BrdU-labeling of the experimental animals for 14 days resulted in the detection of proliferating BrdU$^+$ γ/δ T cells in the epithelium, the lamina propria and the interfollicular area of the PP; their frequencies were of the same order of magnitude (Fig. 3). These observations indicate that many BrdU$^+$ γ/δ T cells are present in the traffic areas of the intestine: in the interfollicular area of the PP and in the lamina propria of the mucosa.

2.4 Clonal characterization of γ/δ T cells in the intestine and the intestinal lymph of the pig

CDR3 length analysis was used to characterize whether, in the intestine and in the lymph, the different γ/δ T cell receptors were used randomly or whether a clonal expansion was present. TCRDV3-expressing and DV5-expressing γ/δ T cells were clonally expanded within the jejunum and the ileum whereas they were polyclonal in the mesenteric lymph nodes, the PBMC and the draining lymph (Fig. 4). CDR3 length analysis of TCRDV1 and DV4 transcripts did not show oligoclonal expansions irrespective of the tissue analyzed. Interestingly the same oligoclonal CDR3 profiles of TCRDV3 transcripts were visible in the jejunum and the PP of the jejunum and ileum (Fig. 4). Identical TCR-δ transcripts were confirmed at those sites when dominant bands of identical length were directly sequenced (data not shown).

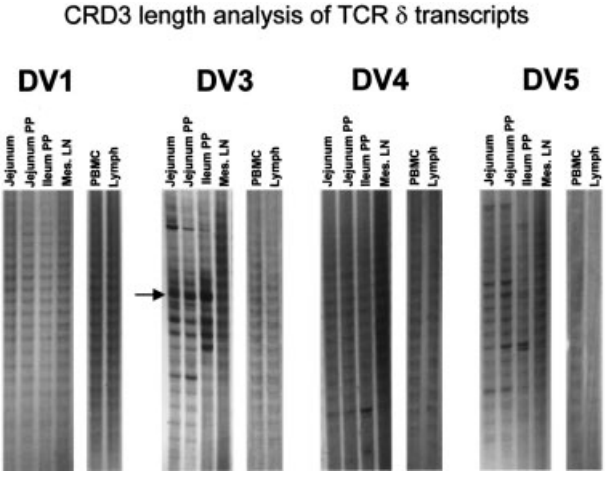

Fig. 4. CDR3 length analysis of TCR-δ transcripts: PCR-amplified TCR-δ transcripts (TCRDV1–DV5) from the intestine, the mesenteric lymph node (Mes. LN) and from the lymph were size-separated on a denaturing gel. The lymph samples were obtained from a different 6-month-old minipig. The intestinal CDR3 profiles from the DV3 and DV5 transcripts showed oligoclonal expansions whereas the CDR3 profiles from the mesenteric lymph nodes, the lymph and the blood were highly polyclonal. The arrow shows the dominant band, present in the jejunum and ileum; sequencing of that band demonstrated an identical TCR-δ transcript in both sites (data not shown).

3 Discussion

In the present study we followed the migration of CD2$^-$ γ/δ T cells, a non-cytotoxic porcine γ/δ subset detected by the marker MAC320 [15, 16]. During the experiments about 1% of the whole lymphocyte pool of the pig was drained via the lymph duct cannula; thus the experiments did not affect the total lymphocyte populations of the experimental animals. The marker analysis of these γ/δ T cells in the intestinal lymph demonstrates that the gut-emigrating γ/δ T lymphocytes belong preferentially to the CD8αα phenotype, which indicates a subset with putative thymus-independent precursors [17]. A small but constant proportion of γ/δ T lymphocytes that had entered the cell cycle in the intestine continuously emigrates via the intestinal lymph. This BrdU$^+$ γ/δ T cell subset represents about 0.5% within all lymph γ/δ T cells, and ~0.04% of all lymphoid cells that leave the intestine via lymph. As BrdU$^+$ γ/δ T cells were preferentially detected in the interfollicular area of the PP and in the lamina propria two or four weeks after a single BrdU injection (Fig. 3), the emigrating BrdU$^+$ γ/δ T cells obviously represent a specific migrating subset. Thus these recirculating BrdU$^+$ T cells may provide a memory function for the intestinal immune system.

Often the epithelial layer is discussed as a preferential site of γ/δ T cell production [9]. Partly in contrast to this hypothesis are the numbers of BrdU$^+$ γ/δ T cells present in the epithelial layer of the gut after a continuous labeling period of 14 days with BrdU: in all compartments proliferating γ/δ T cells were present, although a calculation in absolute numbers of these cell populations was not performed (Fig. 3). Recent data from athymic mice demonstrated γ/δ T cell production in the mesenteric lymph nodes, also supporting the importance of other locations of γ/δ T cell origin in addition to the epithelial layer [18]. Our morphological data cannot document the site of origin of the BrdU$^+$ γ/δ T cells observed in the lymph: the proliferating γ/δ T cells may have their origin in (1) the Peyer's patches, (2) the lamina propria or (3) the epithelium. However, it is concluded that the BrdU$^+$ γ/δ T cells obtained from the intestinal lymph and used for the recirculation study definitively had entered the cell cycle in the gut because the had incorporated the label *in vitro*.

The *in vitro* FITC and BrdU labeling provide the experimental basis to follow a preferential migration of the gut-derived γ/δ T cells through the intestine. Although the subset of BrdU$^+$ γ/δ T cells is small, it demonstrates a unique migratory property. The decreasing numbers of BrdU$^+$ γ/δ cells in gut lymph (Fig. 1B) may be due to the retention of these cells in the different compartments of the gut. Such a migration pattern was observed for the BrdU$^+$ IgA$^+$ cells in animals that had received a single dose of BrdU [14]; these BrdU$^+$ IgA$^+$ cells do not significantly re-enter the intestinal lymph. Although BrdU$^+$ γ/δ T cells and BrdU$^+$ IgA$^+$ cells had a comparable appearance in lymph (Fig. 1B and [14]), their further migration differs completely. This is obvious from the fact that less than 2% of the injected BrdU$^+$ IgA$^+$ cell pool was recovered in the lymph within 72 h (Fig. 2A).

As recently shown, the BrdU$^+$ IgA$^+$ cells accumulate in the intestinal lamina propria [13]. This is in line with initial data on the recirculation of PP-derived lymphoblasts into the intestinal wall [19, 20]. BrdU$^+$ γ/δ T cells, however, quickly exit again via lymph from the gut. More than 22% of the injected BrdU$^+$ γ/δ T cells had been re-collected 72 h after cell transfusion. About 20% of the injected BrdU$^+$ γ/δ T cells had already reached the intestinal lymph again at 36 h, demonstrating their quick recirculation.

Apoptosis might have an influence on the reduction of emigrating proliferating BrdU$^+$ γ/δ T cells shown in Fig. 1B. But the high recovery of this cell subset shown in Fig. 2A demonstrates that apoptosis did not affect this specific population of recollected recirculating BrdU$^+$ γ/δ T cells.

Obviously the intestine is a major site for the entry of newly formed BrdU$^+$ γ/δ T cells as well as a typical emigration site for these cells. Therefore the contribution of these gut-derived BrdU$^+$ γ/δ T cells to the blood pool was analyzed in lymph-duct-cannulated and control pigs. Cannulated pigs showed a depletion of BrdU$^+$ γ/δ T cells; the number of cells was three-times higher in control pigs. The blood pool of BrdU$^+$ γ/δ T cells in control animals is 156×10^6 lymphocytes in absolute number, about 10% of the residual peripheral blood γ/δ T cells observed in totally thymectomized pigs ($\sim2.0\times10^9$ cells) by Licence and Binns [21]. The depletion of BrdU$^+$ γ/δ T cells in the blood in the present experiments — in accordance with the results of Licence and Binns [21] — provides evidence for the origin of this subset in the intestinal wall.

The clonally expanded γ/δ T cells observed in the jejunum and ileum using CDR3 spectratyping of TCR-δ transcripts reflect a specific cell subset. This is a typical situation of the adult pig and similar data have been described in humans, whereas in young individuals no clonal expansion was observed [4, 22]. Since this method analyses all γ/δ T cells, irrespective of their cell cycle, we do not know if clonally expanded γ/δ T cells are proliferating cells and belong to the BrdU$^+$ population. In our experiments it was not possible to selectively analyze clonal expansion of the BrdU$^+$ γ/δ T cells from the intestinal lymph because of the small amount of this subset ($\sim7.5\times10^6$ cells in absolute numbers collected during 36 h).

However, a distribution of the clonally expanded γ/δ T cells along the gut is only possible by efficient cell migration — indicated by the detection of the same dominant γ/δ T cell clones at different sites along the small intestine. A local clonal proliferation without recirculation would not result in such a homogenous distribution. So far we have only indirect evidence that the BrdU$^+$ γ/δ T cells efficiently migrating in the lymph-duct experiments belong to the same population of clonally expanded cells derived from the gut. Further, we do not know yet if recirculating BrdU$^+$ γ/δ T cells reflect a memory subset or a population of thymus-independent γ/δ T cells that are newly formed in the intestine. The finding that most of the gut-emigrating γ/δ T cells express CD8α suggests that these cells have thymus-independent precursors [17].

In conclusion, our experiments demonstrate an extraordinary subset of γ/δ T cells that (1) enter the cell cycle in the intestine, (2) recirculate quickly preferentially through the gut and (3) represent a γ/δ T cell subset comparable to the residual cells observed in thymectomized pigs. Taken together, these observations strengthen the

hypothesis that gut-derived γ/δ T cells are a cell pool that does not stay in the intestinal wall but efficiently recirculates — obviously to support intestinal immunity or oral tolerance along the length of the intestine. In future experiments it has to be tested whether the proliferating γ/δ T cells in intestinal lymph are clonally expanded. This would prove the hypothesis that not only α/β T cells [23] but also selected clones of γ/δ T cells have a specific migration pattern resulting in a distribution especially in the different compartments of the intestine. Such an approach is also necessary for further research on the clonal-selection model of lymphocyte homing [24].

4 Materials and methods

4.1 Animals

All animal experiments were performed with the agreement of the Local Government of Hannover, Lower Saxony (509i – 42502–98/131, dated 26th November 1998). Four groups (A–D) of female Göttingen minipigs (aged 8–12 months) were used; the experimental details are shown in Table 2.

4.2 Lymph-duct cannulation and lymph collection (groups A and B)

To obtain lymph coming directly from the intestinal wall (pseudo-afferent), a recently developed technique was used [14]. For mesenteric lymph node-resected pigs, at about 9 months of age the lymph duct cannulation was performed and the lymph collected continuously [13]. A permanent i.v. cannula was also established in the external jugular vein. The lymph flow per hour was recorded and the hourly output of lymphocytes was determined.

4.3 *In vitro* labeling of cells obtained from intestinal lymph and cell transfusion (group A)

The gut-emigrating lymphocytes were collected for the recirculation study over a period of 56 h (seven 8-h collection periods). During this period the lymph bottles also contained 0.5 μmol BrdU (Sigma, Germany); thus all cells in the S-phase of the cell cycle were labeled *in vitro* directly after draining from the cannula [14]. The collected cells were centrifuged, resuspended in RPMI 1640 and stored at 4°C until the end of the sterile collection period. The lymphocytes were labeled *in vitro* using FITC (Isomer I; Sigma) and a total of $2.80\pm0.64\times10^9$ $FITC^+$ cells (containing 1.22±1.08% $BrdU^+$ γ/δ T cells) were retransfused i.v. as described earlier [13]. Thereafter, the lymph and blood samples were obtained (Table 2), and the animals were killed 1 day, 4 days or 8 days after the cell transfusion.

4.4 Flow cytometry for detection of lymphocyte subsets and of recirculating cells

An indirect immunofluorescence staining method was used to determine the lymphocyte subpopulations in the cell sus-

Table 2. Animals and experimental approach in the four groups (A–D)

Group	Central venous cannula	Lymph duct cannula	Experimental approach	Sampling
Group A (*n*=4)	Yes	Yes	Sterile lymph collection for 56 h, FITC and BrdU labeling of the collected lymphocytes *in vitro*, retransfusion of labeled cells	Lymph and blood samples: 5, 10, 15 and 30 min; hourly from 1 to 6 h; 9 h and 12 h; then 12-h intervals after cell transfusion Organs and final blood and lymph samples after: 1 day (*n*=1) 4 days (*n*=2) 8 days (*n*=1)
Group B: control pigs (*n*=3) cannulated (*n*=4)	Yes	 No Yes	BrdU: single dose i.v. 1 week after establishing the cannuals	Lymph and blood samples: 5, 10, 15 and 30 min; hourly from 1 to 6 h; 9 h and 12 h; then 12-h intervals after BrdU injection
Group C (*n*=8)	Yes	No	BrdU: single dose i.v. (20 mg/kg body weight)	Blood and organs: 1 day (*n*=3) 14 days (*n*=3) 28 days (*n*=2)
Group D (*n*=3)	Yes	No	BrdU: 3 doses i.v. per day over 14 days (5 mg/kg body weight)	Blood and organs: at the end of the BrdU labeling

pensions and in the blood, lymph and organs. Pig-specific antibodies were used: CD3, CD4 and CD8α from VMRD, Pullmann, USA; IgA and IgM from Serotec, Biozol, Eching, Germany; to examine $CD2^-$ γ/δ T cells, we used MAC 320 (a personal gift from R. M. Binns [16]). The MAC320 antibody detects nearly all porcine γ/δ T cells as demonstrated by double-labeling with the anti-pig-δ-chain antibody (PGBL22A), which was not available at the start of the present experiments. As secondary antibodies anti-mouse or anti-rat isotype-specific PE-labeled conjugates (Southern Biotechnologies, Birmingham, AL, USA) were applied. Double labeling was performed to detect the CD8α-low and γ/δ T double-labeled cells [25]. The lymphocyte subset proportions were analyzed using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany) and at least 5000 events in the lymphocyte gate were counted. Positive proportions were determined in histogram plots. For the double-labeling, a dot-plot analysis was used for red and green fluorescence.

Cells that had been labeled with FITC *in vitro* and retransfused were examined in the intestinal lymph, in blood samples and in the suspensions of the various tissues. In the lymphocyte gate of each suspension the proportion of immigrated $FITC^+$ lymphocytes (labeling index) was determined in the green fluorescence channel on the basis of 30,000 events. The subset composition of the retransfused cell population was analyzed by setting a live gate on the FITC-labeled cells. For this part of the study 1000 recovered $FITC^+$ cells were analyzed in the blood and lymph samples.

4.5 Detection of $BrdU^+$ cells among γ/δ T lymphocytes

The migratory properties of the $BrdU^+$ newly formed cell population among the FITC-labeled lymphocytes was studied after retransfusion. Indirect double-color immunocytochemistry was performed on cytospin preparations for the lymphocyte subsets (detecting CD3, γ/δ T cells, IgA and IgM) and the incorporated BrdU to determine the proportion of newly formed lymphocyte subsets in the blood and lymph [12]. The percentage of $BrdU^+$ retransfused cells was determined for the CD3, γ/δ T, IgA and the IgM subpopulations by counting at least 1000 positive cells in the intestinal lymph.

4.6 Detection of $BrdU^+$ cell in the tissue (groups C and D)

The $BrdU^+$ γ/δ T cells were detected in the compartments using double-labeling for γ/δ T cells and the incorporated BrdU [26]. Using an ocular grid, the size of a representative area of the lamina propria and the PP compartments (follicles, interfollicular area and dome) was determined. The occurrence of γ/δ T cells per area was given as cells / mm^2. The frequency of intraepithelial $BrdU^+$ γ/δ T cells was calculated based on the epithelium overlying a defined area of mucosa.

4.7 CDR3 spectratyping

RNA from different tissues and lymph was reverse-transcribed into cDNA. TCRDV1, DV3, DV4 and DV5 transcripts were amplified with *Taq*-Polymerase using Vδ- and Cδ-specific primers as previously described [4]. After an initial hot start, amplification of TCRD rearrangements consisted of 37 cycles of 40 s at 94°C, 50 s at 61°C and 1 min at 72°C, followed by a final extension for 10 min at 72°C. The expected PCR product length was 200–250 bp. Amplification with a DV2-specific primer rarely resulted in a PCR product even when a different set of primers and conditions were used (data not shown). Primers were designed within conserved regions of DV families from published sequences [27]: DV1, 5' CTC ACC ATT TCA GCC TTA CAG 3'; DV3, 5' ACA ATC TCT TCC TTA CAA CTG GC 3'; DV4, 5' GAG TTT GAC ACT GAG TGA CTC GG 3'; DV5, 5' TTC ACT TGG TGA TCT CCT CAG TG 3'; Cδ1, 5' AAC GGA TGG TTT GGA ATT AGG C 3'.

For analysis of CDR3 lengths, 2–3 μl of each PCR mixture was added to formamide-containing loading buffer. PCR products were heat-denatured for 2 min at 95°C, then size-separated on a 6% denaturing polyacrylamide gel and visualized by silver staining (Silver Sequence™ DNA staining reagents) as recommended by the manufacturer (Promega, Madison, WI, USA). Bands were photographed by exposing polyacrylamide gels for 8–15 s to an Automatic Processor Compatible Film (Promega Silver Sequence™).

4.8 Statistics

For all results the mean and standard deviation were calculated, and differences of $p<0.05$ in the Mann-Whitney U-test were taken as significant.

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