Antibody Testing for use in Immunohistochemistry and the Investigation of Precociously Induced Maturation of the Gastrointestinal Tract in Young Nude Rats

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Abstract

Background: Rat pups are born with an immature intestine adapted for the effective uptake of milk nutrients and with high permeability for bioactive molecules from the maternal milk until weaning. During weaning the immune system is activated after stimulation with e.g. dietary antigens, leading to an inflammatory response in the gut. This response is believed to be important for progress of gut maturation which involves changes in the small intestine from a foetal- to adult-like state. Recent studies indicate that immune cells, especially T cells, could play a key role in this and that treatment with immunosuppressive drugs delay gut maturation. The aim of this project was to further investigate the role of the immune system in the maturation of the gastrointestinal (GI) tract using immunodeficient nude young rats as the model.

Methods: At first different antibodies were tested with immunohistochemistry (IHC) to see if they could be used in the investigation of GI maturation. Thymus deficient (NIH-Foxn1^{mu}, nude) suckling rats were treated with a dietary antigen (PHA) or a protease (trypsin) known to induce precocious GI maturation. Whereafter, the maturity of the intestines was investigated, i.e., organ growth and the proportion of adult-like epithelium was studied by morphometric methods and the presence of CD3^{+} cells was studied using IHC techniques.

Results: The α-CD3 antibody was found to be useful for the further IHC studies of GI maturation. My combined results revealed that the treated nude rats showed intestinal maturation, similar to that seen at weaning in euthymic suckling rats. This included increased length of the proximal villi, tendency of increased crypt depth and change from foetal-like vacuolated cells to adult-like non-vacuolated cells in the distal epithelium. Surprisingly, CD3^{+} cells could be observed in the small intestine of the nude athymic young rats, however, in a lower amount than in the euthymic young rats.

Conclusion: This study showed that the intestinal maturation process could be induced in nude young rats. However, since CD3^{+} cells could be detected in the nude rats this indicate that thymus-independent T cells might be involved in maturation of the gut after birth. These discoveries might lead to a better understanding of maturation of the GI tract and hopefully help in the search for treatment of Necrotizing Enterocolitis (NEC) in preterm humans born too early with an immature gut.

Abbreviations

GI – Gastrointestinal

LP – Lamina propria

FcRn – Neonatal Fc Receptor

PHA – Phytohemagglutinin A

MLN – Mesenteric lymph nodes

CyA – Cyclosporin A

IL-2R – Interleukin-2 Receptor

NEC – Necrotizing Enterocolitis

dH2O – Distilled water

IHC – Immunohistochemistry

PBS – Phosphate Buffer Saline

MCT – Mast Cell Tryptase

Iβ7 – Integrin β7

SI – Sucrase-Isomaltase

Thy-1 – Thymocyte differentiation antigen 1

PCNA – Proliferating Cell Nuclear Antigen

SD – Sprague-Dawley strain

Nude_SD – Nude neonates nurtured by their natural dam

Nude_Nude – Nude neonates nurtured by a euthymic dam

TCR – T cell receptor

BIgG – Bovine immunoglobulin G

BSA – Bovine serum albumin

CCK – Cholecystokinin

PAR-2 – Proteinase-activated receptor 2

IEL – Intraepithelial lymphocytes

Introduction

The organization of the small intestine

The small intestine, a part of the gastrointestinal (GI) tract, is divided into three parts: the duodenum, the jejunum and the ileum. The duodenum is the proximal part and is mainly responsible for the digestion of nutrients, using enzymes from the exocrine pancreas and bile from the gallbladder. Jejunum and ileum are the two distal parts of the small intestine and they are mainly responsible for the absorption of nutrients [1, 2]. The wall of the small intestine consists of three layers of tissue: the mucosa, submucosa and muscularis propria, figure 1. The mucosa is the layer facing the lumen and it consists of an outer layer of epithelium that provides the selective uptake of nutrients and rejection of harmful solutes. Beneath the epithelium is a layer of loose connective tissue called the *lamina propria* (LP) containing blood vessels, nerve endings and lymphatic capillaries, as well as a rich variety of immune and inflammatory cells that contribute to the host defense and normal gut physiology. Finally, a thin layer of smooth muscle, called *muscularis mucosae*, ends the mucosa [1, 2].

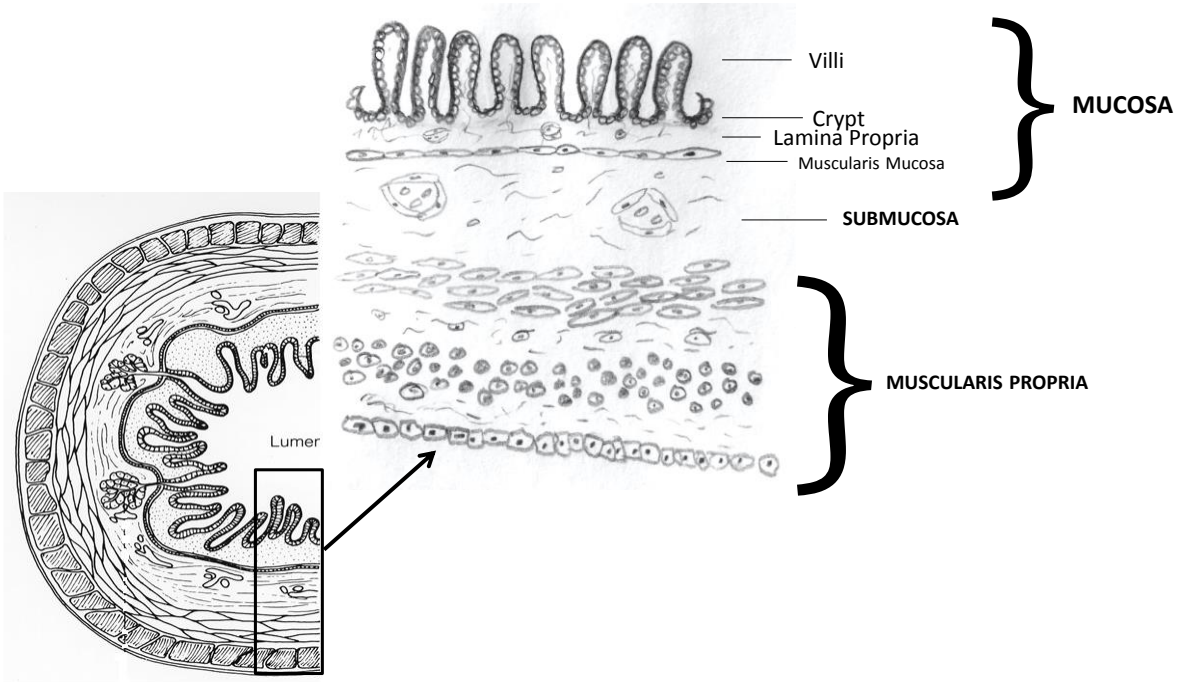

Figure 1. Schematic drawing of the organization of the intestinal wall. *The small intestinal wall is organized into three main layers, Mucosa, Submucosa and Muscularis propria. The Mucosa is the layer closest to the lumen and the epithelial layer is divided into villi and crypts. Beneath the epithelium is a layer of loose connective tissue called Lamina propria (LP). A thin muscle layer, Muscularis Mucosae, ends the Mucosa. (https://upload.wikimedia.org/wikipedia/commons/a/a2/Mucosa.jpg)*

The epithelium is organized into villi and crypts to increase the absorptive area and in the base of the crypts there are permanently anchored stem cells that undergo cell division originating new cells for epithelial cell turnover. The epithelial lineages that emerge from the immature primitive cells are enterocytes, goblet cells and enteroendocrine cells that migrate from the crypts to the villi, and in the villi tips the old cells are shed. There are a few cells that migrate downwards into the bottom of the crypts where they differentiate into long-lived Paneth cells that synthesize and release antimicrobial peptides as a first line of defense against harmful microbes [1-3].

The immune system of the gut

The protection against harmful agents starts with the first line of defense, which are the physical and chemical barriers. In the gut these are, for example, the mucosal barrier and the antimicrobial peptides secreted by the Paneth cells in the crypts[4]. After the mucosal barrier the immune system is the next defense against harmful antigens reaching the gut. As mentioned the LP is rich in a variety of immune cells and among them the T cells. T cells are immune cells of the adaptive immune system that recognize antigens via T cell receptors (TCR) that will bind to the antigen which lead to the activation of the cell. Each T cell has a unique TCR on their surface as a result of the developmental selection during their maturation in the thymus, where they go through positive and negative selection[5]. The TCR are non-covalently associated with CD3 molecules that are involved in intracellular signaling and important for T cell activation[6]. Upon activation T cells will, among other things, secret IL-2 which in turn will bind to IL-2 receptors (IL-2R) which will lead to the activation and proliferation of more antigen specific T cells and other immune cells[4, 7].

Development of the gastrointestinal tract

The development of the mammalian GI tract starts in the safe environment of the mother's womb. This highly organized process result is a specialized intestinal epithelium that has full digestive and absorptive functions, which also includes some endocrine and immunological functions. The ontogenetic development can be divided into different phases, where the earliest phases happen during gestation. This includes morphogenesis, cytodifferentiation and the preparation of the epithelium for the postnatal life. The phases after birth include the suckling period, where the intestine has to adapt to the new environment and be fully responsible for the absorption of nutrients. The last phase is the weaning period when the offspring changes from maternal milk to solid food, and the intestine then has to modify the systems for digestion and transport into more mature properties [1, 3]. Different mammalian species are born at different intestinal maturation stages and this is dependent on the length of the gestation. The longer the gestation period the more mature the foetus is at birth. The precocious species like sheep and pig have long gestational periods and are therefore more mature at birth, which means that their intestine is more developed than altricial species, like mouse and rat. The altricial species have a short gestation period and are born with a high dependence of their dam for thermoregulation, nutrition and evacuation of the bowels [3].

In early postnatal life the absorption of bioactive macromolecules, such as immunoglobulin G (IgG) and growth factors, from the maternal colostrum and milk is very important. Some mammals, like the mouse and rat, are born more or less hypoglobulinemic, meaning that they have few or no immunoglobulins in their blood plasma. They are therefore dependent on the passive transport of IgG from the maternal milk thorough the small intestine. There are two different pathways for transport of macromolecules. The first one is specific receptor-mediated transcytosis, where macromolecules bind to specific receptors, like IgG to the FcRn receptor that shuttles them across the intestinal epithelium. The second pathway is the non-specific transcytosis, where the macromolecules are transported across the intestinal epithelium by vesicular transport. The ability of the epithelium to transport macromolecules by this pathway is related to the presence of large cellular supranuclear vacuoles. The epithelium of the distal part of the small intestine of the altricial species at birth consists of vacuolated foetal-type enterocytes, they have a so called open intestine, which are replaced by mature non-vacuolated cells during weaning (21 days after birth in rats)[3]. In suckling animals the intestinal degradation is low due to low gastric secretion and even though the pancreas appears anatomically and morphologically developed already at

birth, it has a low secretion of enzymes. This leads to incomplete proteolytic digestion letting the intact macromolecules reach the small intestine for absorption. The process leading to the decrease in uptake of macromolecules at weaning is called gut closure and is dependent on increased intraluminal degradation and epithelial maturation with decreased endocytic activity [1, 3, 8, 9].

Animal model: Precociously induced maturation of the gastrointestinal tract

Earlier studies show that the maturation of the GI tract can be precociously induced by a lectin from red kidney beans, phytohemagglutinin A (PHA), and by exogenous proteases in suckling rats. Feeding these exogenous substances will have strong growth promoting effects on the GI tract and its accessory organs and induce gut closure, similar to normal weaning effects. PHA and protease stimulate the replacement of immature vacuolated cells in the distal small intestine to adult-type non-vacuolated epithelial cells, switch the intestinal disaccharides; decrease lactase and increase maltase and sucrase, and also pancreatic growth and increased production and secretion of pancreatic enzymes; amylase and trypsin [10-12].

Connection between gut maturation and immune system

Exposure to PHA and protease may provoke an inflammatory response in the gut, which can also be seen at natural weaning, the so called 'physiological inflammation' [11, 13, 14]. Both normal weaning and induced maturation by PHA are accompanied by the activation of T cells, through the production and secretion of pro-inflammatory cytokines and chemokines, like IL-1, IL-2 and TNF-α, that activate and attract T cells from thymus and peripheral lymphoid organs to the small intestine [11, 15, 16]. At normal weaning an increase in activated T-cells, an increase in the weight of mesenteric lymph nodes (MLN) and an overall increase in immune cells in the LP can be seen. These immunological changes accompanies the morphological changes seen during the maturation of the small intestine; the villous area increases, the crypts get deeper and the epithelium of the distal part of the small intestine changes from the immature type to mature type [13, 17].

The association between activated T-cells and the development of the intestinal epithelium seems to be essential for the maturation process, since treatment with cyclosporine A (CyA), an immunosuppressant drug, leads to delayed development and maturation of the small intestine; reduced intestinal growth including villus area, crypt depth and crypt proliferation [18]. In another experiment it has also been shown that the maturation of the small intestine is associated with the activation of T-cells, since a blockage of interleukin-2-receptor (IL-2R) leads to reduced intestinal growth. Due to this, a theory has been proposed that the T-cells are one of the main factors for the inducement of maturation of the small intestine [13, 17].

Motivation and significance of the project

There is an intense interest in the process of maturation and developmental stages of the GI tract since there is an increase in human preterm birth, resulting in infants with an immature GI tract. This is believed to cause a lot of unwanted passage of antigens which may influence health in later life. In many cases prematurity may lead to necrotizing enterocolitis (NEC), which is a multifactorial disease and it is the most common cause of GI related morbidity and mortality in premature infants [19]. The intestine of preterm birth resembles that of an open intestine of the suckling rat pup. That is the reason why rat pups are a good model for investigating the development of the GI tract [20].

The aim of this master project was to investigate the role of the immune system, especially the role of T cells, in the maturation of the GI tract. The investigation was done in athymic (nude) rat pups that are deficient in T lymphocytes and therefore have impaired T cell functions since they lack a functional thymus [21]. The nude rat pups were treated with PHA or a protease, trypsin, to see if it was possible to induce maturation precociously and the presence of mature T cells was investigated. According to previous data and the theory that T cells have an essential role in the maturation process it was hypothesized that induced maturation would be impaired, and no mature T cells, expressing CD3, would be found in the small intestine. However, to be able to do this the starting point of this project was to test different antibodies and investigate their optimal dilution for the target tissue for further use in the study. Additional antibodies were bought and they also needed to be tested for their specific antigen and be optimized for the target tissue, in this case the small intestine.

Material and methods

Animals

The study was approved by the local Malmö-Lund Ethical Review Committee for Animal Experimentation and conducted in accordance with the European Community regulation concerning the protection of experimental animals. The study were carried out using immunodeficient athymic rats (NIH-*Foxn1*^{rnu}, Charles River Laboratories International Inc., nude), meaning that they lack a functional thymus and are therefore T cell deficient, and euthymic rats of the Sprague-Dawley strain (SD) (Mol: SPRD Han; Taconic M&B, Denmark). They were bred and kept under specific pathogen-free conditions in the department animal facility at Lund University (20±1°C, 50±10 RH%, 12:12 h light-dark cycle). Before parturition, the pregnant dams were moved to separate cages (polycarbonate) with aspen wood bedding (Beekay B & K Universal AB, Solletuna, Sweden), enriched with paper-nesting material (Sizzle-pet, Lillicobiotech). Parturition date was denominated as day 0 and litters were restricted to ≤12 pups for the study. The restriction was kept as a standardized model because if the litter size is too big the rat pups will not get enough maternal milk and they will get deficient and small, and if the litter size is small the rat pups will get too much milk and they will get bigger and the results would be compromised. All rat pups were kept with their dams during the experiments. The rat dams had free access to water and rodent laboratory chow (RM1, SDS, Essex, England) placed on the lid of cages. In order to prevent the pups from eating the solid chow, the cage height was increased using a 7 cm wall extender.

Experimental design

To investigate the implication of T cells on induced maturation during the postnatal development of the small intestine of young suckling rats, experiments were performed in a split-litter manner where the nude rat pups were randomly divided into different treatment groups. There were two sets of experimental animals were the first set consisted of nude rat pups nurtured by their natural dam, 7 individuals from one litter. The second set consisted of nude rat pups nurtured by euthymic SD dams since three to five days after birth, 34 individuals from 3 litters. This transfer could be stressful for the young animals, but the second experimental set was included due to the difficulties experienced to get normal size litters (n=10-12) for the nude pups reared by their nude dams.

The first experimental set, nude rat pups nurtured by their natural dams, were divided into one treatment group and one control group. The treatment group were gavaged with a purified lectin, PHA from red kidney bean (*Phaseolus vulgaris)*, at fourteen days of age, with a single dose 0.1 mg/g.bwt (4 individuals). The control group received distilled water (dH2O) at fourteen days of age and the administration

volume was 0.01 ml/g.bwt single dose (3 individuals). dH2O was used as control since it was used as a vehicle to dissolve the treatments. The problem with using dH2O as a control is that it has an osmotic effect that could cause diarrhea, which is uncomfortable and stressful for the rat pup. However, the doses that were used in this experiment did not cause this effect. The littermates from the second experimental set were divided into different treatment groups and a control group. The first treatment groups were gavaged with PHA, at fourteen days of age, either with a single dose of 0.1 mg/g.bwt (4 individuals), or a repeated dose of 0.05 mg/g.bwt/day, once a day for three days (8 individuals). The other treatment groups were gavaged, at fourteen days of age, with pancreatic trypsin of porcine origin, either with a single dose of 0.6 mg/g.bwt (6 individuals), or 1 mg/g.bwt (4 individuals) or repeated dose of 0.6 mg/g.bwt/day, once a day for three days (7 individuals). The control group received dH2O at fourteen days of age and the administration volume was 0.01 ml/g.bwt single dose (5 individuals), figure 2. The different protocols and concentrations were used due to that induced maturation had not been investigated in young nude rats before.

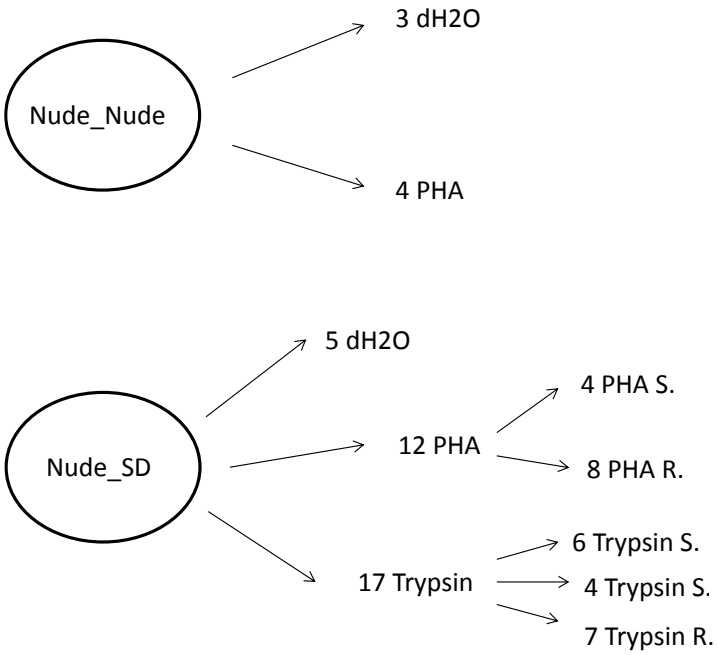

Figure 2. Design of the animal experiment. *Nude_Nude – rat pups nurtured by their normal dam, 3 treated with dH2O and 4 treated with PHA. Nude_SD – rat pups nurtured by euthymic dam since few days after birth, 5 treated with dH2O, 4 treated with PHA single (S.) dose, 8 treated with PHA repeated (R.) dose, 6 treated with trypsin single dose, 4 treated with trypsin single high (S.H.) dose and 7 treated with trypsin repeated dose. All animals were treated at 14 days of age and the ones treated with repeated dose were feed on day 15 and 16 as well. On day 17 of age they were euthanized.*

Animal experiments and sample collection

On day 14 after birth the rat pups were weighed and the proper dosage of treatment was calculated for each individual, then they were gavaged with their specific treatment dose using a plastic gastric tube. The rats that were treated with the repeated doses went through the same procedure on day 15 and 16 after birth as well. On day 17 the rat pups were weighed and then gavaged with a macromolecular marker cocktail, containing BSA (1.25mg/g b.wt) and BIgG (0.25mg/g b.wt). Three hours after the marker cocktail feeding the rat pups were euthanized by inhalation of isoflurane and then opened from the throat to the urine bladder. 1ml blood were collected with direct heart-puncture into a syringe containing a mixture of 1.5 mg EDTA and 20 KIU of a protease inhibitor (Trasylol®, Bayer HealthCare AG, Germany) and it was put on ice before plasma was obtained by blood centrifugation at 3000xg for 15 min at +4°C and stored at -20°C until further analysis of the permeability of the small intestine by measuring the concentrations of the macromolecular markers from the cocktail. Then the pancreas was dissected out, washed in cold saline and weighed, and then it was put in a plastic bag and put on dry ice

and later stored in -70°C until further analysis of the trypsin activity. The small intestine were dissected from the pylorus to the ileo-cecal junction, measured by a ruler and split into equal halves as a proximal and a distal part of the small intestine. The parts were flushed with cold saline, weighed and a piece of approximately 1 cm of each part were taken from the middle and fixed in 10% neutral buffered formalin, which cross-links the protein so the tissue does not degrade, for 24 hours at room temperature and then stored in 70% ethanol, room temperature, until paraffin embedding. After the dissection of the small intestine the cecum, the liver, the spleen and the stomach were dissected out, washed with cold saline and weighed, but not stored. In the euthymic rat pups the thymus was also dissected out washed in cold saline and weighed. When the sample collection were complete the heart artery were cut to ensure that the rats were completely dead.

I started my master project by taking an animal experimental course, *Laboratory Animal Science for Researcher –Rodents and Lagomorphs,* to be able to work with the animals. I did not participate in treatment and sample collection of the animals used in my study. The tissue had already been collected and fixed in formalin and was kept in 70% ethanol in room temperature and from there I started my experiments. However, I was assisting in animal experiments on euthymic rat pups that were performed in the same manner as the experiments on the nude rat pups.

Sample preparation

The small intestinal tissue from nude and euthymic rat pups, and small intestine, thymus and spleen from adult euthymic rats kept in ethanol was embedded in paraffin according to standard procedures. A small piece were cut from the tissue in the 70% ethanol and put into cassettes that were put in ethanol 80% over night. The next day they were incubated in ethanol 95% and 99%, after that they were incubated in xylene (x3), exchanging the ethanol in the tissue with xylene, they were kept in xylene overnight in room temperature. The third day the cassettes with the tissue were then embedded in paraffin with a melting point at 52-54°C to be able to embed the paraffin into the tissue. After that the tissue samples were embedded in embedding paraffin with a melting point of 56-58°C to not melt in hands. The paraffin embedded tissue was then cut in a microtome into 5 μm thick sections that were fixed on microscope slides (Thermo scientific, Polysine Slides), 37°C overnight.

Morphometry

Histology samples of 5 μm thickness were deparaffinised in xylene, ethanol and dH2O and stained with haematoxylin Harris and eosin (H & E) according to standard procedures. Haematoxylin is a positively charged compound that binds to acidic compound containing negative charge, like DNA, thus stain the nuclei in a violet color. Eosin is an acidic compound that binds to positively charged proteins in the cytoplasm and stains them pink. The slides were then dehydrated using ethanol 99% and xylene and mounted under cover slip using Eukitt (Sigma-Aldrich). From each individual pictures of 20 complete villous and 20 crypts both for the proximal and distal parts of the small intestine were taken using an Olympus PROVIS microscope connected to a camera. The pictures were then analysed by measuring the villi width and length and crypt depth of both proximal and distal small intestine. In the distal part of the small intestine the length of the non-vacoulated cells were also measured and put in proportion to the total villi length and estimated in percentage (%), and used as a measurement of maturity.

Immunohistochemistry

Literature search for extrathymic T cell markers

To find good markers for extrathymic T cells a quite extensive literature search was made. Many different markers were considered but in the end the decision fell on Thy-1, which is a marker that is found on thymectocytes and mature T cells in mice, but also on mature T cells in nude mice[22]. The other marker that was chosen was CD3 which is a co-receptor to the TCR, and it is non-covalently associated but important for the activation of the T cells[6]. CD3+ cells in nude rats and mice accumulate with age[23-25], and even if it is a defining feature of the T cell linage CD3+ cells in nude animals seems to have some different features than CD3+ cells from the thymus-dependent pathway[21].

Antibody testing

Antibodies, α-MCT, -MAdCAM, -Iβ7, -IL-2Rα and -CD45 (table 1), available in the lab had to be analysed to see if they were suitable for immunohistochemistry (IHC) analysis and could be further used in this study. They were tested in different dilutions, 1:50, 1:100, 1:200 and 1:400, mixed in 1% BSA in 0.02M phosphate buffer saline (PBS). This was also done to learn the technique of the IHC method and therefore antibodies that were known to work for the target tissue were used as technical controls, table 2. The antibodies were tested on small intestine from young euthymic SD rat pups and on each slide one or two dilutions of the antibody, the technical control and a negative reagent control were tested. The negative reagent control was 1% BSA in 0.02M PBS, without antibody, and it was used to exclude background staining from the detection kit.

New antibodies were bought, α-Thy-1 and α-CD3 (table 1), for the detection of extrathymic T cells in the nude rat tissue. These antibodies had to be tested and optimized for the IHC method and the target tissue. They were first tested on adult euthymic SD rat small intestine and as positive tissue controls thymus and spleen from the same rats were used since this tissue contains T cells for sure. Then the antibodies were tested on the target tissue, nude small intestine, using IHC analysis. The α-CD3 were also tested on tissue from small intestine of euthymic young rat pups from 7 days of age up to 28 days of age with a 7 day interval, to quantify the amount of CD3+ cells during normal development. The quantification was estimated as cells/villi, table 3.

Table 1. *Schedule of the antibody targets, the antibody and company of antibodies used for optimisation.*

Antibody target	**Antibody**	**Company**
Mast Cell Tryptase (MCT)	rabbit polyclonal anti-Mast Cell Tryptase (FL-275):sc-32889	Santa Cruz Biotechnology
Mucosal vascular addressin cell adhesion molecule (MAdCAM)	mouse monoclonal anti-MAdCAM-1 (F-6): sc-374398	Santa Cruz Biotechnology
Integrin β7 (Iβ7)	rabbit polyclonal anti-Integrin β7 (H-120): sc-15330	Santa Cruz Biotechnology
Interleukin-2 receptor α (IL-2Rα)	rabbit polyclonal anti-IL-2R α (M-19): sc-666	Santa Cruz Biotechnology
leukocyte common antigen (CD45)	rabbit polyclonal anti-CD45 (H-230): sc-25590	Santa Cruz Biotechnology
Sucrase-Isomaltase (SI)	rabbit polyclonal anti-Sucrase-Isomaltase (R-125): sc-99174	Santa Cruz Biotechnology
Thymocyte differentiation antigen 1 (Thy-1)	mouse monoclonal anti-CD90/Thy-1 [MRC OX-7] (ab225)	Abcam
Cluster of differentiation 3 (CD3)	rabbit monoclonal anti-CD3 [SP7] (ab16669)	Abcam

Table 2. *Schedule of the antibody targets, the antibody, company and dilution of antibodies used as positive controls.*

Antibody target	**Antibody**	**Company**	**Dilution**
Neonatal-Fc-receptor (FcRn)	rabbit polyclonal anti-FcRn (M-255): sc-66893	Santa Cruz Biotechnology	1: 600
Proliferating cell nuclear antigen (PCNA)	mouse monoclonal anti-PCNA clone PC10	DakoCytomation	1:1200

Table 3. *The quantitative estimation of CD3+ cells per villi expressed in plus signs. A higher number of plus means a larger amount of CD3+ cells.*

+	<1 cell/villi
++	1-2 cells/villi
+++	~5 cells/villi
++++	~10 cells/villi
+++++	>10 cells/villi

IHC method

First the tissue slides were deparaffinised, using xylene, ethanol (99%, 95%, 70%) and 0.01M PBS, and then pap pen circles were drawn on the sample slides, 3-4 per slide each containing two tissue sections. IHC analysis was performed by endogenous peroxidase blocking of the tissue samples, followed by incubation with a blocking reagent, Background sniper or Rodent Blocker R (MACH 1/MACH 4 Universal; Biocare Medical, Llc.; USA) to reduce background staining by blocking the immunoglobulins in the tissue. The tissue was then incubated with primary antibodies, diluted in 1% BSA in 0.02M PBS overnight at +4°C, see table 1 and 2. The second day, the samples were treated with a detection system,

HRP-Polymer Detection kit (MACH 1/MACH 4 Universal; Biocare Medical, Llc.; USA) and the procedure was performed according to the manufacturer´s specifications using DAB as the chromogen substrate. The HRP-polymer will bind to the primary antibody and then the DAB chromogen will react with the peroxides and give an orange/brow colour. Then the tissue samples were counterstained with Mayer's haematoxylin, which gives a weaker staining than Harris haematoxylin due to that Mayer's contain alcohol and Harris does not[26]. The weaker staining was used in IHC to give a clear chromogen staining of the target. Whereafter the sections were dehydrated in ethanol and xylene, and mounted under cover slip using Eukitt (Sigma-Aldrich). To exclude unspecific binding of the HRP-polymer detection kit a negative control were included on all slides, were the primary antibody had been replaced with only 1% BSA in 0.02M PBS.

Antigen retrieval was used to break the cross-linking between the proteins that forms during fixation with formalin[27]. This procedure was done after deparaffination for α-CD3, where the slides were boiled in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), to uncover hidden antigenic sites, according to abcam *IHC antigen retrieval protocol* for microwave procedure. This procedure was not made for all antibodies because they came with specifications, the kit (MACH 4) is supposed to minimize the background staining and since the antibodies were old and had been used before the time it takes to test all the antibodies with antigen retrieval was not worth it, due to the time limit of the project.

Statistics and analyzing methods

All data showing maturation are presented as mean values ± standard deviations (SD). ANOVA analyses, both one-way and two-way, were used to statistically compare the data between the different treatment groups. Dunnett's multiple comparison test was used to compare the treatment groups with the control group. Unpaired T-test was performed when the parameters were too few. The analyses were considered significant when $P<0.05$, expressed as *:$P<0.05$, **:$P<0.01$, ***:$P<0.001$ and ****:$P<0.0001$. All calculations were done using Prism v7.0 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Quantitative analyses were performed using Olympus PROVIS microscope to estimate the differences in expression patterns of the different immune targets. ImageJ software (National Institute of Health USA) was used for morphometric evaluation of the images.

Results

Antibody testing

Different antibodies (table 1) where first analysed to identify which were suitable for immunohistochemistry and their optimal dilution for the target tissue. This was done to decide which antibodies were suitable for further use in the study of the precociously induced maturation of the nude suckling rats. The antibodies were tested in different dilutions, 1:50, 1:100, 1:200 and 1:400, mixed in 1% BSA in 0.02M PBS. The antibodies against Thy-1 and CD3 were newly purchased and therefore tested on small intestine from euthymic adult SD rat, and as positive tissue controls spleen and thymus from the same SD rats were used. The other antibodies had been used before, thus tested on small intestine of euthymic young SD rats. As technical controls FcRn and PCNA (table 2), and as negative reagent control 1% BSA in 0.02M PBS were used.

Figure 3 show representative pictures from the IHC analysis with the rabbit polyclonal antibodies on tissue from proximal small intestine of euthymic young rats. The technical control stained with α- FcRn

show positive staining on the epithelial cells lining the villi, indicating that the staining method worked as expected. The intense staining in the villi tips, can be seen in many of the pictures, is background staining probably due to apoptotic cells that give unspecific binding. The antibodies against MCT, CD45 and SI showed positive staining for all dilutions. The positive staining of MCT showed the clearest staining with dilution 1:200 while the chromogen reaction for dilutions of 1:50 and 1:100 overstained the tissue in a very short time giving an unclear result. The antibodies against CD45 and SI showed clearest result for the dilution 1:50, while the other dilutions gave very weak staining. α - Iβ7 showed unclear and unspecific positive staining for all the dilutions, indicating that it might not work properly. The antibody against IL-2R α showed no positive staining for any dilution.

The results from the IHC with mouse monoclonal antibodies can be seen in figure 4, which shows representative pictures from α -PCNA, the technical control, and α -MAdCAM on proximal small intestine from young SD rat. The technical control showed a positive staining of the antibody indicating that the IHC method worked. The α -MAdCAM showed no positive staining for any of the dilutions. The old antibodies were first tested with the MACH 1 system and it was then realized that a new detection kit was needed that is more specific for the target tissue, in this case the small intestine of young rats. The new detection kit was bought, MACH 4, and it contain a more specific background sniper, the rodent blocker R. This detection kit was used on the new antibodies, α-Thy-1 and α-CD3. The old antibodies were not tested again with the new detection kit due to time limits.

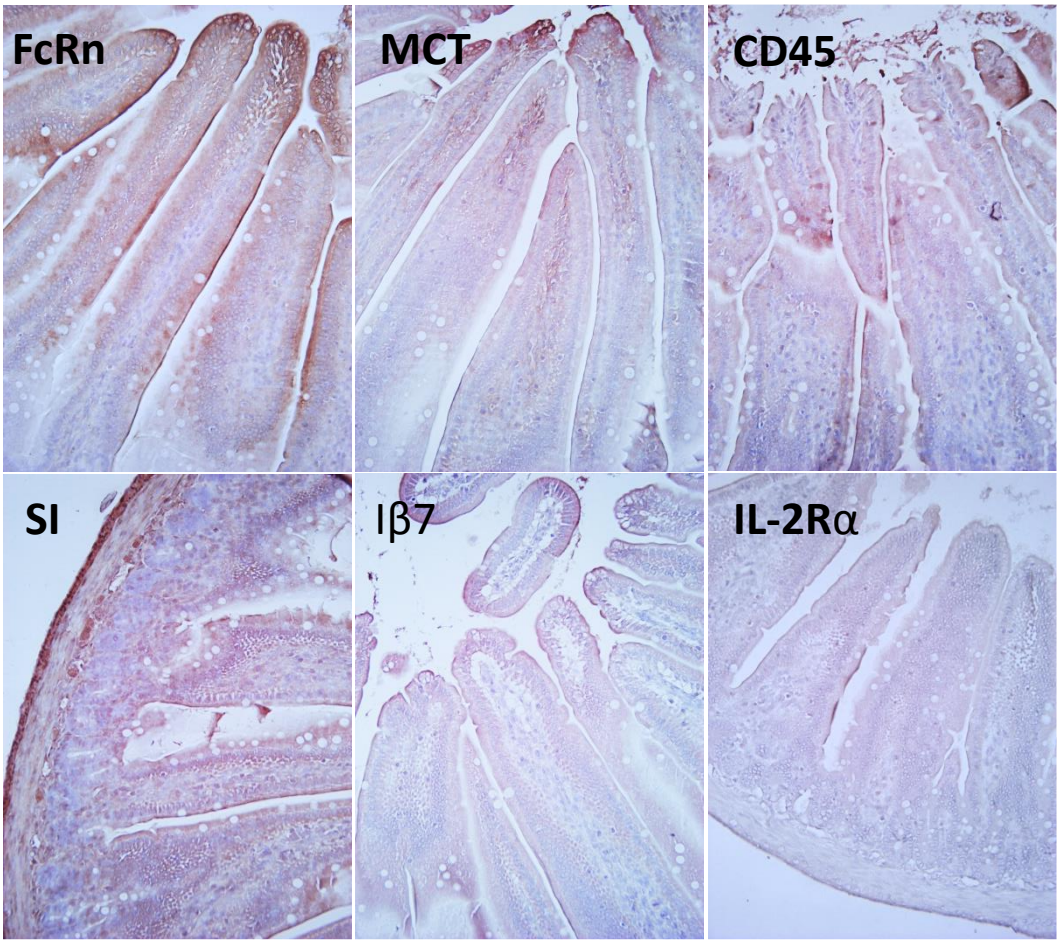

Figure 3. IHC testing of rabbit polyclonal antibodies *in proximal small intestine from 17 days old SD rats, 200x. Tissue stained with α-FcRn 1:600 (technical control), α-MCT 1:200, α-CD45 1:50, α-SI 1:50, α-Iβ7 1:50 and α-IL-2Rα 1:50. FcRn, MCT, CD45, SI and Iβ7 gave positive staining. IL-2Rα gave no staining.*

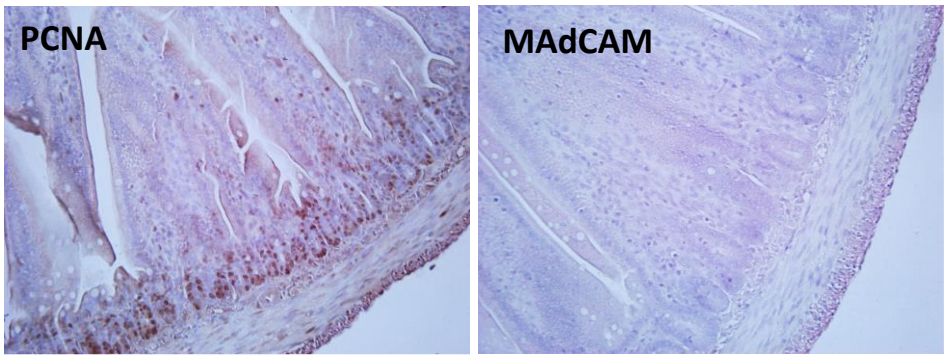

Figure 4. IHC testing of mouse monoclonal antibodies *in proximal small intestine from 17 days old SD rats, 200x. Tissue stained with α-PCNA 1:1200 (technical control) and α-MAdCAM 1:50. PCNA gave positive staining and MAdCAM gave no staining.*

The antibody against Thy-1 was analysed with IHC in adult rat thymus and compared to the negative reagent control stained with only 1% BSA in 0.02M PBS (Fig.5). Positively stained cells were seen in the cortical areas of the thymus while non in the medullar parts. Adult spleen from the same SD rat was also stained with α-Thy-1 but no positive staining was identified. The antibody had to be optimised for the

target tissue, therefore the proximal small intestine of adult euthymic SD rats were also stained. Positively stained cells could be observed in the LP (Fig.6) and the dilution 1:50 gave the clearest result.

To investigate the antibody against CD3, it was first used in analysis of the thymus and spleen from adult euthymic SD rat and positive staining could be observed in both tissues compared to the negative reagent control. In the thymus, the medullar parts where positively stained, and in the spleen, the positively stained areas where placed inside the white pulp, result not shown.

Proximal small intestine of normal adult rat was also stained with α-CD3 to optimise the antibody, but the IHC analysis was not successful (Fig.7 A and B). There was a lot of unspecific staining, and it was impossible to distinguish between positively stained cells and background staining. The α-CD3 antibody was therefore tested after antigen retrieval, which gave clear staining (Fig.7 C). The CD3+ cells were easy to distinguish and the cells could be observed in the tissue.

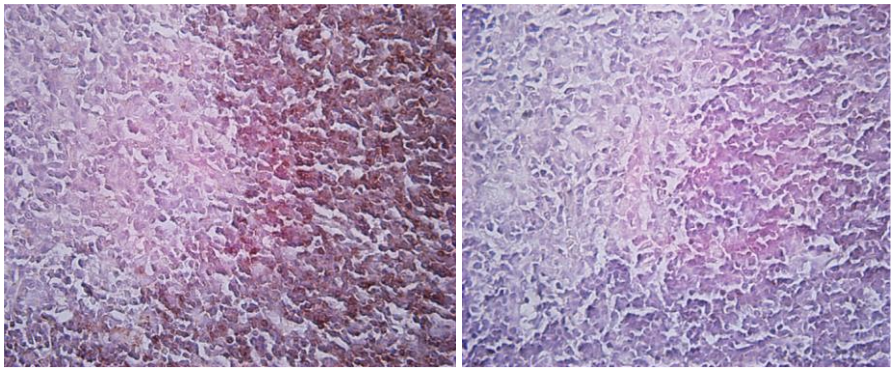

Figure 5. Thymus of adult SD rat stained with α-Thy-1. *Picture to the left stained with α-Thy-1 and picture to the right negative control (1% BSA in 0.02M PBS), 400x. Positive staining of Thy-1 can be seen in the cortical areas of the thymus indicating that it stains T cells in the early developmental process.*

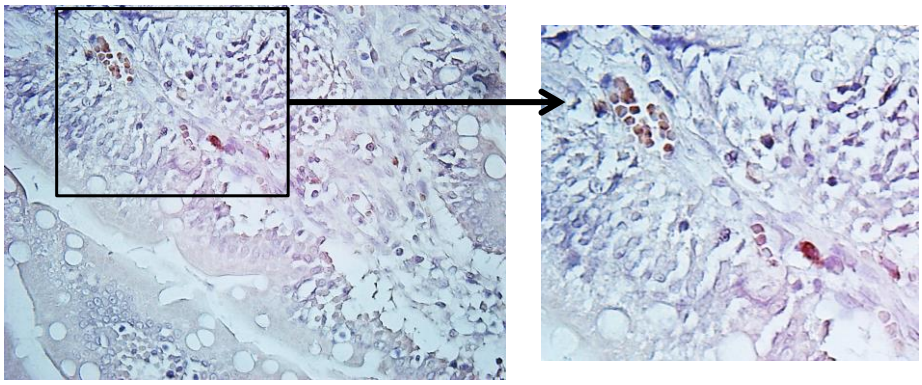

Figure 6. Small intestine stained with αThy-1. *IHC of adult small intestine from SD rats, stained with α-Thy-1, dilution 1:50, 400x. Positive staining could be observed in the LP.*

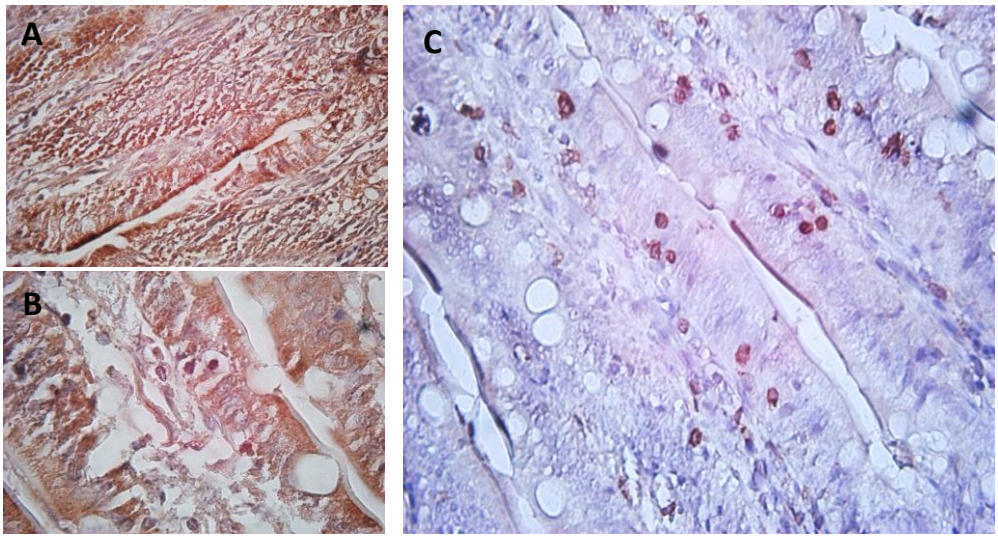

Figure 7. Small intestine stained with αCD3. *IHC of proximal small intestine from SD adult rat, stained with α-CD3.* ***A****. α-CD3 1:50, 400x,* ***B****. α-CD3 1:100, 1000x* ***C****. α-CD3 1:100, 400x, pretreated for antigen retrieval. Without the antigen retrieval the antibody stained unspecifically but after the antigen retrieval the CD3+ cells could be clearly seen in the LP.*

For optimisation of the antibodies not only the optimal dilution was determined but also the antibodies optimal substrate reaction time was determined. In table 4, all of the optimal dilutions and substrate reaction times can be found for each antibody.

Table 4. *Schedule of the antibodies, their optimal dilution and their substrate reaction time.*

Antibody:	Optimal Dilution:	Substrate Reaction Time:
α-MCT	1:200	30 seconds
α-MAdCAM	-	-
α-Iβ7	1:50	1minute
α-IL2Rα	-	-
α-CD45	1:50	2 minutes
α-Sucrase-Isomaltase	1:50	50 seconds
α-Thy-1	1:50	5 min
α-CD3	1:100	3 min

Intestinal morphometry of the Nude_SD rat pups

The investigation of the morphometry of the small intestine was made to establish if maturation could be precociously induced in nude suckling rats. The growth-promoting effects on young nude rats nurtured by euthymic Sprague-Dawely dam (Nude_SD) and orally treated with trypsin or PHA, single or repeated, was analysed on the morphometry of the small intestine by measuring the width and length of the villi and the depth of the crypts. The control groups were treated with dH2O. The analyses were made blinded to minimize human bias in judgement. The correspondence of the individuals with their treatment groups was not revealed until the analyses were completed.

Proximal small intestine

A small increase in the villi length of the proximal small intestine can be observed between the treated groups compared to the control in the H&E stained samples seen in figure 8. It is difficult to know if this increase is due to the effects of the treatment or by stretching of the material when handling it.

There were no significant differences in the width of the villous for the PHA treatments or trypsin treatments compared to the control group of the proximal intestine (Fig. 9 and 10). There were no significant differences in the crypt depth of any of the trypsin treated groups compared to the control. However, the PHA repeated dose had a small significant difference in the crypt depth compared to the control. No difference could be seen for the PHA single group for the crypt depth. There was a clear increase in the villi length in both of the PHA treated groups compared to the control. The villi length of the trypsin treated single high and repeated groups had no significant increase, but trypsin single group had a small significant increase of the villi length. However, this small increase could also be due to the handling of the tissue and the SD for the trypsin treatments are high meaning that there are variations in the treatment groups.

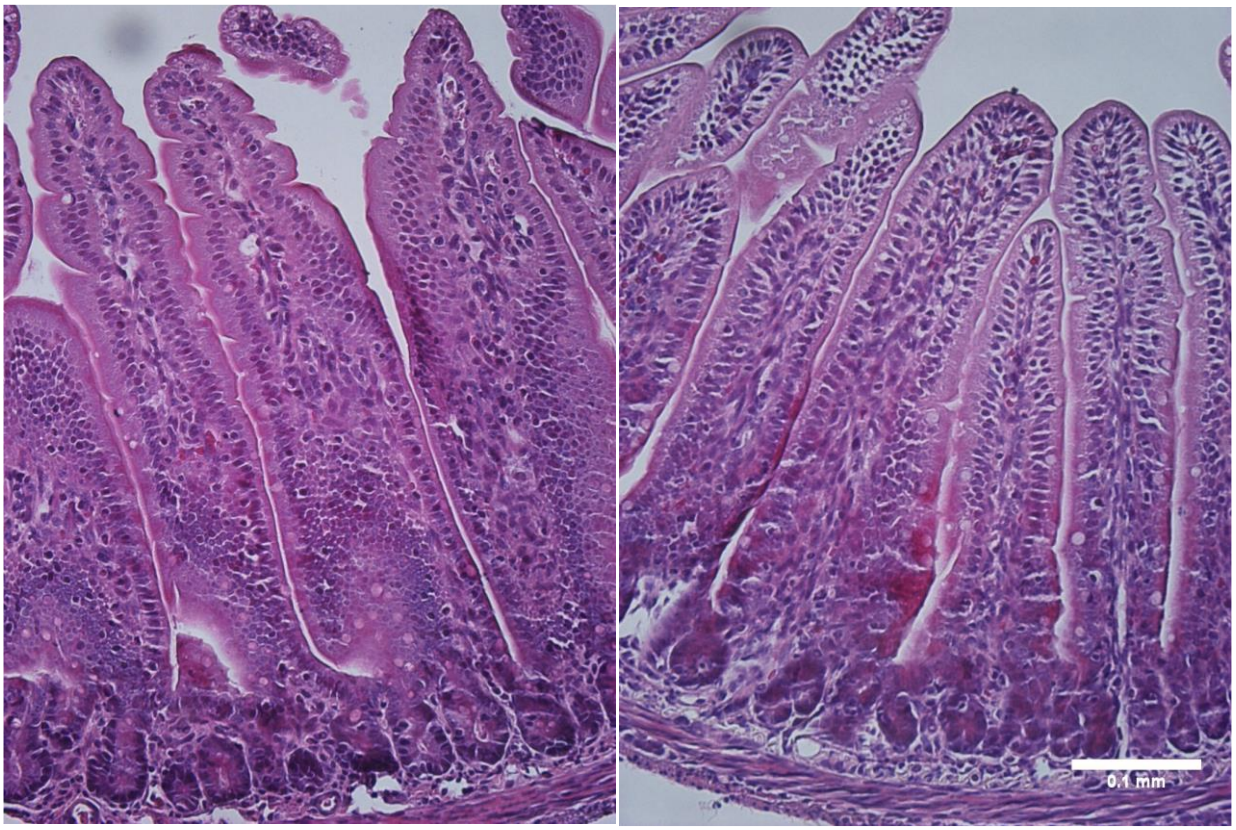

Figure 8. H&E stained small intestine. *The proximal small intestine of Nude_SD rat pups, 17 days of age treated at 14 days of age, stained with H&E, 200x. Picture to the left represents treated group. Picture to the right represent control group treated with dH2O. A small difference of the villi length can be seen between the treated and the control. Scale bar in the right down corner showing 0.1mm.*

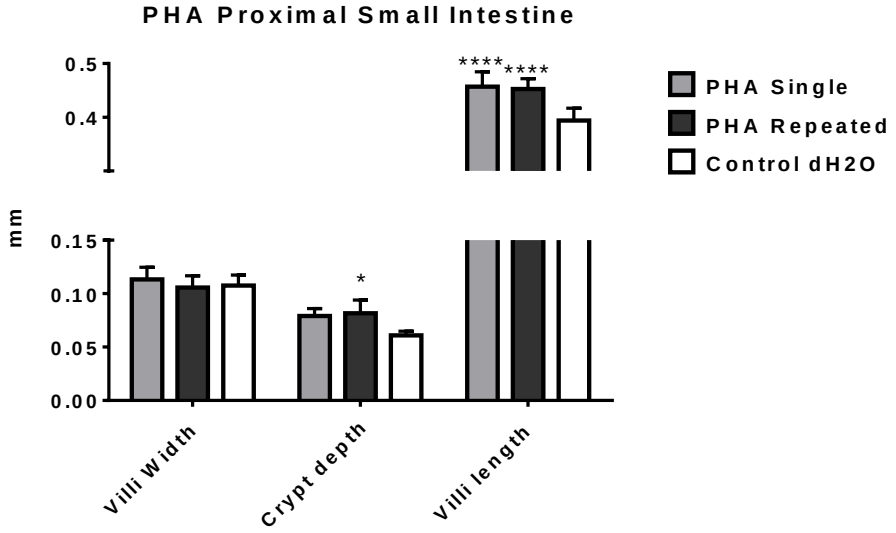

Figure 9. PHA effects on the morphology of the proximal small intestine. *Width and length of villi and crypt depth, measured in mm, of 17 days old Nude_SD rat pups proximal small intestine treated at 14 days of age with PHA single dose 0.1mg/g.bwt (4 individuals), PHA repeated dose 0.05mg/g.bwt/day (8 individuals) for 3 days and a control group treated with dH2O 0.01ml/g.bwt (5 individuals). The results are expressed as means ±SD. Two-way ANOVA test was performed, *= p<0.05, ****=p<0.0001. The villi length of the proximal small intestine had significantly increased in young rats treated with PHA.*

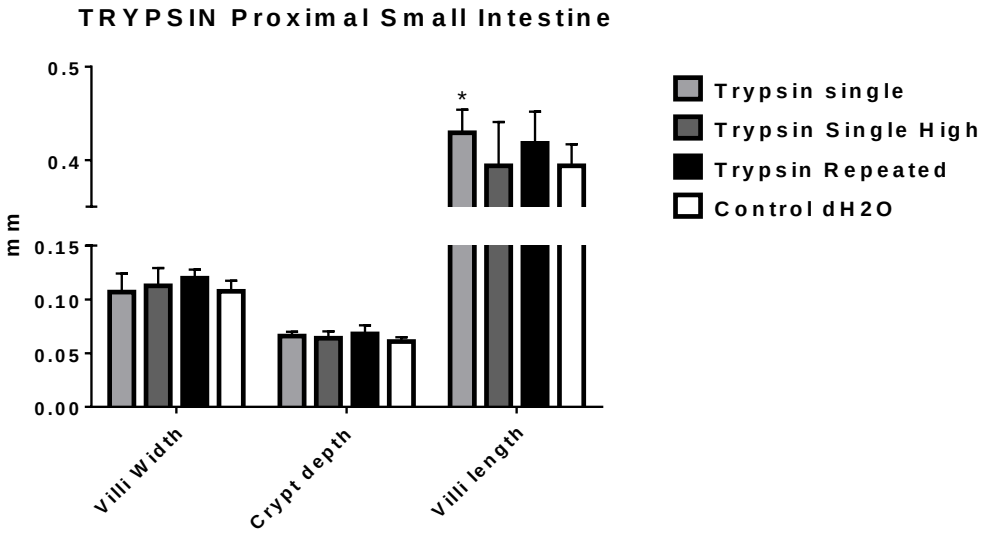

Figure 10. Trypsin effects on the morphology of the proximal small intestine. *Width and length of villi and crypt depth, measured in mm, of 17 days old Nude_SD rat pups proximal small intestine treated at 14 days of age with Trypsin single dose 0.6mg/g.bwt (6 individuals), Trypsin single high dose 1mg/g.bwt (4 individuals), Trypsin repeated dose 0.6mg/g.bwt/day for 3 days (7 individuals) and a control group treated with dH2O 0.01ml/g.bwt (5 individuals). The results are expressed as means ±SD. Two-way ANOVA test was performed, *= p<0.05.A significant increase could only be seen in villi length in young rats treated with trypsin single dose.*

Distal small intestine

The distal part of the small intestine, of the Nude_SD rat pups treated with PHA or trypsin was analysed for the same morphometric parameters as the proximal part. Figure 11, a representative picture of the distal small intestine of treated Nude_SD rat pups compared with a control, stained with H&E, can be seen. The treated rat pup only has vacuolated cells left in the villi tip while the control has vacuolated cells along the whole villi.

No significant differences could be observed for the villi width, length or the depth of the crypts in any of the treatment groups, PHA or trypsin, compared to the control group (Fig.12 and 13). In addition, the proportion of non-vacuolated cells of the total villi length was calculated in percentage as a measure of maturity. Trypsin single dose and trypsin single high dose had a significantly more mature distal small intestine, around 50% and over 80% respectively, compared to the control rat pups, which had less than 20% maturity. While the trypsin repeated dose did not have a significant increase, even though there is an indication of a growth-promoting effect. The PHA treatment both groups had significant increase in maturity, over 50%, compared to the control (Fig. 14). However, the SD for the PHA treatments is high so the result is probably not reliable. Trypsin single high dose had the highest increase in maturation.

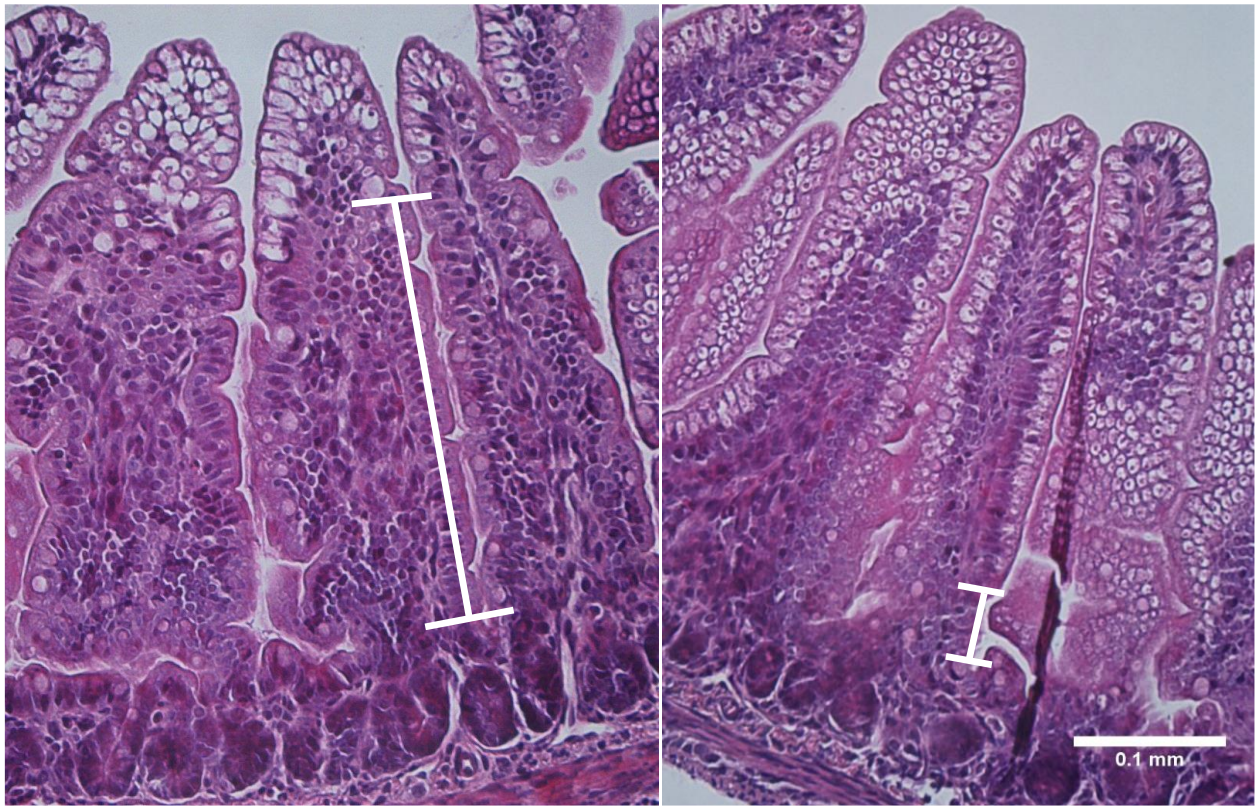

Figure 11. H&E stained distal small intestine. *Pictures of the distal small intestine of Nude_SD rat pups, 17 days of age treated at 14 days of age, stained with H&E illustrating length to vacuolated cells, 200x. Picture to the left represent treated groups. Picture to the right represent control group treated with dH2O. The length of non-vacuolated cells is longer for the treatment groups compared to the control group. Scale bar in the right down corner showing 0.1mm.*

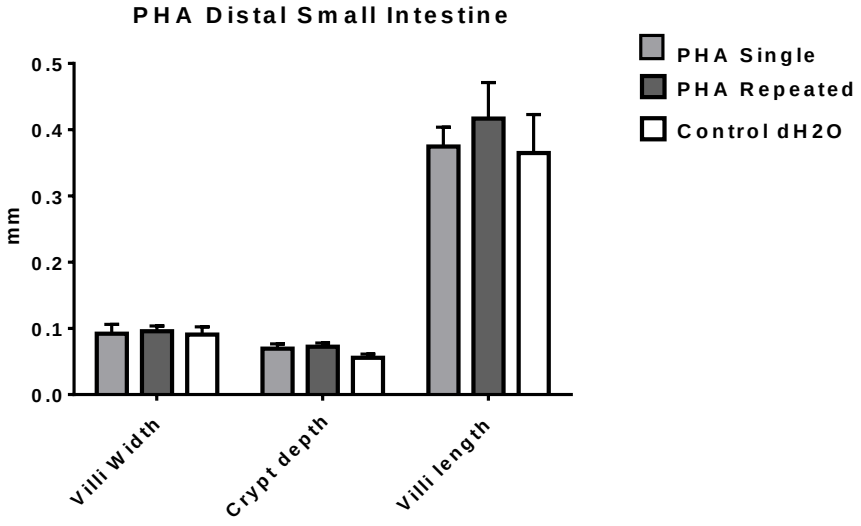

Figure 12. PHA effects on the morphology of the distal small intestine. *Width and length of villi and crypt depth, measured in mm, of 17 days old Nude_SD rat pups distal small intestine treated at 14 days of age with PHA single dose 0.1mg/g.bwt (4 individuals), PHA repeated dose 0.05mg/g.bwt/day for 3 days (8 individuals) and a control group treated with dH2O 0.01ml/g.bwt (5 individuals). The results are expressed as means ±SD. Two-way ANOVA test was performed. No significant differences could be observed for villi width, crypt depth or villi length in distal small intestine of young rats treated with PHA.*

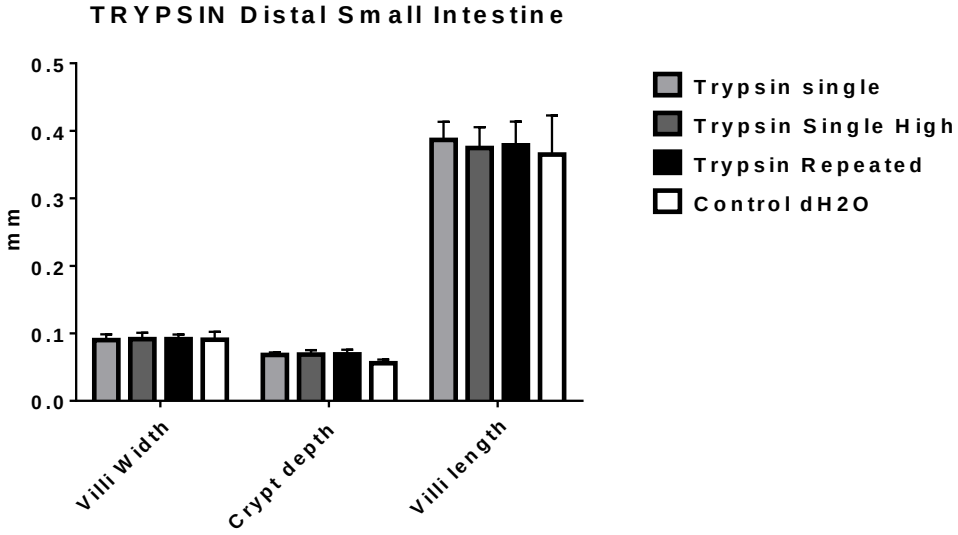

Figure 13. Trypsin effects on the morphology of the distal small intestine. *Width and length of villi and crypt depth, measured in mm, of 17 days old Nude_SD rat pups distal small intestine treated at 14 days of age with Trypsin single dose 0.6mg/g.bwt (6 individuals), Trypsin single high dose 1mg/g.bwt (4 individuals), Trypsin repeated dose 0.6mg/g.bwt/day for 3 days (7 individuals) and a control group treated with dH2O 0.01ml/g.bwt (5 individuals). The results are expressed as means ±SD. Two-way ANOVA test was performed. No significant differences could be observed for villi width, crypt depth or villi length in distal small intestine of young rats treated with trypsin.*

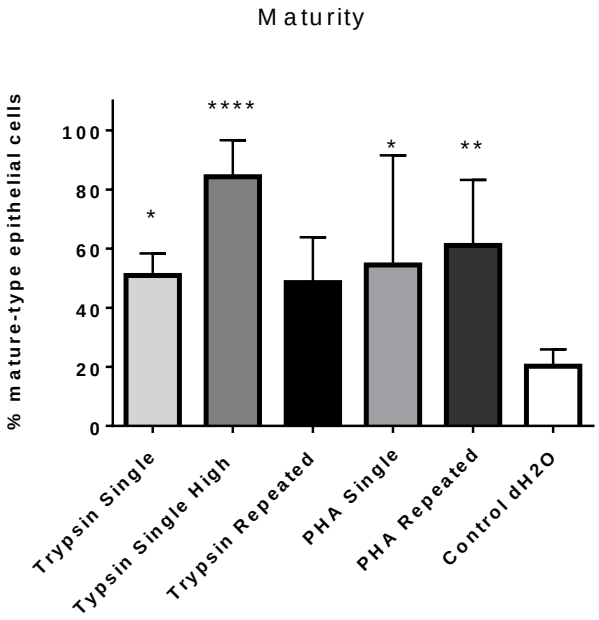

Figure 14. Maturation of the distal small intestine of treated young rats. *Maturity of the epithelium of the distal small intestine estimated in percentage (%) as the length of non-vacuolated cells in proportion to the total villi length, for the different treatment groups. 17 days old Nude_SD rat pups distal small intestine treated at 14 days of age with Trypsin single dose 0.6mg/g.bwt (6 individuals), Trypsin single high dose 1mg/g.bwt (4 individuals), Trypsin repeated dose 0.6mg/g.bwt/day for 3 days (7 individuals), PHA single dose 0.1mg/g.bwt (4 individuals), PHA repeated dose 0.05mg/g.bwt/day for 3 days (8 individuals) and a control group treated with dH2O 0.01ml/g.bwt. The results are expressed as means ±SD. One-way ANOVA test was performed, *= p<0.05, **= p<0.01, ****=p<0.0001. All treatment groups, except trypsin repeated, showed significant increase in maturity of the epithelial cells of the distal small intestine.*

Intestinal morphometry of the Nude_Nude rat pups

The other experimental set with the young nude rat pups nurtured by their natural dam (Nude_Nude) were only orally treated with PHA single dose, but analysed in the same way as the Nude_SD rat pups. The PHA treatment had no effect on the width of the villi or on the crypt depth in either the proximal or the distal parts of the small intestine (Fig. 15 and 16). The treatment had a small significant increase in the length of the villi in the proximal part, but no increase in the distal part.

The maturity of the distal small intestine was calculated as the proportion of non-vacuolated cells of the total villi length showed in percentage. PHA has a clear effect on the inducement of the maturity of the distal small intestine, over 70% maturity compared to less than 20% for control (Fig.17).

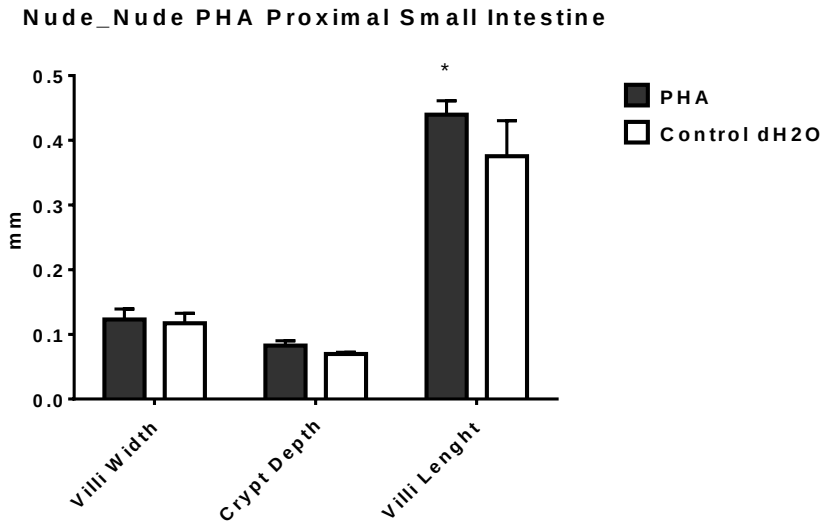

Figure 15. PHA effects on the morphology of the proximal small intestine. *Width and length of villi and crypt depth, measured in mm, of 17 days old Nude_Nude rat pups proximal small intestine treated at 14 days of age with PHA single dose 0.1mg/g.bwt (4 individuals) and a control group treated with dH2O 0.01ml/g.bwt (3 individuals). The results are expressed as means ±SD. Two-way ANOVA test was performed, *= p<0.05. Significant difference could only be observed for villi length of the proximal small intestine of young Nude_Nude rats treated with PHA.*

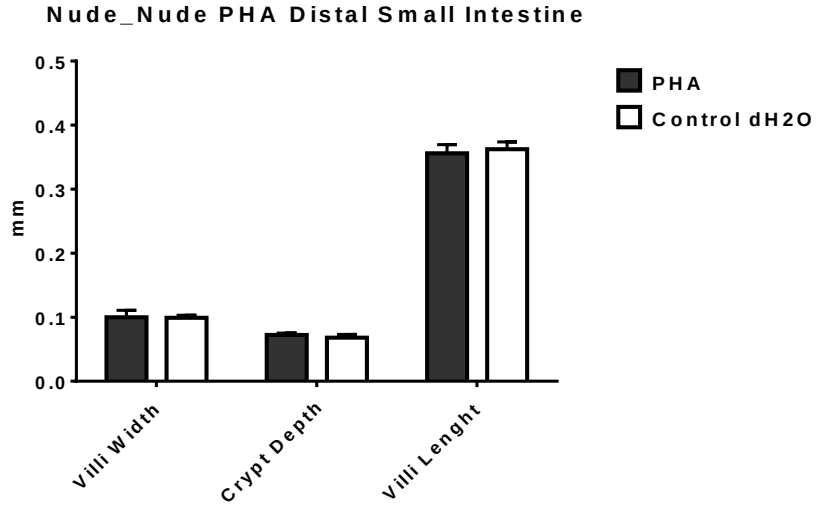

Figure 16. PHA effects on the morphology of the distal small intestine. *Width and length of villi and crypt depth, measured in mm, of 17 days old Nude_Nude rat pups distal small intestine treated at 14 days of age with PHA single dose 0.1mg/g.bwt (4 individuals) and a control group treated with dH2O 0.01ml/g.bwt (3 individuals). The results are expressed as means ±SD. Two-way ANOVA test was performed. No significant differences could be observed for the distal small intestine of young Nude_Nude rats treated with PHA.*

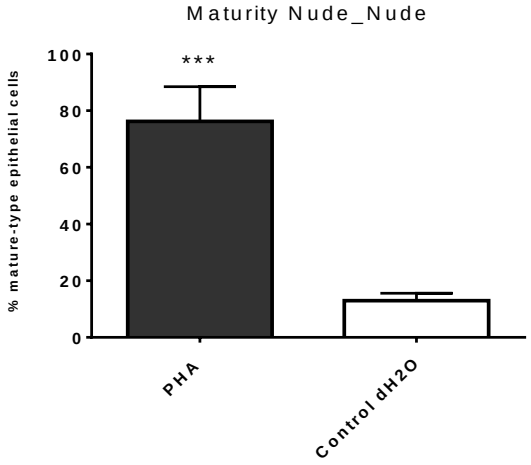

Figure 17. Maturation of the distal small intestine of PHA treated young rats. *Maturity of the epithelium of the distal small intestine estimated in percentage (%) as the length of non-vacuolated cells in proportion to the total villi length, for the different treatment groups. 17 days old Nude_Nude rat pups distal small intestine treated at 14 days of age with PHA single dose 0.1mg/g.bwt (4 individuals) and a control group treated with dH2O 0.01ml/g.bwt (3 individuals). The results are expressed as means ±SD. Unpaired T-test was performed, ***=p<0.001. There is a significant increase in the maturity of the epithelium of the distal small intestine of young Nude_Nude rats treated with PHA.*

Organ weight, trypsin activity and intestinal permeability of nude young rats

The proximal and distal part of the small intestine and the pancreas were weighed, the trypsin activity of the pancreas and the permeability of the small intestine in both Nude_SD and Nude_Nude rat pups was analysed by others in the lab.

Organ weight

The weight of the proximal and distal small intestine of the young nude rats had significantly increased for all treatment groups compared to the control for both Nude_SD and Nude_Nude rat pups, figure 18 and 19. The pancreas weight was also measured during the sample collection and no difference can be seen for the weight between the treatment groups compared to the control group for neither the Nude_SD nor the Nude_Nude young rats, figure 18 and 19.

Trypsin activity

The trypsin activity was analysed in the pancreas for both Nude_SD and Nude_Nude and measured as trypsin units (U) per gram body weight (Fig. 20 and 21). The PHA treatments of both Nude_SD and Nude_Nude young rats had significant increase of the activity compared to the control group. Trypsin single dose and trypsin repeated dose had no significant increase in the trypsin activity of the pancreas in the Nude_SD young rats. However, trypsin single high dose had significant increase in trypsin activity in the pancreas and this might indicate that the effect seen by trypsin treatment was dose related.

Permeability of the small intestine

The concentration of BIgG and BSA was measured from the blood plasma obtained during sample collection and in Nude_SD the concentration of BIgG from all the treatment groups had significantly decreased compared to the control group, indicating a decreased BIgG uptake, figure 22. The concentration of BSA in the plasma had significantly decreased in almost all treatment groups, except for trypsin repeated dose, compared to the control group for Nude_SD rats, indicating a lowered uptake of the macromolecule. The permeability of BIgG and BSA had also significantly decreased in the PHA treated Nude_Nude young rats compared to the control treated rats, figure 23. The low significance of the permeability of BSA could be due to the high variations of the control group. If more individuals were included the variations would probably be smaller.

Figure 18. The organ weight of small intestine and pancreas of Nude_SD young rats. *The weight of proximal and distal small intestine and the pancreas of Nude_SD rat pups treated with trypsin single dose 0.6mg/g.bwt (6 individuals), trypsin single high dose 1mg/g.bwt (4 individuals), trypsin repeated dose 0.6mg/g.bwt/day for three days (7 individuals), PHA single dose 0.1mg/g bwt (4 individuals), PHA repeated dose 0.05mg/g.bwt/day for three days (8 individuals) and a control group treated with dH2O 0.01ml/g.bwt (5 individuals) measured at 17 days of age. The results are expressed as means ±SD. Two-way ANOVA was performed, ***=p<0.001, ****=p<0.0001. The weight if the small intestine, both proximal and distal, had significantly increased for all treatment groups, while no significant difference could be observed of the pancreas.*

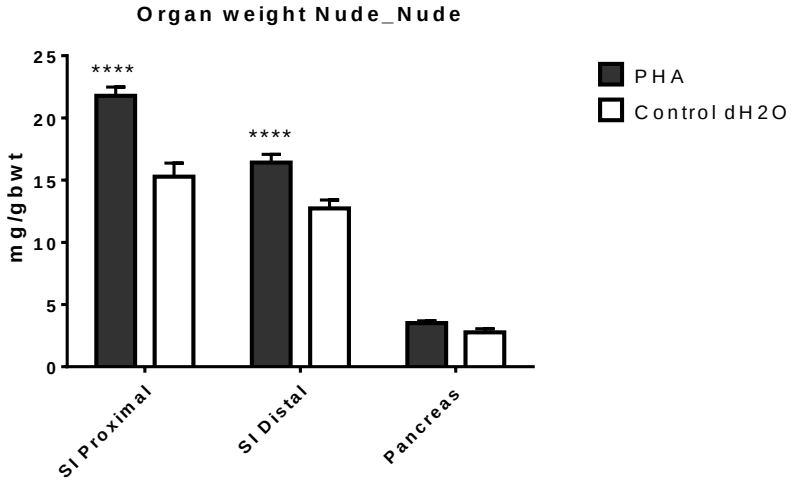

Figure 19. The organ weight of small intestine and pancreas of Nude_Nude young rats. *The weight of proximal and distal small intestine and the pancreas of Nude_Nude rat pups treated PHA single dose 0.1mg/g bwt (4 individuals) and a control group treated with dH2O 0.01ml/g.bwt (3 individuals) measured at 17 days of age. The results are expressed as means ±SD. Two-way ANOVA was performed, ****=p<0.0001. Significant increase of the weight of the small intestine, both proximal and distal, could be obsereved in PHA treated rats, while no significant difference could be seen in the weight of the pancreas.*

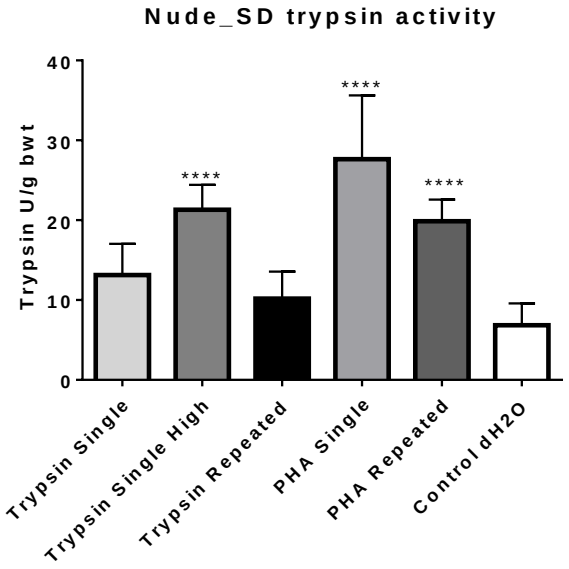

Figure 20. Trypsin activity of the pancreas in Nude_SD young rats. *The trypsin activity of the pancreas was measured at 17 days of age in Nude_SD rat pups treated with trypsin single dose 0.6mg/g.bwt (6 individuals), trypsin single high dose 1mg/g.bwt (4 individuals), trypsin repeated dose 0.6mg/g.bwt/day for three days (7 individuals), PHA single dose 0.1mg/g.bwt (4 individuals), PHA repeated dose 0.05mg/g.bwt/day for three days (8 individuals) and a control group treated with dH2O 0.01ml/g.bwt (5 individuals). The results are expressed as means ±SD. One-way ANOVA was performed,****=p<0.0001. Significant increase in the pancreas trypsin activity could be observed for both PHA treatments, but only for single high dose of the trypsin treated groups.*

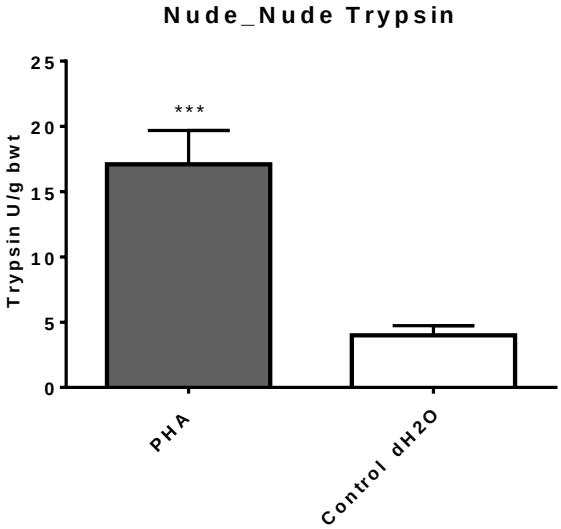

Figure 21. Trypsin activity of the pancreas in Nude_Nude young rats. *The trypsin activity of the pancreas was measured at 17 days of age in Nude_Nude rat pups treated with PHA single dose 0.1mg/g.bwt (4 individuals) and a control group treated with dH2O 0.01ml/g.bwt (3 individuals). The results are expressed as means ±SD. Unpaired t-test was performed,***=p<0.001. Significant increase of the pancreas trypsin activity could be observed in Nude_Nude rats treated with PHA.*

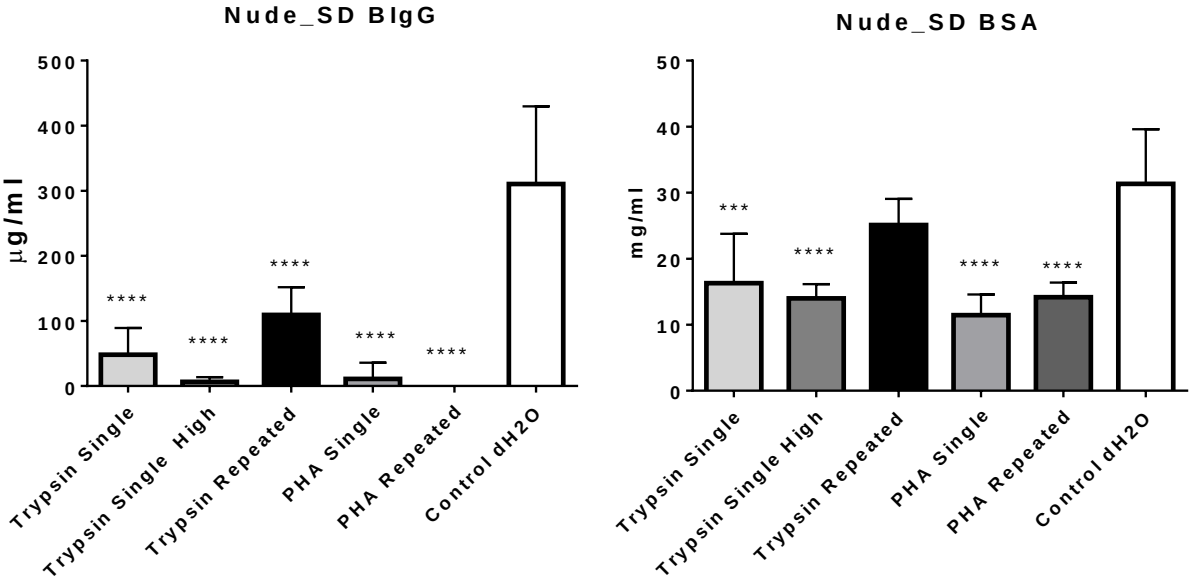

Figure 22. Permeability of BIgG and BSA in the small intestine of Nude_SD young rats. *The plasma concentration of BIgG (µg/ml) and BSA (mg/ml) in Nude_SD treated with trypsin single dose 0.6mg/g.bwt (6 individuals), trypsin single high dose 1mg/g.bwt (4 individuals), trypsin repeated dose 0.6mg/g.bwt/day for three days (7 individuals), PHA single dose 0.1mg/g.bwt (4 individuals), PHA repeated dose 0.05mg/g.bwt/day for three days (8 individuals) and a control group treated with dH2O 0.01ml/g.bwt (5 individuals). The results are expressed as means ±SD. One-way ANOVA was performed***=p<0.001,****=p<0.0001. Significant decrease of plasma BIgG could be observed in all treatment groups and for BSA, except trypsin repeated treatment.*

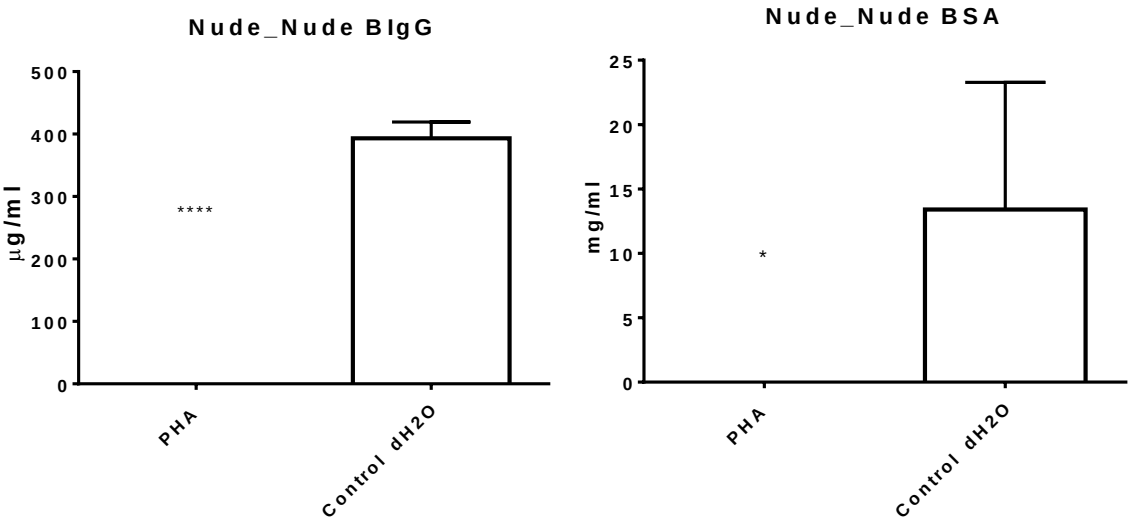

Figure 23. Permeability of BIgG and BSA in the small intestine of Nude_Nude young rats. The concentration of BIgG in the plasma measured in mg/ml and the concentration of BSA measured mg/ml from Nude_SD treated *PHA single dose 0.1mg/g.bwt (4 individuals) and a control group treated with dH2O 0.01ml/g.bwt (3 individuals). The results are expressed as means ±SD. Unpaired t-test was performed,*=p<0.1, ****=p<0.0001. Significant decrease of BIgG and BSA could be observed in PHA treated Nude_Nude rats. PHA induced total closure of the small intestine, no marker molecules were detected in blood after treatment.*

Immunohistochemistry analysis for detection of T cells

The Nude_SD small intestine was stained with α-Thy-1 to investigate the presence of immature T cells. Few Thy-1 positive cells were found distributed between all treatment groups and also in the control group treated with dH2O (result not shown).

The small intestine from Nude_SD, Nude_Nude and euthymic rat pups, during natural development from 7 days to 28 days after birth, were stained with α-CD3 to investigate the presence of mature T cells. In euthymic young rats CD3+ cell could be found in all ages (Fig. 24). In 7 days of age and in 14 days of age the amount of CD3+ cells were low (estimated as +++, table 3), but a small increase of CD3+ cells could be seen from 7 days of age to 14 days of age. Around weaning, 21 days of age, the CD3+ cells increased (++++) until day 28 (+++++).

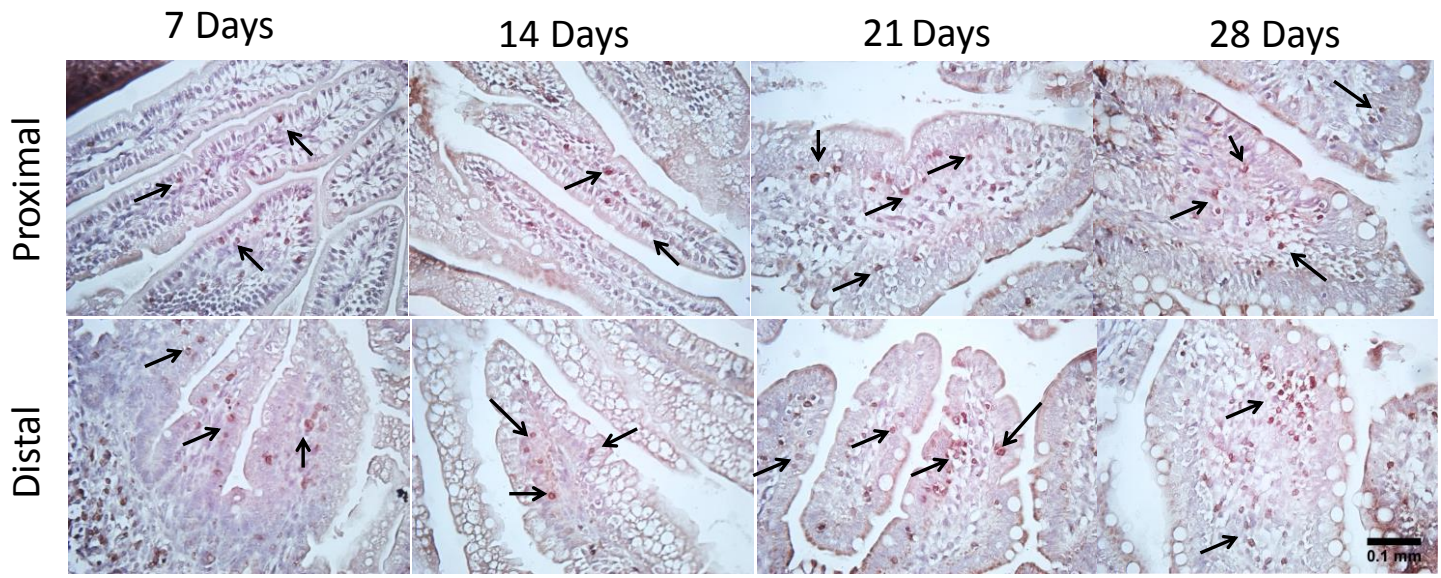

Figure 24. CD3+ cells in natural development. *Proximal and distal small intestine of natural development of SD rats, 7 to 28 days of age, analysed with immunostaining, α-CD3, and counterstained with haematoxylin Mayers, 400x. CD3+ cells could be observed at all ages and the amount of CD3+ cells were estimated to approximately 5 cells/villi (+++) for 7 and 14 days of age, 10 cells/villi (++++) for 21 days of age and >10 cells/villi (+++++) for 28 days of age.*

In the Nude_SD rat pups a small amount of CD3+ cells, less than one positive cell per villi, could be seen in the small intestine in all treatment groups, even in the control group treated with dH2O (Fig. 25). The colored lining of the villi is unspecific reactivity due to the secondary system. The CD3+ cells were also found in the Nude_Nude small intestine of both PHA treated and control (Fig. 26). The nude rat tissue had very few T cells in the small intestine compared to the euthymic rats, even the 7 days old normal rat pup tissue (+++) contained a lot more positive cells than the 17 days nude rat tissue (+). The amount of CD3+ cells in the nude tissue were estimated as less than 1 cell/villi, and no different pattern could be seen between the Nude_SD and Nude_Nude or between the different treatment groups or between proximal and distal parts. The analysis of the CD3+ cells were only made with qualitative analysis due to time limits, to obtain a more specific result a quantification of cells/area could be done.

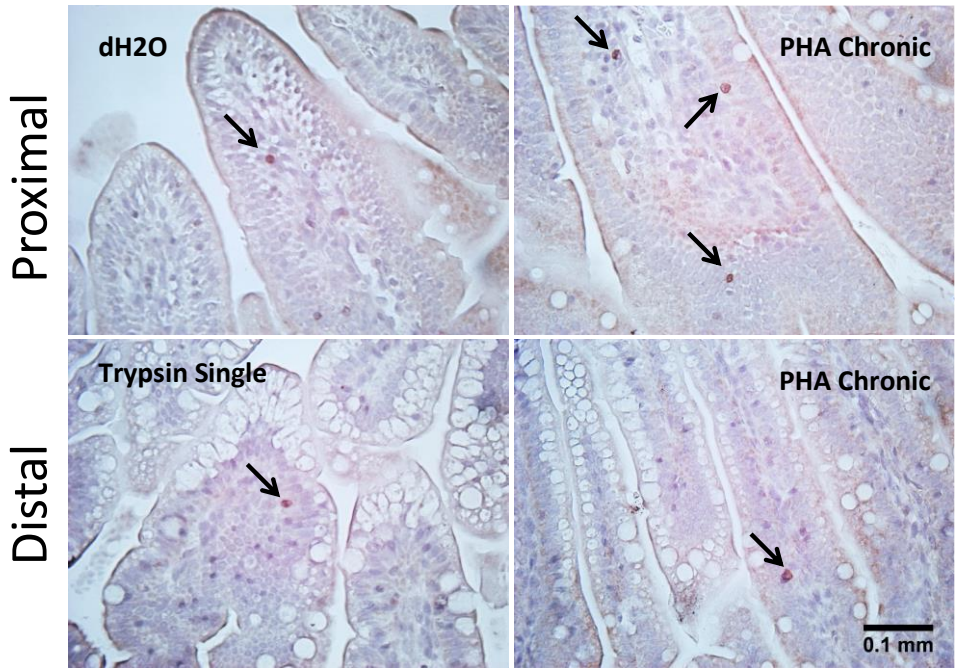

Figure 25. CD3+ cells in Nude_SD young rats. *Proximal and distal small intestine of Nude_SD rat pups treated with either PHA, trypsin or dH2O analysed with immunostaining, α-CD3, and counterstained with haematoxylin Mayers, 400x. CD3+ cells could be found in all treatment groups and the control group. The amount of CD3+ cells were estimated as less than1 cell/villi (+).*

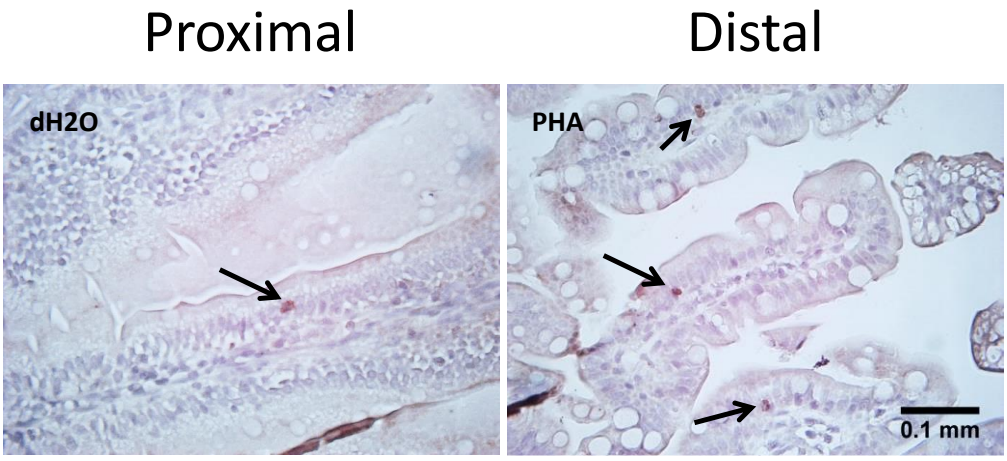

Figure 26. CD3+ cells in Nude_Nude young rats. *Proximal and distal small intestine of Nude_Nude rat pups treated with PHA or dH2O analysed with immunostaining, α-CD3, and counterstained with haematoxylin Mayers, 400x. CD3+ cells could be found in the PHA treatment and control group. The amount of CD3+ cells were estimated as less than 1 cell/villi (+).*

Discussion

Antibody testing for IHC

At first I tested different antibodies to find their optimal dilution (table 4) for the IHC and target tissue. To be sure that the method worked properly previously tested, optimised and established antibodies in the lab, rabbit polyclonal antibody α -FcRn and mouse monoclonal antibody α – PCNA, were used as technical controls of the method. FcRn is an essential receptor for the transfer of maternal IgG to the infant and it is mainly expressed by epithelial cells in the small intestine of suckling rats [28]. PCNA is a protein that acts as a cofactor of DNA polymerase delta, thus it is found in the nucleus of proliferating cells, mainly localised in the small intestinal crypts [29].

The α-MCT, α-CD45 and α-SI antibodies stained clearly without a lot of unspecific staining. MCT and CD45 are targets of the immune system, MCT is a protease stored and secreted by mast cells as an inflammatory response while CD45 is a transmembrane glycoprotein expressed on all leucocytes [30, 31]. Sucrase-isomaltase is a brush border membrane protein which is important for the final stages of carbohydrate digestion and is localized on the apical side of the enterocytes mainly in the proximal small intestine [32]. MAdCAM is a mucosal vascular addressin that acts as a homing receptor for recirculating lymphocytes to the Peyer´s patches and intestinal LP and IL-2Rα is a subunit of the IL-2 receptor, which is a surface receptor with multiple biological processes, like cell-growth and proliferation of activated T- and B-cells [7, 33]. α-MAdCAM and α -IL-2Rα gave no staining in the suckling rat tissue indicating that the antibodies did not function in IHC and were possibly too old to be used. The α-Iβ7 gave positive staining but it was unclear and unspecific and gave a lot of background staining in the target tissue. Iβ7 is a receptor mainly involved in trafficking and retention of lymphocytes, hence expected to be found around blood vessels in the LP [34] but it was not. To get a better result with α-Iβ7 and the other antibodies tested antigen retrieval could have been done before the staining procedure to reveal the hidden antigens. If there would have been more time a positive and a negative control for the antibodies could have been used. As a positive control a tissue that is known to express the wanted antigen could be used and as a negative control the tissue could be treated with peptide blocking of the antigen. If the antibody gives positive staining after peptide blocking the antibody stain unspecifically[35].

Thy-1 is a glycoprotein that is expressed by many different cell types like neurons and thymocytes. It promotes T cell activation and is involved in cell death, migration, cellular adhesion etc. (https://www.bio-rad-antibodies.com/cd90-glycosylphosphatidylinositol-anchored-glycoprotein.html; 10-06-2016). Thy-1 is expressed by T cells in mice and it has been reported that nude mice also express Thy-1 on their mature T cells [22]. In this study the investigation for the optimal dilution for the antibody against Thy-1 observed that Thy-1 is only expressed by precursor T cells in the rat. This was realized since the positive staining of the antibody was seen in the cortical areas of the thymus, which is the location for the earliest events in T cell development [36], in adult euthymic rats. There was no positive staining in the spleen of the adult rat, where there are a lot of mature T cells. These results indicate that Thy-1 is only expressed on precursor T cells in the rat tissue, and it agrees with previous results [37]. The result of the present study showed that Thy-1+ cells could be observed in the small intestine of the adult euthymic rat and this could possibly be precursor T cells that will mature in the gut independent of the thymus.

CD3 is a co-receptor of the T cell receptor (TCR), non-covalently associated, and is important in T cell activation. The CD3 complex is a defining feature of the T cell lineage and thus a marker for T cells (https://www.bio-rad-antibodies.com/minireview-cd3-antibody.html; 10-06-2016). It was found in the medullar

parts of the thymus, which is the location for the later events of the T cell maturation [36], indicating that the antibody properly stains mature T cells. It was also found in the white pulp of the spleen, assumable the T cell rich regions, which confirms that the antibody stains specifically for CD3+ cells.

Mechanisms of action of PHA and trypsin

Some differences can be seen between the treatment with PHA and trypsin, even though no specific pattern has been evident more than the fact that trypsin needs a higher concentration to induce the same maturation state, the differences might be due to different mechanisms of action for the two treatment substances.

After feeding with PHA it will bind to the epithelium and line the whole villi, from the tip to the beginning of the crypts in the small intestine[38]. This will result in the disturbance of the morphology of the small intestine leading to villi shortening, which will later be restored through the increase in crypt cell proliferation [11, 38]. The direct binding of PHA stimulated crypt hyperplasia [11], which might be through the activation of the mitogen-activated protein kinase cascade known to be involved in cell growth and differentiation. It has been shown that PHA activated this cascade in both human and rodent intestinal cell-lines [39]. The crypt hyperplasia lead to the increase in villi length, deeper crypts and the change from vacuolated cells to adult-like non-vacuolated cells in the distal epithelium [10, 11, 38], that could be seen in the treated nude rats. The binding of PHA to enteroendocrine cells have been shown to lead to the release of cholecystokinin (CCK) [40], which is a GI hormone that stimulates the secretion of digestive enzymes from the exocrine pancreas [41], thus the increase of pancreatic trypsin seen in the nude suckling rats might be stimulated by the release of CCK. It has been shown that precociously induced maturation in euthymic suckling rats is associated with the activation of the immune system and an increase in the numbers of intestinal T and B cells [11]. There are theories that binding of PHA to the intestinal epithelium will induce the secretion of cytokines and growth factors, which will stimulate the recruitment of immune cells and their activation. The immune cells will then in turn be involved in the stimulation of growth and the development of the GI tract [11, 15, 42].

During normal weaning there is an increase in the secretion of enzymes from the pancreatic acinar cells, including trypsin [1], and feeding with exogenous trypsin lead to precociously induced maturation of the GI tract of the suckling rat indicating that trypsin is involved in the process of maturation. Studies have shown that trypsin might be a signaling molecule that specifically regulates cells by cleaving and triggering proteinase-activated receptor 2 (PAR-2), which is a G-protein-coupled receptor. PAR-2 receptors can be found in the gastrointestinal tract on the apical and basolateral side of enterocytes in both crypts and villi of the small intestine, on the pancreas and even on T cell lines [43-45]. At concentrations that are normally present in the intestinal lumen trypsin activates PAR-2 at the apical side of the enterocytes which will stimulate eicosanoid secretion, which act in a paracrine and autocrine manner and regulate multiple processes in the intestine [45]. It is possible that the exogenous trypsin given to the suckling rat pups activates PAR-2 signaling in the small intestine that provoke a pro-inflammatory response by the secretion of eicosanoids and thereby stimulate the induced maturation. It has been shown that eicosanoids act locally in the intestine to regulate growth and mediate inflammation [46].

Effects of PHA and trypsin on the small intestine in nude rat pups

The gut of the young rat undergoes the final remodeling process during the third postnatal week, the weaning period. This is the time when the GI tract has to be fully adapted and able to digest an adult diet. The process includes gut growth, change in epithelial cell kinetics and a decrease in gut permeability

[3, 16]. It has been established in previous studies that maturation can be precociously induced in suckling rat pups by oral feeding PHA or protease [10-12]. In this study the precociously induced maturation was investigated in nude rat pups due to their lack in a functional thymus leading to a T cell deficiency and the study aimed to examine the importance of T cells during the maturation process of the GI tract. The direct effects seen in the nude rat pups in this study by PHA treatment were the increase in proximal villi length and the change from vacuolated cells, in the distal small intestine, to an adult-like non-vacuolated epithelium. This coincides with the effects seen in the small intestine of euthymic suckling rat pups treated with PHA and at normal weaning [3, 10, 11, 16]. The trypsin treated nude rat pups showed a similar maturation pattern for the distal part of the small intestine in the change into adult-like epithelium, which was also observed in euthymic suckling rats treated with protease [12]. The increase in villi length in the proximal small intestine was only observed for treatment with trypsin single dose and not for any of the other trypsin treatments compared to the control. In the euthymic rat pups treated with protease the increase in villi length could be seen [12].

The change into an adult functional phenotype of the distal small intestine was in this study measured as maturity of the epithelium, the proportion of the non-vacuolated cells to the total villi length presented in percentage. The results obtained showed a large range between the treatment groups in the maturity, from non-significant to almost 100% maturity (Fig. 14 and 17). Repeated trypsin treatment had no significant increase in the maturation of the epithelium. Trypsin single, PHA single and PHA repeated treatments had a small significant increase, while trypsin single high treatment had a high increase in the maturity. The exogenous substances will temporarily cause a mucosal disturbance that will lead to an accelerated crypt cell proliferation and later the change into a mature epithelium [10, 38]. The trypsin single high dose might have severe effects on the epithelium, leading to severe disturbance and the renewal has to arise quickly. This could be the explanation to the high significance in only this parameter; it is a direct and acute effect of the damage.

In natural weaning and in precociously induced maturation the crypts get significantly deeper due to the accelerated cell proliferation [3, 11, 12, 16]. In the present study only treatment with PHA chronic dose showed increase in crypt depth in the nude rat pups (Fig. 9.). Previous studies have shown that mucosal T cells promote the crypt hyperplasia and regulate enterocyte growth under normal conditions and during weaning [13, 47-49]. It was proven in this study that the nude rat pups had CD3+ lymphocytes in their small intestine, even if the amount observed is lower than euthymic rat pups (Fig. 25 and 26). It might be that this small amount of T cells is enough to stimulate cell proliferation and promote exchange of vacuolated epithelial cells to adult-like non-vacuolated cells, but might not be enough to provoke the rate of cell proliferation that is needed for expanding the crypt depth.

The small intestinal weight of the Nude_SD and Nude_Nude rat pups was measured and analysed (by others) showing that both PHA and trypsin had growth promoting effects on the proximal and distal small intestine compared to the control (Fig. 18 and 19). There were no visible differences between the Nude_SD and Nude_Nude of the PHA single treatment. There are some small differences between the treatment groups in the Nude_SD rat pups, where the PHA treatment and the trypsin single high dose have the highest increase. The results coincide with earlier studies made on euthymic rat pups treated with either PHA or protease [10-12]. Even though the rat pups in this study were immunodeficient they show similarities in the pattern for precociously induced maturation as euthymic rat pups and normal weaning.

In addition to the organ weight, the permeability of the small intestine of the nude rat pups was measured by feeding a marker solution cocktail, containing BIgG and BSA, to the pups three hours before euthanizing. The concentration of the markers was measured in plasma (analysed by others) obtained during sample collection. The results obtained showed decrease in the uptake of macromolecules for almost all treatment groups compared to the control for both BSA and BIgG (Fig. 22 and 23). Trypsin repeated had no significant decrease in the uptake of BSA. This result indicates that the nude rat pups have a similar gut closure as the one seen at weaning. This effect can also be seen in induced maturation in euthymic rat pups [10-12]. The decrease in BSA coincides with the decrease in vacuolated cells in the distal small intestine of the treated nude rat pups, which is responsible for the unspecific transport of macromolecules over the epithelium [3]. The decrease in the uptake of BIgG might be a consequence of decrease in the receptor FcRn, which is a specific receptor mainly for the uptake of IgGs from the colostrum and maternal milk [3, 50]. The presence of FcRn was not considered in this study but according to previous studies the FcRn receptors decrease during precocious maturation in euthymic rat pups [10-12]. Since many similarities were observed between the euthymic and nude rat pups during precocious induced maturation this could be a possible explanation.

During normal weaning the pancreas also undergoes a process of maturation parallel to that of the GI tract, which includes weight gain and increase in enzyme production and secretion [1]. The pancreas of the nude rat pups was investigated by others and no significant increase of the pancreas weight could be observed (Fig. 18 and 19). However, all of the PHA treated groups, including the Nude_Nude rat pups, showed increase in trypsin activity in the pancreas (Fig.20 and 21). This agrees with the studies made on euthymic rat pups treated with PHA, except that the euthymic rat pups had an increase in pancreatic weight [10, 11]. The trypsin treated groups only had significant increase for trypsin activity in trypsin single high group compared to the control group. The results coincide with the previous study made on euthymic rat pups treated with microbial-like pancreatic enzymes, where the protease treated rats showed increase in trypsin activity. The increase in enzyme production of the exocrine pancreas seems to be associated with postnatal changes of the hormone levels, such as glucocorticoids, and the increase of such receptors in young rats at weaning [1, 51].

The present study showed that maturation of the GI tract can be precociously induced in immunodeficient suckling rats, with an impaired T cell function, by feeding with a red kidney bean lectin or a pancreatic protease. These substances stimulated the proliferation of crypt cells leading to the lengthening of the proximal villus and change from vacuolated cells to adult-like non-vacuolated cells in the distal small intestine. The effects of trypsin treatment seem to be dose related since most effects are seen for only trypsin single high dose. Additionally it was shown that there are mature T cells present in the small intestine in nude suckling rats at 17 days of age, despite their dysfunctional thymus. No differences in the maturation pattern could be observed between the Nude_SD and Nude_Nude rat pups treated with PHA.

Thy-1 + and CD3+ cells in the small intestine of nude rats

Normal weaning and precociously induced maturation in euthymic young rats result in an increase in immune cells, especially T cells, while the inhibition of T cells with CyA has shown an impaired and delayed maturation. Blocking of IL-2R also leads to the same outcome [13] all indicating that T cells have an important role in the maturation and development of the GI tract. The intestine of the euthymic rat has CD3+ intraepithelial lymphocytes (IEL) already the first day after birth, which will increase progressively throughout the suckling period [52], this might suggest that CD3+ IELs are not only

important for the immune response during weaning but might also be important for the protection of the neonatal rat from the first day after birth.

It has been proven that adult nude mice and rats have T cells expressing CD3, and they accumulate with age [23-25]. Nude mice at 8 weeks of age did not express any CD3+ cells in the spleen or lymph nodes, but after 8 weeks they started to increase in numbers [24]. The maturation of extrathymic T cells mostly takes place in the MLN and then the T cells migrate to the intestine where they accumulate, mostly as IELs [23]. A study made on nude neonatal rats showed that mature T cells could not be developed and that CD3+ IELs did not appear until four to six month of age [53]. These results lead to the speculation that nude rats would have a delayed maturation during weaning, due to the decrease in mature T cells. The present study did not agree with this speculation, and the presence of CD3+ T cells was observed in the nude suckling rat small intestine, albeit in lower amounts. The nude rat tissue of the small intestine were stained with α-CD3 antibodies and the presence of CD3+ cells could be observed in all treatment groups even the control, indicating that extrathymic T cell maturation takes place in the gut. There was clearly less CD3+ cells in the nude small intestine of 17 days old suckling rats compared to the euthymic suckling rats from 7 days of age up to 28 days after birth. No pattern between the different treatment groups could be observed; they all had a very small amount of CD3+ cells. The CD3+ cells were found in the LP of the villi and close to the crypts. They were also found in the epithelium indicating that these cells are CD3+ IELs. This agrees with another study that also showed the presence of mature T cells in nude young rats [17]. The study investigated mature T cells expressing IL-2R and CD5 in the MLNs during weaning, which showed that there was approximately the same numbers of these positive cells in both euthymic and nude rat pups. Even though they found that the total number of T cells, in the T-dependent interfollicular areas of the MLN, was decreased in the nude rat pups. Due to these findings it is not possible to exclude the role of T-cells in the maturation process in suckling rats during weaning[17].

The presence of mature T cells in the nude rat pups might be explained by the theory that maternal-milk derived T cells can be transported to the neonatal gut mucosa after birth, where they can get activated by antigens from the lumen [17, 54, 55]. The present study investigated both nude rat pups nurtured by euthymic dams and nude rat pups nurtured by their natural dam, no difference in the presence or numbers of CD3+ cells could be observed. This suggests that there is no significant transport of maternal T cells to the offspring from the maternal milk. This agrees with a previous study made on euthymic mice indicating that there is no transport of maternal T cells to the gut after birth [56]. The presence of Thy-1+ cells in the Nude_SD small intestine was also investigated in this study. Few positive cells could be found and they were distributed between the treatment groups including the control group. Too few samples were included in this investigation, thus no significant results can be observed. It can be hypothesized that there are very few Thy-1+ cells because they have differentiated into mature T cells. The increase in pancreas growth, villi width and crypt depth were not significantly clear in the nude suckling rats treated with PHA or trypsin, as it was for euthymic suckling rats treated the same way. The decrease in mature T cells might be the explanation, since an increase in the crypt depth could be seen in all treatment groups even if it was not significant for more than treatment with PHA chronic, and a reduced number of T cells might delay the precociously induced maturation in nude rats.

Conclusion

As can be understood there are many factors playing a part in the complex development of the small intestine including exogenous and endogenous molecules, intracellular communication, hormonal

secretion and physiological inflammation. The immune response seen at natural weaning and during precociously induced maturation is an important feature for the maturation and development of the GI tract, were the effect of T cells seems to play a key role. T cells could be detected in nude suckling rats indicating that the thymus-independent T cell maturation in the gut is stimulated shortly after birth. Even a small amount of T cells seems to be important for the maturation process. More research is needed in many aspects of the induced maturation, the maturation process of the extrathymic T cells and the mechanism of action of PHA and trypsin needs to be further studied to get a clearer picture of the maturation and development process. The discoveries of the presence of T cell in nude suckling rats might lead to better understanding of the key players of the maturation of the GI tract and hopefully help in the search for treatment of NEC in preterm birth infants.

References

1. Walthall K, Cappon GD, Hurtt ME, et al. Postnatal development of the gastrointestinal system: A species comparison. Birth defects research. Part B, Developmental and reproductive toxicology 2005;74:132-56.
2. Barrett KE. In: M.Thomas MWaC, ed. Gastrointestinal Physiology. 2nd ed. United States of America: McGraw-Hill Education, 2014:3-7.
3. Pacha J. Development of intestinal transport function in mammals. Physiological Reviews 2000;80:1633-1667.
4. Abbas AK, Lichtman AHH, Pillai S. Basic Immunology: Functions and Disorders of the Immune System: Elsevier Health Sciences, 2012.
5. Femke Broere SGA, Michail V. Sitkovsky and Willem van Eden. T cell subsets and T cell-mediated immunity. In: Parnham FPNaMJ, ed. Principles of Immunopharmacology. Volume 3rd Springer, 2011:15-27.
6. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol 2009;27:591-619.
7. Miniami Y, Kono T, Miyazaki T, et al. The il-2 receptor complex: Its structure, function, and target genes. In: Paul WE, ed. Annual Review of Immunology. Volume 11: Annual Reviews Inc. {a}, P.O. Box 10139, 4139 El Camino Way, Palo Alto, California 94306, USA, 1993:245-268.
8. Pierzynowski SG, Westrom BR, Svendsen J, et al. Development of exocrine pancreas function in chronically cannulated pigs during 1-13 weeks of postnatal life. Journal of Pediatric Gastroenterology and Nutrition 1990;10:206-212.
9. Pierzynowski SG, Westrom BR, Erlanson Albertsson C, et al. Induction of exocrine pancreas maturation at weaning in young developing pigs. Journal of Pediatric Gastroenterology and Nutrition 1993;16:287-293.
10. Linderoth A, Biernat T, Prykhodko O, et al. Induced growth and maturation of the gastrointestinal tract after phaseolus vulgaris lectin exposure in suckling rats. Journal of Pediatric Gastroenterology and Nutrition 2005;41:195-203.
11. Prykhod'ko O, Fed'kiv O, Linderoth A, et al. Precocious gut maturation and immune cell expansion by single dose feeding the lectin phytohaemagglutinin to suckling rats. British Journal of Nutrition 2009;101:735-742.
12. Prykhodko O, Pierzynowski SG, Nikpey E, et al. Pancreatic and pancreatic-like microbial proteases accelerate gut maturation in neonatal rats. Plos One 2015;10:1-14.
13. Thompson FM, Mayrhofer G, Cummins AG. Dependence of epithelial growth of the small intestine on t-cell activation during weaning in the rat. Gastroenterology 1996;111:37-44.
14. Cummins AG, Thompson FM. Effect of breast milk and weaning on epithelial growth of the small intestine in humans. Gut 2002;51:748-754.

15. Pie S, Lalles JP, Blazy F, et al. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. Journal of Nutrition 2004;134:641-647.
16. Cummins AG, Steele TW, Labrooy JT, et al. Maturation of the rat small-intestine at weaning - changes in epithelial-cell kinetics, bacterial-flora, and mucosal immune activity. Gut 1988;29:1672-1679.
17. Cummins AG, Thompson FM, Mayrhofer G. Mucosal immune activation and maturation of the small-intestine at weaning in the hypothymic (nude) rat. Journal of Pediatric Gastroenterology and Nutrition 1991;12:361-368.
18. Cummins AG, Labrooy JT, Shearman DJC. The effect of cyclosporine-a in delaying maturation of the small-intestine during weaning in the rat. Clinical and Experimental Immunology 1989;75:451-456.
19. Gephart SM, McGrath JM, Effken JA, et al. Necrotizing enterocolitis risk state of the science. Advances in Neonatal Care 2012;12:77-87.
20. Puiman P, Stoll B. Animal models to study neonatal nutrition in humans. Current Opinion in Clinical Nutrition and Metabolic Care 2008;11:601-606.
21. Rolstad B. The athymic nude rat: An animal experimental model to reveal novel aspects of innate immune responses? Immunological Reviews 2001;184:136-144.
22. Hunig T. T-cell function and specificity in athymic mice. Immunology Today 1983;4:84-87.
23. Guy-Grand D, Azogui O, Celli S, et al. Extrathymic t cell lymphopoiesis: Ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. Journal of Experimental Medicine 2003;197:333-341.
24. Kennedy JD, Pierce CW, Lake JP. Extrathymic t-cell maturation - phenotypic analysis of t-cell subsets in nude-mice as a function of age. Journal of Immunology 1992;148:1620-1629.
25. Schwinzer R, Hedrich HJ, Wonigeit K. T cell differentiation in athymic nude rats (rnu/rnu): Demonstration of a distorted t cell subset structure by flow cytometry analysis. European Journal of Immunology 1989;19:1841-7.
26. Avwioro G. Histochemical uses of heamatoxylin - a review. Journal of Pharmacy and Clinical Sciences 2011;1:24-34.
27. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: Past, present, and future. Journal of Histochemistry & Cytochemistry 1997;45:327-343.
28. Roopenian DC, Akilesh S. Fcrn: The neonatal fc receptor comes of age. Nature Reviews Immunology 2007;7:715-725.
29. Strzalka W, Ziemienowicz A. Proliferating cell nuclear antigen (pcna): A key factor in DNA replication and cell cycle regulation. Annals of Botany 2011;107:1127-1140.
30. Payne V, Kam PCA. Mast cell tryptase: A review of its physiology and clinical significance. Anaesthesia 2004;59:695-703.
31. Altin JG, Sloan EK. The role of cd45 and cd45-associated molecules in t cell activation. Immunology and Cell Biology 1997;75:430-445.
32. Sim L, Willemsma C, Mohan S, et al. Structural basis for substrate selectivity in human maltase-glucoamylase and sucrase-isomaltase n-terminal domains. Journal of Biological Chemistry 2010;285:17763-17770.
33. Shyjan AM, Bertagnolli M, Kenney CJ, et al. Human mucosal addressin cell adhesion molecule-1 (madcam-1) demonstrates structural and functional similarities to the alpha 4 beta 7-integrin binding domains of murine madcam-1, but extreme divergence of mucin-like sequences. Journal of Immunology 1996;156:2851-7.
34. Gorfu G, Rivera-Nieves J, Ley K. Role of beta(7) integrins in intestinal lymphocyte homing and retention. Current Molecular Medicine 2009;9:836-850.
35. Bordeaux J, Welsh A, Agarwal S, et al. Antibody validation. Biotechniques 2010;48:197-209.
36. Zuniga-Pflucker JC. Innovation - t-cell development made simple. Nature Reviews Immunology 2004;4:67-72.
37. Crawford JM, Barton RW. Thy-1 glycoprotein - structure, distribution, and ontogeny. Laboratory Investigation 1986;54:122-135.

38. Linderoth A, Prykhod'ko O, Ahren B, et al. Binding and the effect of the red kidney bean lectin, phytohaemagglutinin, in the gastrointestinal tract of suckling rats. British Journal of Nutrition 2006;95:105-115.
39. Otte JM, Chen CX, Brunke G, et al. Mechanisms of lectin (phytohemagglutinin)-induced growth in small intestinal epithelial cells. Digestion 2001;64:169-178.
40. Herzig KH, Bardocz S, Grant G, et al. Red kidney bean lectin is a potent cholecystokinin releasing stimulus in the rat inducing pancreatic growth. Gut 1997;41:333-338.
41. Liddle RA. Cholecystokinin cells. Annual Review of Physiology 1997;59:221-42.
42. Schaeffer C, Diab-Assef M, Plateroti M, et al. Cytokine gene expression during postnatal small intestinal development: Regulation by glucocorticoids. Gut 2000;47:192-198.
43. Nystedt S, Emilsson IE, Wahlestedt C, et al. Molecular-cloning of a potential proteinase activated receptor. Proceedings of the National Academy of Sciences of the United States of America 1994;91:9208-9212.
44. Bohm SK, Kong WY, Bromme D, et al. Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochemical Journal 1996;314:1009-1016.
45. Kong WY, McConalogue K, Khitin LM, et al. Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. Proceedings of the National Academy of Sciences of the United States of America 1997;94:8884-8889.
46. Eberhart CE, Dubois RN. Eicosanoids and the gastrointestinal tract. Gastroenterology 1995;109:285-301.
47. Mowat AM, Felstein MV, Baca ME. Experimental studies of immunologically mediated enteropathy .III. Severe and progressive enteropathy during a graft-versus-host reaction in athymic mice. Immunology 1987;61:185-188.
48. MacDonald TT, Spencer J. Evidence that activated mucosal t cells play a role in the pathogenesis of enteropathy in human small intestine. Journal of Experimental Medicine 1988;167:1341-9.
49. Ferreira RC, Forsyth LE, Richman PI, et al. Changes in the rate of crypt epithelial cell proliferation and mucosal morphology induced by a t-cell-mediated response in human small intestine. Gastroenterology 1990;98:1255-63.
50. Menard S, Cerf-Bensussan N, Heyman M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal Immunology 2010;3:247-59.
51. Sangild PT, Westrom BR, Fowden AL, et al. Developmental regulation of the porcine exocrine pancreas by glucocorticoids. Journal of Pediatric Gastroenterology and Nutrition 1994;19:204-212.
52. Perez-Cano FJ, Castellote C, Gonzalez-Castro AM, et al. Developmental changes in intraepithelial t lymphocytes and nk cells in the small intestine of neonatal rats. Pediatric Research 2005;58:885-91.
53. Helgeland L, Brandtzaeg P, Rolstad B, et al. Sequential development of intraepithelial gamma delta and alpha beta t lymphocytes expressing cd8 alpha beta in neonatal rat intestine: Requirement for the thymus. Immunology 1997;92:447-56.
54. Hale ML, Hanna EE, Hansen CT. Nude mice from homozygous nude parents show smaller pfc responses to sheep erythrocytes than nude mice from heterozygous mothers. Nature 1976;260:44-45.
55. Cabinian A, Sinsimer D, Tang M, et al. Transfer of maternal immune cells by breastfeeding: Maternal cytotoxic T lymphocytes present in breast milk localize in the peyer's patches of the nursed infant. PLoS One 2016;11:e0156762.
56. Torow N, Yu K, Hassani K, et al. Active suppression of intestinal cd4(+)tcralphabeta(+) t-lymphocyte maturation during the postnatal period. Nature Communications 2015;6:7725.