Characterization of canine intestinal intraepithelial lymphocyte population subsets

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PhD Thesis

Submitted by

Nicole Luckschander-Zeller
from Austria

Thesis advisor

Prof. Dr. T. Brunner
Institute of Pathology
Medical/Vetsuisse Faculty of the University of Bern

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Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

TABLE OF CONTENTS

1. SUMMARY

Although lymphocytes seem to play an important role in the pathogenesis of canine inflammatory bowel disease (IBD), little is known about the canine intestinal immune cells. Therefore, the goal of this work was the in-depth phenotypical and functional characterization of IEL in the small and large intestine from a mono-breed population, i.e. Beagle dogs, in order to establish an unbiased "baseline" for future studies on the role and fate of intestinal T cells in the development of canine enteropathies.

In the first study we established a computer-assisted cell counting method for the evaluation of $CD3^+$ T lymphocytes in the intestinal lamina propria and in the epithelium of healthy adult Beagle dogs. Furthermore a comparison between neonatal and adult dogs was performed to investigate, whether distribution changes with increased age. This work demonstrates that $CD3^+$ T cells can be found frequently in canine intestinal epithelium, and can be accessed for further evaluation.

In the second study we established the isolation procedure of intestinal intraepithelial lymphocytes (IEL) from healthy adult Beagle dogs, followed by a multi-parameter flow cytometry analysis. Analysis revealed that canine IEL substantially differ from lymph node T cells and consist of various unconventional lymphocyte subsets, unique to mucosal surfaces. These include γδ T cells, and $CD4^-CD8^-$ and $CD8\alpha\alpha^+$ T cells. IEL populations in adult dogs were also compared to those isolated from neonatal Beagles. A high frequency of undifferentiated $CD4^-CD8^-$ T cells could be found in newborn dogs, whereas in adult dogs mature $CD4^+$ and $CD8^+$ T cells predominated.

In the functional characterization of canine IEL we focused on activation and proliferation characteristics of this cell population. While IEL alone did not show an activation-induced proliferation, they significantly inhibited the proliferation of activated lymph node T cells in a cell number-dependent manner. These findings demonstrate for the first time that canine IEL have an immunoregulatory phenotype, which may contribute to the maintenance of the intestinal immune homeostasis and may be lost in canine chronic enteropathies.

These results give first insights in the characterization of canine intestinal immune cells, which might contribute for a better understanding and future therapies of canine IBD.

2. INTRODUCTION

2.1. The intestine – a complex organ in a challenging environment

2.1.1. The intestine – provider and protector

One of the best known functions of the gastrointestinal tract is the absorption of water and the digestion and absorption of nutrients. The small intestine is mainly responsible for the mixture, digestion and absorption of carbohydrates, proteins and fat, as well as for water absorption. The large intestine accounts for forming and storage of feces, absorbs volatile fatty acids, completes water resorption and absorption of some minerals and trace elements. In addition to food-containing vitamins the large intestine is also responsible for the up-take of some vitamins produced by commensal bacteria. These tasks alone are enough to regard the gastrointestinal tract as an extraordinary organ considering the multitude of mechanical, enzymatic and transportation processes required to fulfill our daily needs regarding calories, nutrients and water uptake. The small and large intestine have developed different surfaces to optimize their respective functions. The small bowel has become the body's largest surface in order to create a maximal contact zone between the lumen containing digested food and absorptive cellular elements of the small bowel. The presence of Kerckring's folds, villi and microvilli in the small intestine results in a 600-fold increase in the surface area as compared with the surface of a simple cylindrical tube [1]. Instead of villi, the large bowel contains only crypts, which also contribute to the surface increment.

This enormous surface is a direct contact zone between the external environment (luminal content) and our body. This fact implies a constant exposure of the intestinal surface to food antigens and commensal bacteria but also to potential pathogens [2]. Therefore the immune system has developed complicated mechanisms to protect our body from possible pathogenic invaders on the one hand but to tolerate food antigens and harmless commensal bacteria on the other hand. A variety of immune cells located in the intestinal mucosa are responsible for maintaining this endangered balance.

In principal, the canine intestine is comparable to the gastrointestinal tract from mice and men concerning the anatomical situation (monogastric animals) and the mucosal

immune system in as much as it is known. Historically, the majority of studies had been carried out in rodents or tissue obtained from human patients. The expanding availability of species-specific tools provides the opportunity to study a wider range of animals, including dogs. Where species comparisons of mucosal immune system had been made, dogs had been shown to bear greatest similarity to that documented for man [3].

The high frequency of gastrointestinal diseases in dogs with often fatal outcome has urged veterinarians to better study immunological processes in the canine gastrointestinal mucosa. Therefore, we will discuss in the following introduction studies performed on canine intestine in comparison to those in mice and men. Furthermore, the work conducted under this PhD thesis, focusing on the detailed characterization of the canine intestinal immune system, should contribute to a better and more detailed understanding of the canine gastrointestinal immune system in health and in disease.

2.1.2. The intestinal epithelium – more than a physical barrier

The epithelial barrier is a dynamic and complex structure, which consists of various components. The first line of defense is built of mucin - glycoproteins produced by goblet cells. Intestinal mucin forms a thick layer, which lines the luminal surface of intestinal epithelial cells and reduces the ability of bacteria and viruses to attach to the epithelium (non-immune exclusion) [4]. It also serves as reservoir for secreted IgA (sIgA) and gives sIgA the possibility to bind to invading pathogens and impair their ability to penetrate the intestinal mucosa. There are several studies concerning intestinal IgA in various species. In contrast to cats, where the highest numbers of plasma cells is found in the ileum [5], in dogs intestinal plasma cells are mainly found in the duodenum [6].

The next line of defense is represented by a monolayer of epithelial cells. In this single cell layer individual cells are firmly connected by tight junctions, built by occludins, claudins and zonula occludens proteins, which makes a passage of macromolecules almost impossible. It is important to imagine the epithelial cells not only as a barrier but furthermore as a dynamic and reactive cell type, which plays an important role in the host defense. Epithelial cells can act as an antigen-presenting cell, but they are also able to produce anti-microbial peptides, chemoattractants and

immunoregulatory cytokines. In general, one can distinguish in the small intestine between four main epithelial cell types: absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells. In contrast to the short living epithelial cells (2-5 days), Paneth cells are long-living (approx. 20 days) and express a number of biological defense peptides and proteins including lysozyme, type II phospholipase 2, and alpha-defensins [7].

In order to detect foreign pathogens epithelial cells express Toll-like receptors (TLRs) (e.g. TLR4, TLR2) and nucleotide-binding oligomerization domains (NOD). These pattern recognition receptors recognize conserved motifs on pathogens and commensal bacteria. In this way epithelial cells are capable to mount immune responses, if necessary [8]. It has been also shown that TLRs expression on epithelial cells can be downregulated, possibly contributing to mucosal tolerance to commensal bacteria [9]. TLRs were recently the main focus of interest in canine research. It has been shown that antibodies specific for human or murine TLRs detect canine TLRs on leukocytes by flow cytometry [10]. Furthermore, it was possible to stimulate a T helper 1 (Th1) response *in vitro* by co-stimulating canine peripheral blood mononuclear cells with immune modulatory oligonucleotides (IMO, synthetic agonists of TLR9) and the lectin Concanavalin A [11]. TLR4 mRNA expression has also been detected in the canine stomach and small intestine [12]. These findings underline on the one hand the importance of TLRs in the intestinal immune system, but also confirm some of the similarities between different species such as mice and dogs.

There is a strong and direct interaction between epithelial cells and intestinal mucosal lymphocytes. First, epithelial cells are in a direct contact with lamina propria lymphocytes (LPL) via basolateral projections through the semi-porous membrane pores. Second, so-called intraepithelial lymphocytes (IEL) are tightly embedded in between the epithelial cells, above the intestinal basement membrane. In this situation intestinal epithelial cells are predisposed to act as “non-professional” antigen-presenting cells, predominantly activating $CD8^+$ T cells via MHC class I. Furthermore, epithelial cells can also function as “professional” antigen-presenting cells, capable of presenting antigen via MHC class II in order to stimulate $CD4^+$ T cells [13]. Another demonstration of the interaction between epithelial cells and lymphocytes is the great influence of epithelial cells on lymphocyte trafficking. For

example, the chemokine CCL25 is constitutively expressed by epithelial cells and leads to the recruitment of $CCR9^{+}$ $\alpha4\beta7^{+}$ T cells to the intestinal mucosa.

2.1.2. From mucosa-associated lymphoid tissues to the immune effector cells of the intestinal mucosa

In principle the intestinal immune cells are located in two major compartments: 1) the inductive compartment, where the initial antigen presentation to cells of the adaptive immune system occurs and 2) the effector compartment, which consists of the lamina propria, where antigen-experienced lymphocytes migrate to and the epithelium, where IEL residue [14]. The mucosal inductive sites are represented by specialized lymphoid tissues, such as Peyer`s Patches (PP), isolated lymphoid follicles (ILFs), and mesenteric lymph nodes (MLN). After oral intake of an antigen, the primary antigen presentation takes place either in the PP or in ILFs, which are generally considered as mucosa-associated lymphoid tissue (MALT). These structures share general features as they are covered with follicle-associated epithelium (FAE), which contains differentiated epithelial cells known as M cells [6]. M cells represent only "ports" for antigens, they do not process the antigen nor do they present antigen to immune cells. M cells have been shown to transport intact bacteria without harming them. Several gateways are possible for an antigen in order to penetrate the intestinal epithelium: 1) antigen uptake by PP-associated M cells, followed by direct delivery to APCs such as dendritic cells (DC) or macrophages located in the PPs; 2) villous M cell-dependent uptake followed by delivery to APCs located in the lamina propria; 3) dendritic cell-dependent sampling of luminal antigens, likely via transepithelial dendrites; 4) antigen uptake by intestinal epithelial cells followed by presentation of the antigen to lymphocytes via MHC class I or class II [15].

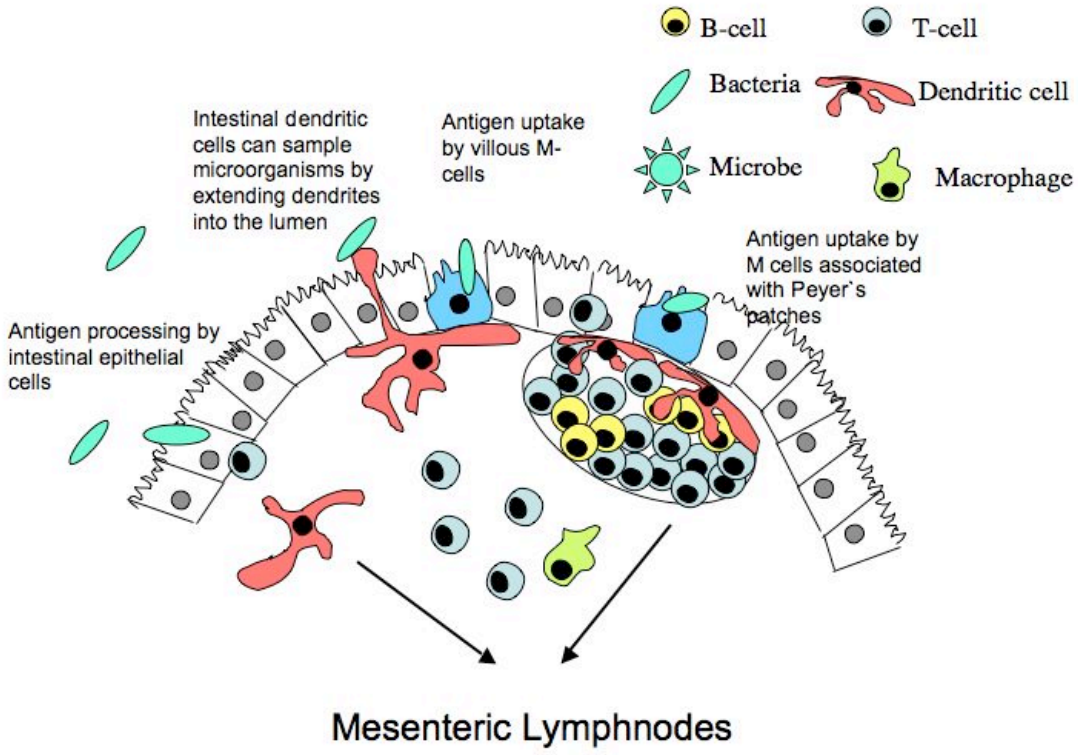

Figure 1: *Schematic presentation of intestinal antigen uptake pathways. Antigens can be taken up by epithelial cells and then presented via MHC class I or class II molecules, or dendritic cells can sample luminal antigen by extending dendrites through the epithelium. M cells can serve as "gates" through the epithelium, most often embedded in the FAE, in strong association with underlying Peyer`s Patches (PP). (adapted from Niess et al. [15])*

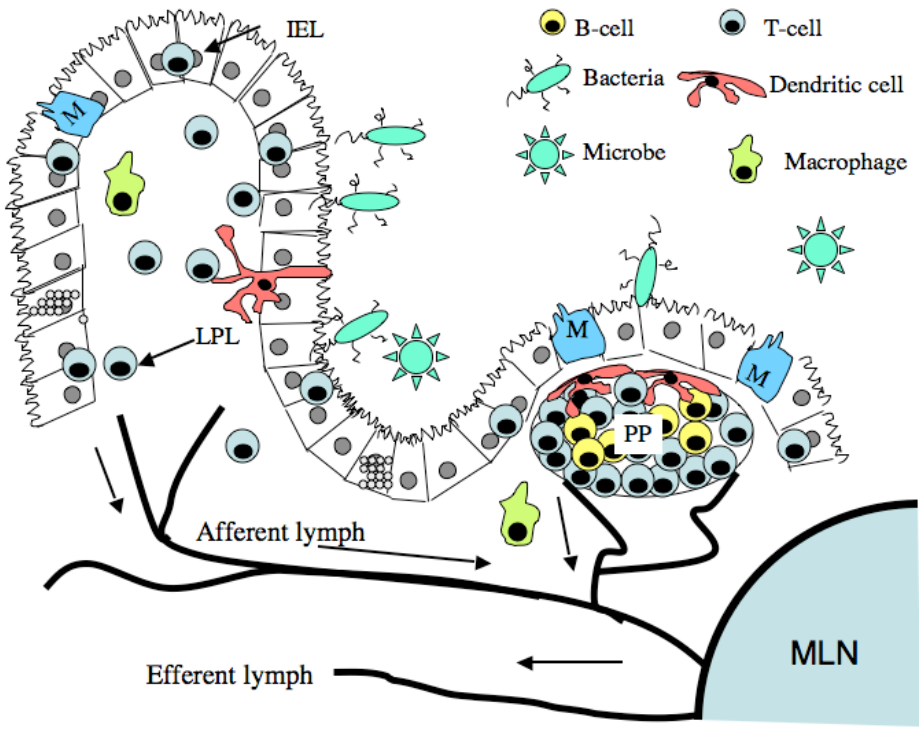

Figure 2: *Schematic diagram showing the major compartments of the gut-associated lymphoid tissue (GALT). Lymphoid cells are embedded in the epithelium (IEL), scattered throughout the lamina propria or concentrated in the Peyer`s Patches (PPs). Lymphocytes, activated by exposure to antigen in the PPs or in the lamina propria, migrate via afferent lymph to the mesenteric lymph nodes (MLN) and enter via efferent lymph the main blood stream.*

MHC class II expression in canine enterocytes has been confirmed by German et al. in 1998. Similar to men and mice, the strongest expression could be found in jejunal and ileal crypt areas [16].

Lymphocytes can be found in the intestine either embedded in the epithelium (IEL) or scattered throughout the lamina propria (LPL). We will discuss the differences between the two lymphocyte populations in a following chapter. In brief, most of the IEL belong to an “unconventional” subset of T cells. This subtype differs substantially from peripheral lymphocytes with respect to their phenotype, function and ontogeny [17]. In contrast, the majority of LPL belongs to the “conventional” T cell subset, which have a lot in common with peripheral lymphocytes. They home to the intestinal mucosa upon activation in the inductive sites such as PP or MLN. In the

following chapter on IEL the differences between distinct mucosal lymphocyte subsets will be discussed more in detail.

Although this work focuses on lymphocytes it should be mentioned that DC located in intestinal inductive sites (PP and MLN) are reported to have an important role in the priming of gut-homing lymphocytes [18,19].

2.1.3. Oral tolerance: Intestinal homeostasis and antigen-specific regulatory T cells

The induction of oral tolerance has been extensively studied in rodents, while fewer studies have been performed in dogs. However, it is clear that oral tolerance can be induced in dogs [20].

In principal, there are two primary effector mechanism of oral tolerance: 1) the induction of clonal anergy or deletion and 2) the induction of regulatory T cells that mediate active suppression. Generation of an immune response generally requires ligation of the T cell receptor with peptide MHC complexes in the presence of appropriate co-stimulatory molecules. Presentation of high doses of orally administered antigen induces primarily anergy / deletion of specific T cells due to the absence of co-stimulators or in the presence of inhibitory ligands [21]. It is reported, that high doses of oral antigen can lead to deletion of antigen-reactive T cells in PP of mice transgenic for the antigen-specific T cell receptor. The deletion was mediated by apoptosis, and was dependent on dosage and frequency of antigen feeding [22]. Feeding high doses of antigen to normal animals also increases the susceptibility of their lymphocytes to die by apoptosis after systemic challenge with antigen in adjuvant [23].

TGF-β production seems to be a link between tolerance induced by deletion / anergy and the induction of regulatory T cells. The immunosuppressive cytokine TGF-β is usually produced by T cells and stroma cells in the gut. It is an important factor for IgA class switching and induces FoxP3 expression in conventional T cells in order to turn these cells into regulatory T cells [24]. It has been reported that apoptotic cells are usually taken up by macrophages, and the phagocytosis of these dying cells results in the induction of TGF-β production. Also apoptotic T cells can release TGF–β in latent and bioactive form [25]. Thus, TGF-β production seems to have a central role in oral tolerance. For dogs, assays for the detection of cytokine expression by real-time RT-PCR have been developed for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18,

IFN-γ, TNF-α and TGF-β, and applied in healthy dogs. Transcripts of TGF-β, IL-18 and TNF-α were expressed 10 time more than those of IL-10 and IFN-γ [26].

Also a field of extensive investigations is the role of DC in oral homeostasis. The proposed route of conventional antigen uptake in the intestine is via M cells in the FAE, followed by presentation to DC located in the PP. DC of the PP comprise several distinct subsets, including some that produce IL-10 and polarize naïve T cells to a regulatory phenotype. Furthermore, it is likely that DC loaded with orally administered antigen migrate from the intestinal mucosa to meet naïve $CD4^+$ T cells in the MLN [27]. Due to the few species-specific reagents there is a lack of studies focusing on intestinal DC in dogs. However, there are studies in dogs, which describe the presence of MHC class II-expressing cells with a dendritic type of morphology distributed throughout the intestinal mucosa, most obviously at the villous base [16,28].

Regulatory T cells (Tregs) are in the center of interest in the field of oral tolerance. Tregs can be functionally defined as T cells that suppress immune responses either by producing IL-10 or TGF-β or by cell-cell contact [29]. There are different regulatory T cell subsets involved in the gut homeostasis and oral tolerance. Naturally arising Tregs (nTregs) are T cells, which arise from the thymus and enrich in the $CD4^+CD25^+$ subset. Although CD25 is a useful marker for Tregs, not all Tregs express CD25 and not all $CD25^+$ cells are Tregs. At the moment the most specific marker of nTregs is the transcription factor FoxP3. Deficiency or mutation in FoxP3 is associated in human patients (IPEX syndrome) and in mice (scurfy mouse) with severe autoimmune syndromes, which includes involvement of the small bowel, indicating that these cells are important for the maintenance of gut homeostasis [30]. There is further evidence that Foxp3 expression can be induced in the GALT. $CD103^+$ DCs seem to induce $FoxP3^+$ T cells in an antigen–specific manner, through a mechanism depending on TGF-β and retinoic acid [31]. There are also other induced Treg subsets. One of the first subsets described are Th3 cells, which mainly produce TGF-β. As already discussed, TGF-β is important in inducing peripheral Tregs, the presence of Th3 cells can also influence Tregs development in neighboring cells (infectious tolerance). Another well-described Tregs population are IL-10-secreting Tr1 cells. These cells are induced in vivo following mucosal administration of antigen and both their development and their function depends on IL-10 [32]. In order to

underline the importance of T cells with regulatory functions in the intestinal homeostasis it should be mentioned that knockout mice for TGF-β and CTLA-4 die rapidly due to multi-organ autoimmune disease. Furthermore IL-10-deficient mice develop spontaneous intestinal inflammation in the presence of *Helicobacter hepaticus* [33]. Of course Tregs are also of high interest in veterinary medicine. Until now, studies were hampered due to the lack of species-specific antibodies. However, recently a conjugated murine FoxP3 antibody was reported to be cross-reactive with canine lymphocytes. With the use of this antibody Biller et al. were able to demonstrate a significant increase of Tregs in the blood and peripheral lymphnodes in dogs with cancer compared to healthy control dogs [34].

Finally, it should be mentioned that besides the classical Tregs there are also intestinal $CD4^-$ T cell populations, which show a regulatory phenotype. These cells, which are mainly $CD8^+$ or $TCR\gamma\delta^+$, are located in the epithelium and are the subject of the following chapter.

2.2. Intraepithelial lymphocytes (IEL) – possible preservers of the intestinal homeostasis

2.2.1. IEL subsets in the small and large intestine

Intestinal intraepithelial lymphocytes (IEL) are mostly T cells, which are embedded in between the intestinal epithelial cells. In mice IEL comprise approx. 10–15% of all cells in the epithelial layer [35], in humans their frequency is even higher (16-20%) [36]. Immunohistochemical studies in the dog have shown that the numbers of IEL is in a similar range (12-20%), with higher frequencies in the villus than in the crypts [37]. Given their strategic location between the gut lumen and intestinal mucosa containing a large arsenal of immune cells, it is essential that IEL posses different skills that may allow to enhance immune responses or suppress inflammatory reactions, depending on the needs. Likely as a result of these different needs, multiple subpopulations of IEL are found with diverse effector functions. Many of these IEL subsets differ substantially from peripheral lymphocytes.

Peripheral T lymphocytes can be divided into MHC class II-restricted $CD4^+$ T-cell receptor (TCR)$\alpha\beta^+$ T cells and MHC class I-restricted $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ T cells. The IEL compartment consists predominantly of $CD8^+$ T cells, while $CD4^+$ T cells are rather under-represented in the epithelial layer of mice, humans and dogs. Approx.

10% of IEL in the murine small bowel, and also a substantial portion of canine IEL [38], has a $CD4^-$ $CD8^-$ (double negative) phenotype, which are usually absent in the peripheral circulation. Whereas almost all peripheral $CD8^+$ T cells express the CD8αβ heterodimer, a substantial portion of the $CD8^+$ IEL express the homodimer CD8αα. Whereas peripheral $CD8\alpha\beta^+$ T cells express predominantly the TCRαβ, the TCR usage in the IEL compartment is more complex. While a large number of $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ cells are found, $TCR\gamma\delta^+$ T cells, which often also express the CD8αα co-receptor, are frequently found in the epithelial layer. Although distinct IEL subsets show different phenotype, function and ontogeny, they all show an "activated but resting" immune cell phenotype [39]. For example, freshly isolated IEL show constitutive cytotoxic activity, but proliferate poorly in response to TCR stimulation [40].

In general, IEL can be subdivided into two major subpopulations based on the TCR and the co-receptors they express. Conventional or "type a" mucosal T cells express CD4 or CD8αβ together with TCRαβ. Unconventional or "type b" mucosal T cells include $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ T cells, $TCR\gamma\delta^+$ T cells with either CD8αα or CD8αβ co-receptor and $CD4^-$ $CD8^-$ T cells.

Conventional "type a" IEL share similarities with splenic or lymphatic $CD4^+$ and CD8αβ cells. These $TCR\alpha\beta^+$ T cells primarily recognize antigens presented by conventional MHC class I and II. In contrast to MLN T cells, which have a highly diverse TCRαβ repertoire, the TCRαβ repertoires of IEL is only oligoclonal and non-overlapping between $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ T cells [41]. "Type a" IEL are reported to share various TCR gene rearrangements with lamina proria and thoracic duct T cells [42]. These findings support the current idea that "type a" IEL are primed by antigen in the PP, are drained via mesenteric lymph node, enter the blood stream via the thoracic duct and home back into the epithelial layer. Their intestinal homing is dependent on α4β7 integrin, which binds to the mucosal adressin MADCAM 1. Further translocation into the epithelium requires a switch to αEβ7 expression, which binds to the epithelial adhesion molecule E-cadherin and allows the embedment of IEL into the epithelial layer.

Unconventional or "type b" mucosal T cells include $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ T cells, $TCR\gamma\delta^+$ T cells with either CD8αα or CD8αβ co-receptor, and $CD4^-$ $CD8^-$ T cells.

Although these diverse "type b" lymphocytes differ in some effector functions (e.g. the production of keratinocyte growth factor), they also share many similar features, which clearly distinguishes them from "type a" IEL (table1). Recent analysis of their gene expression profiles demonstrated that "type b" IEL are very similar but differ from "type a" IEL [43].

Properties		**Type a**	**Type b**
Gene expression	CD2	++	-
	CD5	++	+/-
	CD28	++	-
	CTLA4	++	-
	Ly6C	++	+/-
	Ly49E	-	++
TCRαβ chain gene usage overlaps with lamina propria and thoracic duct		++	-
Conventional MHC restriction		++	-
Representation in athymic mice		-	+
Reconstitution from peripheral lymph nodes		++	+/-
Immunological memory of infection		++	-

Table 1: General features of type a and type b IEL adapted from Hayday et al. [43]

"Type b" IEL are usually permanent residents in the intestinal epithelium. These cells do not recognize antigens presented by MHC class I or class II molecules, but are rather activated and primed directly by epithelial cells via a different set of molecules. In mice, a novel and specific ligand for the CD8αα homodimer was identified: the thymic leukemia (TL) antigen, a non-classical MHC class I molecule. TL is constitutively expressed on intestinal epithelial cells, suggesting that this receptor-ligand pair might play a role in the cross-communication between the intestinal epithelium and the neighboring IEL. Also TCRγδ$^+$ cells are reported to respond directly to non-classical MHC class I-like molecules (MHC class Ib). Mouse T10 or T22 or human MICA/MICB represent such non-classical MHC class Ib molecules. Several of these self-activating proteins can be induced by heat shock in gut epithelial cells or are upregulated in a variety of carcinomas [44].

A limited number of previous studies have investigated the distribution and quantification of IEL in the canine intestine. Immunohistochemical studies have shown that in the intestinal epithelial compartment mainly T cells can be found, which express TCRαβ and TCRγδ [45]. It was also demonstrated that $CD8^+$ IEL outnumber $CD4^+$ IEL in the canine intestinal epithelium [37]. Only 2 studies have used flow cytometry analysis for the further characterization of canine IEL subpopulations [38,46]. These studies also show, that the canine IEL compartment mainly consists of $CD8^+$ T cells. Sonea et al. could further show that a substantial portion of IEL consist of $CD8^-CD4^-$ T cells and she demonstrated for the first time $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ T cells in the canine epithelium. However, the lack of a multi-parameter flow cytometrical analysis in these studies makes the simultaneous analysis of the TCR usage of $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ IEL subsets impossible. Therefore an in depth characterization of canine IEL, as in mice and human tissue, is currently still missing and represents the major aim of this study.

2.2.2. Extra-thymic origin of "type b" IEL – still an unsolved issue

The evidence that "type b" IEL can be found in the gut of athymic mice (*nu*/*nu* mice), while they are absent in the spleen or peripheral lymph nodes, was the underlying reason to suggest that IEL may develop independent and outside of the thymus. In *nu/nu* mice, or neonatally thymectomized mice a considerable population of $TCR\gamma\delta^+$ IEL and a reduced number of $TCR\alpha\beta^+$ IEL could be detected in the intestine [47]. In addition, several groups have shown that fetal liver or bone marrow progenitors can reconstitute "type b" IEL in thymectomized recipients [48,49]. These findings suggest that maturation and selection of IEL is substantially different from that of peripheral T cells.

Most of the knowledge on the thymic selection process comes from the H-Y TCR transgenic mouse model. H-Y TCR transgenic mice express a transgenic TCR that recognizes a Y chromosome–encoded *smcy* peptide. Female mice, which do not express the H-Y antigen, show normal numbers of antigen-specific "type a" T cells in the periphery, whereas they harbor a diminished number of "type b" intestinal IEL. In contrast, in male mice that express the H-Y antigen all peripheral antigen-specific T cells are depleted, yet a high number of unconventional $CD8\alpha\alpha^+$ intestinal IEL is

maintained [50]. These data suggest that CD8αα^{+} IEL are not negatively selected and may develop outside the thymus.

Cryptopatches (CPs) are an accumulation of lymphocyte progenitors in the intestinal mucosa of newborn mice. With the discovery of these cryptopatches in the murine small intestine, the hypothesis developed that these serve as the first site for "type b" IEL development [51]. In contrast to these initial finding, it was found later on that lymphopoiesis occurs mainly in the MLN, but less in PP and not in CPs [47]. Thus, it is currently unclear to which extent CPs contribute to the development of "type b" IEL, and further investigations are needed to clarify this issue.

A current hypothesis suggests that unconventional IEL develop from double negative (DN) thymic progenitors that escape to the periphery before MHC-based selection in the thymus occurs. It has been shown that the negative selection of auto-reactive γδ T cells is very efficient in the adult thymus, but is markedly attenuated in the neonatal thymus. As a consequence γδ T cells escape negative selection, undergo extra-thymic differentiation, and find sanctuary in the intestinal epithelium [52]. This finding is consistent with the high number of unconventional IEL early in life. While some data support extra-thymic development of IEL, others do not. Thus, the true mechanism of "type b" IEL development is still under debate and awaiting further studies.

2.2.3. IEL effector functions

"Type a" IEL generally undergo efficient antigen- and MHC-driven positive and negative selection in the thymus. In general "type a" IEL show an effector/memory T cell phenotype, as they are likely primed outside the epithelium and homed back into the intestinal epithelium.

Many of the CD4^{+} IEL have regulatory functions, and have been implicated in the induction of oral tolerance and in the maintenance of intestinal homeostasis. Their importance in the prevention of intestinal inflammatory disorders has been demonstrated in various mouse models. For example, the transfer of naïve CD4^{+} CD45RB^{hi} T cells into immunodeficient recombination activation gene (Rag)2$^{-/-}$ recipients rapidly leads to the development of colitis. In contrast, co-transfer of CD4^{+} CD25^{+} or CD4^{+} αEβ7^{+} T cells can prevent disease development due to their

immunoregulary effector functions [53]. "Type a" CD8αβ$^+$ TCRaβ$^+$ IEL primarily represent antigen-specific cytotoxic T cells. They are able to kill via perforin/granzymes or engagement of the Fas receptor [54]. Adoptive transfer of "type a" antigen-specific intestinal IEL into a naïve host can protect against challenge. This was shown by a murine model, where IEL obtained from the intestines of mice infected with *Toxoplasma gondii*, provided long term protection to naïve hosts [55].
"Type b" IEL differ substantially from "type a" IEL with respect to expression of their cell surface markers, function and ontogeny – as already discussed earlier.
CD8αα$^+$ TCRαβ$^+$ IEL have been described as auto-reactive but not self-destructive. This finding is illustrated in TCR-antigen double transgenic mice. These mice express an LCMV (lymphocytic choriomenigitis virus) epitope-specific TCR transgene and the cognate antigen transgene driven by an MHC class I promoter. Upon systemic LCMV infection CD8αα$^+$ TCRαβ$^+$ T cells show clear signs of activation, but in contrast to conventional TCRαβ$^+$ T cells their activation does not result in cytolytic effector functions and tissue destruction [56]. TCRγδ$^+$ "type b" IEL are described to have different functions. They are not MHC class I- or class II-restricted and are able to recognize non-classical MHC class Ib molecules in order to act as a first line of defense [57]. Furthermore, TCRγδ$^+$ T cells promote epithelial wound healing by the production of keratinocyte growth factor (KGF), indicating another protective function of unconventional IEL.

2.3. Intestinal immunopathology

2.3.1. Intestinal inflammatory reactions – failure of the gut homeostasis

The intestine builds the largest surface of the body exposed to the external environment. The normal gut microflora comprises mainly bacteria but viruses, fungi and protozoa can also be present. The small bowel contains only few bacteria, while the highest number of bacteria can be found in the colon. Most of them are obligate anaerobes such as *Bacteroides, Eubacterium, Bifidobacterium, Fusobacterium, Peptostreptococcus* and others [58]. Intestinal microbiota represent not only a threat to our organism, but they also actively metabolize undigested carbohydrates and produce essential vitamins. Thus, the intestinal immune system must defend our body form invading pathogens and at the same time support symbiosis with commensals.

Failure of this highly sophisticated system can lead to inflammatory responses, tissue destruction and functional impairment.

As discussed in the chapter of oral tolerance the intestine is in a state of "suppressed inflammation". In general, under the influence of TGF-β and IL-10 innate and adaptive immune cells are kept suppressed after eradication of pathogens.

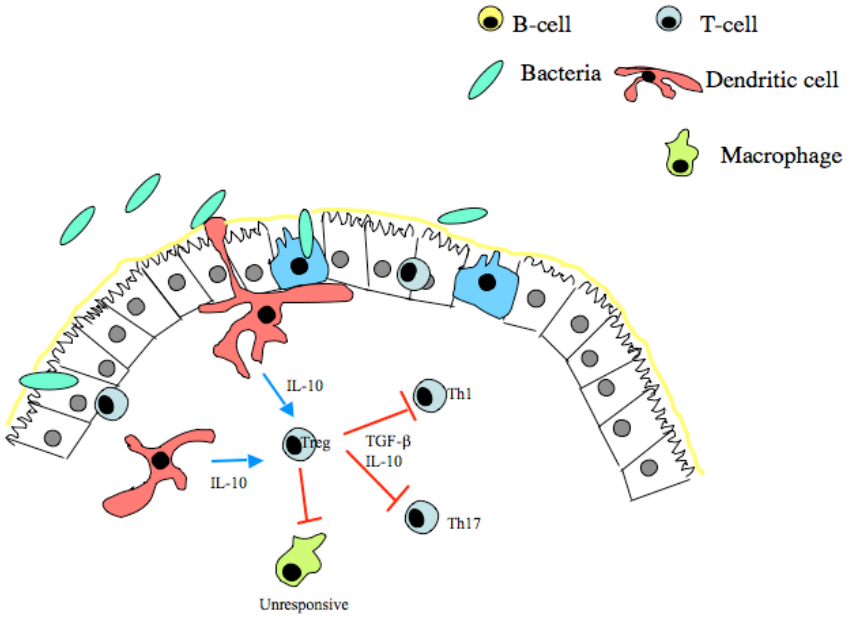

Figure 3: *Suppressed inflammatory state of the gastrointestinal mucosa (adapted from Sartor et al. [59]). In this state there is a high production of IL-10 and TGF-β, blocking overreactions of inflammatory (Th1 / Th17) responses*

However, if there is an epithelial injury, bacterial invasion can occur and induce the production of IL-12 and IL-23 in order to stimulate a Th1 or Th17 type of immune response. Th1 responses are mainly implicated in the pathogenesis of inflammatory bowel disease and a number of other autoimmune and inflammatory disorders. More recently IL-23 has attracted interest as a suggested key driver of chronic intestinal inflammation in humans and mice. IL-23 was shown to promote a novel subset of $CD4^+$ helper T cells (Th17 cells), which are able to produce pro-inflammatory cytokines as TNFα and IL-17 [60]. Several mouse models demonstrate the important role of IL-23 in the induction of intestinal inflammation. In $Rag^{-/-}$ mice, administration of an agonistic CD40 monoclonal antibody led to a systemic and intestinal inflammation. Functional analyses showed that the systemic response was driven by IL-12, whereas the intestinal inflammation required the presence of IL-23.

These results identify IL-23 as a key effector cytokine within the intestinal immune system [61].

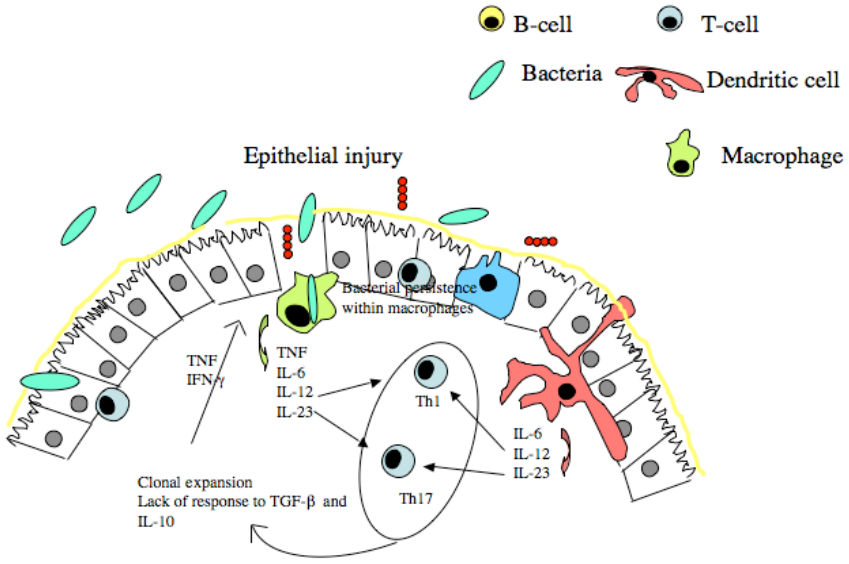

Figure 4: *Epithelial injury with bacterial invasion is followed by antigen presentation and IL-12 / IL-23 production. This leads to Th1 and Th17 clonal expansion and secretion of TNF and IFN-γ. The results are phagocyte activation, ongoing inflammation and mucosal damage (adapted from Sartor et al. [59]).*

Antigen stimulated $CD4^+$ helper cells are also able to secrete small amounts of IL-4, which promotes a Th2 response. Th2-driven cytokines are important for the development of ulcerative colitis (UC), as demonstrated in diverse mouse models. Intrarectal administration of the hapten oxazolon to mice without prior sensitization induces a Th2-driven colitis, characterized by the production of IL-4 and IL-5. In chronic forms of oxazolone colitis, the initial IL-4 response is super-imposed by an IL-13 response [62].

2.3.2. Inflammatory bowel disease (IBD)

There is general agreement among human and veterinary investigators that the external environment, the patient's genetic background, the intestinal microflora and the immune system are all involved and functionally integrated in the generation of IBD [63].

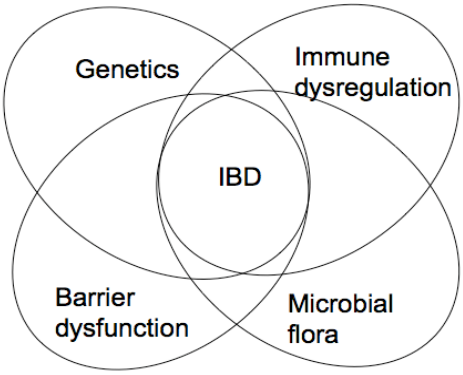

Figure 5: *The IBD pathogenesis includes several factors such as genetic background, environment, microbial flora and the immune system (adapted from Kucharzik et al. [64])*

The field of genetics has opened new insights into the pathogenesis of IBD. Several genetic loci have been linked with IBD. The most explored and described so far are the *NOD2/CARD 15* mutations. NOD2 belongs to a family of intracellular proteins that recognize bacterial muramyl dipeptide, a major component of the bacterial wall. Variants of NOD2 are not only present in some phenotypic subtypes of patients; they are also associated with some clinical aspects of the disease (e.g. early onset, fistula development) [63,64]. The precise mechanisms through which NOD2 mutations may predispose to the development of Crohn`s Disease are still under debate. Nevertheless, the discovery of these mutations in IBD patients strengthened the hypothesis that in IBD an aberrant immune response is directed against components of the intestinal microflora. In dogs, the genetic background of IBD is fairly unknown, although pure-bred dogs (e.g. Soft-coated Wheaten Terriers and Lundehunds), suffering from IBD and protein-losing enteropathy, seem to be over-represented in the veterinary literature [65,66].

The intestinal bacterial flora plays a major role in the initiation and perpetuation of chronic IBD. Animal models clearly demonstrated that colitis does not develop in IL-10 gene-deficient mice in a germ-free environment [67]. Furthermore, in human IBD, inflammation is present in parts of the gut containing the highest bacterial concentrations [68]. Also enhanced mucosal permeability may contribute to chronic intestinal inflammation. Recent data from animal models show that inflammation in the gastrointestinal tract can be initiated by molecular defects restricted to the

epithelium in the presence of normal flora and normal underlying innate and adaptive immune responses [69].

In human patients, IBD can be subdivided into Crohn`s Disease and ulcerative colitis (UC). The two forms of the disease do not only differ in their clinical picture, there are also very different immune processes at play. Crohn`s Disease is a marked transmural granulomatous process, which can affect any part from the gastrointestinal tract from the mouth to the anus and it is usually driven by a Th1/Th17 response. UC is a more superficial disease, which usually affects the colon and is driven by a Th2 response.

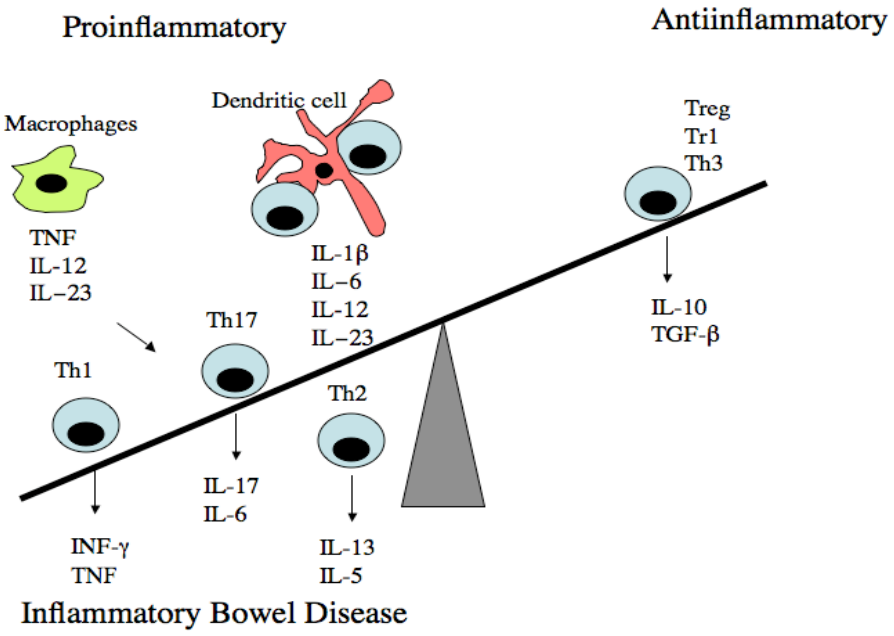

Figure 6: *Intestinal mucosal homeostasis demonstrates a fragile balance between effector cells and regulatory cells. In case of inflammatory bowel disease, there is an imbalance towards the inflammatory response. Depending on the type of the disease a Th1/ Th17 immune response or a Th2 immune response can dominate. (adapted from Sanchez-Munozet al. [70])*

Canine IBD still lacks these exact definitions. In dogs, the two major forms of IBD cannot be distinguished and IBD is common in both, the small and large intestine [71]. Despite the high frequency of IBD in dogs, the diagnosis is still based on the presence of chronic gastrointestinal symptoms, the exclusion of underlying infectious or neoplastic diseases and the histological evidence of benign intestinal inflammation. In dogs the most common form is a lymphocytic-plasmacytic infiltration in the

intestinal walls, although other cell infiltrates are also described (neutrophilic, eosinophilic) [72]. Increased numbers of $CD3^+$ T lymphocytes in the epithelium and in the lamina propria have been documented in the colon and in the duodenum of dogs with IBD [73,74]. These findings demonstrate the implication of T lymphocytes in canine IBD, however, so far no difference in cytokine expression could be found between healthy and IBD-affected dogs [26].

Although major progress has been made in the characterization of human IBD, the pathomechanisms of canine IBD are fairly unexplored. So it seems to be conclusive, that therapies, successfully established in human medicine, often fail in the treatment of canine patients or lead to unbearable side effects. This situation demonstrates the need for further and detailed studies in order to better understand canine intestinal pathomechanisms. The in-depth characterization of canine intestinal intraepithelial lymphocytes in this work gives new insights into the canine intestinal immune system. Therefore, this work may contribute to a better understanding and a more precise characterization of canine IBD into the future.

3. AIMS

The incidence of canine inflammatory bowel disease (IBD) increased in the last years, unfortunately often with a fatal outcome. Despite the high frequency of IBD in dogs, the diagnosis is still based on clinical signs of chronic diarrhoea, vomiting and weight loss, combined with histological evidence of inflammatory infiltration of the intestinal mucosa. Although uncontrolled activation of intestinal lymphocytes has been suggested one of the underlying reasons for canine IBD, little is known about the lymphocyte population in normal and diseased intestine due to the lack of species-specific reagents and standard protocols.

Therefore, the main goal of this project is the in-depth phenotypical and functional characterization of intestinal T cells in newborn and adult dogs to establish an experimental system for future studies on the role of intestinal T cells in the development of canine enteropathies.
Various steps seem to be necessary to reach this final goal.

I) PHENOTYPICAL CHARACTERIZATION

1) Immunohistochemical verification of the **distribution and quantification** of intestinal T cells (CD3+) in the epithelium and in the lamina propria in a monobreed standard. Furthermore a comparison between neonatal and adult dogs will be peformed to investigate, whether distribution changes with increased age.

2) Thorough phenotypical characterization of canine intraepithelial lymphocytes (IEL) in adult and neonatal dogs. The use of multiparameter flow cytometric analysis will allow the demonstration of differences between peripheral and IEL lymphocytes of the different **IEL subpopulations**. Furthermore, using this method the differentiation into conventional type a IEL and the until now never described unconventional type b IEL should be possible .

II) FUNCTIONAL CHARACTERIZATION

Analysis of canine IEL in regard of their **activation and proliferation** characteristics.

3) So far, no functional characterization of canine intestinal T cells exists. Thus, this part of the study will give important insights into whether intestinal T

cells vary substantially from their peripheral counterparts with regards to their activation and differentiation profiles.

After isolation of intestinal intraepithelial lymphocytes, cells will be further purified on a FACS Vantage cells sorter. Isolated T cells will be stimulated in vitro and T cell activation will be assessed by measuring proliferation (^{3}H - thymidine uptake). These experiments should reveal whether intestinal T cells form different parts of the canine intestine have a different activation pattern than their peripheral counterparts.

4) Analysis of **cytokine expression** of IEL by RT-PCR in regard of immunoregulatory cytokines (IL-10, TGF-β). Measurement of mRNA levels (IL-10, TGF-β, IFN–γ) upon stimulation of isolated and purified IEL in comparison to peripheral lymphocytes will be perfomed, investigating differences in the cytokine expression patterns.

4. DISSERTATION EQUIVALENTS

4.1. DISSERTATION EQUIVALENT I

Horizontal and vertical distribution of $CD3^+$ T lymphocytes in the intestine of healthy adult and neonatal dogs

N.S. Pfammatter, N. Luckschander, S. Jakob, S. Hartnack, T. Brunner, I.A. Burgener.

Despite the high frequency of canine inflammatory bowel disease and the often fatal outcome of this serious chronic enteropathy, the pathomechanisms of this disease are fairly unknown. Lymphocytes are suggested to play an important role in the pathogenesis of IBD in other species.

Therefore the main goal of this work was the description of the **distribution and quantification** of intestinal T cells ($CD3^+$) in the epithelium and in the lamina propria in a monobreed standard. Furthermore a comparison between neonatal and adult dogs was peformed to investigate, wheter distribution changes with increased age.

Full thickness biopsies were obtained from 7 different localizations (stomach, descending and ascending duodenum, jejunum, ileum, ascending and descending colon) from 6 adult and 4 neonatal healthy beagles. The paraformaldehyde-fixed, paraffin-embedded tissue was stained with an anti-CD3 antibody proven to recognize canine $CD3^+$ lymphocytes. Cell counts were made computer-assisted to obtain more objective results. The $CD3^+$ T lymphocytes were counted in an area of minimally 200,000 μm^2 in the villus and crypt region in the small bowel, whereas in the stomach and large bowel, they were counted in top and base layers.

In general, there was a maximum of $CD3^+$ T lymphocytes in the duodenum and jejunum, whereas a decline was seen towards the ileum and colon. Significantly more T cells could be found in the lamina propria than in the epithelium. The intraepithelial lymphocyctes (IEL) accumulated in the villi, only few IEL were located in the crypts. Compared to the adult dogs, the neonatal dogs showed a similar distribution pattern, but on average ten times less $CD3^+$ T lymphocytes in the analogous localizations and weaker differences between localizations.

First of all, this study provides an accurate and objective method for the quantification of intestinal lymphocyte numbers. The use of an image analyzing software program supports the objective analysis of the $CD3^+$ T cell count per area. The examined area (200,000 μm^2) was 3.5 to 20 times larger than those examined in previous studies [15,27,28], providing more reliable results.

Furthermore this study demonstrates, that $CD3^+$ T cells can be found frequently in canine intestinal lamina propria and epithelium. Also a specific regional distribution of intestinal T cells could be shown, which might be accommodated to the special needs of this region. For example, the accumulation of the T lymphocytes in the tip of the villi probably reflects their high exposure to luminal antigens. The decline of the number of T cells from the small intestine to the colon is somehow surprising, taking in account the increasing numbers of intestinal bacteria, specially commensal bacteria, but could be explained with the decreasing food and external antigen load as a consequence of the degradation of ingesta. The distribution pattern of $CD3^+$ T cells in neonates was similar to adults, but on average, ten times less $CD3^+$ T lymphocytes were found in the analogous localizations, indicating age-dependent changes.

It is clearly demonstrated, that $CD3^+$ cells are embedded between the epithelial cells outside of the basal membrane, with a frequency of 1-2 per every 10 epithelial cells. These data indicate that $CD3^+$ lymphocytes, i.e. IEL, are present in the epithelial layer of the small and large bowel of Swiss Beagle dogs and can be isolated for further characterization.

HORIZONTAL AND VERTICAL DISTRIBUTION OF CD3+ T LYMPHOCYTES IN THE INTESTINE OF HEALTHY ADULT AND NEONATAL DOGS

N.S. Pfammatter[a], N. Luckschander[a,b], S. Jakob[b], S. Hartnack[c], T. Brunner[b,*], I.A. Burgener[a,*,#].

[a] Department of Clinical Veterinary Medicine, Division of Small Animal Internal Medicine, Vetsuisse Faculty, University of Bern, Länggassstrasse 128, CH-3012 Bern, Switzerland

[b] Division of Immunopathology, Institute of Pathology, University of Bern, Murtenstrasse 31, CH-3010 Bern, Switzerland

[c] Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, CH-3012 Bern, Switzerland

[*] Contributed equally as senior authors

[#] Corresponding author: tel: + 41 31 631 29 09; fax: + 41 31 631 22 75; e-mail address: iwan.burgener@kkh.unibe.ch (I.A. Burgener)

Abstract

T lymphocytes are considered to play an essential role in the pathogenesis of inflammatory bowel disease (IBD). Therefore, it is important to know the distribution of these cells in the intestine of healthy dogs and their changes from birth to adolescence. The aim of this study was to define the distribution pattern of CD3+ T lymphocytes in the intestine of healthy adult dogs and to compare it to neonatal puppies.

Full thickness biopsies were obtained from 7 different localisations (stomach, descending and ascending duodenum, jejunum, ileum, ascending and descending colon) from 6 adult and 4 neonatal healthy beagles. The paraformaldehyde-fixed, paraffin-embedded tissue was stained with an anti-CD3 antibody proven to recognize canine CD3+ lymphocytes. The positive cells were counted in an area of total 200,000 μm^2 in the designated 7 localisations and at 4 different sites per localisation (villi and crypts in lamina epithelialis {LE} and lamina propria {LP}, respectively).

The horizontal distribution in adult dogs showed a maximum of CD3+ T lymphocytes in the duodenum and jejunum, whereas a decline was seen towards the ileum and colon. In the stomach, almost no positive cells were observed. The vertical distribution revealed an accumulation in the villi, but only few positive cells in the crypts. In the villi of the duodenum, jejunum, and ileum, there were

significantly more CD3+ T lymphocytes in the LP than in the LE. Compared to the adult dogs, the neonatal dogs showed a similar distribution pattern, but on average ten times less CD3+ T lymphocytes in the analogous localisations and weaker differences between localisations. This finding suggests that after birth the intestinal canine mucosa is gradually being populated by T cells.

Keywords: inflammatory bowel disease, CD3, T Lymphocytes, Dog, canine

1. Introduction

The intestinal immune system is constantly exposed to a vast array of luminal antigens, including those derived from food, components of the commensal microbial flora, and pathogenic organisms. While protective immune responses against intestinal pathogens are critical to avoid the invasion of the host, uncontrolled responses against food antigens or commensal bacteria may lead to devastating inflammatory diseases. Thus, the intestinal immune homeostasis must be tightly regulated to avoid a state of chronic uncontrolled inflammation (German et al., 2003). Current data in human patients suggest that inflammatory bowel disease (IBD) is the result of various predisposing genetic and environmental factors acting on the immunoregulatory system (Sartor, 1997), eventually leading to its over-activation and excessive inflammation of the gastrointestinal mucosa. Furthermore, IBD is likely also the result of an imbalance between effector and regulatory T cell responses (Strober et al., 2002). In humans, IBD is classified in Crohn's Disease and Ulcerative Colitis. Crohn's Disease can affect the whole gastrointestinal tract from the mouth to the anus with the terminal ileum and the right colon being affected most commonly, whereas Ulcerative Colitis is an inflammatory disease of the large intestine. While Ulcerative Colitis is limited to the mucosal surface, Crohn's Disease involves the entire bowel wall. (Isaacs et al., 2005). In dogs, these two major forms cannot be distinguished and IBD is common in both, the small and large intestine (Guilford, 1996).

Canine IBD has been defined as a spectrum of gastrointestinal disorders with persistent or recurrent diarrhea and/or vomitus, failure to respond to symptomatic therapies alone (parasiticides, antibiotics and gastrointestinal protectants), failure to document other causes of gastroenteritis by thorough diagnostic evaluation, and histologic evidence of benign intestinal inflammation. This inflammation is further characterised by the type of cellular infiltrate (neutrophilic, eosinophilic, lymphocytic, plasmacytic, granulomatous), where lymphocytic-plasmacytic infiltration is the most common form in dogs (Hall and German, 2005). Increased numbers of CD3+ T lymphocytes in lamina propria (LP) and lamina epithelialis (LE) have been documented in lymphocytic colitis (Leib et al., 1989; Stonehewer et al., 1998; Jergens et al., 1999; German et al., 2001), in the duodenum of dogs with IBD (Jergens et al., 1992; German et al., 2001) and in histiocytic ulcerative colitis (German et al., 2000). In contrast to these studies, Jergens (1996) found a reduced numbers of CD3+ cells in the small intestine of dogs with IBD. When comparing CD3+ cells in dogs with chronic enteropathies, Schreiner et al. (2008) did not find significant differences before and after therapy. Furthermore, decreased lymphocyte apoptosis has been documented in the duodenum of dogs with IBD (Dandrieux et al., 2008). Given the important role of T lymphocytes in IBD and the somewhat conflicting results, further studies on absolute numbers and distribution pattern of intestinal T cells are needed for clarification.

Therefore, the aim of this study was to determine the distribution of T lymphocytes in LP and LE from stomach to colon in healthy dogs of a single breed to allow subsequent comparison with dogs suffering from chronic enteropathies like IBD. The additional comparison of intestinal T cells in adult versus neonatal dogs should further assess how the intestinal mucosa is populated with lymphocytes during development.

2. Materials and methods

2.1. Dogs

Full thickness biopsies of clinically healthy Beagles were obtained at necropsy immediately after euthanasia. Six adult clinically healthy dogs from a research facility were eliminated for reasons unrelated to the gastrointestinal tract, whereas four neonatal puppies (1-2 days old) were super-numerous at a laboratory animal breeding facility. All experimental procedures were approved by the Cantonal Committee for Animal Experimentation, Bern, Switzerland. The group of adult dogs had a median age of 11 years (range 9-11 years). Immediately after euthanasia by intravenous barbiturate injection, full thickness sections from 7 different localisations were obtained: stomach (pyloric region), descending and ascending duodenum (at the end of the pancreatic limb and at the transition to the jejunum), jejunum (middle part), ileum (close to the ileocaecal junction), and ascending and descending colon (next to the caecocolic junction and at the transition to the rectum; see Fig. 1). From all localisations and all dogs, routine stainings with hematoxylin and eosin were performed.

2.2. Antibodies

Preliminary work revealed that the mouse anti-canine CD3 (clone CA17.2A12, Serotec, Oxford, GB) is not suitable for staining paraffin-embedded tissue (results not shown). In contrast, a rabbit anti-human CD3 antibody (Neomarkers, Fremont,

USA) has been shown to work on paraffin-embedded tissue and to crossreact with canine T cells. To prove specificity of anti-human CD3 for canine CD3, antibodies were tested on cytospins of highly purified canine peripheral blood CD4+ T cells. Briefly, mononuclear cells were isolated from peripheral blood of a healthy dog using a Ficoll paque plus density gradient (GE Healthcare, Uppsala, Sweden) as described by the manufacturer. Mononuclear cells were then stained with rat anti-canine CD4 RPE Ab (clone YKIX 302.9, Serotec) and sorted for CD4+ T cells on a FACSVantage cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). After cytospin (3 minutes, 300g), the CD4+ cells were either stained with rabbit Immunoglobin G (Sigma, St. Louis, USA) as isotype control, mouse anti-canine CD3, or rabbit anti-human CD3, followed by the adequate biotinylated secondary Abs (goat anti-mouse or goat anti-rabbit, Dako, Glostrup, Denmark). The CD4+ T lymphocytes were positively stained with both Abs, whereas the isotype control was negative (Fig. 2 a-c). Another control with the rabbit anti-human CD3 was performed by immunohistochemistry. The Ab stained canine cells in well known localisations of CD3+ cells, as around the vessels in the spleen (periarteriolar lymphoid sheaths; PALS) and in the lymph follicles in the ileum of neonatal puppies (Fig. 2 d-f).

2.3. Tissue preparation and immunohistochemistry

After obtaining the tissue, it was transported in gauze tinctured with 0.9% sodium chloride solution in plastic tubes on ice. Tissue samples were then fixed in 4% paraformaldehyde and embedded in paraffin. The sections were cut in 5 µm slices and used for immunohistochemical examination. The tissue sections were deparaffinised, rehydrated and boiled for 10 min in a steamer. After rinsing in Tris-buffered saline (TBS, pH 7.4), the endogenous peroxidase activity was blocked by incubation with endogenous peroxidase blocking solution (S2001, Dako) for 20 minutes in a moist chamber at 20°C. After a washing step with TBS, the sections were incubated with 5% goat serum supplemented with 0.5% casein and 0.1% sodium azide in TBS (Ab dilution buffer) for 30 minutes at 20°C. The tissue was then stained with the primary Ab (rabbit anti-human CD3, diluted 1:100 in Ab dilution buffer with 5% goat serum (adult dogs) or with 2.5% goat serum and 2.5% dog serum (neonatal dogs)) for 60 min in a moist chamber at 20°C. The dog serum was added to the Ab dilution buffer for the neonates to avoid background staining of the phagocyted maternal Abs in the LE due to the polyclonal character of the rabbit anti-human CD3 Ab. Purified rabbit immunoglobulin was used at 1 µg/ml as staining control. Sections were then washed for 2x3 min in TBS. The secondary biotinylated goat anti-rabbit Ab (1:100) was applied for 60 min in a moist chamber at 20°C. Sections were washed 2x3 min in TBS prior to incubation with the pre-aggregated avidin-biotinylated

horseradish peroxidase complex (K 0376, Dako: 1:200 in TBS) for 40 minutes. After washing in TBS, immunohistochemistry was developed by incubating sections for 7-8 min in the dark with horseradish peroxidase substrate solution (0.2 mg/ml 3,3´-diaminobenzidine tetrahydrochloride DAB, 0.012% H_2O_2, 20 mM citric acid monohydrate, 100 mM imidazole, 100 mM NaCl, pH 7.0) in a glass cuvette. Slides were washed and counterstained for 30 seconds with hematoxilin, followed by rinsing in tab water. Sections were then dehydrated (2x20 s 35% ethanol, 2x20 s 70% ethanol, 2x20 s 95% ethanol, 2x2 min 100% ethanol, 2x5 min xylol) and mounted with Eukitt mounting medium (Electron Microscopy Sciences, Hatfield, GB) and appropriate cover slips (Jakob et al., 2008).

2.4. Evaluation of sections

All sections were analysed by a single investigator (NSP). Initial analysis of the distribution of positive cells was made with the x5, x10 and x20 objectives. Images were taken with a microscope (Axioplan 2, Zeiss, Feldbach, Switzerland) and a digital camera (Axiocam HR, Zeiss) with the x20 objective and were then transferred to a computer. Cell counts were made with a computer image analysis system (Image Pro Plus, Version 1.3.1., MediaCybernetics, Maryland, USA). The CD3+ T lymphocytes were counted in the villus and crypt region in the small bowel, whereas in the stomach and large bowel, they were counted in top and base

layers (Fig. 3). The space between the crypts and the muscularis mucosae was not taken into evaluation. In each area, the LE and the LP were delineated on the computer screen and the positively stained cells were identified by the investigator in an area of minimally 200,000 μm^2. The results were expressed as positive cells per 200,000 μm^2 in top and base of LE and LP, respectively, for each localisation.

2.5 Statistical analysis

In order to find differences between the seven localisations repeated measures ANOVAs with Bonferroni correction for multiple comparisons were performed for the four different sites in the adults and the neonates with the software package SPSS (SPSS® version 14.0 SPSS Inc., Chicago, IL, USA). For the comparison of LE versus LP and top versus base a Wilcoxon signed rank test was performed.

3. Results

3.1. Histology

Hematoxylin and eosin stained sections were initially examined by a single pathologist. The majority of sections had normal architecture and cellular distribution, but abnormalities were occasionally observed. In all cases, these changes were focal. Thus, cell counts were not made in the abnormal areas (German et al., 1999).

3.2. Adult dogs

3.2.1. Horizontal distribution

An example of the different localisations of an adult dog is presented in Fig. 4. Taken the six adult dogs together, there was a decline in CD3+ cells in top LE and LP from the small intestine to the colon, whereas the stomach contained almost no CD3+ cells (Fig. 5). In top LP, the ileum showed similar numbers of CD3+ cells than the rest of the small intestine, whereas in top LE of the ileum, the numbers were similar to those of the colon. The obvious differences between small and large intestine from the top where not evident anymore in the base LE and LP, where in general less CD3+ were found.

With regard to significant differences, the stomach expressed less CD3+ cells in top LE compared to the descending duodenum ($p = 0.024$), the ascending colon (p

= 0.034) and the descending colon (p = 0.008). In top LP, the stomach expressed significantly less CD3+ cells than the descending duodenum (p = 0.001), the ascending duodenum (p = 0.004), the jejunum (p = 0.009) and the descending colon (p = 0.027). Furthermore, the ascending colon revealed less CD3+ lymphocytes than the descending duodenum (p = 0.004), the ascending duodenum (p = 0.020), and the jejunum (p = 0.033), whereas the descending colon expressed less CD3+ cells than the descending duodenum (p = 0.003).

In base LE, the stomach expressed significantly less CD3+ cells than the ileum (p = 0.045) and the ascending colon (p = 0.039), whereas the ascending duodenum revealed less CD3+ lymphocytes than the ascending colon (p = 0.045). In the base LP, there were no significant differences detectable.

3.2.2. Vertical distribution

The vertical distribution revealed a decrease from the top of the villi to the base for the small intestine, whereas there was no obvious difference between top and base in the colon. In the tips of the villi, there was an obvious compartmentalisation of T lymphocytes, with a gradual decline towards the base of the villi and the crypts. When comparing the 4 layers together, the variation of CD3+ cells was higher in top LE compared to top LP, whereas it was minimal in the basal layers (Fig. 5).

A significant decrease from top LE to base LE was found in descending duodenum, ascending duodenum, jejunum (for all p = 0.028), ileum (p = 0.046)

and descending colon (p = 0.028). In the LP, a significant decrease from top to base was seen throughout all regions of the intestine except the stomach.

3.2.3. Absolute numbers

The absolute numbers of CD3+ cells in top LE and top LP were lowest in the colon, whereas they were approximately twice as high in duodenum and jejunum in top LE, and ~3 x higher in the small intestine in top LP. The absolute numbers in the basal layers throughout the whole gastrointestinal tract were similar to the values in the colon in the top layers with the exception of the small intestine LE, where the average values were even lower.

3.3. Neonatal dogs

3.3.1. Horizontal distribution

An example of the different localisations of a neonatal dog is presented in Fig. 6. Taken the four neonatal dogs together, there was a decline in CD3+ cells in top LE and LP from duodenum to jejunum, ileum and colon, whereas the stomach contained almost no CD3+ cells (Fig. 7). In the top LE, the ileum showed less CD3+ cells than the colon. Unlike in adult dogs, the jejunum revealed less

had positive cells in top LE and LP than the duodenum, whereas the ascending colon a similar density of CD3+ cells as the jejunum. The obvious differences between duodenum and the rest disappeared in the base LE and LP, where in general less CD3+ were found.

With regard to significant differences, the jejunum expressed less CD3+ cells in top LE than the descending duodenum ($p = 0.007$). In top LP, the stomach revealed significantly less CD3+ lymphocytes than the ileum ($p = 0.044$), whereas there were no significant differences detectable in the basal layers.

3.3.2. Vertical distribution

The vertical distribution revealed a decrease from the top to the base of the duodenum, whereas there was no obvious difference between top and base in the jejunum (Fig. 7). In the ileum, a comparison between top and base was not possible due to the fact that the base region contained numerous lymph follicles (Fig. 6e). In the ascending colon, a decline from top to base was seen, whereas there was no obvious difference in the descending colon. Statistically no significant differences were found.

3.3.3. Absolute numbers

The absolute numbers of CD3+ cells in top LE and top LP were similar and highest in the duodenum, whereas the jejunum and ascending colon revealed approximately 50% less positive cells than the duodenum, but still more than the ileum. The absolute numbers in the basal layers were highest in the jejunum and the colon in the base LE, whereas there were more CD3+ cells in LP in the small intestine than in the colon.

3.4. Comparison between adult and neonatal dogs

Compared to the adult dogs, the neonatal dogs showed a similar distribution pattern, but on average ten times less CD3+ T lymphocytes in the analogous localisations (Fig. 5 and 7). Nevertheless, differences were found in top LE and LP in the jejunum, ileum and ascending colon: the jejunum in neonates yielded less CD3+ cells than the duodenum, the ileum showed less CD3+ cells than the ascending colon, and the ascending colon expressed more positive cells than the descending colon. Furthermore, the amount of CD3+ cells in top LE and LP of the small intestine was similar in neonatal dogs, whereas in adult dogs, there were significantly more positive cells in the LP. In the basal layers, there were two major differences between neonatal and adult dogs. In the stomach of neonatal dogs, the difference between top and base could not be determined because there

was no obvious base region (Fig. 6 b). The second major difference was found in the ileum, where the base region was full of lymphoid follicles with a B cell nucleus and CD3+ T lymphocytes around it (Fig. 6e), which made comparative counting impossible.

4. Discussion

IBD is a common cause of chronic diarrhea in dogs, were lymphocytic-plasmacytic infiltration is the most common histological finding. The diagnosis of LP cellularity relies on the experience and the bias of the pathologist, even though attempts for standardisation of histology have been made (Day et al., 2008). In this investigation, a systematic comparative analysis of CD3+ T lymphocytes in the intestinal LE and LP of neonatal and adult healthy dogs was carried out to allow subsequent comparison with dogs suffering from chronic enteropathies like IBD. Interestingly, the horizontal distribution of CD3+ T cells in neonatal and adult dogs showed a decline from the small intestine to the large intestine, whereas the stomach yielded almost no positive cells. Furthermore, the T lymphocytes gradually decreased from the villus tip towards the crypts. Compared to the adult dogs, the neonatal dogs showed on average ten times less CD3+ T lymphocytes in the analogous localisations. Previous studies in mice have shown that the colonization of the intestine with commensal bacteria also triggers the expansion and distribution of intestinal T cells (Bauer et al., 2006). While an increase of CD3+ T cells from neonates to adults, and stomach to small intestine is in agreement with this notion, the strong decline from the small intestine to the more heavily with bacteria colonized large intestine may not follow the same rules.

Different studies in the past have analysed the distribution of CD3+ lymphocytes in the intestinal mucosa. All but one of these studies (Kleinschmidt et al., 2007)

used a rabbit anti-human CD3 Ab from Dako, crossreacting with canine CD3. In our own study, a different rabbit anti-human CD3 Ab from Neomarkers was used. Therefore, the specificity of this antibody for canine CD3 had to be proven. This was demonstrated by sorting canine CD4+ T cells from peripheral blood and simultaneously staining them with an anti-canine CD3 Ab (not suitable for paraffin sections) and the rabbit anti-human CD3 Ab (suitable for paraffin sections). Both antibodies specifically stained CD4+ T cells (Fig. 2b,c). Furthermore, the rabbit-anti human CD3 Ab stained canine cells in well known localisations of CD3+ cells, as around the vessels in the spleen (PALS) and in the lymph follicles in the ileum of a neonatal puppies (Fig. 2e,f).

The main goal of this study was to establish a bench mark for CD3+ lymphocyte distribution in healthy dogs for further analysis and comparison with dogs suffering from IBD. Therefore, we wanted to establish a cell counting system where the values of CD3+ lymphocytes in LP (LP lymphocytes = LPL) and LE (intraepithelial lymphocytes = IEL) are determined the same way and the two compartments can be compared. In previous studies, CD3+ lymphocytes in the LE were specified by numbers of IEL per 100 enterocytes (Elwood et al., 1997; Stonehewer et al., 1998; German et al., 1999; Kleinschmidt et al., 2007). In contrast, counting the CD3+ cells per area using an image analysing software program or a grid may prove to be easier to use in diagnostical analysis. Furthermore, by counting the cells in defined total areas (i.e. 200,000 μm^2), the

results should be less susceptible to a sampling bias. To count T cells in at least 200,000 μm^2 2-5 images of the gut (with a 20 x objective) had to be analysed in adult dogs and 8-10 images in neonatal dogs. This chosen area is 3.5 to 20 times bigger than those examined in previous studies with the idea to get more accurate and stable results for the LPL as well as the IEL. In previous studies, the LPL were specified as positive cells per 100 mononuclear cells in an area of 60,500 μm^2 (Stonehewer et al., 1998), per area of 10,000 μm^2 (German et al., 1999; Kleinschmidt et al., 2007) or 5-10 x 2500 μm^2 (Elwood et al., 1997). Due to the different counting methods used in these studies, CD3+ IEL and LPL could not be compared together.

The different studies in the past revealed partially conflicting results when investigating the CD3+ T cell distribution. In the study presented herein, a higher number of localisations over the whole gastrointestinal tract were taken into account, with 7 localisations from the stomach to the colon. The stomach yielded almost no CD3+ cells. This finding is expected because of the minimal bacterial flora due to the acidic gastric juice and the surface protection by mucus (Kenneth, 2008). The decline of the number of T cells from the small intestine to the colon could be explained with the decreasing antigen load as a consequence of the degradation of ingesta (Kleinschmidt et al., 2007). Although different regions and localisations evaluated, the same vertical distribution pattern was documented in previous studies (Elwood et al., 1997; German et al., 1999; Kleinschmidt et al., 2007). Jergens (1996) described a uniform distribution in 3 different localisations

in the villi of the duodenum. The compartmentalisation of the T lymphocytes in the tip of the villi in the small intestine probably reflects an increasing level of exposure to luminal antigens (Elwood et al., 1997). Furthermore, T cell responses depend on presentation of antigens by molecules of the major histocompatibility complex (MHC) on antigen-presenting cells (APC). MHC class II+ APC are predominantly dentritic cells, which in dogs are located in subepithelial regions of the villus LP (Elwood et al., 1997; Kleinschmidt et al., 2007).

The absolute numbers of CD3+ cells observed in LP of adult dogs in this study were similar to those described in the study of German et al. (1999). When comparing the results for adult dogs with Elwood and co-workers (1997), they found much more CD3+ LPL in the top and center of the villi (~4 times more in duodenum, jejunum and ileum), but similar numbers in the base of the villi and the crypts. Kleinschmidt et al. (2007) on the other hand had much lower numbers of CD3+ cells, which he explained with the use of a monoclonal anti-CD3 antibody compared to polyclonal antibodies used by Elwood (1997) and German (1999). Nevertheless, given the much higher area accounted for in the study described herein, the authors expect their results to be more accurate and less susceptible to bias. As already described above, the absolute numbers of CD3+ IEL are not comparable to other studies due to a different counting method used. In the small intestine, the top LE yielded less CD3+ cells and a greater variation than the top LP in adult dogs, but not neonates. It has been suggested that T cells migrate from the LP towards the lumen in response to antigenic stimulatio

(Jergens et al., 1996) and that LPL function as a reservoir for the IEL population. Therefore, it would be very interesting to compare healthy dogs with IBD dogs, and IBD dogs before and after therapy.

The distribution pattern of CD3+ T cells in neonates was similar to adults, but on average, ten times less CD3+ T lymphocytes were found in the analogous localisations. The immune system of neonates is immunologically competent at the time of birth, but any immune response in infancy must necessarily be a primary response (Chappuis, 1998). According to Chappuis (1998), the maturation of the immune response will occur during the first 2-3 weeks. With this in mind, a continuous augmentation of the number of T lymphocytes should take place in the first month of life. Kleinschmidt et al. (2007) examined the intestine of young dogs (1 month to 1 year) and compared it with adult and senior dogs. As discussed above, their results were not comparable with our results, partially due to the use of another anti-CD3 Ab. Nevertheless, the distribution in the LP of the villi and the base of the crypts was similar.

In a small group of 19 dogs with chronic enteropathies, Schreiner et al. (2008) did not find significant differences in CD3+ cells in duodenal LP before and after therapy in 10,000 μm^2. At the end of our study, the new protocol with 200,000 μm^2 was used to investigate endoscopical biopsies from dogs with chronic enteropathies. Biopsies of duodenum and colon of 3 dogs with food-responsive diarrhea (FRD), 2 dogs with IBD, and duodenum of 3 dogs with protein-losing enteropathy (PLE) secondary to severe IBD were analysed (Fig. 8).

Histologically, the surface and architecture looked similar to that of the clinically

healthy dogs. The decline of CD3+ T cells from the small intestine to the colon and from the villi to the crypts was similar as in healthy dogs. In the villi, a compartmentalisation as described in healthy dogs could not be seen, and the T lymphocytes were more disseminated over the whole villus. The biopsies of dogs with PLE revealed a lot more T lymphocytes in the villi than biopsies from healthy dogs or dogs with FRD or IBD. However, due to the limited number of samples, these results have to be considered preliminary and further studies with a higher number of diseased dogs are needed to obtain conclusive and statistically significant results.

In conclusion, the evaluation of the distribution of CD3+ T lymphocytes in healthy adult dogs can be used as bench mark to be compared with the distribution in dogs with enteropathies like IBD. Furthermore, the comparison with the neonatal puppies may help to understand the process of maturation of the immune system. Whether the tenfold increase of CD3+ T lymphocytes is a continuous event or occurs in the first few weeks of life is currently not known.

Acknowledgements

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Figure captions

Fig.1. Localisations of full-thickness biopsies.

A: stomach (pyloric region); B + C: descending (at the end of the right pancreatic limb) and ascending duodenum (transition to jejunum); D: jejunum (middle part); E: ileum (1-5 cm from the ileocaecal junction); F + G: ascending (1-5 cm from the caecocolic junction) and descending colon (transition to rectum).

Fig.2. Test of specificity of anti-CD3 staining on isolated T cells and tissue sections.

a, b, c: Lymphocytes were isolated from peripheral blood from a healthy dog and sorted for CD4+ cells. Cytospin preparations were stained with an isotype control (rabbit IgG; a) (bar 20 µm; x 100 oil), mouse anti-canine CD3 (b), and rabbit anti-human CD3 (c). d, e, f: Examples of anti-CD3 immunohistochemistry from healthy dogs with isotype control (rabbit IgG; spleen; d) (bar 50 µm; x 40) and rabbit anti-human CD3 in the spleen (e) and the ileum (f).

Fig.3. Detection of CD3+ cells in lamina epithelialis and lamina propria and top and base.

A. Schematic representation of the distinction between lamina epithelialis (LE) and lamina propria (LP) in top and base. A typical example is shown for the small intestine and the colon. B. Examples for CD3 staining in top and base of the intestinal mucosa of neonates and adult dogs (x 40). Intraepitelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) are shown.

Fig.4. Detection of CD3+ cells at different gastrointestinal localisations in adult dogs.

Representative anti-CD3-stained tissue sections are shown: a. duodenum isotype control (rabbit IgG; bar 50 µm; x 40); b. stomach; c. descending duodenum, villus; d. descending duodenum, crypt region; e. ascending duodenum, villus; f. jejunum, villus; g. ileum; h. ascending colon, top; i. descending colon, base.

Fig.5. Distribution of CD3+ T cells in different localisations of the gastrointestinal tract of adult dogs.

Intraepithelial lymphocytes and lamina propria lymphocytes of top and base in the localisations indicated are shown. Numbers and median of CD3+ cells were calculated per 200,000 μm^2 from 6 adult dogs.

Fig.6. Detection of CD3+ cells at different gastrointestinal localisations in neonatal puppies.

Representative anti-CD3-stained tissue sections are shown: a. jejunum isotype control (rabbit IgG; bar 50 µm; x 40); b. stomach; c. ascending duodenum, villus; d. ileum, villus; e. ileum, lymph follicle; f. descending colon.

Fig.7. Distribution of CD3+ T cells in different localisations of the gastrointestinal tract of neonatal puppies.

Intraepithelial lymphocytes and lamina propria lymphocytes of top and base in the localisations indicated are shown. Numbers and median of CD3+ cells were calculated per 200,000 μm^2 from 4 neonatal puppies.

Fig.8. Comparison of anti-CD3 immunohistochemistry on endoscopic biopsies of the duodenum from dogs with chronic enteropathies.

a. food-responsive diarrhea (FRD); b. inflammatory bowel disease (IBD); c. protein-losing enteropathy (PLE) secondary to a severe IBD. Note that the epithelium in FRD and IBD has normal architecture, whereas the epithelium of the dog with PLE is mildly injured (Day et al., 2008). The number of intraepithelial lymphocytes is normal in FRD, mildly increased in IBD, and markedly increased in PLE. (bar 50 μm; x 40)

Pfammatter NS, Luckschander N, Jakob S et al. 32

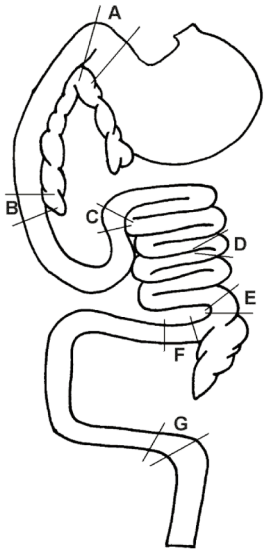

Figure 1

Pfammatter NS, Luckschander N, Jakob S et al. 33

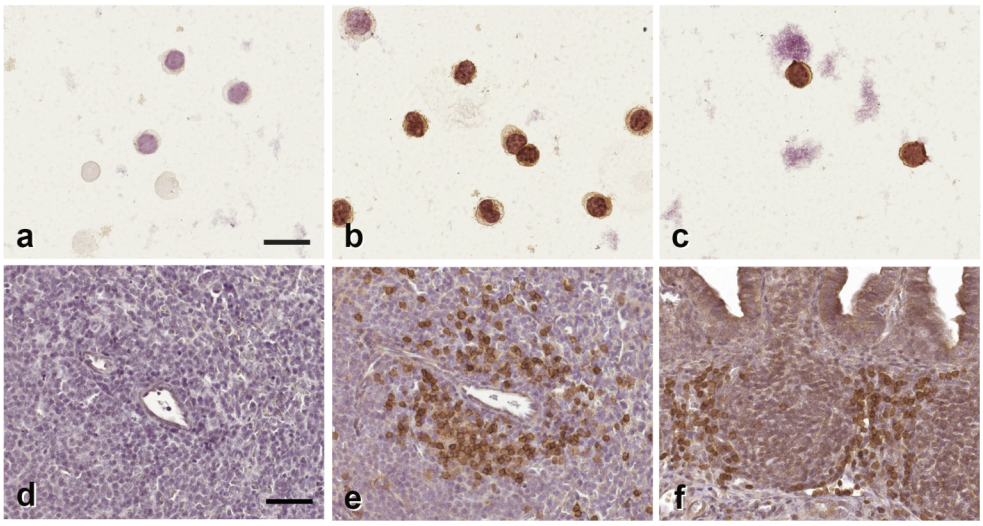

Figure 2

Pfammatter NS, Luckschander N, Jakob S et al. 34

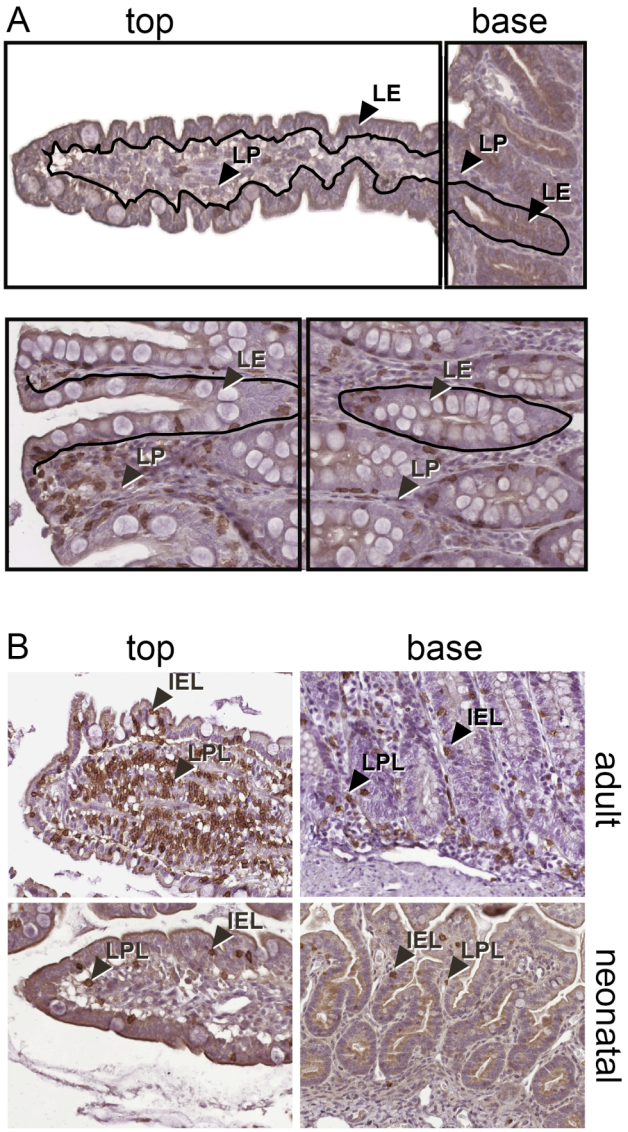

Figure 3

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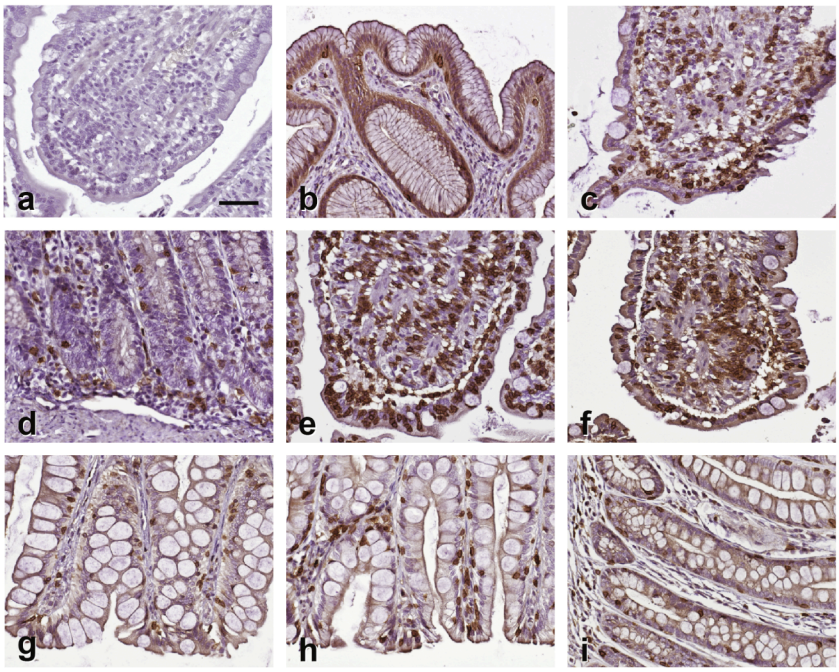

Figure 4

Pfammatter NS, Luckschander N, Jakob S et al. 36

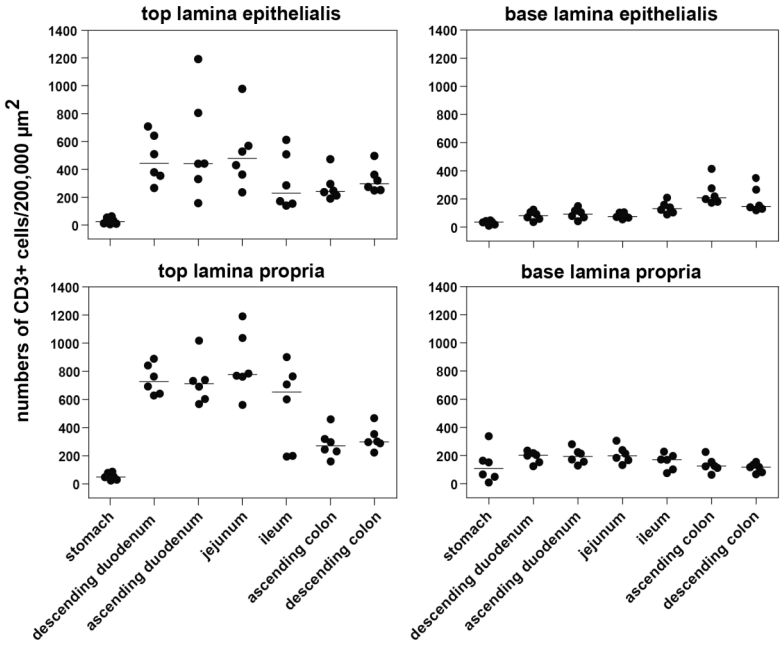

Figure 5

Pfammatter NS, Luckschander N, Jakob S et al. 37

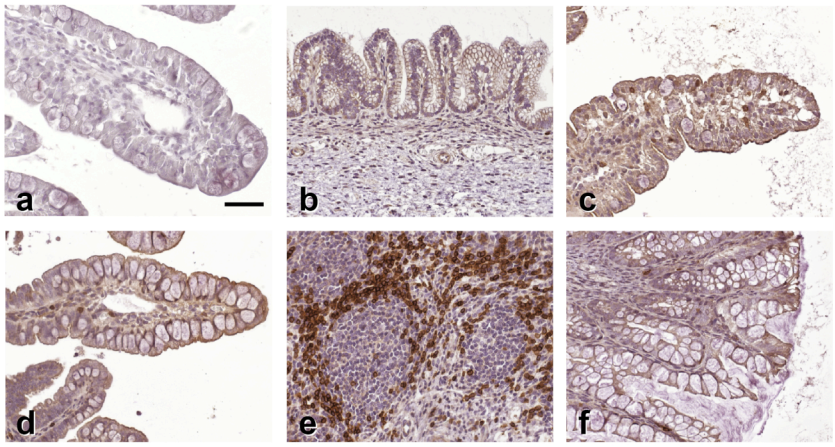

Figure 6

Pfammatter NS, Luckschander N, Jakob S et al. 38

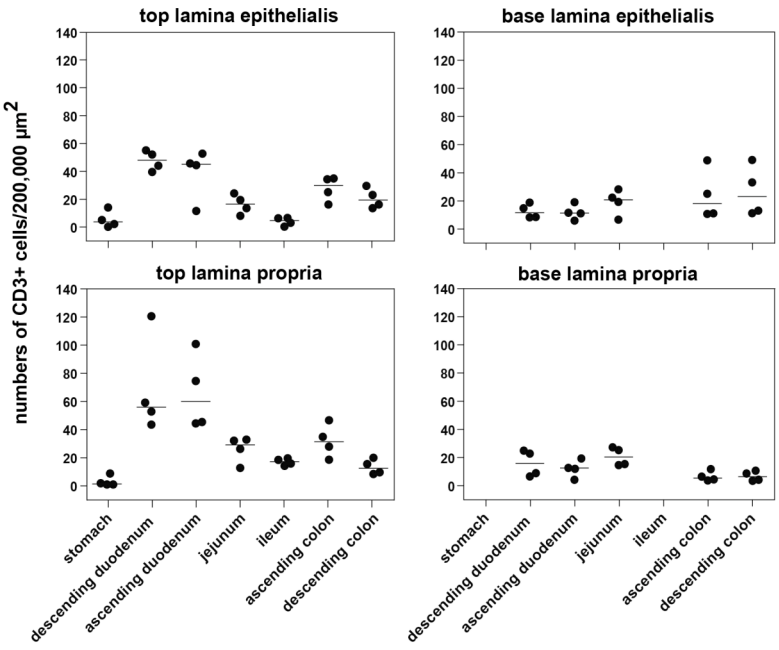

Figure 7

4.2. DISSERTATION EQUIVALENT II

Phenotypical and functional characterization, and developmental changes of canine intestinal intraepithelial lymphocytes

Nicole Luckschander[1, 2], Nadia S. Pfammatter[1, 2], Daniel Sidler[1], Sabine Jakob[1], Iwan A. Burgener[2], Peter F. Moore[3], Andreas Zurbriggen[2], Nadia Corazza[1], and Thomas Brunner[1*]

[1]Division of Immunopathology, Institute of Pathology, University of Berne, Switzerland, [2]Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Berne, Switzerland, and [3]Veterinary Medicine Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, USA

Veterinary Research, submitted

Despite the increasing frequency of canine IBD, and the important role of intestinal lymphocytes in the pathomechanisms of this disease, little is currently known about the canine lymphocytes in the normal and diseased gut. Intestinal IEL are localized in an important strategic position, between the outside world and the inner sterile body core. IEL not only act as a first line of defense against possible pathogenic invaders, they also play a regulatory role in oral tolerance. Thus, the phenotypical composition of IEL may represent a sensitive surrogate marker for pathological changes in the intestine, e.g. as observed during IBD.

The aim of this study was the thorough phenotypical and functional characterization of canine intestinal IEL. The cells were isolated from full-thickness biopsies of 15 adult Swiss Beagle dogs (mean age 8.2 $\pm$ 2.8 years) and compared to lymph node cells. The phenotypical characterization by multi-parameter flow cytometry revealed that canine IEL substantially differ from lymph node T cells. In the peripheral lymphoid organs the T cell compartment is composed of a mixed population of $CD4^+$ and $CD8^+$ T cells. In marked contrast, in the intestinal epithelium only few $CD4^+$ cells (<15%) were found, with slightly higher numbers in the small bowel. Instead, most of the IEL of the small and large bowel expressed the CD8α antigen. In the peripheral

immune system most T cells are TCR$\alpha\beta^+$, whereas only very few TCR$\gamma\delta^+$ cells are found. In the intestinal epithelial compartment TCR$\alpha\beta^+$ T cells were found to be the dominant population, however, a substantial portion of IEL are TCR$\gamma\delta^+$ T cells, representing the more unconventional "type b" phenotype. For the first time a description of the differential TCR usage of CD8$\alpha\beta^+$ and CD8$\alpha\alpha^+$ IEL in the canine gut was possible, due to the simultaneous detection of three parameters. Their abundance in the canine intestinal epithelial layer suggests an important role of these T cell subsets in the maintenance of intestinal immune homeostasis. After having established the phenotypical characterization of intestinal IEL in a monobreed standard, IEL populations of adult dogs were further compared to those isolated from neonatal Beagle dogs. Results indicated that there are significant differences between the two age groups of dogs in the distribution of different lymphocyte subsets. In newborn dogs a high frequency of undifferentiated CD4$^-$CD8$^-$ T cells are found, whereas in adult dogs mature CD4$^+$ and CD8$^+$ T cells predominate, indicating a maturation of the intestinal immune system during development. As IEL in other species have been previously suggested to exhibit regulatory functions we investigated the role of IEL on lymph node T cell proliferation. Results indicated that canine IEL differ significantly from other T cell populations with regard to activation-induced proliferation. They do not strongly proliferate in response to stimuli that are very effective for the induction or proliferation in other T cell population (^{3}H-thymidine incorporation). Furthermore, they significantly inhibited the proliferation of activated lymph node T cells in a cell number-dependent manner. These findings demonstrate for the first time that canine IEL have a more immunoregulatory phenotype, which may contribute to the maintenance of the intestinal immune homeostasis and its loss in canine IBD.

In summary, we could establish a "gold standard" of flow cytometric analysis for the phenotypical characterization in canine intestinal IEL in a monobreed standard. For the first time the unconventional T cell subsets, including CD8$\alpha\beta^+$ TCR$\alpha\beta^+$ and CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ / TCR$\gamma\delta^+$ T cells, could be exactly described. Significant differences in the IEL population in comparison between neonatal and adult dogs could be shown, indicating age dependent changes in the IEL composition. For the first time a functional characterization of canine IEL was studied, indicating a more

regulatory phenotype of canine IEL. These data show important insights in the characterization of intestinal intraepithelial lymphocytes and might offer a better understanding and new therapeutic strategies for the treatment of canine IBD.

Phenotypical and functional characterization, and developmental changes of canine intestinal intraepithelial lymphocytes

Nicole Luckschander[1, 2], Nadia S. Pfammatter[1, 2], Daniel Sidler[1], Sabine Jakob[1], Iwan A. Burgener[2], Peter F. Moore[3], Andreas Zurbriggen[2], Nadia Corazza[1*], and Thomas Brunner[1*]

[1]Division of Immunopathology, Institute of Pathology, University of Bern, Murtenstrasse 31, 3010 Bern, Switzerland

[2]Departement of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Länggasstrasse 128, 3012 Bern, Switzerland

[3]Veterinary Medicine Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Rm.1126 Haring Hall, Davis, CA 95616, USA

* To whom correspondence should be addressed: Nadia Corazza (nadia.corazza@pathology.unibe) or Thomas Brunner (tbrunner@pathology.unibe.ch)

Short title: Characterization of canine intestinal intraepithelial lymphocytes

Abstract - Little is currently known about the lymphocyte populations in the normal and diseased canine gut. The aim of this study was thus the phenotypical and functional characterization of canine intraepithelial lymphocytes (IEL). IEL were isolated from full-thickness biopsies of 15 adult Swiss Beagle dogs (mean age 8.2 $\pm$ 2.8 years) and compared to lymph node cells. The phenotypical characterization by multi-parameter flow cytometry revealed that canine IEL substantially differ from lymph node T cells and consist of various unconventional lymphocyte subsets, unique to mucosal surfaces. These include γδ T cells, and $CD4^-CD8^-$ and $CD8\alpha\alpha^+$ T cells. IEL populations in adult dogs were also compared to those isolated from neonatal Beagle dogs. Analysis revealed that in newborn dogs a high frequency of undifferentiated $CD4^-CD8^-$ T cells are found whereas in adult dogs mature $CD4^+$ and $CD8^+$ T cells predominate, indicating a maturation of the intestinal immune system during development. As IEL in other species have been previously suggested to exhibit regulatory functions we investigated the role of IEL on the activation-induced proliferation of lymph node T cells. While IEL alone did not show an activation-induced proliferation, they significantly inhibited the proliferation of activated lymph node T cells in a cell number-dependent manner. These findings demonstrate for the first time that canine IEL have an immunoregulatory phenotype, which may contribute to the maintenance of the intestinal immune homeostasis and may be lost in canine chronic enteropathies.

1. Introduction

The gastrointestinal tract plays the key role in the uptake of fluids and nutrients, and at the same time it forms the main protective barrier between the sterile environment of the body and the outside world [11]. To protect the body from invading pathogens the intestinal mucosa has developed one of the biggest and most complex organ-associated immune systems [21]. Under normal physiological conditions the intestinal immune system is tightly regulated to avoid overshooting reactions. In marked contrast, during inflammatory bowel disease (IBD) a loss of regulatory control mechanisms leads to perpetuating inflammatory and infiltrative responses. For example, in experimental mouse models of colitis absence of the immunoregulatory cytokines IL-10, TGFβ or IL-2 leads to severe inflammatory responses in the intestinal mucosa due to overstimulation by the intestinal flora [12]. Not only in human patients, but also in dogs, the development of inflammatory disorders of the intestinal mucosa, such as IBD, is observed with increasing frequency [21]. Despite the high incidence of IBD in dogs the mechanistic events leading to these disease entities are poorly understood. The diagnosis is still based on clinical signs of chronic diarrhoea, vomiting and weight loss, combined with histological evidence of inflammatory infiltration of the intestinal mucosa [6]. Although several lines of evidence point towards an important role of intestinal lymphocytes in the development of canine IBD the precise patho-mechanisms are currently not understood. In part this lack

of understanding is based on the so far poor characterization of the canine intestinal immune system.

In the intestinal mucosa immune cells are found in various compartments, i.e. the Peyer's patches as organized inductive sites, the lamina propria and even within the epithelial layer. In this study we focussed on the characterization of the so-called intestinal intraepithelial lymphocytes (IEL), a developmentally and functionally unique lymphocyte compartment strategically located in the intestinal epithelial layer as a first line of defense. IEL are readily accessible by simple dissociation from the epithelial cells, and changes in the composition of the IEL compartment likely reflect ongoing immunological responses in the intestinal mucosa. For example, during intestinal viral infections the epithelial layer is infiltrated by virus-specific cytotoxic T cells [18]. Thus, the phenotypical composition of IEL may represent a sensitive surrogate marker for pathological changes in the intestine, e.g. as observed during IBD. A limited number of previous studies have investigated the distribution and quantification of IEL in the canine intestine. Most of these descriptive studies were done by immunohistochemistry, which however does not allow the simultaneous analysis of multiple lymphocyte markers [7,8,9,15]. Similarly, intestinal tissue samples from various breeds were used, likely leading to a high variability of the results due to inter-breed differences in the composition of IEL. Therefore, the goal of this study was the in-depth phenotypical and functional characterization of IEL in

the small and large intestine from a mono-breed population, i.e. Beagle dogs, in order to establish an unbiased "baseline" for future studies on the role and fate of intestinal T cells in the development of canine enteropathies. Development-dependent changes in the IEL compartments were analyzed by comparing lymphocyte subsets in the epithelial layer of adult dogs versus newborn dogs with an average age of 1 day. Phenotypic changes were analyzed by multi-parameter flow cytometry, whereas functional parameters consisted of activation-induced proliferation and cytokine expression profiles. Our present data describe for the first time so far unrecognized lymphocyte subsets in the canine intestinal epithelium and their developmental changes from newborn to adult dogs. Furthermore, we provide evidence that in the healthy dog IEL have a regulatory phenotype as they potently inhibit the activation-induced proliferation of lymph node T cells.

2. Materials and Methods

2.1. Animals

Tissue samples were either obtained from adult (n = 15) or neonatal (n = 6) healthy Swiss Beagle dogs. All dogs were provided from the Novartis Centre de Recherche Santé Animale (CRA) (St. Aubin, Switzerland) or the RCC Ltd Laboratory Animal Service (Füllinsdorf, Switzerland). The adult dogs (mean age 8.2 $\pm$ 2.8 years, 8 male, 7 female dogs) were clinically healthy animals, which

were eliminated for reasons unrelated to the gastrointestinal tract. The neonatal puppies (average 1 day, 5 male, 1 female) were culled from a laboratory animal breeding facility due to excessive numbers. Immediately after euthanasia by barbiturate injections full thickness sections from intestine (ascending duodenum, mid jejunum, mid colon) and mesenterial lymph nodes were taken, and further processed for cell isolation or immunohistochemistry. All animal experiments were conducted according to the guidelines of the Animal Experimentation Committee of the canton of Berne.

2.2. Antibodies

All monoclonal antibodies (mAb) used for flow cytometry were specific for canine leukocyte antigens. mAb against CD4 (clone YKIX302.9, PE conjugate), CD3 (clone CA17.2A12, FITC conjugate), CD8β (clone CA15.4G2), CD8α (clone YCATE55.9 FITC conjugate) and CD45 (clone YKIX 716.13, biotinylated) were purchased from Serotec (Morphosys AbD GmbH, Düsseldorf, Germany). Monoclonal antibodies against TCRαβ (clone CA15.8G7), TCRγδ (clone CA20.8H1) were generated as previously described (8) and purified on protein G columns. Antibodies against TCRαβ and TCRγδ were labeled with biotin according standard protocols. Unlabeled antibodies were detected using R-PE-conjugated goat anti-mouse IgG F(ab)2 fragment (BD Becton Dickinson, Allschwil, Switzerland), biotinylated antibodies with Cy5-

conjugated streptavidin (THP Medical Products, Vienna, Austria). Rabbit anti-human CD3 antibody for immunohistochemical detection of IEL was obtained from Neomarkers (Fremont, CA).

2.3. Immunohistochemistry

Intestinal samples were fixed in 4% paraformaldehyde in PBS and then embedded in paraffin. Five μm sections were dewaxed and pretreated by boiling sections for 10 min in 10 mM citrate buffer, pH 6.0, in a steamer to retrieve antigen. Endogenous peroxidase activity was blocked with peroxidase blocking solution (S2001, Dako) and unspecific binding was blocked with 5% goat serum, 0.5% casein, 0.1% sodium azide in TBS (Ab dilution buffer). Tissue sections were then stained with a rabbit anti-human CD3 antibody, (1:200) in Ab dilution buffer or a control antibody for one hour at RT in a moist chamber. Purified rabbit immunoglobulin was used at 1 μg/ml as a staining control. The specificity of the anti-human CD3 antibody has been previously tested on sorted $CD4^+$ canine T cells. After 3 washes in TBS, sections were stained with biotinylated goat anti-rabbit Ig antiserum (1:100 in Ab dilution buffer, Dako) for one hour at RT. Thereafter, sections were washed and incubated with a streptavidin-biotin/horse radish peroxidase conjugate (1:200 in TBS, Dako) for 40 min. Finally, sections were washed and developed with a peroxidase substrate solution (0.2 mg/ml 3,3´-diaminobenzidine tetrahydrochloride DAB, 0.012% H_2O_2, 20 mM citric acid

monohydrate, 100 mM imidazole, 100 mM NaCl, pH 7.0) for 7 minutes in the dark and counterstained with hematoxilin. Sections were then dehydrated with an increasing concentration of ethanol and finally mounted.

2.4. IEL isolation

The IEL were isolated by previously published protocols, which were adapted to canine lymphocytes [2,16]. In brief, IEL were dissociated from small and large intestinal tissue by gently stirring tissue pieces in Ca^{2+}- and Mg^{2+}-free HBSS containing 2% horse serum, 1 mM DTT, and 0.5 mM EDTA for 2 x 30 min at 37°C. Isolated cells were sequentially passed through a 70 μm and a 40 μm pore size nylon mesh cell strainer (Becton Dickinson, San Jose, CA). The IEL were then separated from enterocytes by a 40%/70% Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation (15 min, 900 x g, RT). IEL at the 40%/70% Percoll interphase were harvested and washed twice in PBS, 2% horse serum. Cell numbers and viability were estimated using a hemocytometer and trypan blue dye exclusion. IEL preparation generally contained between 15 and 70% (57 $\pm$ 21%) $CD45^+$ leukocytes.

2.5. Isolation of lymph node lymphocytes

Mesenteric lymph nodes were mechanically disrupted, passed through a 40 μm pore size nylon mesh cell strainer, washed once with PBS, 2% horse serum and then further used for analysis.

2.6. Flow cytometry

Mesenteric lymph node lymphocytes and IEL from small and large bowel were stained with differentially labeled antibodies as indicated in the results. For double stainings, 100'000 viable cells were incubated with primary fluorescent dye-labeled antibodies (anti-CD3-FITC,1:100 dilution; anti-CD4-PE, 1:1000 dilution; CD8α-FITC, 1:20 dilution) or biotinylated antibodies (anti-CD45-biotin, 1:200 dilution; anti-TCRαβ, 1:200 dilution; anti-TCRγδ, 1:200 dilution) in a 96-well V-bottom plate for 15 min at 4°C in the dark. After two washes with PBS/2% horse serum cells were resuspended in 100 μl PBS/2% horse serum containing Cy5-conjugated streptavidin and incubated for 15 minutes at 4°C in the dark. Cells were then washed again, fixed and analyzed by flow cytometry.

For the simultaneous detection of CD8α, CD8β and TCR triple stainings were used as follows. Viable cells were first incubated with rat anti-CD8α-FITC (1:20 dilution) and mouse anti-CD8β (1:100 dilution) antibodies. After two washes with PBS/2% horse serum cells were resuspended in 100 μl PBS/2% horse serum containing goat anti–mouse-PE (1:300 dilution) and incubated for 15 minutes at 4°C in the dark. Cells were washed and unsaturated goat anti-mouse antibody was

blocked by incubation with 1 mg/ml mouse IgG at 4°C in the dark for 10 minutes. After an additional wash biotinylated mouse anti-canine TCRαβ (1:200 dilution) or TCRγδ (1:200 dilution) antibodies were added for 15 minutes. Cells were then washed, and biotin was detected with Cy5-conjugated streptavidin (1:500 dilution). After a final wash all samples were fixed in 1% phosphate-buffered formaldehyde and analyzed by flow cytometry on a FACScan (BD Biosciences). Data were analyzed using the Flow Jo (Tree Star, Ashland, OR) software package. For analysis, cells were normalized to $CD45^+$ cells. Data were reported as the mean $\pm$ SD. Statistical analysis was done using the Mann Whitney U test or paired t-test, as indicated in the text.

2.7. Proliferation assay

IEL from duodenum and lymphocytes from mesenteric lymph nodes were isolated from 8 adult healthy Beagle dogs by above described protocols, and either directly (n = 5) used for proliferation assays or stored overnight in cell culture medium (iscove`s modified dulbeccos`s medium, IMDM), 2 mM L-alanyl glutamine, 5% FCS, 50 μg/ml gentamycine) at 4°C for further purification by cell sorting (n = 3). IEL were separated from contaminating epithelial cells based on forward/side scatter properties (lymphocyte gate) using a FACStar cell sorter (BD Biosciences). The purity of the sorted IEL was confirmed by CD3 staining and usually contained >99% $CD3^+$ cells. IEL and lymph node-derived lymphocytes

were plated at a density of 1 x 10^5 cells/well in 96-well U-bottom microtiter plates in complete medium in triplicates. In some experiments lymph node lymphocytes were co-cultured with decreasing numbers of IEL, starting at a 1:1 ratio (unsorted cells) and a 1:3 ratio (sorted cells) respectively, followed by subsequent three-fold dilutions. Unspecific effects of contaminating epithelial cells in unpurified IEL preparations on lymph node T cell proliferation were assessed by sorting epithelial cells on a cell sorter and identical co-culture with lymph node cells. Cells were then stimulated with medium control, or PMA (50 ng/ml) plus ionomycin (500 ng/ml) as a polyclonal T cell stimulus. Cultures were incubated for 24 h at 37°C in 5% CO_2 and then pulsed with 1 μCi/well ^{3}H-thymidine for 6 h. Cells were harvested on glass fiber filters and ^{3}H-thymidine incorporation was measured on a Topcount β counter (Perkin Elmer, Schwerzenbach).

2.8. Real time PCR

IEL from duodenum and lymphocytes from the mesenteric lymph nodes of 8 adult healthy Beagle dogs were isolated and sorted by flow cytometry as described above. Cells were then stimulated for 6h with PMA (50 ng/ml) plus ionomycin (500 ng/ml), harvested and lyzed in TRI reagent (Sigma Aldrich). Total RNA was isolated and cDNA was generated using a high capacity cDNA Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an Applied Biosystems real-time PCR 7500 machine using SYBR green and the canine-

specific Quantitech primer sets (Qiagen, Hilden, Germany) for GAPDH, IFNγ, TGFβ and IL-10. Data were normalized to the expression levels of the house-keeping gene GAPDH.

3. Results

3.1. Immunohistochemical detection of intraepithelial lymphocytes (IEL) in the canine small and large intestine

Tissue sections from small and large canine intestine were stained with an anti-CD3 antibody, demonstrating numerous $CD3^+$ cells in the epithelium and in the lamina proria. (Fig. 1B and 1C). Control reagents did not reveal any positive staining (Fig. 1A). Higher magnification showed that $CD3^+$ cells are embedded in between the epithelial cells outside of the basal membrane, with a frequency of 1-2 for every 10 epithelial cells.

3.2. Canine intestinal IEL consist predominantly of $CD45^+$ $CD3^+$ T cells

Analysis by flow cytometry of isolated intestinal IEL demonstrated a clearly distinguished lymphocyte gate. Staining of these cells for the common leukocyte marker CD45 and the T cell marker CD3 revealed that >95% of the cells within the lymphocyte gate were $CD45^+$ and $CD3^+$. (Fig. 2).

3.3. Canine intestinal IEL consist of conventional and unconventional T cell subsets

In the peripheral lymphoid organs the T cell compartment is predominantly composed of $CD4^+$ and $CD8^+$ T cells. A similar distribution was found in the mesenteric lymph node of adult Beagle dogs. In marked contrast to the lymphnode, in the intestinal epithelium only few $CD4^+$ cells (<15%) were found, with slightly higher numbers ($p=0.0279$) in the small bowel (Fig. 3A). In the peripheral immune system most T cells are $TCR\alpha\beta^+$, whereas only very few unconventional $TCR\gamma\delta^+$ cells are found. In our results, in the intestinal epithelial layer and in the lymphnodes $TCR\alpha\beta^+$ T cells were found to be the dominant population, however, a substantially greater number (TCRγδ MLN vs. IELSB $p<0.0001$, MLN vs IELLB $p<0.0001$) in unconventional $TCR\gamma\delta^+$ T cells (small bowel 22.9 $\pm$ 6.3%; large bowel 27.6 $\pm$ 9.9%) was observed in the intestine compared to the mesenteric lymph node (1.7 $\pm$ 1.6%) (Fig. 3B). The comparison between the expression of the CD8αβ heterodimer with the more unconventional CD8αα homodimer showed that the vast majority of $CD8^+$ T cells in the canine mesenteric lymph node, as well as intestinal IEL expressed the CD8αβ heterodimer (small bowel: 49.7 $\pm$ 8.6%, large bowel: 59.3 $\pm$ 12.2%). In the mesenteric lymphnode $CD8\alpha\alpha^+$ T cells were basically absent , whereas in the intestinal epithelium we frequently observed relatively high numbers of unconventional T cells with the CD8αα homodimer (small bowel: 8.6 $\pm$ 1.5%,

large bowel: 14.3 ± 6.7%) (CD8αα MLN vs IEL SB p=0.0032, MLNvsIELLB p=0.0001) (Fig. 3C).

3.4. Unconventional canine CD8αα$^+$ IEL express both the αβ and the γδ T cell receptor

In a multi-parameter flow cytometric analysis, it could be shown, that both the CD8αβ$^+$ and the CD8αα$^+$ subsets were clearly distinguishable within the IEL lymphocyte gate (Fig. 4A). Analysis of the TCR expression of these distinct IEL subsets revealed that the conventional CD8αβ$^+$ subsets predominantly express the TCRαβ heterodimer, whereas within the unconventional CD8αα$^+$ subset a high percentage of cells expressed also TCRγδ. Detailed analysis of CD8αα$^+$ and CD8αβ$^+$ IEL from 4 dogs revealed that TCRγδ$^+$ T cells are only a minor portion of CD8αβ$^+$ IEL, whereas TCRγδ-expressing cells are concentrated in the CD8αα$^+$ IEL subset (Fig. 4B, C) (CD8αα TCRαβ vs TCRγδ p=0.01, CD8αβ TCRαβ vs TCRγδ p= 0.008).

3.5. Developmental changes in IEL composition in neonatal versus adult dogs

Significant differences could be found in the IEL subpopulations comparing neonatal and adult dogs. CD4$^+$ T cell receptor (TCR)αβ$^+$ IEL (Fig. 5A) and CD8$^+$ TCRαβ$^+$ IEL (Fig. 5B) were detectable only at low levels in newborn dogs but were dramatically increased in the small and large bowel of adult Beagle dogs.

CD4TCRαβ NB vs AD SB p= 0.0016, CD4TCRαβ NB vs adult dogs LB p=0.0021, DNTCRαβ NB vs AD SB p= 0.0005; DN TCRαβ NB vs AD LB p=0.0005; CD8TCRαβ NB vs AD SB p=0.0005; CD8TCRαβ NB vs AD LB p= 0.0032. Substantial numbers of $CD4^{-}CD8^{-}$ $TCR\alpha\beta^{+}$ IEL (Fig. 5C) and even more remarkable up to 50% of IEL in the small bowel and up to 60% in the large bowel of IEL in neonates were defined as $CD4^{-}CD8^{-}$ IEL $TCR\gamma\delta^{+}$(Fig. 5D).

3.6. Canine IEL respond poorly to mitogenic stimulation but display suppressive activities

Figure 6A shows a high proliferative response of the mesenterial lymphnode lymphocytes (LN), while intestinal IEL proliferated poorly upon stimulation unsorted $p<0.0001$, sorted $p=0.0004$. Figure 6B confirms the data reported above in regard to proliferation. However, LN in co-culture with a decreasing LN/IEL ratio, IEL seemed to dose-dependently inhibit the proliferation of LN ($p=0.0002$). In a following experiment using highly purified sorted IEL and intestinal epithelial cells as negative control the previous data above were confirmed as sorted IEL failed to proliferate upon stimulation, but strongly inhibited the proliferative response of LN ($p=0.02$). In marked contrast, highly purified epithelial cells did not show such suppressive behavior, and co-culture with mesenteric lymph node cells rather resulted in increased proliferation of the latter ($P=0.0113$); Fig. 6C).

3.7. Expression of immunoregulatory and inflammatory cytokines in canine IEL

Expression of mRNA of the cytokines IL-10, IFN-g and TGF-β in mesenterial lymphocyte lymphocytes (LN) and purified intestinal intraepithelial lymphocytes (IEL) upon stimulation was analyzed by quantitative PCR. Stimulated IEL expressed higher levels of IFNγ ($p=0.02$) and IL-10 ($p=0.08$) paired t test one tailed (Fig. 7A,7B). However, IEL did not seem to express higher levels of the suppressive cytokine IL-10 and lower levels of the pro-inflammatory cytokine IFNγ, compared to mesenteric lymph node cells, but rather expressed higher levels of both cytokines. Interestingly, both lymphocyte populations expressed lower levels ($p=0.07$) of TGFβ upon stimulation (Fig. 7C).

4. Discussion

Intestinal intraepithelial lymphocytes are residents of the gut epithelium, embedded at high frequency in between intestinal epithelial cells. This highly strategic location may enable IEL to serve as a first line of defense to protect our nearly sterile core body against possible invaders from the antigen-rich gut lumen. On the other hand IEL are also able to tolerate harmless luminal antigens and even actively contribute to oral and systemic tolerance by regulating antigen-specific immune responses. This notion illustrates a potentially critical role of IEL in the maintenance of gut homoeostasis, and their development and composition is adapted to these needs. In mice and man intestinal IEL are generally subdivided

into two major subpopulations, based on their TCR usage and co-receptor expression [10]. The conventional or "type a" mucosal T cells comprise $CD4^+$ or $CD8\alpha\beta^+$ lymphocytes with the αβ TCR, whereas the unconventional or "type b" subsets contain $CD8\alpha\alpha^+$ expressing TCRαβ and TCRγδ as well as $CD4^-CD8^-$ T cells. These IEL subpopulations do not only differ in their co-receptor and TCR usage but also show major functional and potentially developmental differences. "Type a" mucosal T cells have much in common with peripheral $CD4^+$ or $CD8\alpha\beta^+$ cells, but also show some gut-specific regulatory functions. Thus, $CD8\alpha\beta^+$ TCRαβ IEL may exert some cytolytic functions [4], whereas resident (in contrast to infiltrating) $CD4^+$ TCRαβ cells often show a regulatory phenotype [3]. "Type b" TCRγδ cells likely play a dual role in the regulation of protective immune responses and the regulation of epithelial layer integrity via the release of keratinocyte growth factor [1]. Clearly, various IEL subsets play a critical role in the regulation of health and disease in the intestinal mucosa, including the pathogenesis of inflammatory bowel disease.

Their abundance in the canine intestinal epithelium (Fig. 1) suggest that canine IEL may have a similarly important role in the regulation of intestinal immune homeostasis. Unfortunately to date the canine intestinal immune system is still poorly characterized. Furthermore, studies on the role of intestinal immune cells in the regulation of the pathogenesis of canine IBD are severely hampered by the lack of species-specific reagents, in particular antibodies against leukocyte

markers. So far, most studies on the distribution and characterization of intestinal immune cells of healthy and diseased dogs mostly employed immunohistochemistry in a descriptive manner [5,7,8,9]. Critically, these studies did not prove the presence of various IEL subsets due to the lack of multi-parameter analysis.

The goal of this study was thus the in-depth phenotypical characterization of canine IEL using multi-parameter flow cytometrical analyses. Up to now only very few flow cytometry-based studies on canine intestinal immune cells have been described [20,23]. As not more than two parameters were detected simultaneously, these studies only allowed only a relatively superficial description of canine IEL phenotypes. The simultaneous detection of three parameters, on the other hand, enabled us to demonstrate for the first time the differential TCR usage of CD8$\alpha\beta^+$ and CD8$\alpha\alpha^+$ IEL in the canine gut. As these distinct subsets have been previously shown in mice to exert substantially different roles in intestinal immune responses their identification is likely relevant for the investigation of canine IBD [17]. Another major difference between previous and the present study is the selection of the study material [20,23]. While in previous studies endoscopical tissue biopsies from various breeds were used, our study focussed on full-thickness biopsies from Beagle dogs only in order to avoid inter-breed variability and to develop a mono-breed standard. These methodological differences may also explain some of the discrepancies between our and previous

results. For example, our study revealed that in the lymphocyte gate >95% of the cells were $CD45^+$ and $CD3^+$, with basically no $CD21^+$ B cells (<1%), indicating that solely IEL and not contaminating lamina propria lymphocytes were assessed. This high percentage of T cells in the IEL compartment is well known in mice [14,19] and was also suggested by immunohistochemical studies in dogs [8,15]. However, these findings stand in contrast to those of Sonea et al., in which only about 60% of the cells in the lymphocyte gate stained positive for CD45, among which only 50% were positive for CD3. As no density gradient centrifugation was performed in these studies, the low percentage of $CD45^+$ cells in the lymphocyte gate may be due to a contamination with (dying) epithelial cells. On the other hand the low number of $CD3^+$ cells may be explained by TCR downregulation of IEL, likely due to prolonged incubation cycles during the isolation. Clearly, our own results demonstrate that canine IEL show a similar phenotypical pattern as in mice and man [13,14,19], suggesting similar functions in the regulation of local immune responses. Despite the many similarities between canine and rodent IEL, we also observed quite distinct features of canine IEL. Notable is the high frequency of double-negative ($CD4^-CD8^-$) IEL in adult dogs, and more pronounced, in newborn dogs. While we can currently only speculate about their ontogeny and function, their higher frequency in newborn and lower frequency in adult dogs suggests a rather immature phenotype of this IEL subset. Also surprising was the high percentage of $CD4^+$ TCR$\alpha\beta^+$ T cells. In mice an increase

in $CD4^+$ T cells is usually indicative of ongoing immune responses and reflects the antigen-driven infiltration of peripheral T cells. Furthermore, the percentage of $CD4^+$ T cells in the IEL compartment is generally higher in the large bowel due to the higher antigen load and bacterial colonization. In contrast, we observed that in the canine small bowel $CD4^+$ T cells are found at higher frequency compared to the large bowel. Critically, this high percentage (up to 15%) was observed in the absence of any signs of inflammation. Thus, canine $CD4^+$ IEL could represent a regulatory T cell subset, rather than pro-inflammatory T helper cells.

While a phenotypical characterization of IEL may be indicative of certain functional properties, based on comparison with studies in other species, it does not prove that IEL or certain IEL subsets have regulatory activities. In this study we provide, to our knowledge, first-time evidence that canine IEL have a potent immunoregulatory potential. While mesenteric lymph node T cells strongly proliferated in response to mitogenic stimuli, IEL failed to do so as reported in other species [22]. However, co-culture of IEL with mesenteric lymph node cells even at low ratios substantially inhibited the proliferation of lymph node cells. This clearly indicates that IEL, or at least certain IEL subsets, lack conventional T cell effector functions and represent regulatory T cells. When analyzing the underlying mechanism(s) of IEL-based suppression of mesenteric lymph nodes, we failed to identify clear candidates. While there is good evidence that in mice the immunoregulatory cytokine IL-10 and TGFβ are critical effector molecule of

IEL regulatory functions and oral tolerance induction [12], we did not observe a clear expression pattern of these cytokines in regulatory IEL compared to mesenteric lymph node cells. Part of the problem may however also be that both mesenteric lymph node cell and IEL preparations contained various T cells subsets with differential expression of these regulatory cytokines. For example, $CD4^+$ regulatory T cells may also be present in mesenteric lymph node cells, as pro-inflammatory T cells may also exist in the epithelial layer. Similarly, analysis of mRNA expression does only partially reflect the actual protein production of these suppressive cytokines. Due to the lack of canine IL-10- and TGFβ-specific antibodies for the use of RT-PCR we can currently only estimate the capacity of canine IEL to synthesize these regulatory factors.

In conclusion, the present study represents the first multi-parameter characterization of intestinal IEL in a mono-breed population. This methodology allowed the first description of $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ and $CD8\alpha\alpha^+$ $TCR\gamma\delta^+$ IEL subsets. Their abundance in the canine intestinal epithelial layer suggests an important role of these T cell subsets in the maintenance of intestinal immune homeostasis. Our study also demonstrates for the first time that canine IEL have potent suppressive activities by suppressing mitogen-driven proliferation of peripheral T cells. The comparison of this “gold standard” in a healthy mono-breed population of Beagle dogs with other breeds as well as diseased dogs may

promote the development of multi-parameter flow cytometry as a future diagnostic modality in the diagnosis of canine IBD.

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Figure legends

Figure 1: Immunohistochemical detection of $CD3^+$ cells in the small and the large canine intestine

Paraffin sections of canine small and large intestine were stained with control rabbit IgG (**A**) or anti-CD3 antibody (**B, C**). Lower (20x) and higher magnifications (40x) of representative stainings of small (A, B) and large intestine (C) are shown. Inserts show enlargements of the epithelial layer with IEL (arrow). Bars indicate 50 μm.

Figure 2: Characterization of canine intestinal intraepithelial lymphocytes

IEL were isolated from the small bowel of an adult Beagle dog as described in Materials and Methods, stained with anti-canine CD45 and anti-canine CD3, followed by flow cytometrical analysis. The distinct forward/side scatter properties of IEL and their staining for CD45 and CD3 in a typical isolation are shown.

Figure 3: Lymphocyte subset composition of the canine intraepithelial compartment

Mesenteric lymph node cells (MLN), and IEL from the small (SB) and large bowel (LB) from 15 healthy adult Beagle dogs were isolated and analyzed for

their cell surface marker expression by flow cytometry. **A)** Analysis of $CD4^+$ and $CD8^+$ lymphocyte subsets. **B)** TCRαβ and TCRγδ usage in MLN and IEL. **C)** Detection of the $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ lymphocyte subsets. Mean values +/- SD of n = 15 are shown. Numbers were normalized to $CD45^+$ cells.

Figure 4: T cell receptor usage of canine intraepithelial $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells

IEL from small bowel of healthy adult Beagle dogs were isolated and stained for CD8α and CD8β, and TCRαβ or TCRγδ. **A)** Example of $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ subset detection and analysis of their TCR expression (TCRαβ and TCRγδ). **B), C)** Analysis of TCRαβ and TCRγδ expression in the $CD8\alpha\beta^+$ (B) and the $CD8\alpha\alpha^+$ (C) subsets. Numbers were normalized to $CD8\alpha\beta^+$ cells (B), respectively $CD8\alpha\alpha^+$ cells (C). Mean values +/- SD of n = 4 are shown.

Figure 5: Differences in IEL subpopulations in small and large bowel from newborn and adult dogs

IEL were isolated from the small (SB) and large bowel (LB) of 15 healthy adult (AD) and 6 newborn (NB) Beagle dogs and their leukocyte marker expression was characterized by flow cytometry. **A)** $CD4^+$ $TCR\alpha\beta^+$ IEL, **B**) $CD8^+$ $TCR\alpha\beta^+$ IEL, **C**) $CD4^-CD8^-$ $TCR\alpha\beta^+$ IEL, **D)** $CD4^-CD8^-$ $TCR\gamma\delta^+$ IEL. ** $p < 0.005$, *** $p \leq 0.0005$.

Figure 6: Canine IEL inhibit proliferation of mesenteric lymph node T cells

A) Mesenteric lymph node cells (LN) or IEL from the small bowel of adult Beagle dogs were isolated, and stimulated with medium control or PMA plus ionomycin. The proliferative response was measured by [^{3}H]-thymidine incorporation after 48h. Mean vaules $^+$/- SD of n=5 experiments are shown. **B)** Unsorted mesenteric lymph node cells (LN) or IEL were either cultured alone with medium control or PMA plus ionomycin, or Mesenteric lymph node cells (LN) were co-cultured with decreasing LN/IEL ratios. Mean values $^+$/- SD of triplicates of typical experiments out of 5 are shown. **C)** Same experimental approach using sorted IEL and intestinal epithelial cells (EP) as negative control. Mean values $^+$/- SD of triplicates of typical experiments out of 3 are shown. Values in B) and C) were normalized to the maximal proliferation of stimulated lymph node cells (100%).

Figure 7: Cytokine expression profile of canine IEL

Mesenteric lymph node cells (LN) and IEL from small intestine of 8 adult Beagle dogs were isolated, and stimulated for 6 h with medium control (ctrl.) or PMA plus ionomycin (stim.). Total RNA was isolated and the expression of IFNγ **(A)**, IL-10 **(B)**, and TGFβ **(C)** was analyzed by real-time RT-PCR. Bars indicate mean values, symbols shown values of individual samples.

Luckschander N, Pfammatter NS, Sidler D et al. 30

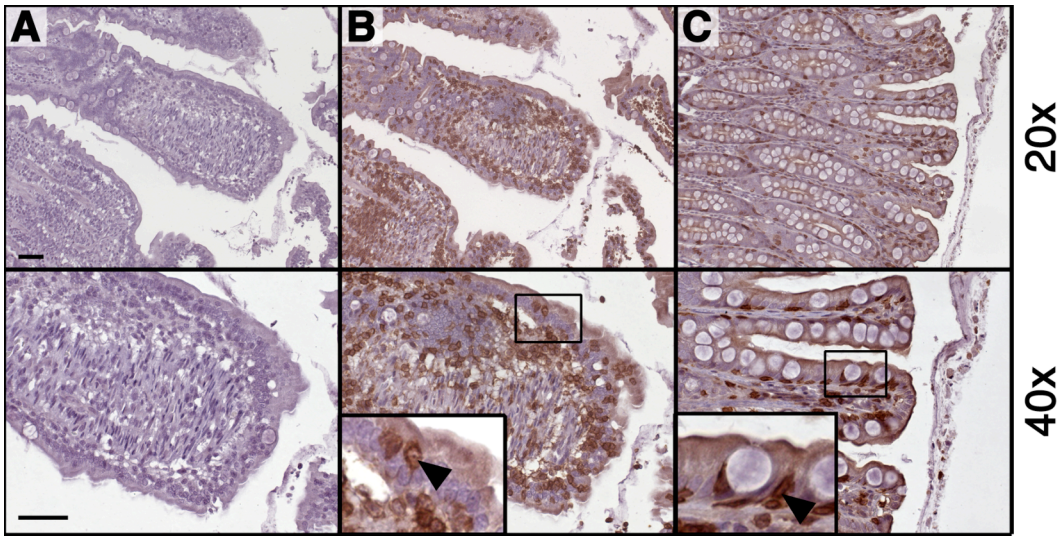

Figure 1

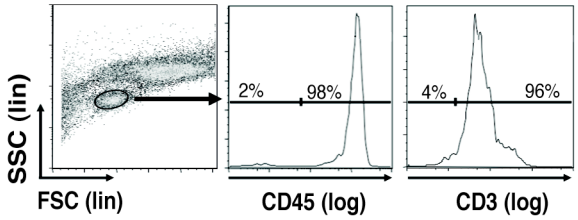

Figure 2

Luckschander N, Pfammatter NS, Sidler D et al. 32

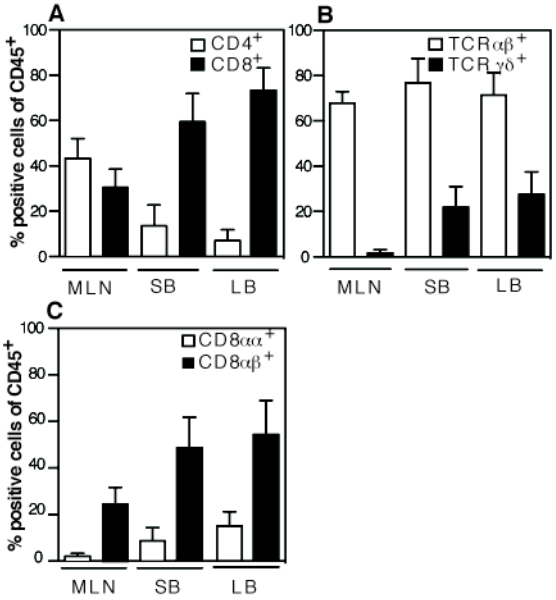

Figure 3

Luckschander N, Pfammatter NS, Sidler D et al. 33

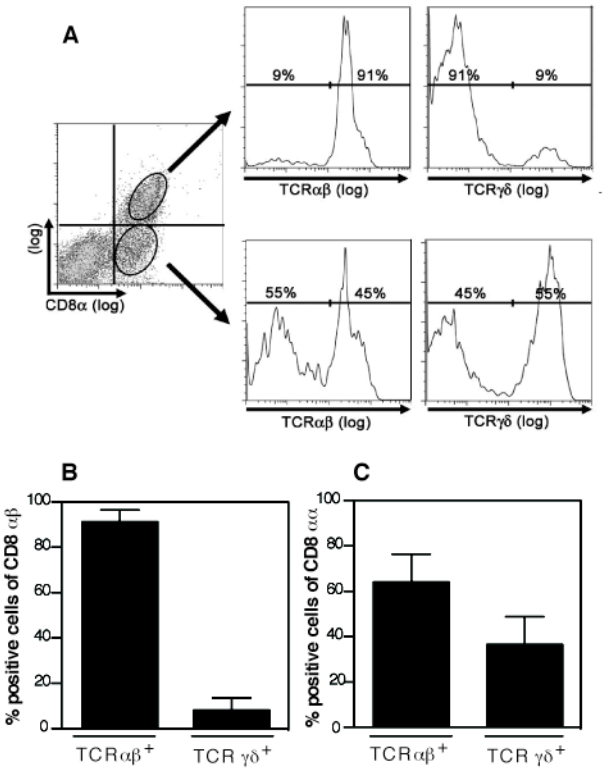

Figure 4

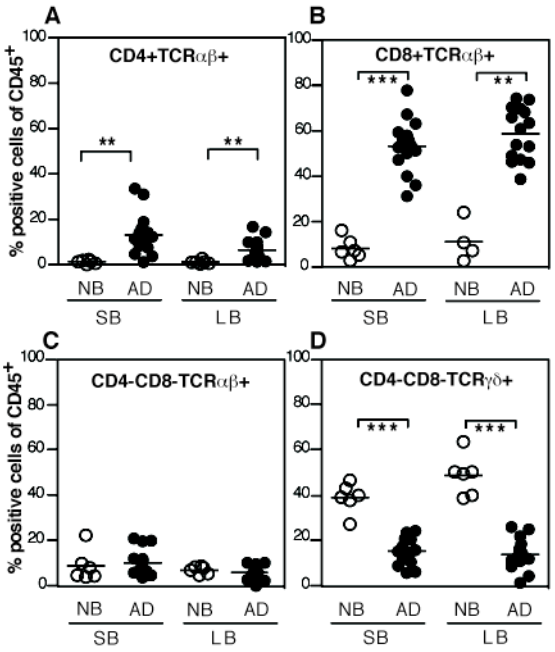
A
CD4+TCRαβ+
B
CD8+TCRαβ+
C
CD4-CD8-TCRαβ+
D
CD4-CD8-TCRγδ+
% positive cells of CD45+
NB AD NB AD
SB LB

Figure 5

Luckschander N, Pfammatter NS, Sidler D et al. 35

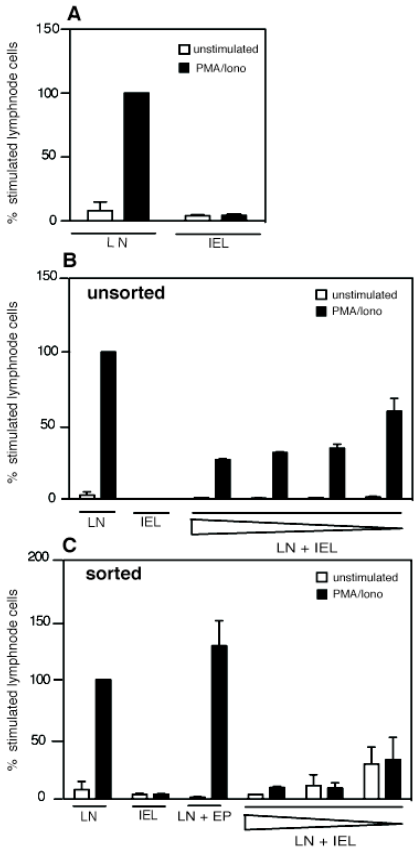

Figure 6

Luckschander N, Pfammatter NS, Sidler D et al. 36

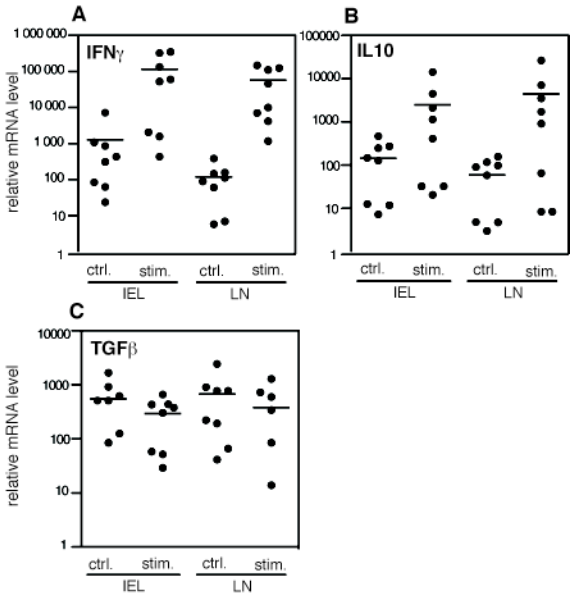

Figure 7

5. DISCUSSION AND OUTLOOK

Chronic diarrhea, vomiting and weight loss are common clinical conditions in small animal veterinary practice. The underlying reason for these severe gastrointestinal symptoms in canine patients is often IBD. Unfortunately, despite the high frequency of this severe disease in dogs, the exact pathomechanisms are fairly unknown. Furthermore, the current state of our collective knowledge regarding the canine mucosal immunology is largely based on studies carried out in rodents or in human tissue. Due to the lack of species-specific reagents, canine studies have contributed only little to clarifying the pathogenesis of IBD. Although the overall mechanisms to control mucosal surfaces are likely very similar in mono-gastric animals, therapies, adopted from human medicine, often show severe side effects or lack efficacy in dogs. In order to provide the best medical care for canine patients and their owners, there is a clear need for species-specific studies to gain greater understanding of the canine mucosal immune system and its regulation.

Several points of evidence support an important role of intestinal lymphocytes in the pathogenesis of IBD. In this work, we therefore focused on the phenotypical and functional characterization of canine IEL, which are easily accessible and may function as a surrogate marker for ongoing immunological processes in the underlying intestinal tissue. All our investigations were performed on full thickness biopsies of healthy adult Beagle dogs providing a mono-breed standard in order to reduce the heterogeneity of different breeds.

In the first work a CD3 staining was performed on different intestinal locations (stomach, descending and ascending duodenum, jejunum, ileum, ascending and descending colon) from 6 adult and 4 neonatal healthy beagles. The positive cells were counted in an area of total 200,000 μm^2 in the designated 7 localizations and at 4 different sites per localization (villi and crypts in lamina epithelialis and lamina propria, respectively). The computer–assisted cell count provided an objective method to assess the regional distribution of the T cells in the lamina propria and in the epithelium. A distinct intestinal T cell distribution in the different parts of the intestinal mucosa could be demonstrated. Furthermore, age-dependent differences (based on comparison between neonatal and adult dogs) of IEL and lamina propria

lymphocytes were observed. This work illustrated the frequent presence of T lymphocytes in the canine intestinal epithelium allowing the isolation and further investigation of these cells.

In the second study our first aim was the characterization of the different lymphocyte subpopulations in the epithelial compartment by using a multi-parameter flow cytometrical analysis. Therefore, IEL were isolated from full-thickness biopsies of 15 adult and 6 neonatal Swiss Beagle dogs and compared to lymph node cells. For the first time an in-depth phenotypical characterization of intestinal canine IEL, including the description of unconventional ”type b” lymphocytes, could be established. Furthermore, significant differences in the IEL subpopulations of adult and newborn dogs could be shown. This study established a “ gold standard” in a healthy mono-breed population. A future comparison with other breeds as well as diseased dogs may further show inter-breed or disease-based differences in IEL subset distribution and may help to promote flow cytometry as a tool for the diagnosis and therapy follow-up of canine IBD.

In the present study, we used pooled IEL from two different localizations (ascending duodenum and mid jejunum) for the phenotypical characterization of small bowel lymphocytes. In future studies it would be interesting to isolate IEL from distinct and individual localizations of the small and large bowel in order to investigate the horizontal distribution of different IEL sub-populations along the intestine.
Also the detailed characterization of the lamina propria lymphocytes by multi-parameter flow cytometric analysis would be very interesting, as these cells are mainly involved in the antigen-directed immune defense of the intestine. The lamina propria is an important effector side of the intestinal immune system, to which numerous antigen-experienced lymphocytes migrate, following initiation at the inductive sites. Therefore, the immune cells in the lamia propria consist primarily of immunoglobulin A (IgA) $^{+}$ plasma cells and fairly equal numbers of $CD4^{+}$ and $CD8\alpha\beta^{+}$ T cells. Most of the T lymphocytes belong to the conventional “type a” mucosal T cells, which express an αβ TCR together with CD4 or CD8αβ as TCR coreceptor. While the CD8αβ “type a” mucosal T cells display all characteristics of cytolytic effector memory T cells, the $CD4^{+}$ type a mucosal T cells can display a

regulatory or regulated function with suppressive activity [14]. In case of IBD, the chronic inflammatory response is represented by an infiltration of lymphocytes and macrophages in the lamina propria. While in human patients the mucosal immune response is polarized into a Th1 or Th2 response, this distinct differentiation cannot be made in dogs. Canine IBD is mainly characterized by a lympho-plasmacytic infiltration in the lamina propria, which reflects the importance of lamina propria lymphocytes (LPL) in the pathomechanisms, but also in the diagnosis of IBD. The isolation of LPL would allow correlation of phenotypes and functions and the application of flow cytometric analysis. However, the purity of the preparations and any effects of the isolation procedure always limit the study of isolated lymphocytes. In our experience, the isolation procedure of canine intestinal LPL (based on three more EDTA treatments and digestion with collagenase V / DNAse) implicates a high risk of contamination due to the IEL compartment. Also a high variability of $CD21^+$ cells in the lamina propria compartment represented a not efficient digestion procedure. (Luckschander et al. unpublished data). Therefore further investigations of canine LPL must be attentive to factors that may artificially alter the results. Further studies are needed for the improvement of the LPL isolation techniques. Also a combined application of different techniques (flow cytometry and immunohistochemistry) seems to be important in future studies of canine LPL in order to increase the reliability of the flow cytometric results.

As IEL in other species had been previously suggested to exhibit regulatory functions we investigated the role of IEL on the activation-induced proliferation of lymph node T cells. While IEL alone did not show a mitogenic response, they significantly inhibited the proliferation of activated lymph node T cells in a cell number-dependent manner. First, this clearly indicates that canine IEL are substantially different from peripheral lymphocytes in respect to their activation behavior. Furthermore, IEL or certain IEL subsets seem to lack conventional effector functions of peripheral T cells and exert more immunoregulatory functions. Surprisingly, our study represents the first functional characterization of canine IEL, demonstrating their immunoregulatory phenotype. Interestingly, however, our data obtained in dogs correlate with other studies generated in mice and man, and further support the regulatory characteristics of IEL [75].

Of course it would be highly interesting to further analyze the immunoregulatory functions of canine IEL. Recently, an antibody cross-reactive with murine and canine FoxP3 has been described and used for the identification of canine regulatory T cells in blood and lymph nodes from healthy dogs and dogs with cancer. [34] The isolation and identification of $FoxP3^+$ regulatory T cells in the canine intestine would help to clarify their importance in the gut homeostasis and their possible role in the regulation of canine IBD. Furthermore, specific isolation would allow their functional characterization with respect to proliferation and cytokine expression. Not only the regulatory functions of IEL but also their cytotoxic potential has never been described so far. In contrast, the cytotoxic capacity of murine and human IEL is well investigated. For example, Lundqvist et al. reported that human IEL are not spontaneously cytotoxic, but are able to kill target cells in an anti-CD3-mediated redirected cytotoxicity assay [76]. Establishing a cytotoxicity assay for canine IEL, either antigen-specific or via antibody-mediated activation, they could demonstrate that canine IEL express cytotoxic molecules and are able to exert not only regulatory but also cytotoxic functions.

Although we were able to demonstrate a regulatory function of canine IEL, we did not observe a clear expression pattern of "regulatory" cytokines (e.g. IL-10, TGF-β) in IEL compared to mesenteric lymph node cells. Part of the problem may, however, also be that both mesenteric lymph node cells and IEL preparations contain various T cells subsets with differential expression patterns of these regulatory cytokines. For example, $CD4^+$ regulatory T cells may also be present in mesenteric lymph node preparations, as pro-inflammatory T cells may also exist in the intestinal epithelial layer. Furthermore, analysis of mRNA expression does only partially reflect the actual protein expression of these regulatory cytokine. In our present work we used an RT-PCR-based method to investigate the cytokine expression in *ex vivo* cultured IEL. If a commercial ELISA kit would be available for canine IL-10, IFN-γ and TNF-α these cytokines could be detected in *ex vivo* cell culture supernatant upon IEL stimulation. This assay would further allow investigating the regulatory potential of canine intestinal IEL.

Although these investigations represent only the beginning of canine mucosal immunology and much more remains to be done, this study represents a new step in the characterization of the canine intestinal immune system and provides new hypotheses into potential pathomechanisms of canine enteropathies. It further promotes the development of multi-parameter flow cytometry as a future standard protocol in the diagnosis of canine IBD.

6. APPENDIX

6.1. Flow cytometric analysis of intestinal IEL as a diagnostic tool for canine IBD

The increasing incidence of canine IBD enforces veterinarians to face this difficult gastrointestinal disease more often. Therapies, successfully established in human medicine, often fail in canine patients or are associated with severe side effects. The diagnosis of canine IBD is still based on chronic gastrointestinal symptoms, exclusion of any infectious or neoplastic disorders and the presence of intestinal lympho-plasmacellular infiltration as a sign of inflammatory reactions. Despite the obvious importance of lymphocytes in the diagnosis and pathogenesis of this disease, most studies on the distribution and characterization of intestinal lymphocytes of healthy and diseased dogs employed immunohistochemistry in a descriptive manner [28,37,45].

In our previous work we aimed at the establishment of a "gold standard " in the phenotypical characterization of intestinal canine IEL by flow cytometric analysis. In this project the goal was the evaluation of a multi-parameter flow cytometrical analysis of intestinal IEL as a diagnostic tool of canine IBD. In order to reach this goal multiple steps were necessary. First, we compared IEL isolated from intestinal full thickness and endoscopical biopsies (n=5 per group) from the same healthy Beagle dog, for the validation of endoscopical biopsies as a reasonable source for the interpretation of flow cytometrical analysis. In a next step, we compared intestinal endoscopical biopsies from healthy Beagle dogs with dogs suffering from chronic enteropathies (CE) (n = 11), evaluating flow cytometry as a diagnostic tool in canine IBD.

No differences in the T cell subsets between full thickness and endoscopical biopsies

IEL from small bowel were isolated from full-thickness and endoscopical biopsies from the same healthy control Beagle dog (n=5). The method of IEL isolation had been previously established and published by Brunner et al. [77], and has been adapted to canine intestine (Luckschander et al., unpublished data). After isolation, a flow cytometric analysis was performed using species-specific antibodies against canine T cell markers (CD45, CD3, TCRαβ, TCRγδ, CD4, CD8α, CD8β) in order to characterize the IEL compartment.

In full thickness and endoscopical biopsies, IEL were distinguished from contaminating epithelial cells based on forward and side scatter properties, and could be easily identified in the lymphocyte gate. There was no significant difference in the quality of isolation. In general between 20 and 30% of acquired cells were in the lymphocyte gate, (full thickness biopsies 26.2% $\pm$ 6.2, endoscopical biopsies 23.3%$\pm$3.2). Staining of these cells for the common leukocyte marker CD45, the TCRs $\alpha\beta$ or $\gamma\delta$ and the B cell marker CD21 revealed that >98% of the cells within the lymphocyte gate were $CD45^+$, the sum of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells was > 98% and <1 % of the cells were B cells. These results demonstrated that IEL found in the indicated gate are mainly T cells. The isolation of full thickness and endoscopical biopsies showed a distinct lymphocyte gate, in which predominantly T cells could be found and allowed a further analysis by flow cytometry. (Figure 7)

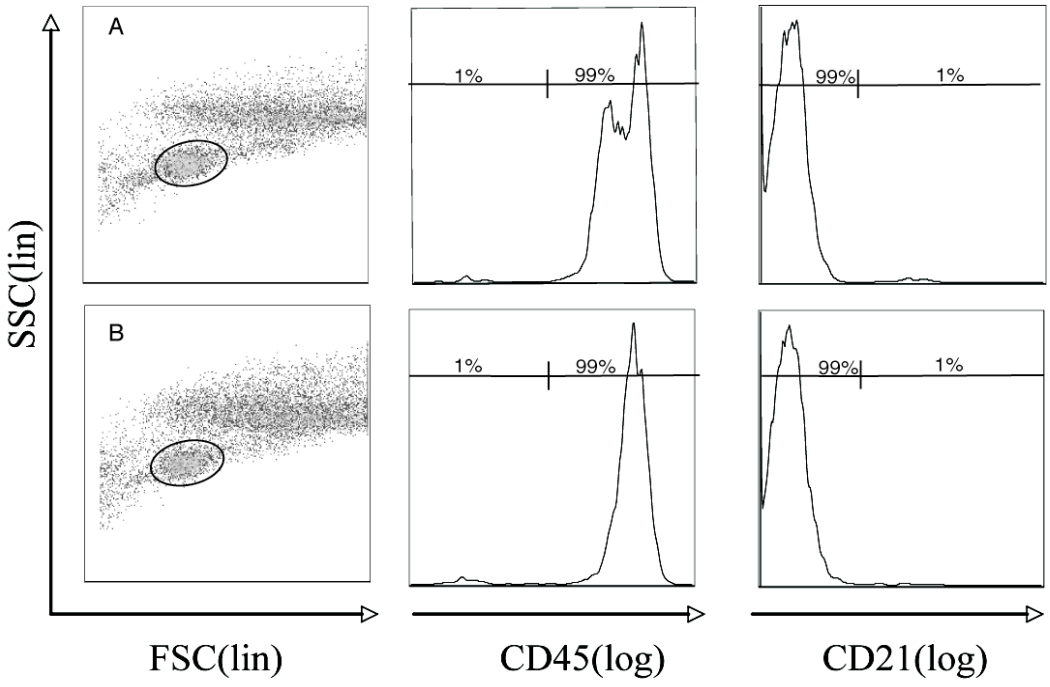

Figure 7: *IEL were isolated from full thickness biopsy (A) and from endoscopical biopsy (B) of the small bowel of an adult healthy Beagle dog as previously described, stained with anti-canine CD45 and anti-canine CD21, followed by flow cytometric analysis. The distinct forward/side scatter properties of IEL and their staining for CD45 and CD21 in a typical isolation are shown. Staining of these cells for the common leukocyte marker CD45 and the B cell marker CD21 revealed that within the lymphocyte gate >98% of the cells are leucocytes, no B cells could be found within the gate.*

In a next step IEL subpopulations were characterized, using a multi-parameter flow cytometry analysis. The canine intestinal IEL compartment mainly consists of a mixed T cell population of $CD8^+$ and $CD4^+$ T cells, while the $CD8^+$ T cells greatly outnumber the $CD4^+$ T cells. In the present study we could reproduce previous results as in the intestinal epithelial layer $TCR\alpha\beta^+$ T cells were found to be the dominant population. All of the $CD4^+$ T cells express $TCR\alpha\beta$, while a substantial portion of the $CD8^+$ T cells express $TCR\gamma\delta$, representing the more unconventional "type b" phenotype of IEL. Not unusual for dogs, a high percentage of $CD8^-CD4^-$ cells could be found, from which the majority was $TCR\gamma\delta^+$. This fraction represents a more immature phenotype.

We could not find any significant differences ($p<0.05$) in the IEL subsets, isolated from either full thickness or endoscopical biopsies. (Figure 8) These results validated intestinal endoscopical biopsies for the use of flow cytometric analysis.

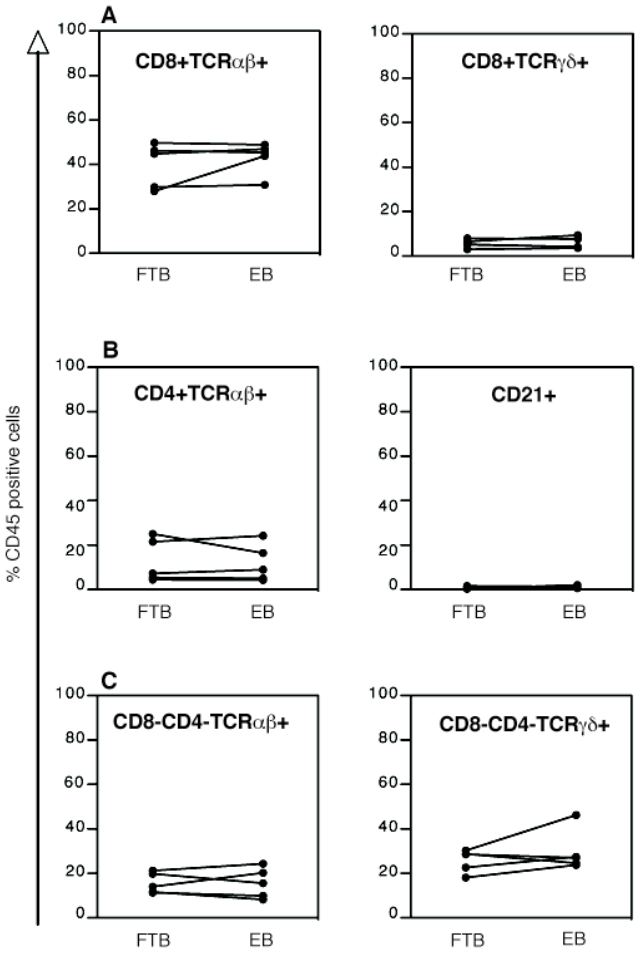

Figure 8: *IEL from full thickness biopsies (FTB) and endoscopical biopsies (EB) from 5 healthy adult Beagle dogs were isolated and analyzed for their cell surface marker*

expression by flow cytometry. ***A)*** *Analysis of $CD8^+$ $TCR\alpha\beta^+$ and $CD8^+$ $TCR\gamma\delta^+$ lymphocyte subsets.* ***B)*** *CD4 and CD21 usage in FTB and EB in the IEL compartment.* ***C)*** *Detection of the $CD8^-CD4^-TCR\alpha\beta^+$ and $CD8^-CD4^-TCR\gamma\delta^+$ lymphocyte subsets. Numbers were normalized to $CD45^+$ cells. No significant differences in the IEL subset composition between FTB and EB, isolated from the same dog could be found.*

No significant differences in the IEL T cell subsets between endoscopical biopsies of healthy control dogs (HC) and dogs with chronic enteropathies (CE)

Eleven dogs with chronic gastrointestinal signs were entered into a prospective clinical study. Infectious, endocrinologic or neoplastic diseases as a reason for gastrointestinal symptoms were excluded in all dogs. Animals were assessed clinically using the CIBDAI score by Jergens et al. and the CCECAI score by Allenspach et al. before and after therapy [78,79]. Dogs were separated into two groups according to their initial response to treatment with elimination diet. Dogs, which improved clinically after 10 days were called food-responsive dogs (FRD) (n=7). The dogs that did not respond to elimination diet were given oral prednisolone (2 mg/kg, once a day) for 10 days followed by a tapering dosage over 10 weeks. These dogs belonged to the IBD group (n=4).

During duodenoscopy, 5 endoscopical samples were taken from the proximal small intestine with a 2.8 mm serrated endoscopic biopsy forceps and were placed in ice-cold phosphate buffered saline (PBS). The biopsy specimens were isolated as described previously (Luckschander et al. unpublished data) and stained using species-specific antibodies (CD45, CD3, TCRαβ, TCRγδ, CD4, CD8α, CD8β). Staining of these cells for the common leukocyte marker CD45, TCRs αβ or γδ and the B cell marker CD21 revealed that >98% of the cells within the lymphocyte gate were $CD45^+$, the sum of the $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells was > 98% and <1 % of the cells were B cells. This shows that in the indicated gates mostly T cells can be found. No differences in the light scatter properties could be demonstrated between endoscopical biopsies from healthy dogs and endoscopical biopsies from dogs with CE. (Figure 9)

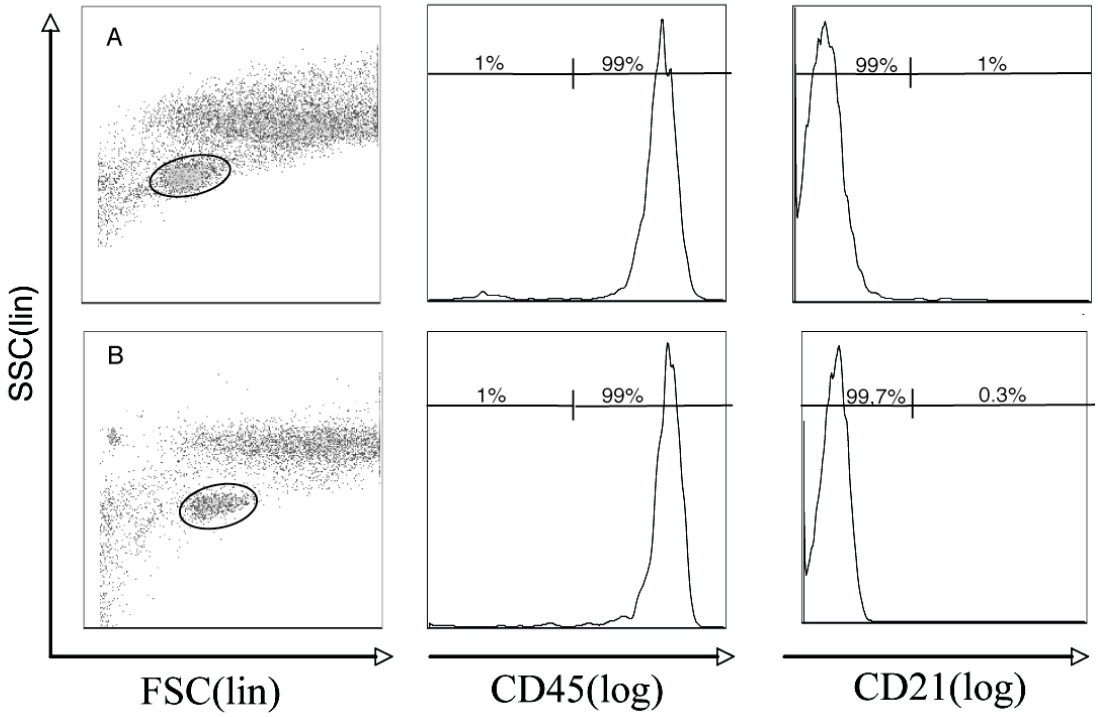

Figure 9: *IEL were isolated from small bowel endoscopical biopsies from HC dogs (A) and from dogs with CE (B) as previously described, stained with anti-canine CD45 and anti-canine CD21, followed by flow cytometrical analysis. The distinct forward/side scatter properties of IEL and their staining for CD45 and CD21 in a typical isolation are shown. In the indicated gate more than 98% of the cells were CD45$^+$, no B cells could be found.*

In the comparison of IEL subsets isolated from the HC and CE group, no significant differences could be found, although a significant decrease of $CD4^+TCR\alpha\beta^+$ T cells was obvious comparing the FRD with IBD group. Despite the overall improvement of the CE dogs after therapy (significant decrease of the CIBDAI and CCECAI), there were no significant differences in the IEL subsets before and after therapy.

IEL isolated from FTB or EB of the same healthy control dog did not show any significant differences with respect to the isolation and the T cell subsets. These results clearly show that it is possible to isolate IEL from canine intestinal endoscopical biopsies and further evaluate these cells with flow cytometrical analysis. In the comparison of EB from HC Beagle dogs and dogs suffering from CE, several difficulties were found. First of all, we had established a mono-breed IEL characterization standard in our previous work. The further comparison of the IEL population between healthy Beagle dogs and sick dogs of variant breeds contains the

risk of not having excluded interbreed variances, which could be falsely interpreted as a consequence of the underlying disease. Although it was possible to isolate IEL from endoscopical biopsies of sick dogs, no significant differences between the HC and the CE group with respect to the T cell subsets could be found. Although there was a significant decrease of the $CD4^+$ $TCR\alpha\beta^+$ IEL in the IBD group (compared to the FRD group), this finding is hard to interpret. The decrease of these cells seems to be not reliable as in general canine IBD is defined by a mural lympho-plasmacellular infiltration. Nevertheless, we should consider that the IBD group also shows a significant decrease in albumin ($p<0.05$). So this finding might reflect the architectural destruction of the epithelium in this severe form of canine IBD, which is also associated with a decreased absorption and an increased gastrointestinal loss of albumin. It was previously described that a low albumin in CE dogs at the beginning of therapy is a bad prognostic factor [79]. In our current study all the dogs with a significant decrease of $CD4^+$ $TCR\alpha\beta^+$ had a low albumin (<20g/L), the loss of this cell subset as a bad prognostic factor in canine CE might be worth further evaluation.

Flow cytometrical analysis of the intestinal lymphocyte populations as a diagnostic tool still seems to be an appealing goal. In this study, we could prove that the procedure of the lymphocyte isolation and the lymphocyte staining of endoscopical biopsies is feasible in healthy and sick dogs. Nevertheless, for the further evaluation of flow cytometry as a diagnostic or prognostic tool in canine IBD, the isolation of LPL should be included as these cells are mostly not affected by the loss of the architecture and therefore would probably better reflect the pathomechanisms during intestinal inflammatory diseases.

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6.3. Abbreviations

APC	antigen presenting cell
CTLA	cytotoxic lymphocyte associated antigen
CARD	caspase recruitment domains
CCECAI	canine chronic enteropathy clinical activity index
CE	chronic enteropathies
CIBDAI	canine IBD activity index
CPs	cryptopatches
DC	dendritic cell
EB	endoscopical biopsies
E-cadherin	epithelial calcium dependant adhesion molecules
EDTA	ethylenediaminetetraacetic acid
FAE	follicle-associated epithelium
FoxP3	forkhead box P3
FRD	food-responsive dogs
FSC	forward scatter
FTB	full thickness biopsies
GALT	gut-associated lymphoid tissue
HC	healthy control dogs
IBD	Inflammatory bowel disease
IEL	intraepithelial lymphocytes
IFN-γ	Interferon-gamma
IgA	immunoglobuline A
IL	Interleukin
ILFs	isolated lymphoid follicles
IMO	immune modulatory oligonucleotides
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
KGF	keratinocyte growth factor
LCMV	lymphocytic choriomenigitis virus
LPL	lamina propria lymphocytes
mRNA	messenger ribonucleic acid
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
NOD	nucleotide-binding oligomerization domains
PPs	Peyer`s Patches
RT-PCR	Real Time Polymerase Chain Reaction
Rag	Recombination activating gene
TCR	T-cell receptor
TNF-α	Tumor necrosis factor alpha
Tregs	regulatory T cells
TLRs	Toll-like receptors
UC	Ulcerative colitis
SSC	sideward scatter

Declaration of Originality

Last name, first name: Luckschander-Zeller Nicole

Matriculation number: 05-119-177

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

Bern, 02.04.2009……

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6.6. Curriculum vitae

NICOLE LUCKSCHANDER-ZELLER

ADRESS: Small Animal Clinic University of Bern
Länggasstrasse 124, 3012 Bern Switzerland
Tel: 0041 31 631 23 15
Fax: 0041 31 631 22 75
e-mail: Nicole.Luckschander@kkh.unibe.ch

DATE OF BITH: 18.05.1971

MARTIAL STAUS: married, 1 child (Conner, born 29.08.2007)

LANGUAGES: German, English, French (fluent spoken and written)

PROFESSIONAL EDUCATION:

Diplomate ECVIM-CA, 2006	European College of Veterinary Internal Medicine Companion Animals
Diplomate ACVIM Baltimore, 2005	American College of Veterinary Internal Medicine
Post Graduate Degree Doctor of Veterinary Medicine Vienna, 1998	University of Veterinary Medicine Vienna, Austria
Veterinary Degree Vienna, 1996	University of Veterinary Medicine Vienna, Austria

PROFESSIONAL EMPLOYMENT

PhD student, clinical internist 2005 - current	Small Animal Hospital Faculty of Veterinary Medicine University of Bern, Switzerland
Residency Small Animal Internal Medicine 2001-2004	Small Animal Hospital Faculty of Veterinary Medicine University of Bern, Switzerland
Faculty Veterinarian 1999 - 2001	Hospital for Small Animals University of Veterinary Medicine Vienna, Austria
Staff Veterinarian 1996 - 1999	Hospital for Small Animals University of Veterinary Medicine Vienna,Austria

SABBATICAL / ADVANCED STUDIES

Resident Small Animal Internal Medicine	Hospital for Small Animals

01/02-07/02	Louisiana State University Baton Rouge, USA
Small Animal Internal Medicine 01/01– 03/01 and 03/99-04/99	Foster Hospital for Small Animals Tufts University North Grafton, MA 01536

PRIZES & AWARDS

Graduate school for cellular and biomedical sciences financial support at the ECVIM-CA meeting 2008

Second prize of the ECVIM-CA research communication
Correlation between serologic markers of diseases (pANCA) and response to treatment in dogs with IBD
Luckschander N, Allenspach K, Doherr M, Groene A, Seibold F, Gaschen F
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ORIGINAL SCIENTIFIC ARTICLES IN PEER-REVIEWED JOURNALS

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