Butyrophilin and Butyrophilin-like genes and their role in epithelial cell-intraepithelial T lymphocyte cross-talk

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Cover illustration: Gut with magnifying glass showing the intestinal epithelium, where the interaction between the intraepithelial T lymphocytes and the neighboring epithelial cells is poorly defined, by Cristina Lebrero Fernández.
iEC: intestinal epithelial cell; IEL: intraepithelial lymphocyte.

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To my family

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ABSTRACT

More than 50% of our immune system is located in the gut. The intestinal epithelium, which forms an interface between the organism and the environment, harbors intraepithelial lymphocytes (IELs) that comprise a mixture of conventional αβ T cells and unconventional αβ- and γδ T cells. IELs play important roles in regulation of gut epithelial integrity and in recognition of stressed and infected epithelial cells, and thus, are critical effector components of mucosal immunity. However, the understanding of the IEL function and their interaction with the neighboring epithelial cells is still limited. The aim of this thesis was to investigate how the Butyrophilin (Btn) and Butyrophilin-like (Btnl) molecules are involved in the epithelial cell – IEL cross-talk and hence, to characterize their role in regulating local T cell mediated immune responses in the intestinal mucosa.

Btn and Btnl proteins have over the past decade emerged as novel regulators of T cell functions both in periphery and locally in the tissue, and have been shown to be genetically associated with various inflammatory and proliferative disorders. We have reported the ability of intestinal epithelial cell (iEC)-specific Btnl proteins to induce IEL activation and proliferation in conditions without exogenous stimulation, which may contribute to the upkeep of the intestinal IEL pool. We have furthermore identified novel intestinal epithelial cell expressed Btnl- heteromeric protein complexes, and demonstrated that one of them, the Btnl1-Btnl6 heteromeric complex, specifically enhances the expansion of intestinal IELs bearing the Vγ7Vδ4 receptor *in vitro*. We have additionally explored how iEC-specific Btnl proteins are regulated in the neonatal murine small intestine and found that Btnl- protein expression is delayed in the ontogeny and that the expression of the *Btnl* genes is regulated on post-transcriptional level. Our data demonstrate that the proteins are not detectable in the small intestinal epithelium of mice before 3 weeks of age, and that the appearance of Btnl1 and Btnl6 proteins correlates with the expansion of intestinal Vγ7Vδ4 IELs, further adding strength to our *in vitro* results. Since γδ IELs are essential for

the maintenance of the homeostasis in the gut, our findings suggest that Btnl proteins have implications in the intestinal immune response. To increase the understanding of the Btn and Btnl molecules' role in intestinal disorders, we have characterized the expression of human and mouse Btn and Btnl genes in colonic inflammation and intestinal tumors. Our results show an altered expression of the *BTN* and *BTNL* genes in these diseases and indicate an association between Btn and Btnl genes and ulcerative colitis and colon cancer.

In summary, this thesis work has demonstrated that iEC-specific Btnl proteins can regulate the function of intestinal intraepithelial lymphocytes in the gut, and that Btn and Btnl genes are associated with bowel pathology. Nonetheless, further studies are necessary to identify the complete immunomodulatory implication of the Btn and Btnl family members in healthy and inflamed/infected gut mucosa.

Keywords: butyrophilin-like, butyrophilin, intraepithelial lymphocytes, mucosal immunity, intestinal epithelial cells, γδ T cells, intestinal inflammation, colon cancer, ulcerative colitis.

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SAMMANFATTNING PÅ SVENSKA

Mer än 50% av våra immunceller finns i tarmen. Tarmslemhinnan, som utgör en viktig barriär mot vår omgivning och som skyddar kroppen från skadliga organismer, innehåller s.k. intraepiteliala T lymfocyter (IELs). Dessa lokala immunceller har en unik sammansättning av αβ TCR och γδ TCR T celler som skiljer sig från kroppens övriga T lymfocyter och är viktiga för igenkänning av stressade och infekterade celler samt för reglering av inflammation i tarmen. Förståelsen kring funktionen av IELs samt konsekvensen av deras interaktion med andra celler i tarmslemhinnan, framför allt epitelceller som de är i direkt kontakt med, är dock fortfarande begränsad. Syftet med projektet var att kartlägga hur Butyrophilin (Btn) och Btn-like (Btnl) molekyler medverkar vid kommunikationen mellan epitelceller och intestinala IELs och därmed karaktärisera deras roll vid regleringen av tarmens immunsvar. Btn och Btnl proteiner har de senaste åren uppmärksammats som nya regulatorer av immunförsvaret och har visat sig kunna reglera T lymfocyter och även vara associerade med inflammatoriska sjukdomar och cancer. Vi har visat att funktionen av intestinala IELs, och därmed också immunresponsen i tarmen, kan moduleras av Btnl proteiner som uttrycks av tarmens epitelceller, och att interaktionen mellan Btnl och IEL i tarmslemhinnan leder till proliferation och aktivering av IELs. Detta peka på att Btnl proteinerna är inblandade i upprätthållande av IEL poolen i tarmen. Vi har vidare identifierat nya biologiska proteinkomplex i tarmepitelet som är uppbyggda av olika Btnl proteiner, och visat att dessa komplex ökar proliferationen av en specifik IEL population som uttrycker Vγ7Vδ4 TCR. Eftersom γδ T celler är viktiga för upprätthållande av homeostasen i tarmen, tex genom att medverka vid nygenerering av epitelceller, samt kan känna igen och eliminera stressade epitelceller, är det viktigt att veta hur dessa T celler regleras. Vi har även tittat på hur de lokala Btnl proteinerna regleras i den neonatala tarmen. Våra data demonstrerar att uttrycket av de vävnadsspecifika Btnl proteinerna är fördröjt i tarmen de första veckorna efter födseln och att proteinerna inte är detekterbara i tarmslemhinnan före 3 veckors ålder i mus. Detta kan ha konsekvenser för expansionen av IEL populationen i den neonatala tarmen och därmed också för tarmens immunrespons tidigt i livet. För att öka förståelsen för Btn och Btnl genernas funktion vid tarmrelaterade sjukdomar har vi karaktäriserat uttryck av humana och mus Btn och Btnl gener vid inflammation och cancer i tarmen. Våra resultat demonstrerar att uttrycket av flera av dessa gener är förändrat vid dessa sjukdomar och visar på en

association mellan Btn och Btnl gener och ulcerös kolit och colon cancer. Sammanfattningsvis har vår forskning visat att Btn och Btnl proteiner kan reglera funktionen av intestinala T lymfocyter och därmed immunresponsen i tarmslemhinnan, samt att Btn och Btnl gener är associerade med tarmrelaterade sjukdomar. En fortsatt kartläggning av Btnl proteinernas betydelse för immunregleringen i tarmslemhinnan är viktig för att ytterligare öka förståelsen för hur immunsystemet regleras i frisk och inflammerad/infekterad tarm.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Cristina Lebrero-Fernández, Joakim H. Bergström*, Thaher Pelaseyed* and Anna Bas-Forsberg.
Murine Butyrophilin-like 1 and Btnl6 form heteromeric complexes in small intestinal epithelial cells and promote proliferation of local T lymphocytes.
Front Immunol. 2016 Jan 19; 7: 1. doi: 10.3389/fimmu.2016.00001

II. Cristina Lebrero-Fernández and Anna Bas-Forsberg.
The ontogeny of Butyrophilin-like (Btnl) 1 and Btnl6 in murine small intestine.
Submitted for publication

III. Cristina Lebrero-Fernández, Thaher Pelaseyed and Anna Bas-Forsberg.
Butyrophilin-like (Btnl) 4 forms heteromeric intra-family complexes and its expression is delayed in the intestine during ontogeny.
Manuscript

IV. Cristina Lebrero-Fernández, Ulf Alexander Wenzel, Paulina Akeus, Ying Wang, Hans Strid, Magnus Simrén, Bengt Gustavsson, Lars G. Börjesson, Susanna L. Cardell, Lena Öhman*, Marianne Quiding-Järbrink* and Anna Bas-Forsberg.
Altered expression of Butyrophilin (*BTN*) and BTN-like (*BTNL*) genes in intestinal inflammation and colon cancer.
Immun Inflamm Dis. 2016 April 1. doi: 10.1002/iid3.105

* The authors contributed equally to this study

Reprints were made according to the journal requirements

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ABBREVIATIONS

7AAD	7-aminoactinomycin D
Apc	Adenomatous polyposis coli
APC	Antigen presenting cell
B7-H3	B7 homologue 3
BSA	Bovine serum albumin
BTN	Butyrophilin
BTNL	Butyrophilin-like
CAR	Coxsackie and adenovirus receptor
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
cDNA	Complementary DNA
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTLA	Cytotoxic T lymphocyte antigen
CV	Conventional
CXCL	CXC-chemokine ligand
DAPI	4',6'-diamidino-2-phenylindole
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DMEM	Dulbecco's modified essential medium
DN	Double negative
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ERMAP	Erythroblast membrane associated protein
FasL	Fas ligand
FCS	Fetal calf serum
GF	Germ-free
HEK	Human embryonic kidney
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICOS	Inducible co-stimulator
iEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IF	Immunofluorescence
IFN-γ	Interferon-γ
Ig	Immunoglobulin

IGF-1	Insulin-like growth factor-1
IL	Interleukin
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T
IP	Immunoprecipitation
JAML	Junctional adhesion molecule-like
KGF	Keratinocyte growth factor
KLH	Keyhole limpet hemocyanin
LN	Lymph node
LPL	Lamina propria lymphocyte
MAIT	Mucosal-associated invariant T
MHC	Major histocompatibility complex
Min	Multiple intestinal neoplasia
MIP	Macrophage inflammatory protein
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MS	Mass spectrometry
Muc	Mucin
MZB	Marginal zone B
NHS	Normal horse serum
NK	Natural killer
pAg	Phosphoantigen
PD-L	Programmed death-ligand
PEI	Polyethylenimine
PI3K	Phosphatidylinosytol 3-kinase
qPCR	Quantitative polymerase chain reaction
RAG	Recombination-activating gene
RNA	Ribonucleic acid
SIGN	Specific intercellular adhesion molecule-3-grabbing non-integrin
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell
TRIM	Tripartite motif
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
WB	Western blotting
WT	Wild-type

1. INTRODUCTION

Our body is protected from pathogens and other harmful substances, and the damage they cause, by a variety of effector cells, tissues and molecules that together make up the immune system.

The immune system is traditionally classified in two types: innate and adaptive. The innate immune system is the early line of host defense, and consists of biochemical and cellular mechanisms that are in place even before infection and that provide a rapid non-specific response to invading pathogens. The main components of the innate immune system are physical and chemical barriers, phagocytic cells, dendritic cells (DCs), circulating plasma proteins and innate lymphoid cells (ILCs) like natural killer (NK) cells. In contrast, the response of the adaptive immune system is antigen-specific, being effective only after undergoing clonal expansion and differentiation, which takes several days, and includes memory that makes future responses against a specific antigen more efficient. There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes.

Recently, several studies have described the existence of cell populations that possess features of both innate and adaptive immunity, suggesting a concept of a continuum of the immune response. These populations have a restricted repertoire of antigen receptors, they are primarily located in mucosal tissues (particularly near epithelia), and they can be functionally grouped by their capacity to respond to infection during the period between activation of the phagocytic cells of the innate immunity and the T and B cells of the adaptive immunity. These bridge populations are known as innate-like cells and include: gamma delta (γδ) T cells, intraepithelial lymphocytes (IELs), invariant natural killer T (iNKT) cells and mucosal-associated invariant T (MAIT) cells, which express T cell receptors for antigen; and B1-B cells and splenic marginal zone B (MZB) cells, which express B cell receptors for antigen [1, 2].

Although most studies on the biology of T cells focus on systemic T lymphocytes, the recently developed interest for how epithelia, being the primary targets of infection and other forms of damage, may influence lymphocyte activation, regulation and function, has drawn attention to local intraepithelial lymphocytes and their interaction and communication with epithelial cells.

1.1 Intraepithelial lymphocytes

IELs are T lymphocytes that reside in the skin and in the mucosal epithelia of the intestine, the biliary tract, the oral cavity, the upper respiratory tract and lungs, and the reproductive tract. IELs represent a significant fraction of the epithelium, with an average of about one IEL per 5-10 epithelial cells in the murine or human small bowel [3]. Intestinal IELs are located intercalated between the epithelial cells (**Figure 1**), and constitute the largest lymphocyte population in the whole body due to the expanded surface of the small intestine epithelium formed by multiple villi and microvilli. The murine skin also harbors an extensive network of IELs, known as dendritic epidermal T cells (DETCs) for their unique dendritic morphology, which do not seem to have exact human counterparts [4, 5].

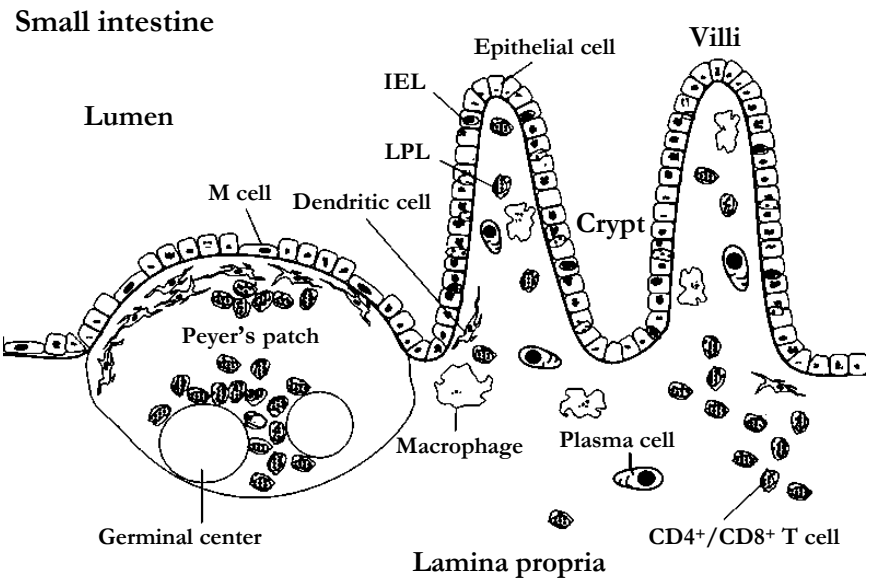

Figure 1. Small intestinal epithelium (modified from reference [6]).
IEL: intraepithelial lymphocyte; LPL: lamina propria lymphocyte.

IELs heterogeneity and phenotype

Murine and human IELs differ from the systemic T cells in their subset composition. Unlike the spleen, peripheral blood and lymph node T cells that can be subdivided into major histocompatibility complex (MHC) class

II-restricted $CD4^+$ $TCR\alpha\beta^+$ T cells and MHC class I-restricted $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ T cells, the IEL population is more heterogeneous and can be classified into two major subpopulations based on the expression of the T cell receptors (TCRs) and co-receptors: the first group, called conventional or type a IELs and more recently named as induced IELs, consists of conventional $CD4^+$ and $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ T cells, while the second subset, referred to as unconventional, type b or natural IELs, is made up of $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$, $CD4^-$ and $CD8^-$ (double negative, DN) $TCR\gamma\delta^+$, and $CD8\alpha\alpha^+$ $TCR\gamma\delta^+$ IELs [7, 8]. These IEL types are found in both humans and mice, however, $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs are present in human fetal intestine but have not been formally identified in adults [9, 10]. In contrast to the small bowel, the murine and human large intestine, which harbors the greatest microbial antigen load, is mainly composed by conventional IELs [7], and the murine skin DETCs are DN $TCR\gamma\delta^+$, which belong to the unconventional IEL group [11, 12].

Unconventional IELs typically express a CD3 complex composed of CD3ζ-FcεRIγ heterodimers or FcεRIγ-FcεRIγ homodimers instead of CD3ζ- CD3ζ homodimers, express by conventional IELs [13].

Furthermore, intestinal IELs are $CD69^+$ and $CD44^+$, but they do not show markers of recently activated cells such as CD25. They are heterogeneous in terms of the expression of conventional T cell markers such as CD2, CD5 and CD28. In contrast to the unconventional IELs, conventional IELs express a typical phenotype of memory T cells namely $CD2^+CD5^+CD28^+$cytotoxic T lymphocyte antigen (CTLA)-$4^+Thy1^+LyC6^+$ [14, 15].

Thus, IELs appear as activated effector cells but require additional activation to manifest a full functional potential, which suggests that the IEL compartment has an "activate yet resting" constitutive state [14, 16].

IELs development and ontogeny

IEL subsets are progeny of precursor cells located in the bone marrow or in the fetal liver, however, their development and maturation is not uniform. Conventional IELs are restricted to MHC and are thymus derived as they are absent in athymic mice [17, 18]. They acquire an activated phenotype in response to antigens in peripheral lymphoid tissue and migrate into mucosal epithelia, presumably awaiting a second exposure to antigen, akin to memory effector cells in other tissues. Unconventional IELs can be either thymus-independent – they are detected in athymic mice [17-22] and they are

believed to develop in gut-associated cryptopatches located in crypt lamina propria [23], or thymus-dependent like murine DETCs and genital tract TCRγδ$^+$ IELs, which are primarily generated in the fetal thymus [24]. Unconventional IELs are not dependent on classical MHC molecules.

Intestinal IELs constitutively express CD103 (also known as αEβ7 integrin) and CC-chemokine receptor 9 (CCR9), which interact with E-cadherin and CC-chemokine ligand 25 (CCL25), respectively, on intestinal epithelial cells, resulting in gut-homing [25].

Ontogeny studies of intestinal IELs demonstrate that newborn rodents have resident IELs in the small bowel, being mainly TCRγδ$^+$ IELs, and that the population increases until weaning age. By contrast, TCRαβ$^+$ IELs are infrequent early in life, but expand with age in response to external antigens [26-28]. Moreover, in the absence of microbiota (germ-free mice) and dietary proteins (antigen-free mice), the IEL subsets are notably reduced with the exception of TCRγδ$^+$ IELs [29, 30].

IELs function

IELs are situated within the epithelium, which not only offers obvious opportunities for direct epithelial cell - T cell interaction, but also an immediate response against pathogenic infection, cell transformation and uncontrolled infiltration by systemic cells in order to preserve the epithelial integrity [7, 8, 12].

IELs exhibit potent cytotoxic capacities mediated by the release of granzyme and perforin located in cytoplasmic granules [31], and by activation-induced expression of Fas ligand (FasL), which induces apoptotic cell death upon ligation with Fas on target cells [16]. IELs additionally produce a variety of chemokines, e.g. macrophage inflammatory protein 1α (MIP-1α) and MIP-1β; cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-13 or IL-17 [14, 32]; and growth factors [33, 34]. Furthermore, IELs express NK cell receptors and can lyse target cells in a TCR-independent manner [35]. This artillery of effector molecules is essential for the maintenance of the epithelial integrity and the protection from severe infection and inflammation. Indeed, IELs have shown to display protective functions against enteric viral, bacterial or parasitic infection, where they secrete perforins, granzymes, IFN-γ, TGF-β and TNF-α, which are essential for host immunity to the pathogens [36-40]. IELs can also prevent or reduce the severity of some inflammatory diseases like colitis or celiac disease, by

expressing anti-inflammatory cytokines such as TGF-β and IL-10, and keratinocyte growth factors (KGFs), which allow to preserve the epithelium and restore tissue integrity after injury [33, 41, 42]. Moreover, DETCs can express high levels of IFN-γ and granzymes, but they also express IL-13, which can regulate B cells [43].

Although IELs show beneficial roles, they can exert uncontrolled cytotoxicity and enhance immune responses, which may initiate or exacerbate inflammatory diseases. Thus, several reports demonstrate a direct correlation between the number of IELs in the intestinal mucosa and disease severity in patients with intestinal inflammatory bowel disease (IBD) [44-46], where these IELs can be responsible for the colitis induction through the secretion of IL-17 [47]. Furthermore, IL-15, which is over-expressed by intestinal epithelial cells from individuals with celiac disease, is known to trigger potent cytotoxic responses by IELs [48].

1.2 Intraepithelial γδ T cells

γδ T cells, together with αβ T cells and B cells, represent the three lymphocyte lineages found in all vertebrates [49]. Similar to B cell receptors and αβ T cell receptors, the variable region of the γδ TCRs is generated through somatic rearrangement of V (variable), D (diversity) and J (joining) segments, through the activity of the recombination-activating genes encode enzymes, RAG-1 and RAG-2. Structural diversity of γδ TCRs depends on combination of different sets of V, D and J segments, and addition or loss of nucleotides in joining sites [50-52]. Some γδ T cell subsets in particular tissue locations, such as the skin and the uterine epithelia, have no junctional diversity and thus, they express invariant TCRs with canonical junctional sequences [53]. These invariant γδ T cell subsets are derived from fetal γδ T cells, unlike peripheral subsets that express more diverse γδ TCRs [54].

The antigen-receptor repertoire of human and mouse γδ T cells is encoded by Vγ and Vδ genes. The most common Vγ chains in mice are: Vγ1, Vγ2, Vγ4, Vγ5, Vγ6 and Vγ7. Invariant Vγ5Vδ1 DETCs and Vγ6Vδ1 T cells are located in the epidermis and in the uterovaginal epithelia, respectively [55]. γδ T cells predominantly use: Vγ1 or Vγ7 chains combined with various TCRδ chains in the gut [56], Vγ1 or Vγ4 chains in the spleen, Vγ1, Vγ4 or Vγ6 chains in the liver, and Vγ4 or Vγ6 chains in the lung epithelia [52, 57]. In humans, the most prevalent Vδ chains are: Vδ1, Vδ2 and Vδ3. The best defined are the Vγ9Vδ2 T cells, which reside in the peripheral blood [58] (the

TCR nomenclature is according to Heilig and Tonegawa for mouse γδ T cells [59] and Lefranc and Rabbitts for human γδ T cells [60]).

In mouse, γδ T cells are exported from the fetal thymus to epithelia-rich tissues in programmed waves (**Figure 2**). The first wave of γδ T cells populates the epidermis, the second wave homes the epithelia of the reproductive tract and lung, and the third wave colonizes the gut, the spleen and the lymph nodes. After these initial waves, αβ T cells predominate, making up more than 95% of the T cells [51, 52, 61].

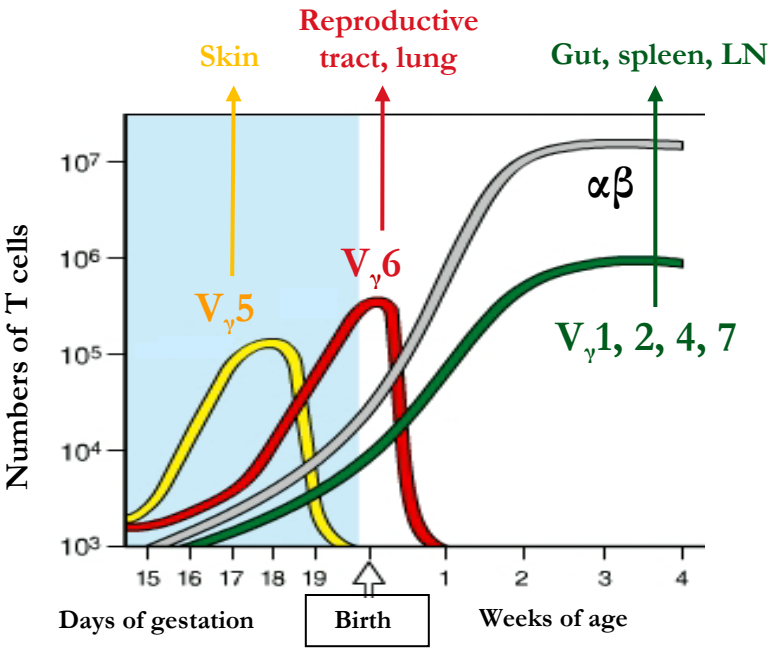

Figure 2. T cell development occurs in waves.
Modified from Janeway et al. Immunobiology. NY: Garland Science; 2001.
LN: lymph node.

In the periphery, γδ T cells play an important role in the immunity of a broad range of infectious stresses [62] and in tumor immune surveillance [63]. γδ T cells directly lyse and eliminate infected or stressed cells through the production of granzymes, and produce a vast variety of cytokines and chemokines to regulate other immune or non-immune cells [64, 65]. Moreover, they also trigger DC maturation [66-68], provide help for B cells and promote the production of immunoglobulin E (IgE) [69, 70], and

present antigens for αβ T cell priming [71-73]. Furthermore, γδ T cells are implicated in protection against cancer, killing tumor cells and secreting potent anti-tumor cytokines such as IFN-γ [74-78]. However, they are also involved in tumor promotion. Thus, γδ T cells producing IL-17 [79, 80], IL-4 [81], IL-10 and TGF-β suppress anti-tumor immune responses by inhibiting effector functions of γδ T cells and $CD4^+$ and $CD8^+$ αβ T cells [63, 82, 83], and by recruiting immunosuppressive myeloid cells that promote angiogenesis, tumor cell growth and regulatory T cells (Treg) differentiation [84, 85].

Although γδ T cells can be found in the periphery, they mainly reside in epithelial tissues being part of the IEL compartment. Intraepithelial γδ T cells, also known as γδ IELs, play unique roles in homeostasis and disease. They participate in tissue repair regulating epithelial cell turnover and differentiation, and producing epithelial growth factors, cytokines and chemokines [33, 34, 86]. At the same time, they are involved in protection from malignancy, recruiting inflammatory cells to the site of damage and killing diseased epithelial cells through their high cytolytic potential [87-89]. Several studies have reported deficiency in wound healing [55, 90], tumor rejection [89], recovery from colitis [33], lung injury [91] and homeostatic regulation of the epithelia [92] in the absence of γδ IELs (γδ $TCR^{-/-}$ mice).

Intraepithelial γδ T cells during homeostasis

Intestinal and skin intraepithelial γδ T cells have been shown to be essential in tissue homeostasis and repair. In the gut, intraepithelial γδ T cells regulate the regeneration and differentiation of intestinal epithelial cells (iECs), controlling the epithelial cell growth and differentiation [86, 93]. In the skin, DETCs contribute to wound healing through secretion of distinct growth factors including KGFs and insulin-like growth factor-1 (IGF-1) [34, 92, 94, 95]. IGF-1 is involved in reverse the epidermal apoptosis and it is constitutively expressed by DETCs [92]. Furthermore, DETCs express chemokines such as MIP-1α, MIP-1β, RANTES and lymphotactin, to recruit specialized inflammatory cells, and cytokines including IL-2, IFN-γ, TNF-α and IL-17 upon activation [96, 97]. This suggests that DETCs not only regulate epidermal homeostasis, but also immune responses during stress or damage.

The cytokines IL-7 and IL-15 are essential for development, localization and survival of γδ T cells, as well as for their homeostasis [98-101]. The development and survival of the epidermal γδ IELs (DETCs) is dependent

on IL-7, but not IL-15 [102], whereas the generation and maintenance of intestinal intraepithelial γδ T cells relies on the presence of both IL-7 and IL-15 [103]. Furthermore, normal γδ IEL development is also dependent on the development of conventional αβ IELs [104].

Cross-talk between intraepithelial γδ T cells and epithelial cells

In some epithelial tissues, like the murine skin, intraepithelial γδ T cells comprise the main T cell population, whereas in other epithelial sites, such as the intestinal epithelium, they coexist with αβ T cells [24]. Intraepithelial γδ T cells are in close contact with the neighboring epithelial cells, and although the communication between them is considered as essential, few molecular inter-cell interactions have been identified [105, 106]. The best characterized examples of epithelial cell - IEL interaction are:

- **NKG2D**

The activating receptor, NKG2D, is a transmembrane protein that belongs to the family of the C-type lectin-like receptors, which is expressed as a homodimer on NK, γδ and $CD8^+$ T cells [107, 108]. In humans, NKG2D is engaged by MICA and MICB, as well as by members of the ULBP family [107, 109], and in mice, by Rae-1, H60 and Mult1 [107, 109]. All NKG2D ligands are homologous to MHC molecules, and they are absent or present at low levels under homeostatic conditions, but are up-regulated by infected, transformed and stressed epithelial cells [108] .

NKG2D has been shown to provide important co-stimulatory signals for intraepithelial γδ T cell activation and function in damaged intestinal and skin tissues: in humans, intestinal intraepithelial γδ T cells expressing the Vδ1 γδ TCR can recognize the NKG2D ligands MICA and MICB, and may serve as an immune surveillance mechanism or may be involved in the maintenance of epithelial homeostasis [87, 88, 110], and in mice, the engagement of NKG2D with its ligands activates DETCs [89, 111-113].

- **JAML**

JAML, or Junctional Adhesion Molecule-Like, is a transmembrane glycoprotein expressed on neutrophils, monocytes and memory T cells [114]. Low levels of JAML were also found on mouse epidermal γδ IELs under steady-state conditions and up-regulated upon activation [115]. JAML is

engaged by the Coxsackie and Adenovirus receptor (CAR), expressed on keratinocytes (epidermal epithelial cells) and intestinal epithelial cells [116].

Binding of JAML to its ligand provides co-stimulation that results in proliferation, through the recruitment of phosphatidylinosytol 3-kinase (PI3K), and activation of DETCs, as well as in production of cytokines such as IL-2, IFN-γ and TNF-α, and expression of KGF-1 by DETCs. Thus, the cross-talk between JAML and CAR is a crucial component in epidermal wound repair [115, 117].

- **CD100**

CD100, also known as Semaphorin 4D, is a member of the semaphorin family, which is expressed on B and T cells, including intraepithelial γδ T cells [118-120]. Engagement between CD100 and one of its ligands, plexin B2, is critical for activation of intraepithelial γδ T cells [120]. Interaction between CD100 on DETCs and plexin B2 on keratinocytes plays an important role in response to keratinocyte damage in the epidermis [120]. In colon, interaction between CD100 on intestinal γδ IELs and plexin B2 on epithelial cells is vital for mediating healing of the colon epithelium during colitis [121]. Thus, the cross-talk between CD100 and plexin B2 is a key component in the regulation of wound healing and inflammation.

- **Skint-1**

Skint-1 is a transmembrane protein that belongs to the Skint Ig superfamily, which is expressed by epithelial cells in the thymus and the skin [122]. Skint-1 determines the repertoire of the epidermal IEL, being essential for the selection of the murine Vγ5Vδ1 intra-epidermal T cell compartment [122-124]. Furthermore, it has been described that only upon engagement by Skint1, Vγ5Vδ1 DETCs are able to express IFN-γ, suggesting that this interaction is vital for the maturation of DETCs [125].

Butyrophilin (Btn) and Butyrophilin-like (Btnl) gene family members have structural relatedness to Skint-1 and are one of the closest relatives outside the Skint family [122, 126]. Btn and Btnl proteins have over the past decade emerged as essential regulators of T cell functions, both in periphery and locally in the tissue [126-130].

1.3 Butyrophilin and Butyrophilin-like molecules

Btn and Btnl genes belong to the family of co-stimulatory molecules

T cell activation requires two signals. The first signal is the interaction between the peptide-antigen-MHC present on the antigen presenting cell (APC) and the T cell receptor. A second signal, known as co-stimulation, which is crucial to achieve full T cell activation or tolerance, is provided by the interaction between co-stimulatory or co-inhibitory molecules, expressed on APC, and the T cell [131].

One of the best characterized families of co-stimulatory molecules is the B7 superfamily, which has a pivotal role in the regulation of T cell responses. This family includes positive co-stimulatory molecules such as B7-1 (CD80 in humans), B7-2 (CD86 in humans) and inducible co-stimulator ligand (ICOS-L), and negative co-stimulatory molecules such as programmed death-ligand 1 (PD-L1), PD-L2, B7 homologue 3 (B7-H3) and B7-H4, expressed by APCs [132, 133].

B7-1 and B7-2 can bind to CD28 or CTLA-4, which are expressed on the T cell surface, delivering activatory or inhibitory signals to T cells, respectively. In addition, ICOS-L interacts with ICOS providing activatory signals, and PD-L1/PD-L2 and B7-H3/B7-H4 bind to PD-1 and B7-H3/B7-H4 T cell expressed-receptors, respectively, inducing inhibitory responses that are crucial for immune tolerance [132, 133].

Btn and Btnl molecules share strong homologies with the B7 family and independent studies over the past 10 years have demonstrated immunological functions for several of the Btn and Btnl family members [126-130, 133, 134].

Butyrophilin and Butyrophilin-like family

The *BTN* and *BTNL* genes are clustered on human chromosomes 5 and 6, and on mouse chromosomes 11, 13 and 17. Several are located within the MHC-locus and are conserved in mice and humans [135, 136].

To date, 11 members (7 BTN and 4 BTNL) have been identified in humans. The BTN molecules are divided in three phylogenetic associated subfamilies: BTN1, BTN2 and BTN3. The BTN1 group is composed only of BTN1A1, while the BTN2 and BTN3 subfamilies contain three molecules BTN2A1,

BTN2A2 and BTN2A3 (pseudogene), and BTN3A1, BTN3A2 and BTN3A3, respectively. The 4 BTNL molecules found in humans are BTNL2, BTNL3, BTNL8 and BTNL9. In mouse, 9 members (2 Btn and 7 Btnl) have been described. The Btn molecules include Btn1a1 and Btn2a2, whereas the Btnl molecules include Btnl1, Btnl2, Btnl4, Btnl5 (pseudogene), Btnl6, Btnl7 (pseudogene) and Btnl9. Among all these members, only BTN1A1, BTN2A2, BTNL2 and BTNL9 are clear orthologues between human and mouse [126, 127, 130].

In addition to the BTNL molecules mentioned above, there are other Butyrophilin-like molecules described. One of them is BTNL10 (BTN4), however, it appears unclear if it produces a full-length transcript [129]. Others have received non-BTNL names: erythroblast membrane associated protein (ERMAP or BTN5) and myelin oligodendrocyte glycoprotein (MOG or BTNL11), both found in human and mouse [129]. While ERMAP is involved in the development of erythroid cells [137, 138], MOG is a glycoprotein involved in the myelination of nerves in the central nervous system and has been linked to immune-related functions [139, 140].

Like the B7 family, the structure of the Btn and Btnl family members consists of two extracellular Ig-like domains (IgV and IgC), a transmembrane domain and a cytoplasmic domain. Additionally, most of the family members, except for Btnl2, BTN3A2 and MOG, contain a B30.2 intracellular domain (**Figure 3**) [126-130, 136].

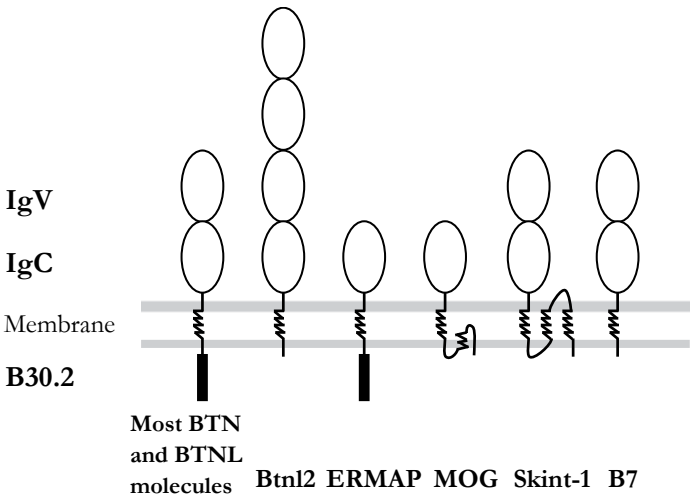

Figure 3. Structural organization of the Btn and Btnl family members, which are structurally related to the Skint and B7 families.

The B30.2/SPRY domain, present in several protein families, covers a wide range of functions. Proteins with B30.2/SPRY domain are involved in RNA metabolism (DDX1, hnRNPs) [141], intracellular calcium release (RyR receptors) [142], regulatory and developmental processes (HERC1, Ash2) [143, 144], and regulation of cytokine signaling (SOCS) [145]. A recent evolutionary adaptation, comprising the combination of SPRY and PRY to produce B30.2 domain, is found in Btn/Btnl and tripartite motif (TRIM) molecules [146, 147]. The members of the TRIM family have a variety of functions, such as viral restriction factors (TRIM5α) and immune signaling (TRIM21), in which the B30.2 domain appears to be involved in multimerization and binding to ligands [148-150].

Btn and Btnl molecules are expressed at the RNA level in a broad spectrum across human and mouse tissues [126, 128]. Whereas some members are highly restricted to a specific tissue, such as murine *Btnl4* and *Btnl6*, which are limited to intestinal epithelial cells [151], others are widely expressed in lymphoid and non-lymphoid tissues, e.g. *BTN2A1* [152] and *Btn2a2* [153]. Moreover, the transcripts' expression is not always reflected at the protein level, for example, while *Btn1a1* transcripts are broadly detected, Btn1a1 protein is only found in lactating mammary tissue and in thymic stroma [153].

Immunological functions of human BTN and BTNL molecules

Over the recent years, several human BTN and BTNL members have been genetically associated with various immunological diseases. Thus, polymorphisms in the human ***BTNL2*** have been linked to a growing number of inflammatory disorders, all of which are characterized by inappropriate T cell activation. Thus, single nucleotide polymorphisms (SNPs) in *BTNL2* have been reported to be associated with the following diseases: sarcoidosis [154-163], ulcerative colitis (UC) [164-166], rheumatoid arthritis [167, 168], spontaneous inclusion body myositis [169], systemic lupus erythematosus [167], type I diabetes [167], tuberculosis [166, 170, 171], leprosy [166] and antigen-specific IgE responsiveness [172]. As these diseases are defined by improper T cell activation, the genetic linkage between Btn and Btnl genes and the inflammatory disorders suggests the family's implication in T cell regulation.

Several studies have additionally identified an association between BTN and BTNL members with cancer. Thus, ***BTNL2*** has been associated with

increased susceptibility to prostate cancer [173] and **BTN3** with ovarian cancer [174-176]. Furthermore, ***BTN3A2*** has been linked to type I diabetes [177], and ***BTN2A1*** to metabolic syndrome [178], myocardial infarction through an effect of dyslipidemia [178-182] and hypertension [183].

In addition, several human BTN and BTNL members have been reported to possess immunomodulatory potential by controlling the biological activity of immune cells, mainly peripheral T cells.

Studies on human **MOG (BTNL11)** have shown that the interaction of MOG with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), expressed on brain microglia and dendritic cells, is involved in the control of immune homeostasis in the healthy human brain [184]. Moreover, binding of **BTN2A1** to the lectin DC-SIGN was reported to modulate immature monocyte-derived dendritic cells. However, this binding required high mannose glycosylation of BTN2A1, glycosylation typical of transformed cells, suggesting that BTN2A1 could have a role in immune surveillance of tumors [152].

The **BTN3** (also known as CD277) subfamily, which is expressed by most human immune cell subsets, including T cells, B cells, monocytes, dendritic cells and NK cells [185], has been largely studied. Many different functions have been attributed to the BTN3 members, including modulation of T cell function, immune evasion and antigen presentation [128]. Use of distinct anti-BTN3 monoclonal antibodies for elucidating the role of BTN3 on the regulation of T cells has led to different biological outcomes. Whereas 232.5 antibody, which binds and phosphorylates BTN3 on the T cell surface [186], and 103.2 antibody, which sterically blocks the association of proteins engaged by BTN3 during activation [187], inhibited T cell activation; 20.1 antibody, which binds to a different epitope on BTN3 that results in cross-linking of the BTN3 molecules [187, 188], triggered T cell activation. Binding of distinct antibodies leads to changes in the organization of BTN3 molecules on the cell surface and thus, it is likely that these structural and biophysical differences contribute to the different functional outputs of these antibodies. Furthermore, it has been reported that BTN3 is highly up-regulated in tumor cells in ovarian cancer by soluble mediators present in the tumor microenvironment, including CCL3 and vascular endothelial growth factor (VEGF), and that its engagement on the surface of activated T cells attenuated anti-tumor T cell responses [174]. Additionally, recent studies have demonstrated that **BTN3A1** can present phosphoantigens (pAgs) to Vγ9Vδ2 T cells, predominantly located in the human blood, and hence, can

act as an antigen-presenting molecule to activate unconventional T cells, which will be recruited to the sites of infection developing their killing potential [188-193].

Although most data suggest an inhibitory role of BTN and BTNL molecules in immune cell activation, there are recent data showing that Butyrophilin-like molecules can also trigger T cell activation. Studies have suggested that **BTNL8**, expressed in neutrophils, binds to resting but not activated T cells, and that the addition of BTNL8-Fc fusion protein to T cell cultures co-stimulated proliferation and cytokine production *in vitro* [194].

Immunological functions of murine Btn and Btnl molecules

The Btn and Btnl family members are characterized by their similarity to the first identified Btn protein, **Btn1a1**, which is involved in the regulation of milk lipid droplets production and secretion during lactation [195, 196]. Recent studies have, however, identified novel immunoregulatory functions for Btn1a1. Thus, Btn1a1, expressed in mammary glands, thymic stromal cells and B cells, has also been reported to be capable of inhibiting T cell responses in *in vitro* assays using Btn1a1-Fc fusion protein [153].

Immunological functions have additionally been identified for several of the other family members. Different mouse studies have documented the influence of **Mog (Btnl11)** in the neuroinflammatory diseases multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) [197, 198]. *Mog*$^{-/-}$ mice revealed that the lack of immune tolerance to Mog in wild-type mice is responsible of the Mog-induced EAE [199]. Furthermore, it was detected that treatment of mice suffering from EAE with Btn1a1 protein can suppress the disease progression due to molecular mimicry and antibody cross-reactivity between Mog and Btn1a1 [200-202].

Studies on mouse **Btn2a2** have demonstrated similar inhibitory effects as for Btn1a1 [153]. Btn2a2 is expressed on thymic epithelial cells, as well as on dendritic cells, monocytes and B cells [153]. Binding of Btn2a2-Fc to activated T cells inhibited TCR activation and induced *de novo* expression of Foxp3 in T cells [153, 203]. Moreover, *Btn2a2*$^{-/-}$ mice revealed enhanced T cell responses, potentiated anti-tumor responses, and exacerbated Mog-induced EAE, increasing the evidence of a negative immunomodulatory role of Btn2a2 [204].

Much of attention has been focused on **Btnl2**, due to the genetically association of human *BTNL2* polymorphisms with several inflammatory disorders [154-172]. Btnl2 is largely expressed on intestinal epithelial cells, dendritic cells and macrophages, in mucosal and lymphoid tissues [205, 206]. Several *in vitro* studies revealed that Btnl2-Fc fusion protein can inhibit T cell proliferation and cytokine production in response to a TCR activating signal in peripheral T cells [205, 206]. Furthermore, Btnl2-Fc was demonstrated to promote expression of Foxp3, a transcription factor necessary for the development and function of Tregs, and thus, to be able to promote the development of regulatory T lymphocytes [207]. Additionally, over-expression of *Btnl2* gene was reported in *Mdr1a*$^{-/-}$ mice, a mouse model of IBD [206], suggesting that Btnl2 is involved in down-modulation of immune responses and thus, in the control of inflammation.

Characterization of **Btnl1** expression demonstrated RNA and protein expression limited to intestinal epithelial cells, but no expression in intestinal lymphoid cells such as IELs or lamina propria lymphocytes (LPLs) [151]. Although a study by another group reported a broader RNA expression, presenting transcripts in a broad spectrum of lymphoid and non-lymphoid tissues, and in CD8$^+$ T cells, B cells, DCs and macrophages, the expression was not confirmed on the protein level [208]. Characterization of Btnl1 function demonstrated the ability of Btnl1 to inhibit the effects of T cells. Studies on peripheral T lymphocytes showed that Btnl1-Fc fusion protein inhibited T cell proliferation via cell cycle arrest and IL-2 production, and that mouse treatment with anti-Btnl1 antibodies enhanced T cell immune responses and exacerbated both Mog-induced EAE and allergic asthma [208]. Another study investigating local effects of Btnl1 in tissue demonstrated an effect of Btnl1 in modulation of IEL - epithelial cell interactions in the murine small intestinal mucosa. Epithelial cell expression of Btnl1 was involved in attenuating the ability of these cells to produce pro-inflammatory cytokines and chemokines of the NFκB pathway, such as IL-6, CXC-chemokine ligand 1 (CXCL1) and MIP-1β (CCL4), in response to activated TCRαβ$^+$ and TCRγδ$^+$ IELs [151].

Btn and Btnl proteins and their counter-receptors

Butyrophilin and Butyrophilin-like proteins mediate complex interactions between different cell types, acting through yet unidentified counter-receptors. The similarity of the domain organization in the Btn/Btnl family to that of the B7 family, together with the observation that Btn-Fc and Btnl-Fc fusion proteins bind to a variety of immune cells (whereas BTN3

members, Btnl1, Btnl2, Btnl9, Btn1a1 and Btn2a2 bind to activated T cells [134, 153, 205, 208], and BTNL8 to resting T cells [194], Btnl1, Btnl2 and Btnl9 also interact with B cells, dendritic cells and macrophages [205, 208]), suggested that they may share binding interactions and partners with the B7 family. This notwithstanding, several studies have shown that Btn-Fc and Btnl-Fc fusion proteins do not interact with known B7 family receptors [134, 153, 194, 206, 208].

Only one binding partner has been identified so far for the Btn/Btnl family. DC-SIGN, expressed by monocytes and dendritic cells, has been shown to interact with the human MOG and BTN2A1 proteins [152, 184].

Also of relevance in this regard is the B30.2 protein domain, which has attracted increasing attention by its possible involvement in T cell interaction [189, 192, 209]. Although the interaction between BTN3A1 and Vγ9Vδ2 T cells is not conclusively established, two major hypotheses have been presented. One suggests a direct binding of pAg to the external IgV domain of BTN3A1, conferring the ability of BTN3A1 to present pAgs on the cell surface to Vγ9Vδ2 T cells [189], whereas the other suggests an indirect pAg presentation, where pAg binds to the intracellular B30.2 domain of BTN3A1, altering the conformation of the extracellular BTN3A1 and thus, driving the activation of Vγ9Vδ2 T cells [192, 210]. However, if the BTN3A1 is the ligand for the γδ T cells itself or if it requires other molecules for TCR engagement, remains unknown.

Taken together, multiple data demonstrate a role of the Btn and Btnl molecules in driving modulation of the immune responses. Much of attention has been focused on the implication of the Btn and Btnl molecules in immune responses in the periphery, however, assessing their role in the local tissues is equally important for a complete understanding of their biological effects. Furthermore, the identification of the counter-receptors for Btn and Btnl molecules is also critical for a full insight into the family's immunomodulatory functions.

In conclusion, further and more detailed studies to decipher a comprehensive view of the role of Btn and Btnl molecules are essential to, in the longer run, use them as potential targets for diagnosis and therapeutics.

2. AIMS

The overall aim of this thesis was to explore the role of the Butyrophilin-like molecules in regulating local T cell mediated immune responses in the gut. The characterization of novel determinants controlling the function of IELs, as well as the identification and exploration of novel IEL – epithelial cell interaction pathways, provides new insights into regulation of T cell mediated immune responses in the intestinal mucosa and thus, into the immune activation and also immune dysregulation in a variety of physiopathological contexts associated with intestinal inflammation and carcinogenic stress.

The specific aims were:

- To further characterize the Btnl1, -4 and -6 molecules, defining their protein expression pattern and identifying their biological form.
- To investigate the ability of the gut resident Btnl proteins to regulate intestinal IELs.
- To define how the expression of the Btnl molecules is regulated in the small intestine during ontogeny and in the absence of gut colonization.
- To determine how *BTN* and *BTNL* genes are regulated in intestinal inflammation and cancer.

3. KEY METHODOLOGY

This section provides a general description of the main experimental procedures used in this thesis work. More detailed protocols can be found in the Materials and Methods section of Papers I-IV.

3.1 Mice

Mice used in this thesis included common wild-type (WT) strains, i.e. C57BL/6 and C3H/HeN, germ-free (GF) mice and knock-out strains, i.e. *Muc2*$^{-/-}$ and *Apc*$^{Min/+}$.

Mouse models of spontaneous colitis (*Muc2*$^{-/-}$) and intestinal tumorigenesis (*Apc*$^{Min/+}$) were used in **paper IV**. *Muc2*$^{-/-}$ mice constitute a relevant animal model to study inflammatory bowel diseases. In mouse colon, bacteria are separated from the epithelial cells by the inner mucus layer formed by Muc2 mucin [211]. *Muc2* deficient mice lack secreted mucus, which allows bacteria to penetrate and reach the epithelium, leading to inflammation of the colon and development of spontaneous colitis. Likewise, humans with active ulcerative colitis have an inner mucus layer that is penetrable [212, 213]. *Apc*$^{Min/+}$ mice constitute a powerful animal model of intestinal carcinogenesis in humans. Min (multiple intestinal neoplasia) is a mutant allele of the murine *Apc* (adenomatous polyposis coli) tumor suppressor gene, encoding a non-sense mutation. Like humans with germline mutations in *APC*, *Apc*$^{Min/+}$ mice are predisposed to intestinal adenoma formation [214, 215].

C57BL/6 and C3H/HeN mice (**paper I**) were purchased from Harlan Laboratories (Netherlands) and Janvier Labs (France), respectively. GF (**paper II**) and conventional (CV) C57BL/6 mice (**papers II and III**), and *Muc2*$^{-/-}$ and *Apc*$^{Min/+}$ mice (**paper IV**), all on the C57BL/6 background, were bred in the Laboratory of Experimental Biomedicine (EBM), Gothenburg University (Gothenburg, Sweden). All animals were housed at EBM, University of Gothenburg. Protocols were approved by the government animal ethics committee (permits no. 335-2012, 310-2010, 280-2012 and 110-2013), and institutional animal care and use guidelines were followed.

3.2 Patients and specimen collection

Colon biopsies from 16 patients with UC, 8 patients with irritable bowel syndrome (IBS) and 17 patients with colon cancer were included in **paper IV**. Parameters analyzed in UC and IBS patients were compared to 18

healthy subjects who served as controls in the study, while parameters analyzed in the tumor of colon cancer patients were compared to unaffected mucosa from the same individuals. Patients were recruited at Sahlgrenska University Hospital, Gothenburg, and Södra Älvsborgs Hospital, Borås, Sweden. The study was performed according to the Declaration of Helsinki and approved by the Regional Ethical Review Board in Gothenburg. All volunteers gave a written informed consent before participation.

Intestinal biopsies were collected and placed immediately in RNAlater (Ambion®) for 24 hours before freezing at -80°C and subsequent RNA extraction.

3.3 Cell lines and generation of transiently and stably transfected cells

HEK 293 cell line, derived from human embryonic kidney cells grown in tissue culture [216], 3T3 fibroblast cell line, derived from murine embryonic tissue [217], and murine intestinal epithelial cell line MODE-K, derived from C3H/He mice [218] were used in **papers I-III**. Cells were maintained at 37°C, 5% CO_2 in Dulbecco's modified essential medium (DMEM; Gibco®, Life Technologies) plus 10% fetal calf serum (FCS; PAA Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.292 mg/ml glutamine and 1× non-essential amino acids (Gibco®, Life Technologies).

Cell transfection is a technique commonly used to introduce exogenous DNA to cells. There are two categories: transient transfection, in which the introduced DNA persists in cells for a limited period of time; and stable transfection, in which the cells pass the introduced DNA to their progeny, because the transfected DNA has been incorporated into the genome.

In **papers I and III**, HEK 293 and MODE-K cells were transiently transfected with Btnl1-, Btnl4-, Btnl6-, Btnl4- + Btnl1-, Btnl6- + Btnl1-, Btnl6- + Btnl4-pMX-IRES-GFP or pMX-IRES-GFP (empty vector) using polyethylenimine (PEI; Polysciences) or lipofectamine (Invitrogen™, Life Technologies) according to standard procedures.

In **papers I-III**, MODE-K cells were stably transfected with Btnl1-, Btnl4-, Btnl6-, Btnl6- + Btnl1-pMX-IRES-GFP or pMX-IRES-GFP, by transduction with viral supernatants, and sorted for GFP^{hi} cells on a BD FACSAria™ II cell sorter (BD Bioscience) or an iCyt Synergy™ cell sorter (BioLegend).

Untagged Btnl-pMX-IRES-GFP and N-terminal FLAG-tagged or HA-tagged Btnl-pMX-IRES-GFP constructs were used based on the experiments' requirements.

3.4 Generation of polyclonal antibodies

Antibodies are produced by the immune system in response to the presence of a specific antigen. Depending on the method of production, the antibodies can be classified into polyclonal and monoclonal. A polyclonal antibody represents a collection of antibodies from different B cell lineages that recognize multiple epitopes on the same antigen, whereas a monoclonal antibody represents an antibody from a single B cell lineage and therefore only binds to one unique epitope.

Btnl1 and Btnl6 polyclonal antibodies used in **papers I and II** were made by Moravian-Biotechnology (Brno, Czech Republic), while Btnl4 polyclonal antibody used in **paper III** was produced by Agrisera AB (Vännäs, Sweden). A synthetic peptide from the extracellular murine Btnl1 or Btnl6 protein sequence was conjugated to an immunogenic carrier protein, keyhole limpet hemocyanin (KLH). Before immunization, the recombinant protein derived from the murine Btnl4 protein sequence was emulgated in Freund's adjuvant. These constructs were injected into New Zealand White rabbits or "Agrisera crossbreed" rabbits (a crossbreed between New Zealand White and Aries French rabbits), respectively. Pre-immune serum was collected from each rabbit and purified using a protein A or G column to serve as negative control. The immune-sera were collected post-immunization and specific antibodies were isolated from sera components by affinity purification on a specific peptide/protein column. Enzyme-linked immunosorbent assay (ELISA) against the original peptide/protein was performed to test the reactivity of these antibodies.

3.5 Preparation of cell suspensions and cell culture

In **papers I-III**, intestinal epithelial cells, intraepithelial lymphocytes and lamina propria lymphocytes were isolated from murine small intestine according to previously described procedures [219-222]. iECs were recovered at the interface between 40% and 20% Percoll (GE Healthcare Bio-sciences AB), and IELs and LPLs were recovered at the interface between 80% and 40% Percoll. Negative and positive selections with anti-CD45 microbeads (Miltenyi Biotec) were performed in some of the

experiments for purification of iECs and IELs or LPLs, respectively, using an auto-MACS separator (Miltenyi Biotec).

The isolated IELs were either analyzed directly or cultured, making use of a long-term culture system for intestinal IELs that permits IELs to be rested and then rapidly re-activated when stimulated via the TCR [222]. IELs were cultured in the presence of 1 μg/mL anti-CD3ε (clone 145-2C11, BD Pharmigen) and a cytokine mixture containing IL-2, IL-3, IL-4 and IL-15 for 48 hours, and thereafter transferred to fresh wells and cultured only in the presence of IL-2. Cells were maintained in 96-well round-bottom plates at 37°C and 10% CO_2. Medium was replaced every 3–4 days.

In **paper I**, splenocytes were obtained from murine spleen and depleted of B cells by negative selection with anti-CD19 microbeads (Miltenyi Biotec) using an auto-MACS separating system (Miltenyi Biotec).

3.6 *In vitro* T cell proliferation assay

The proliferation method used in **paper I** relies on the ability of the carboxyfluorescein diacetate succinimidyl ester (CFSE) highly fluorescent dye to penetrate cell membranes and covalently label intracellular molecules. Due to this covalent coupling reaction, the CFSE can be retained within cells for extremely long periods. The progressive halving of CFSE fluorescence within daughter cells following each cell division allows tracing multiple generations by flow cytometry.

MODE-K cells transduced with Btnl1-, Btnl6- + Btnl1-pMX-IRES-GFP or pMX-IRES-GFP were co-cultured with CFSE-labeled IELs in the presence of anti-CD3 (clone 145-2C11, BD Pharmigen), or in the absence of activation with or without IL-2 (10 U/ml; Roche) or IL-15 (50 ng/ml; R&D Systems); or with CFSE-labeled splenocytes in the presence of anti-CD3 (clone 145-2C11, BD Pharmigen) and anti-CD28 (clone 37.51, BD Pharmigen), or in the absence of activation with IL-2 (10 U/ml; Roche). Lymphocytes were left to proliferate, and cell division and activation was monitored after 72 and 96 hours by flow cytometry. Culture supernatants were collected at 96 hours and used for cytokine protein analysis.

3.7 Flow Cytometry

Flow cytometry was used in **papers I-III** for analysis of the expression of cell surface and intracellular molecules, allowing identification and quantification of specific cell types in a heterogeneous cell population. Cell

components were fluorescently labeled and then excited by a laser to emit light at varying wavelengths. CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes®, Life Technologies) was used for assessment of lymphocyte proliferation in **paper I**.

Cell surface and intracellular antigen expression was analyzed using the following fluorochrome-conjugated anti-mouse antibodies: anti-FLAG- or anti-HA-APC (PerkinElmer), rabbit polyclonal anti-Btnl1 and pre-immune serum (Moravian-Biotechnology), anti-CD45-Alexa Fluor 700 (30-F11; eBioscience), anti-CD3ε-FITC (145-2C11; BD Pharmigen™), anti-pan TCRγδ-eFluor 450 (eBioGL3; eBioscience), anti-TCRβ-APC or APC-Cy™7 (H57-597; eBioscience), anti-TCR Vγ1.1/Cr4-PE (2.11; BioLegend), anti-TCR Vδ4-eFluor 660 (GL2; eBioscience), anti-TCR Vγ7-biotin (kindly provided by Dr. Pablo Pereira, Institut Pasteur) and anti-CD25-PerCPCy5.5 (PC61.5, eBioscience). APC-conjugated AffiniPure F(ab')$_2$ fragment donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) and streptavidin-APC-Cy™7 (BD Biosciences) were used as secondary antibodies. 7-aminoactinomycin D (7AAD; Sigma-Aldrich) and LIVE/DEAD® Fixable Red Dead Cell Stain (Molecular Probes®, Life Technologies) were used to exclude non-viable cells. For detection of intracellular molecules, cells were permeabilized using a cytofix/cytoperm kit (BD Biosciences). Cell samples were acquired on a BD™ LSR II cytometer, and the analysis was performed using the FlowJo Software version 7.6.5 (BD Bioscience).

3.8 Cytokine Assay

Mouse cytokines were measured in supernatants obtained from co-culture experiments in **paper I**, using Mouse Th1/Th2/Th17/Th22 13plex Kit FlowCytomix™ (eBioscience). This method allows the simultaneous detection and quantification of multiple analytes (13 cytokines) in one sample. Samples were acquired on a BD™ LSR II flow cytometer, and data were analyzed using the FlowCytomix™ Pro Software (eBioscience).

3.9 Immunofluorescent staining

The immunofluorescence (IF) is a robust tool to detect the location and expression levels of proteins of interest based on the use of fluorochromes bound to antibodies. IF can be used on cells or tissue sections.

In **paper I**, MODE-K cells transiently transfected with FLAG-tagged-Btnl6-, FLAG-tagged-Btnl6- + HA-tagged-Btnl1-pMX-IRES-GFP or pMX-IRES-

GFP, were plated on collagen-coated coverslips. Cells were fixed in 4% paraformaldehyde, and stained with rabbit anti-HA (Sigma-Aldrich) followed by a goat anti-rabbit-Cy5 (Jackson ImmunoResearch) used as secondary antibody, and with anti-FLAG-PE (Prozyme).

In **paper II**, murine small intestinal sections were fixed in methanol-Carnoy's solution and embedded in paraffin. Sectioning was performed using a cryostat. Sections were deparaffinized, antigen-retrieved, stained with rabbit polyclonal anti-Btnl1 or pre-immune serum, and incubated with TRITC-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch).

Cells and tissue sections (**papers I and II**) were blocked using 10% normal horse serum (NHS) to prevent unspecific binding of antibodies, and mounted in Prolong® Gold antifade reagent containing 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes®, Life Technologies) to visualize nuclei. Images were recorded using the confocal microscope Zeiss LSM700 Inverted available at the Centre of Cellular Imaging at the University of Gothenburg (Gothenburg, Sweden), and analyzed with ZEN lite 2011 microscope software (Carl Zeiss).

3.10 Western Blotting

Western blotting (WB) was used in **papers I-III** to detect the presence of Btnl proteins in tissue or cell lysates.

Murine tissues, isolated primary cells or Btnl- transfected MODE-K cells were homogenized in cell lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100) containing complete protease inhibitor cocktail tablets (Roche Diagnostics). Lysates were clarified by centrifugation, and total protein quantification was performed with BCA Protein Assay Kit (Pierce), where bovine serum albumin (BSA) is used as protein standard. A specific amount of protein was then denatured in reducing or non-reducing sample buffer (NuPAGE® LDS 4x, Novex®, Life Technologies; or SDS-PAGE loading buffer) ± 1 M dithiothreitol (DTT) (Sigma-Aldrich) at 95°C for 5 minutes. Incubation of samples with peptide N-glycosidase F (R&D Systems) at 37°C overnight was used in **paper III** for removal of N-glycans. Samples were loaded onto NuPAGE® 4-12% Bis-Tris Gels (Novex®, Life Technologies) or 6% SDS-PAGE gels. Separated proteins in gels were blotted by wet or semidry transfer to nitrocellulose membranes (Merck Millipore), or Coomassie-stained with Imperial™ Protein Stain (Thermo Scientific) to visualize the protein bands.

Anti-mouse antibodies used to immunoblot the membranes were: anti-FLAG (Sigma-Aldrich), anti-HA (Sigma-Aldrich), anti-GFP (Sigma-Aldrich), rabbit polyclonal anti-Btnl1, -Btnl4 or -Btnl6 and their pre-immune sera (Moravian-Biotechnology and Agrisera AB), and anti-β-actin (Sigma-Aldrich). Specific proteins were then detected using HRP-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch). Streptavidin-HRP was used to detect surface proteins which were biotinylated with non-cleavable EZ-link Sulfo-NHS-LC-Biotin (Thermo Scientific) prior to cell lysis. Membranes were developed with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore), and analyzed with the Fujifilm LAS-4000 Mini luminescence imager.

3.11 Immunoprecipitation

Immunoprecipitation (IP) is one of the most widely used methods for purification of proteins from cells or tissue lysates. Proteins are precipitated using specific antibodies and subsequently detected by western blotting or mass spectrometry. When the antibody targets a known protein that is believed to be a member of a complex of proteins, it is possible to pull down the entire complex and thereby identify unknown members of the complex. This technique, known as co-immunoprecipitation, was applied in **papers I and III**.

Two different IP protocols were used:

- Magnetic bead-based separation, using Dynabeads® Protein G (Novex®, Life technologies) cross-linked to FLAG M2 monoclonal antibody (Sigma-Aldrich) or Dynabeads® M-270 Epoxy (Invitrogen™, Life Technologies) cross-linked to rabbit anti-Btnl1 polyclonal antibody or pre-immune serum (Moravian-Biotechnology). Cell lysates from FLAG-tagged-Btnl-pMX-IRES transduced MODE-K cells or from isolated murine small intestinal epithelial cells, were incubated with the coupled beads. Bound material was collected on a magnet and eluted.

- Protein G PLUS-Agarose (Santa Cruz Biotechnology). Cell lysates from HA-tagged-Btnl-pMX-IRES transduced MODE-K cells were incubated with anti-HA polyclonal antibody (Sigma-Aldrich). Thereafter, the immune complex was captured on a support to which the complex was immobilized (Protein G PLUS-Agarose). Finally, the immunoprecipitates were eluted from the support.

Immunoprecipitated samples were analyzed by western blotting or mass spectrometry.

3.12 Mass Spectrometry

Mass spectrometry (MS) is an invaluable technique in proteomics that measures the mass-to-charge ratio of ions to identify molecules in complex mixtures. In **paper I**, mass spectrometry was used for complex detection in lysates of freshly isolated small intestinal epithelial cells. Cell lysates were subjected to IP using anti-Btnl1 polyclonal antibody or pre-immune serum (Moravian-Biotechnology), run on SDS-PAGE gel, and Coomassie-stained with Imperial™ Protein Stain (Thermo Scientific) for band excision and mass spectrometry analysis.

The proteins were in-gel digested with trypsin (Promega), and the eluted peptides were analyzed by nanoflow liquid chromatography tandem mass spectrometry (nLC-MS/MS) using an Easy-nLC™ 1000 system (Thermo Scientific) coupled to a Q-Exactive™ mass spectrometer (Thermo Scientific) through a nanoelectrospray ion source. Data were analyzed against the *Mus Musculus* NCBI database (29-May-2015) using the Mascot protein identification program (Matrix Science), which identifies proteins from peptide sequence databases.

3.13 Quantitative real-time PCR

Quantitative polymerase chain reaction (qPCR) allows the quantitation of genes in biological samples. In combination with reverse-transcription PCR, which performs complementary DNA (cDNA) synthesis from RNA, qPCR can be used to quantitate changes in gene expression. In **papers II-IV**, qPCR was used to measure gene expression of human and murine *BTN* and *BTNL*, and human *IL-6* genes. qPCR uses fluorescent reporter molecules to monitor the amplification of products during each cycle of the PCR reaction. In these studies, GoTaq® qPCR Master Mix (Promega) containing the double-stranded DNA-intercalating dye agent BRYT Green® was used.

Before qPCR analysis, RNA extraction from the tissues of interest (murine intestinal tissue in **papers II-IV**, and colon biopsies from patients in **paper IV**), and cDNA preparation were performed. Human and murine tissues were lysed and homogenized (Tissuelyser II, Qiagen), and total RNA was isolated using RNeasy® mini kit (Qiagen), including DNAse I digestion. RNA concentration and purity was determined using the spectrophotometer NanoDrop ND-1000. The Omniscript® Reverse Transcriptase kit (Qiagen) and the SuperScript™ III Reverse Transcriptase kit (Invitrogen™, Life

Technologies) were used for cDNA synthesis for human and murine samples, respectively.

Quantitative PCR was performed using GoTaq® qPCR Master Mix according to manufacturer's instructions (Promega) on a LightCycler480 thermal cycler (Roche). Each qPCR analysis was run in duplicate. The sequences of the PCR primers (Eurofins MWG Operon and Applied Biosystems) used in **papers II-IV** are listed in **Table 1**. Gene expression was assessed with the $2^{-\Delta Ct}$ method using human *HPRT1* or murine *β-actin* as housekeeping genes.

Table 1. Primer sequences used for qPCR.

	Primer sequence	
Gene	**Forward**	**Reverse**
Human		
HPRT1	Primers were purchased from Applied Biosystems (Hs99999909_m1)	
BTN1A1	5´-ggatggaagctacgaagaagc-3´	5´-tgcatactgatgtgagggtca-3´
BTN2A1	5´-aggagaccagatttcgtttcct-3´	5´-agggcagcagctgattccat-3´
BTN2A2	5´-gaaggcaggtcctacgatga-3´	5´-tgggccttgatttcaatgag-3´
BTN3A1	5´-tcagaggggaatgctaagagg-3´	5´-caagtatggtgaccgaagaaga-3´
BTN3A2	5´-ctccaatgggaataccaagg-3´	5´-gggaacttgccattttcatcta-3´
BTN3A3	5´-actcaagtggaggaaaatccagt-3´	5´-tggcagatcccgcggctct-3´
BTNL2	5´-agaaggggtcggtcatcag-3´	5´-gctgtatatcttctcccactctgac-3´
BTNL3	5´-tcagtttctacgagctggtgtc-3´	5´-ccaaggcctggacaaactt-3´
BTNL8	5´-gctctcatgctcagtttggtt-3´	5´-gtctggcccaaacacctg-3´
BTNL9	5´-tcttgtcttcctcatgcacct-3´	5´-gcctagcaccttgacctctg-3´
IL6	Primers were purchased from Applied Biosystems (Hs00985639_m1)	
Murine		
β-actin	5´-cttctttgcagctccttcgtt-3´	5´-aggagtccttctgacccatgc-3´
Btn1a1	5´-tactggccttaggatttctcacc-3´	5´-gacgtgaatcttccaatcgaact-3´
Btn2a2	5´-tggagacgaaccctcttacatg-3´	5´-cacatggacggcagtcaaatc-3´
Btnl1	5´-tgaccaggagaaatcgaagg-3´	5´-caccgagcaggaccaatagt-3´
Btnl2	5´-ttcacaatgccagaacttcg-3´	5´-ttccatctctgtccctccac-3´
Btnl4	5´-cattctcctcagagacccacacta-3´	5´-gagaggcctgagggaagaa-3´
Btnl6	5´-atccttggagatccacagtgaa-3´	5´-gggagagaccttgggaaaga-3´
Btnl9	5´-ccccttagagggaggtga-3´	5´-aatactgagaaatctgccatctgtc-3´

3.14 Statistical analysis

Two statistical methods, parametric and non-parametric, were applied in data analysis. Parametric tests assume a normal distribution of the data, whereas non-parametric tests rely on no assumptions of the data's distribution.

In **papers I-III**, parametric statistics were used. The unpaired two-tailed t-test was used for comparison between two independent groups, while One-Way ANOVA followed by Holm-Sidak's multiple comparisons test was applied to evaluate differences between three or more groups. Correlation between parameters was determined using Pearson correlation test.

In **paper IV**, non-parametric statistics were applied. The unpaired two-tailed Mann-Whitney test was used for comparison between two independent groups, while Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied to evaluate differences between three groups. Statistical significance between two paired groups was determined by Wilcoxon matched-pairs signed-ranks test. Correlation between parameters was determined using Spearman correlation test.

Differences were considered statistically significant when $P<0.05$ (*$P\leq0.05$, **$P \leq0.01$, ***$P\leq0.001$, and ****$P\leq0.0001$). All data were generated using GraphPad Prism version 6.04.

4. RESULTS AND DISCUSSION

For many years, body surface epithelia was viewed to primarily contribute to host protection through its physicochemical barrier functions, however, there is emerging evidence that epithelial cells are able to stimulate IELs and hence, to regulate immune responses. This notwithstanding, few molecules used by epithelial cells to instruct immune cells in the intestine have been identified. Defining the interactions involved in the epithelial cell – IEL cross-talk is therefore crucial, not only to improve our understanding of the biology of T cells that reside in intestinal mucosa, but also to give new insights into the immune activation and perhaps more importantly, into immune dysregulation in infectious-, inflammatory- and carcinogenic stress in the gut.

The text below summarizes the findings of the four papers included in this thesis. The results from **Papers I-III**, which focus on the study of the murine, intestine localized Btnl family members, will first be described. This will be followed by discussion of data in **Paper IV** that presents a comprehensive expression analysis of human and murine *BTN* and *BTNL* genes in colonic inflammation and cancer.

Bas et al. previously reported that the expression of *Btnl1*, *-4* and *-6* transcripts is largely restricted to small intestinal epithelial cells, and that Btnl1 protein, detected on the surface of iECs and located in direct juxtaposition with IELs, is implicated in the regulation of activated intestinal IELs by suppressing local pro-inflammatory signals [151]. Therefore, in **papers I-III**, we focused on the characterization of Btnl4 and Btnl6 as possible novel epithelial immune regulators.

4.1. Btnl protein expression and the proteins' biological forms (Papers I and III)

To study the expression of Btnl4 and Btnl6 proteins, we generated rabbit polyclonal anti-Btnl4 and anti-Btnl6 antibodies. Despite several attempts to generate antibodies recognizing the native form of the Btnl4 and Btnl6 proteins, the developed anti-sera only recognized the proteins in their reduced form and thus, could not be used for *in situ* studies. To overcome this obstacle, we turned to molecular biology and generated tools that allowed us to study proteins' expression and function *in vitro*. Thus, we constructed Btnl1, -4 and -6 cDNAs that included a FLAG or HA epitope C

terminal to the putative signal cleavage site, and that were cloned into bicistronic pMX-IRES-GFP expression vectors. Murine small intestinal epithelial MODE-K cells, which do not ordinarily express Btnl proteins, were then transfected with these constructs.

Using the generated antibodies in western blotting under reducing conditions, we demonstrated that Btnl6 protein is exclusively expressed in the intestine, and that its expression in the small intestine is confined to iECs. We further demonstrated that also Btnl4 protein is expressed in epithelial cells in the small intestine. These data are consistent with the previously published mRNA data by Bas et al. [151].

Moreover, the anti-Btnl4 antibody detected two bands in lysates from primary small intestinal epithelial cells and from Btnl4 transfected MODE-K cells. Size-reduction of the bands upon N-glycosidase F treatment of Btnl4 transfected MODE-K cell lysates indicated the existence of two glycosylated forms of the Btnl4 protein. Protein glycosylation has been reported to be involved in biological recognition, where their structure diversity provides signals for protein targeting and cell - cell interactions [223]. Intriguingly, binding of human BTN2A1 to DC-SIGN, which modulates immature monocyte-derived dendritic cells, was revealed to be dependent on tumor-specific glycosylation of the BTN2A1 protein [152]. Thus, distinct glycosylation of the Btnl4 protein may lead to different protein's interactions and hence, to different outcomes depending of local conditions such as intestinal homeostasis or stress.

Whereas Btnl4, like Btnl1, is readily expressed on the surface of small intestinal epithelial MODE-K cells, we showed that cell surface expression of Btnl6 is specifically dependent on the presence of Btnl1. While determining if this Btnl1-dependent expression of Btnl6 was mediated by Btnl1-Btnl6 interaction and using immunoprecipitation techniques, we identified a previously unknown Btnl1-Btnl6 complex displayed on the cell surface of small intestinal MODE-K cells. Mass spectrometry of anti-Btnl1 immunoprecipitated lysates from primary small intestinal epithelial cells revealed a non-reduced Btnl1 homodimer complex of ~130 kDa, and a high molecular mass Btnl1-Btnl6 heteromeric complex and thus, identified the presence of Btnl1-Btnl6 protein complex formation *in vivo*. We additionally demonstrated that Btnl4 can exist both as a homomer and as a Btnl1-Btnl4 heteromeric structure on the surface of small intestinal MODE-K cells. The observed Btnl1-Btnl4 and Btnl1-Btnl6 heteromerizations may explain the inability of the generated antibodies, in particular the anti-Btnl6 antibody, to

recognize the native form of the proteins, most likely due to epitope masking after complex formation.

Altogether, our data demonstrate the presence of multiple Btnl forms comprising various combinations of the iEC-specific Btnl proteins, which may result in different or even divergent functions of the Btnl proteins determined by their composition. However, the high homology between Btnl4 and Btnl6 (88% amino acid identity in the ectodomain [126]) and their capacity to form heteromeric complexes with Btnl1, may also imply redundant functions of the Btnl molecules in the intestinal epithelium.

4.2. Btnl expression in the absence of gut microbiota and in the ontogeny (Papers II and III)

During early neonatal life, namely at birth and at weaning, important changes occur in the gut. The infant's immature intestinal immune system develops as it comes into contact with microbial and dietary antigens. Thus, both microbial colonization and diet have a decisive role in the complete development of the mucosal immune system [224].

To assess the impact of the gut microbiota on Btnl protein expression, we examined the presence of Btnl1 and Btnl6 proteins in germ-free mice. We found that the expression of Btnl1 and Btnl6 proteins in the neonate gut is not dependent of microbial colonization, as Btnl1 and Btnl6 proteins are present in germ-free mice at comparable levels to those detected in conventional mice.

Furthermore, to gain insight into how the weaning event regulates the expression of Btnl proteins, we investigated the presence of Btnl1, Btn4 and Btnl6 proteins in the small intestine of newborn, 1-4 week-old and compared the expression to adult mice. We found that the expression of Btnl1, -4 and -6 proteins is delayed during ontogeny and appears in the small intestinal epithelium of 2-3 week-old pre-weaning pups. This delay was not reflected at the RNA level, where the *Btnl* expression is already detected in the newborn small intestine, suggesting post-transcriptional regulation during mouse intestinal maturation. Although the appearance of Btnl proteins occurs before weaning, where pups are mainly fed with milk, we cannot determine whether an increased exposure to pelleted food and thus, to dietary antigens, is involved in the expression of Btnl proteins, or if the expression is regulated by an unknown developmental factor.

4.3. Immunological role of Btnl proteins (Papers I and II)

The primarily restricted expression of Btnl proteins in gut epithelium and the reported suppression of co-stimulation-induced IEL activation by Btnl1 [151], suggest that other iEC-specific Btnl molecules may have similar capacity to effect IEL - epithelial cell communication.

To further study immunomodulatory roles of the Btnl molecules on IEL response, we performed *in vitro* T cell proliferation assays, exploiting a culture system that overcomes the rapid apoptosis of IELs *ex vivo* [225, 226] and that permits IELs to be rested and then re-activated when stimulated via the TCR [151, 222]. *Ex vivo* IELs, which lack or have low levels of CD25 [16], or splenocytes were co-cultured with Btnl1-, Btnl1-Btnl6- or pMX- (empty vector) transfected small intestinal epithelial MODE-K cells. The reliability of the co-culture system was verified by confirming previously reported suppressive effect of Btnl1-Fc on CD3-activated peripheral T cells [208]. Thus, we proved that under anti-CD3 and anti-CD28 stimulation, splenocyte proliferation is reduced in the presence of both Btnl1 and Btnl1-Btnl6 complex.

In contrast, we found no significant reduction or increase of IEL proliferation by either Btnl1 or Btnl1-Btnl6 complex in the presence of anti-CD3 stimulation. Instead, we demonstrated the capacity of Btnl1 and Btnl1-Btnl6 complex to induce IEL proliferation in the absence of exogenous activation. This ability is dependent of the presence of IL-2 or IL-15, as no proliferation was detected in the absence of these cytokines, and is specific for IELs, as no proliferation was observed in unstimulated splenocytes. Although the Btnl1-Btnl6 complex is not critical for promoting IEL proliferation, we revealed that the Btnl1-Btnl6 complex specifically enhances the expansion of IELs bearing the Vγ7Vδ4 receptor. Altogether, our data imply the contribution of the epithelium-specific Btnl proteins to the upkeep of the intestinal IEL pool. Proliferation of intestinal IELs *in situ* in the absence of activation had been demonstrated by previous studies [227, 228], however, the mechanisms behind the homeostatic expansion of IELs has to our knowledge not been fully defined.

In addition, we found that Btnl- transfected MODE-K cells up-regulate CD25 expression on both TCRαβ$^+$ IELs and TCRγδ$^+$ IELs in the absence of TCR stimulation, and that the observed proliferation is restricted to IELs up-regulating CD25. Although CD25 expression was significantly up-

regulated on IELs in the presence of Btnl proteins and exogenous IL-2, CD25 induction could additionally be observed in conditions with IL-2 and MODE-K cells transfected with pMX. In contrast, in conditions with exogenous IL-15, CD25 expression was only up-regulated on IELs in the presence of Btnl proteins, and was not observed when IELs were co-cultured with pMX- transfected MODE-K cells. In view of the fact that co-cultures with exogenous IL-15, which is constitutively expressed by iECs [229-231], will better reproduce homeostatic conditions in the small intestine than IL-2, which is only available at low levels in the gut under steady state-conditions [232], our data suggest that the IEL proliferation is dependent on the synergy between IL-15 and Btnl proteins. Additionally, we demonstrated that this effect is reliant on direct iEC - IEL contact, as no CD25 expression was found in co-cultures where MODE-K cells and IELs were separated by transwells.

Furthermore, we reported that Btnl proteins can also induce IFN-γ secretion by IELs in the absence of anti-CD3 activation and in the presence of exogenous IL-15. Taking into account that some studies have shown a protective role of IFN-γ in the removal of transformed epithelial cells under steady-state conditions [74], this IFN-γ secretion by IELs in the absence of TCR stimulation may contribute to the maintenance of the homeostasis in the intestinal epithelium. As the IFN-γ production by IELs was significantly higher in the presence of Btnl1 compared to Btnl1-Btnl6 complex, this may reflect different efficiency in regulating IEL function, or even indicate that Btnl6 counter-act the effect of Btnl1.

The identified iEC-expressed Btnl1-Btnl6 heteromeric complex has, as discussed above, a specific biological role particularly elevating the proliferation of intestinal intraepithelial Vγ7Vδ4 T cells under "steady-state" conditions *in vitro*. To assess the association between the Btnl proteins and the IEL repertoire in the small intestine *in vivo*, we examined the γδ expressing IEL repertoire during the first weeks of neonatal development in the murine small intestine, and found that the expansion of Vγ7Vδ4 IELs in the neonate gut correlates with the appearance of the Btnl1 and Btnl6 proteins at 3 weeks of age. Our data are consistent with studies demonstrating an increase in the percentage of γδ IELs detected at 2-3 weeks of age in the small intestine of neonatal mice [26-28]. Moreover, observations reporting extra-thymic origin of γδ IELs [17, 20, 22], and the reported linkage between the intestinal γδ TCR repertoire and the MHC class II locus [56, 233], which intriguingly contains the *Btnl1* and *Btnl6* genes, imply that the gut population of IELs may be governed by gut micro-

environmental factors rather than by immigration of thymus-derived T cells and thus, support our data.

Furthermore, our results showing comparable levels of Btnl1 and Btnl6 proteins in GF and CV mice are supported by earlier publications demonstrating a significant difference in the number of αβ IELs, but no differences in the frequency of γδ IELs between GF and CV mice [29, 30].

Taken together, and in accordance with the suggestion that the IEL colonization process may be regulated by gut micro-environmental factors, our data suggest that the homeostatic expansion of IELs in the gut is driven by iEC-specific Btnl proteins. Curiously, our finding describing that Btnl1-Btnl6 complex enhances the expansion of Vγ7Vδ4 IELs is similar to the one observed for the skin epithelium resident Skint-1, a close relative to Btnl1 and Btnl6 that regulates epidermal Vγ5Vδ1 IEL development [122-124].

4.4. Human and murine *BTN* and *BTNL* gene expression in normal colon (Paper IV)

Although significant progress has been made in understanding the role of Btn and Btnl molecules in modulation of T cell mediated immune responses, little is still known about the molecules' implication in inflammatory and proliferative disorders. Hence, in **paper IV**, we investigated how the *BTN* and *BTNL* genes are regulated in intestinal inflammation and cancer.

We first assessed the expression of *BTN* and *BTNL* genes in normal colon from human and mouse by quantitative real-time PCR. Variable *BTN* and *BTNL* mRNA expression levels were identified. In human colon, *BTN2A1*, *BTN2A2*, *BTN3A1*, *BTN3A2*, *BTN3A3*, *BTNL3* and *BTNL8* genes showed relatively high expression levels, whereas *BTN1A1*, *BTNL2* and *BTNL9* genes were present at low levels. In murine colon, *Btnl1* and *Btnl4* genes were expressed at relatively high levels, *Btn1a1*, *Btnl2* and *Btnl6* genes were found at intermediate levels, and *Btn2a2* and *Btnl9* transcripts were on the limit of detection.

Btnl9 mRNA expression pattern had not been characterized before and thus, we examined its expression in a panel of mouse tissues. We found that liver and mesenteric lymph nodes had the highest expression of *Btnl9* compared to thymus and spleen, and that *Btnl9* was not expressed in small intestine.

4.5. Human *BTN* and *BTNL* gene expression in intestinal inflammation and cancer (Paper IV)

Over the recent years, BTN and BTNL molecules have been genetically linked to various immunological diseases. Polymorphisms in the human *BTNL2* gene have been associated with inflammatory disorders such as sarcoidosis [154-163], ulcerative colitis [164-166], rheumatoid arthritis [167, 168] and myositis [169]; and to prostate cancer [173]. Furthermore, a few studies have identified an association between human *BTN3* and ovarian cancer [174-176].

As several human *BTN* and *BTNL* genes are expressed in the intestine, they may be involved in gastrointestinal disorders. We used qPCR to map their expression in colon samples from patients with ulcerative colitis, irritable bowel syndrome and colon tumors. Expression in UC and IBS patients was compared to the expression in healthy subjects with no prior history of gastrointestinal diseases, while expression in the tumor of colon cancer patients was compared to adjacent unaffected mucosa from the same individuals.

Our analysis revealed a significant up-regulation of *BTN1A1*, *BTN2A2*, *BTN3A2* and *BTN3A3* genes in UC patients compared to healthy subjects. By contrast, the expression of most of the *BTNL* genes was unchanged, with the exception of *BTNL8* that was significantly down-regulated (**Table 2**). Our data showing unchanged levels of *BTNL2* in UC patients suggest that the reported *BTNL2* SNPs associated with susceptibility to UC [164-166] affects the encoded BTNL2 protein instead of the *BTNL2* gene expression, as in the case of sarcoidosis, where the resulting protein lacks the C-terminal IgC domain and the transmembrane helix, thereby disrupting the membrane localization of the protein [154]. Moreover, our data presenting an opposite expression pattern of *BTN3* genes and *BTNL8* gene in UC patients correlate with the divergent ability of these molecules to modulate peripheral T cell activation. If BTN3, reported to inhibit T cell proliferation and cytokine secretion [174, 186], and BTNL8, described to trigger T cell activation [194], exhibit analogous functions in the intestine, the outcome of an up-regulation of BTN3 and a down-regulation of BTNL8 in an inflamed scenario, would be to attenuate the T cell mediated immune response and thus, to limit progression to chronic inflammation. In addition, we demonstrated an inverse correlation between *BTN3A3* and *IFNγ*, where increased expression of *BTN3A3* associates with decreased expression of *IFNγ*. IFN-γ has

previously been reported to be increased in UC patients [234], thus, our results further suggest a feedback mechanism to limit the effect of inflammation in the colon of UC patients.

Whereas inflammatory bowel diseases, namely Crohn's disease and UC, are characterized by macroscopic signs of inflammation or ulceration in the small and large intestine, such changes are not present in irritable bowel syndrome [235]. Our data showed normal *BTN* and *BTNL* gene expression in IBS patients compared to healthy individuals, implying that the altered expression of *BTN* and *BTNL* genes in UC patients is driven by inflammation.

Furthermore, our analysis indicated unchanged expression levels of *BTN* genes in tumor tissue of colon cancer patients compared to adjacent unaffected tissue from the same subjects. By contrast, *BTNL2*, *BTNL3*, *BTNL8* and *BTNL9* genes were significantly down-regulated (**Table 2**). In view of recent studies demonstrating the ability of BTNL molecules to regulate T cell mediated immune responses, where BTNL8 was reported to enhance T cell activation [194], it is logical to speculate that a down-regulation of *BTNL* genes in the tumor may have implications in immune surveillance and tumor promotion.

Table 2. Human *BTN* and *BTNL* gene expression data.

	UC patients	**Colon cancer patients**
BTN1A1	↑↑↑	↔
BTN2A1	↔	↔
BTN2A2	↑↑↑	↔
BTN3A1	↔	↔
BTN3A2	↑	↔
BTN3A3	↑↑↑↑	↔
BTNL2	↔	↓↓
BTNL3	↔	↓↓
BTNL8	↓↓↓	↓↓↓
BTNL9	↔	↓

↑ indicates significant up-regulation (↑ P≤0.05, ↑↑ P≤0.01, ↑↑↑ P≤0.001, and ↑↑↑↑ P≤0.0001).
↓ indicates significant up-regulation (↓ P≤0.05, ↓↓ P≤0.01, ↓↓↓ P≤0.001, and ↓↓↓↓ P≤0.0001).
↔ indicates no significant up- or down-regulation.

4.6. Murine *Btn* and *Btnl* gene expression in intestinal inflammation and cancer (Paper IV)

Humans and mice are surprisingly similar, sharing between 95-98% of our genomes and getting most of the same diseases. The ease with which the murine genome can be manipulated has provided a powerful tool to create useful mouse models of IBD and intestinal cancer.

To better understand the role of Btn and Btnl molecules in inflammatory and proliferative disorders, we examined the expression of murine *Btn* and *Btnl* genes in intestinal samples from mouse models of spontaneous colitis (*Muc2*$^{-/-}$) and intestinal tumorigenesis (*Apc*$^{Min/+}$). Expression in distal, middle and proximal large intestine of *Muc2*$^{-/-}$ mice was compared to the expression in *Muc2*$^{+/-}$ mice, which served as controls. Expression in the small intestinal polyps derived from *Apc*$^{Min/+}$ mice was compared to adjacent unaffected tissue from the same mice.

Our analysis revealed a significant down-regulation of *Btn1a1*, *Btnl1*, *Btnl4*, *Btnl6* and *Btnl9* genes in the distal part of the colon of *Muc2*$^{-/-}$ mice compared to control mice. By contrast, no differences were found in the expression levels in the proximal part (**Table 3**). In both UC patients and *Muc2*$^{-/-}$ mice, signs of inflammation and destruction of colon architecture increase proximally from the distal part, where the inflammation is most pronounced [212, 213, 236, 237]. Thus, our data suggest that the altered expression of *Btn* and *Btnl* genes is related to inflammation. The down-regulation of *Btnl1*, *Btnl4* and *Btnl6* genes was particularly intriguing since these genes are essentially restricted to intestinal epithelia. Btnl1 has been reported to attenuate the epithelial response to activated IELs, resulting in reduced production of pro-inflammatory mediators such as IL-6 and CXCL1, which are involved in promoting influx of monocytes and neutrophils [151, 238, 239], and our data have demonstrated the ability of Btnl1 and Btnl6 to promote IEL proliferation. Hence, as intestinal γδ IELs contribute to preservation and restoration of the gut integrity in colitis [33, 41, 86, 93, 95], a down-regulation of the intestine-specific *Btnl1* and *Btnl6* genes may contribute to progression of the ongoing inflammation and to impair tissue integrity's repair.

The analysis of *Btn* and *Btnl* genes in *Apc*$^{Min/+}$ mice revealed significantly increased expression levels of *Btn1a1* and *Btn2a2* genes in isolated small intestinal polyps compared to adjacent unaffected tissue from the same mice. In contrast, levels of *Btnl1* transcripts were significantly decreased (**Table 3**).

The consequence of an up-regulation of *Btn1a1* and *Btn2a2*, reported to inhibit T cell activation [153] and to induce Treg development [203], may be to contribute to immune evasion and tumor promotion. This is consistent with a study revealing potentiated anti-tumor responses in *Btn2a2*$^{-/-}$ mice [204]. In addition, a down-regulation of *Btnl1*, described to suppress epithelial cell production of pro-inflammatory IL-6 and CXCL1 in response to activated intestinal IELs [151], may further contribute to tumor progression as enhanced levels of both IL-6 and CXCL1 have been reported to participate in development of intestinal cancer [240-242]. Attenuated levels of *Btnl1*, which promotes IEL proliferation, may result in a reduced number of IELs and consequently in reduced IEL-cytotoxic activity to kill transformed epithelial cells [87-89], farther promoting tumor development.

Table 3. Murine *Btn* and *Btnl* gene expression data.

	Muc2$^{-/-}$ mice			*Apc*$^{Min/+}$ mice TUM
	Proximal	**Middle**	**Distal**	
Btn1a1	↔	↔	↓	↑
Btn2a2	↔	↔	↔	↑↑
Btnl1	↔	↓↓↓	↓↓↓↓	↓
Btnl2	↔	↔	↔	↔
Btnl4	↔	↓	↓↓	↔
Btnl6	↔	↓	↓↓	↔
Btnl9	↔	↔	↓	ND

↑ indicates significant up-regulation (↑ P≤0.05, ↑↑ P≤0.01, ↑↑↑ P≤0.001, and ↑↑↑↑ P≤0.0001).
↓ indicates significant up-regulation (↓ P≤0.05, ↓↓ P≤0.01, ↓↓↓ P≤0.001, and ↓↓↓↓ P≤0.0001).
↔ indicates no significant up- or down-regulation.

In conclusion, our results showing an altered expression of the *BTN* and *BTNL* genes in colonic inflammation and intestinal tumors, further attest these genes as active players in the orchestration of immune responses and thus, affirm the importance of these genes in the immune system both in health and in disease.

5. CONCLUDING REMARKS

Btn and Btnl proteins have over the past decade emerged as essential regulators of T cell functions in mice and humans. Heretofore, much of attention has been focused on assessing the proteins' biological effects on systemic T cells, and only few studies have attempted to understand their capacity to regulate the activity of local intraepithelial T cells. Their elucidation is an important step in understanding tissue-specific inflammatory diseases and associated carcinoma, as well as host defense.

Bas et al. previously showed that the expression of *Btnl1*, *Btnl4* and *Btnl6* genes is largely restricted to epithelial cells in the gut, and that Btnl1 is a novel tissue-specific regulator of intestinal epithelial cell - intraepithelial T cell cross-talk, being able to attenuate the epithelial response to activated TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs. This observation, together with our data demonstrating an up-regulation of IEL activation and proliferation dependent of Btnl proteins in conditions without exogenous activation, suggest that the interaction between IELs and Btnl proteins may lead to different outcomes depending on local conditions, e.g., intestinal homeostasis or inflammatory stress. While attenuation of epithelial response to activated IELs may be important in controlling an inflammatory response and progression to chronic inflammation, induction of TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IEL activation and proliferation in the absence of exogenous stimulation may contribute to the maintenance of the intestinal IEL pool. Moreover, we have identified the existence of previously unknown multimeric complexes comprising various combinations of the iEC-specific Btnl proteins, which may have different functions determined by their form, and demonstrated that the Btnl1-Btnl6 heteromeric complex, expressed in primary small intestinal epithelial cells, has a specific biological role particularly enhancing the expansion of IELs bearing the Vγ7Vδ4 receptor in *in vitro* studies. In addition, we examined the $\gamma\delta$ expressing IEL repertoire during the first weeks of neonatal development in the murine small intestine, and found that the expansion of Vγ7Vδ4 IELs in the neonate gut correlates with the appearance of the Btnl1 and Btnl6 proteins at 3 weeks of age. Although further experiments, for example using Btnl$^{-/-}$ approaches, will be necessary to confirm the association between the Btnl1-Btnl6 complex and the *in vivo* proliferation of Vγ7Vδ4 IELs, these *in situ* data further add strength to our *in vitro* results.

Although T cell regulation by Btn and Btnl molecules in now unfolding, the molecules' implication in inflammatory and proliferative disorders is poorly defined. Therefore, we present a comprehensive expression analysis of human and murine *BTN* and *BTNL* genes in colonic inflammation and intestinal tumor. We demonstrated a substantial and significant modulation of several of the genes in ulcerative colitis and colon cancer and hence, our data suggest an inflammation driven- regulation of *BTN* and *BTNL* genes. Altogether, these data represent a valuable resource proposing several Btn and Btnl candidates to further investigate UC and colon cancer susceptibility.

In conclusion, this thesis work has demonstrated that iEC-specific Btnl proteins are involved in the regulation of IEL activity in the gut, and that Btn and Btnl genes are associated with bowel pathology. These findings shed new light on the elucidation of local immune regulation in the gut and the contribution of the Btn and Btnl molecules to pathophysiology. Nonetheless, further studies are necessary to identify the family's full immunomodulatory capacity, e.g. by identifying their binding partners, to, in the future, be able to use them as clinical targets.

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