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The role of HVEM-LIGHT stimulatory signals in intestinal inflammation

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1 Summary

The mucosal immune system is constantly challenged by the luminal contents and by potentially invasive and pathogenic microorganisms. Therefore, the local immune responses need to be tightly regulated. Abnormal immune responses against commensal microorganisms result in chronic inflammatory reactions and tissue destruction as observed in inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC). Various cell types and signaling processes are involved in IBD and a better understanding of how these are regulated is key to the development of improved therapeutics.

TNFSF members regulate important processes in cell proliferation, survival and differentiation and are therefore crucial for the balance between homeostasis and inflammatory responses. Several members of the tumor necrosis super family (TNFSF) are closely associated with IBD. Thus, they represent interesting new targets for therapeutic treatment of IBD. Stimulatory interaction between the two recently described TNFSF members LIGHT and Herpes virus entry mediator (HVEM) has been shown to contribute in intestinal inflammation. Additionally, LIGHT expression was directly associated with human IBD.

The main focus of this thesis was to investigate the role of LIGHT or HVEM stimulatory interactions during immune homeostasis and intestinal inflammation. In the first part of this thesis, we sought to clarify the role of LIGHT and HVEM in the development, activation and differentiation of T and B cells during intestinal homeostasis. For this purpose, we made use of naïve genetically modified mice, which are either deficient for the LIGHT or the HVEM gene. We found that the absence of LIGHT or HVEM signaling does not compromise the development of T cells, B cells, NK cells or NKT cells.

Furthermore, neither LIGHT nor HVEM deficient mice exhibited an altered antibody response in the serum. T helper cell differentiation capacities *in vitro*, in the presence of exogeneous cytokines, of C57BL/6, LIGHT$^{-/-}$ and HVEM$^{-/-}$ mice derived T cells were comparable. By contrast, T cells isolated from the small intestine lamina propria and PPs of LIGHT deficient mice exhibited and increased ratio of regulatory T cells (Treg) to Th17 T cells. Interestingly however, HVEM$^{-/-}$ mice exhibited increased Treg frequency in almost all lymphoid organs except the small intestine and did not show an altered Th17 cell population. Thus, indicating that interactions with other cognate partners likely play a role in immune cell homeostasis.

We next investigated the importance of LIGHT or HVEM on intestinal inflammation using a model of T cell-dependent colitis in immunodeficient mice. The absence of LIGHT or HVEM stimulatory signals on T cells led to attenuated intestinal inflammation in this model. Disease attenuation was more pronounced in the absence of HVEM, indicating that LIGHT signalling through HVEM may only partially contribute to disease outcome. Impaired expansion and altered differentiation pattern of transferred HVEM$^{-/-}$ CD4 T cells was not reversed by the simultaneous co-transfer of C57BL/6 CD4^{+}CD45RB^{high} T cells and was instead found to correlate with decreased responsiveness of HVEM$^{-/-}$ CD4 T cells to IL-6 and IL-23. HVEM stimulatory signals were thus found to be essential for the proper expansion and effector function of pathogenic CD4^{+} T cells.

Furthermore, we assessed the impact of LIGHT or HVEM signaling on innate cells during intestinal inflammation. Therefore, we have used the dextran sodium sulfate (DSS) model of colitis, in which the activation of innate immune cells promotes intestinal inflammation. Upon DSS administration, HVEM$^{-/-}$ mice exhibited significantly reduced intestinal inflammation and pro-inflammatory cytokine production. LIGHT$^{-/-}$ mice exhibited an intermediate

phenotype as compared to C57BL/6 or HVEM$^{-/-}$ mice. These data show that HVEM stimulatory-interactions are also required for the full activation of innate cells and consequent intestinal inflammation, and further indicate that these are likely to be derived in part through LIGHT.

Taken together, these findings emphasize an important role for HVEM-mediated stimulatory signals in promoting intestinal inflammation and indicate that these may be mediated in part by LIGHT.

2 Zusammenfassung

Das Immunsystem der Darmschleimhäute ist einer enormen Anzahl von potenziell gefährlichen Mikroorganismen ausgesetzt, die entweder im Darm leben oder durch die Nahrung aufgenommen werden. Um eine unnötige Immunantwort gegenüber harmlosen darmansässigen Mikroorganismen zu verhindern, muss die lokale Immunabwehr fest reguliert werden. Eine krankhaft gesteigerte Immunabwehr gegen harmlose Darmmikroorganismen resultiert in chronischen Entzündungsreaktionen und damit verbundenen chronisch-entzündlichen Darmerkrankungen, wie Morbus Crohn und Colitis ulcerosa. Eine Vielzahl verschiedener Zellen und Signalprozesse ist in die Entstehung dieser chronisch-entzündlichen Darmerkrankungen involviert. Ein besseres Verständnis über die Regulierung der Immunabwehr im Darm würde helfen neue und verbesserte Therapien zu entwickeln.

Die Mitglieder der Tumor-Nekrose-Faktor (TNF) Familie regulieren Proliferation, Überleben und Differenzierung von Immunzellen und sind folglich enorm wichtig für die Aurechterhaltung der Balance zwischen regulierten und überbordenden Immunantworten. Mehrere Mitglieder der TNF Familie sind in die Entstehung chronischer Darmerkrankungen involviert und stellen daher interessante neue Ziele für deren medikamentöse Behandlung dar. Zwei kürzlich beschriebene neue Mitglieder der TNF Familie, LIGHT und Herpes virus entry mediator (HVEM), tragen zu chronischen Darmentzündungen bei.

Der Schwerpunkt dieser Doktorarbeit war, die Funktion der zell-stimulierenden Interaktionen von LIGHT oder HVEM während Immunhomeostase und Darmentzündung zu untersuchen. Im ersten Teil dieser Arbeit wollten wir die Funktion von LIGHT und HVEM in der Entwicklung, Aktivierung und Differenzierung von T-und B-Zellen während der

intestinalen Homeostase klären. Zu diesem Zweck haben wir naïve genetisch veränderte Mäuse verwendet, die eine Mutation im LIGHT- oder HVEM-Gen tragen. Wir konnten zeigen dass die Abwesenheit von LIGHT oder HVEM in naïven Mäusen die Entwicklung von T-Zellen, B-Zellen, NK-Zellen oder NKT-Zellen kaum beeinträchtigt. Darüber hinaus stellten wir fest dass weder LIGHT noch HVEM defiziente Mäuse veränderte Antikörpertiter im Serum aufweisen. Auch die T-Helfer-Zell-Differenzierung *in vitro*, in Gegenwart von exogenen Zytokinen, war vergleichbar zwischen C57BL/6, LIGHT$^{-/-}$ und HVEM$^{-/-}$ Mäusen. Im Gegensatz dazu zeigten LIGHT$^{-/-}$ T Zellen die aus der Lamina Proria des Dünndarms und der Peyer-Plaques isoliert wurden ein erhöhte Anzahl von regulatorischen T-Zellen (Treg) gegenüber Th17 Zellen. Interessanterweise fanden wir in HVEM$^{-/-}$ Mäusen eine erhöhte Anzahl von Treg Zellen in praktisch allen lymphatischen Organen mit Ausnahme des Dünndarms, und eine unveränderte Th17 Zellpopulation. Dieser Unterschied zwischen LIGHT$^{-/-}$ und HVEM$^{-/-}$ Mäusen deutet darauf hin, dass mögliche Interaktionen von LIGHT und HVEM mit anderen TNF Familenmitgliedern zur Regulierung der intestinalen Homeostase beitragen.

Als nächstes untersuchten wir die Bedeutung und Funktion von LIGHT oder HVEM während einer chronischen Darmentzündung. Dazu verwendeten wir ein T-Zell-abhängigen Mausmodell in immundefizienten Mäusen. Die Abwesenheit von LIGHT oder HVEM auf den T-Zellen führte zu einer abgeschwächten Darmentzündung in diesem Modell. Die Abschwächung der Darmentzündung war stärker in Abwesenheit von HVEM als in Abwesenheit von LIGHT, was darauf hinweist, dass LIGHT vermittelte Signalprozesse durch HVEM nur teilweise zum Krankheitsverlauf beitragen. Die beeinträchtigte Expansion und Differenzierung von HVEM$^{-/-}$ CD4 T-Zellen konnte nicht durch den gleichzeitigen Co-Transfer mit C57BL/6 CD4 T-Zellen rückgängig gemacht werden. Die verminderte Fähigkeit von HVEM$^{-/-}$ CD4 T-Zellen zu expandieren und differenzieren korrelierte stattdessen mit einer

reduzierten Empfindlichkeit gegenüber IL-6 und IL-23 vermittelten Signalen. HVEM ist deshalb unerlässlich für eine ordnungsgemäße Expansion und Effektorfunktion von pathogenen CD4 T-Zellen.

Ferner beurteilten wir die Auswirkungen von LIGHT oder HVEM auf natürliche, unspezifische Immunzellen während einer Darmentzündung. Dafür haben wir das Dextran Natriumsulfat (DSS) Modell eingesetzt, in dem die Aktivierung der natürlichen, unspezifischen Immunzellen zur Darmentzündung führt. Nach der Verabreichung von DSS, zeigten die HVEM$^{-/-}$ Mäuse eine deutlich reduzierte Entzündung des Darms sowie eine stark reduzierte Produktion von proinflammatorischen Zytokinen. In LIGHT$^{-/-}$ Mäusen war die Darmentzündung weniger abgeschwächt als in den HVEM$^{-/-}$ Mäusen. Diese Daten zeigen, dass stimulierende HVEM Signale für die volle Aktivierung der natürlichen, unspezifischen Immunzellen und damit für die DSS induzierte Darmentzündung erforderlich sind.

Zusammengefasst unterstreichen diese Ergebnisse eine wichtige Rolle für HVEM vermittelte Signalprozesse in der Förderung von Darmentzündungen, welche zum Teil durch LIGHT vermittelt werden.

3 Introduction

3.1 The intestinal immune system

The gastrointestinal tract (GIT) is the largest interface between the immune system and the outside world. Its huge mucosal surface area ($100m^2$) is constantly exposed to the external environment and is therefore highly susceptible to colonization and invasion by pathogenic microorganisms. In addition to the constant exposure to dietary antigens and environmental antigens, the human intestine harbors approximately 10^{14} commensal bacteria living in a mutalistic relationship with their host. To discriminate between harmful and non-pathogenic microorganisms and to maintain a homeostatic balance between tolerance and immunity, mammals have evolved a complex gut-associated lymphoid tissue (GALT) equipped with specialized cells of the innate and adaptive immune system. A permanent surveillance of non-pathogenic intestinal microorganisms, and induction of an appropriate non-inflammatory or toleragenic response against these organisms is essential to prevent excessive immune responses, which may be detrimental for the host. Dysregulation of the mucosal equilibrium between tolerance and immunity can contribute to the pathogenesis of inflammatory conditions, such as inflammatory bowel disease (IBD) or allergies (1, 2). This thesis focuses on uncontrolled inflammatory reactions in the gut.

3.1.1 The gut-associated lymphoid tissue (GALT)

The intestine represents a major immune organ with several organized lymphoid structures and specialized cell types present throughout the length of the intestinal tract. This complex network of organized lymphoid structures and cells along the intestinal tract - including small intestine and large intestine - compose the GALT. The GALT includes Peyer's patches (PPs) and

isolated lymphoid follicles (ILFs). Efferent lymphatics leaving these organs then drain to the mesenteric lymph nodes (MLNs). Together, these structures enable a tight surveillance of the contents of the luminal intestinal flora by sampling and transmitting information to the gut immune system. Under homeostatic conditions inflammatory immune responses to the intestinal flora are dampened and the balance between inflammation and tissue homeostasis is maintained. However, changes in the composition of the commensal flora or pathogenic invasion may trigger a rapid and specific inflammatory immune response (3, 4).

Antigen sampling of intestinal luminal bacteria largely occurs through the PPs, which are located directly under the epithelial cell layer. PPs sample antigens or whole microorganisms through the microfold (M) cells of the follicle-associated epithelium (FAE), which overlie them (5). PPs harbor large aggregates of B cells surrounded by dendritic cells (DCs) and T cell-rich areas. The B cell-rich aggregates contain the germinal centers, where antibody hypermutation takes place. Formation of PPs occurs during embryogenesis and is dependent on interleukin-7 receptor α (IL-7Rα) and lymphotoxins (LT). Genetically modified mice, deficient in either the IL-7Rα, lymphotoxin α/β or lymphotoxin β receptor (LTβR), completely lack PPs as well as other secondary LN (6, 7). PPs are only found along the small intestine, whilst ILFs are spread throughout the small and large intestines. ILFs are populated largely by B cells, with an outer ring of DCs and few T cells. They are only ever represented by a single B cell follicle and may possess germinal centers (8).

3.1.2 Intestinal epithelial cells

The intestinal epithelium forms an essential element of the mucosal barrier, separating the host tissue from the external environment. A single

layer of intestinal epithelial cells (IECs), displaying a number of specialized physical and biochemical adaptations, limits free penetration of commensal bacteria and luminal antigens. For the proper integrity of the intestinal epithelial barrier, two main structural components are indispensable: the microvilli of the brush border and the tight junctions (9, 10). The microvilli are apical extensions of enterocytes that create the brush border that physically protects the intestinal barrier from microbial attachment and invasion. Intercellular tight junctions regulate barrier permeability and are linked to the microvilli via actin filaments (11). The secretion of mucin by goblet cells strengthens the epithelial barrier by formation of a thick mucin-rich layer, called glycocalix. Besides forming a protective barrier, mucins actively trap bacteria in the glycocalix via interaction with polysaccharides and proteins on their surface, so that bacteria can be easily washed away in the mucus flow by intestinal peristalsis (12). IEC can also secrete a variety of antimicrobial peptides, including defensins (α and β defensins), cathelicidins and calprotectins. These antimicrobial molecules lyse the bacteria by forming pores in their cell wall and therefore protect the IEC from bacterial invasion (13).

3.1.3 Innate immune recognition of commensal microbial antigens

Occasionally, commensal and pathogenic bacteria may succeed in crossing the epithelial barrier either by transport through the gut epithelium (via M cells) or by damaging the barrier itself. In order to prevent an excessive inflammatory response directed against these intruders, rapid recognition and elimination is needed.

The first sensors of danger are IECs and DCs, which recognize bacterial antigens through the expression of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or nucleotide-binding

oligomerization proteins (NOD). TLRs recognize many different bacteria-associated molecules such as lipopolysaccharides (LPS) (TLR4), flagellin (TLR5) or CpG (TLR9). Whereas TLRs mainly sense extracellular pathogen-associated molecular patterns (PAMPs), the NOD-like receptors (NLR) predominantly recognize intracellular PAMPs. Both, the TLRs and NOD receptors have been reported to be crucial for the maintenance of intestinal immune homeostasis, as dysregulated function in either of these has been associated with increased barrier permeability, together with overt immune responses and chronic inflammation (14).

Under homeostatic healthy conditions expression of PRRs on IECs is negligible and the IECs are relatively unresponsive to intestinal bacteria. Upon recognition of danger signals TLR expression on IECs is upregulated and secretion of various cytokines and chemokines is induced (15). Subsequently activation of surrounding DC and recruitment of other innate cells, such as macrophages (Mϕ) and neutrophils to the site of infection will occur. Additionally, T cells and B cells are activated through antigen-derived peptides, presented on major histocompatibility complex II (MHCII) by DC or Mϕ. Relative unresponsiveness due to low expression of PRRs is also an integral feature of intestinal Mϕs (16, 17). Several phenotypically different DC subtypes can be found in the intestine, of which the exact origin and function is not yet completely understood. It appears that some DCs specifically promote regulatory function by the induction of IL-10 producing regulatory T cells, whereas others are involved in mediating inflammatory responses to pathogenic microorganisms. Some DCs can even directly sample antigen from the intestinal lumen, by the extension of dendrites through the epithelium (18).

3.1.4 Adaptive immune surveillance in the intestine

Large populations of T cells can be found in all compartments of the intestine, namely the GALT, lamina propria (LP) and the epithelium. Organized GALT mainly contains naïve T cells, whereas T cells residing in the LP and epithelium display characteristically an effector/memory phenotype. Although highly heterogeneous, two major T cell subsets can be distinguished based on their T cell receptor (TCR) expression and co-receptor expression. The first subset of T cells consists of TCR$\alpha\beta^+$ MHCII$^+$ CD4$^+$ and MHCI$^+$ CD8$\alpha\beta$ T cells, which are predominantly found in the LP throughout the intestine. The second subset of T cells express either TCR$\alpha\beta$ or TCR$\gamma\delta$ often together with CD8$\alpha\alpha$ and are mainly found in the mucosal epithelium (19). As the discussion of the different features and functions of all the T cell subtypes found in the intestine would go beyond the scope of this thesis, this section will focus on the classical CD4$^+$ T cells abundant in the LP.

CD4$^+$ T cells can be subdivided into different subsets according to their specific cytokine profile and distinct functionality. Currently, at least five different T helper (Th) cell subsets are known: Th1, Th2, Th17, regulatory T cells (Treg) and T follicular helper cells (Tfh). Th1 cells mediate responses towards intracellular pathogens, whilst Th2 cells are involved in the clearance of helminths. Th17 cells appear to be important in the control of extracellular bacteria and fungi and are additionally associated with autoimmune disorders (20). By contrast, Treg cells and Tfh cells function as regulators of inflammatory responses by suppressing T cell activation and promoting antibody class switching, respectivley (21). Intestinal homeostasis requires the proper induction and function of Treg cells and the LP contains a relatively high frequency of these cells as compared to other effector sites. However, Th1 and Th17 cells are also detectable in the normal LP, and Th17 cells are enriched here as compared to other sites. By contrast, Th2 cells are rare or completely absent in the normal intestine.

Studies in human as well as in mice have shown an important role for Treg cells in maintaining immuno-quiescence in the face of intestinal bacteria, as is evidenced by severe intestinal inflammation in patients suffering from polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in which a mutation in the FOXP3 gene causes a loss of Treg cells (22). Both, thymus-derived naturally occurring CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells and those that are induced in the periphery from Foxp3$^-$ precursors, preferentially accumulate in the intestine. Conversion of Foxp3$^-$ T cells into Foxp3$^+$ Treg cells is dependent on transforming growth factor β (TGFβ) and retinoic acid, produced locally at this site (23). Treg cells mediate their suppressive activity through surface-expressed inhibitory receptors by cell-to-cell contact and the release of anti-inflammatory cytokines, such as TGFβ and interleukin-10 (IL-10) (24). By acting on DCs and Mφs, IL-10 inhibits the development of Th1-type responses and additionally acts in a positive feedback loop enhancing the differentiation of de novo Treg cells, which in turn provide further secretion of IL-10. TGFβ along with its unique ability to induce Foxp3$^+$ Treg cells, also contributes to the production of Treg cell-derived IL-10 (25).

Th17 cell development in the LP is dependent on the presence of commensal microorganisms, as mice raised under germ free conditions were devoid of Th17 cells (26). However, this finding remains controversial as another report shows similar frequencies of Th17 cells in the LP of germ free-housed mice (27). Interestingly, TGFβ, in addition to its essential function in Treg cell differentiation is also required for the induction of Th17 cells acting together with the pro-inflammatory cytokine interleukin-6 (IL-6) (28). This emphasizes a central role of TGFβ in orchestrating the pro-inflammatory and anti-inflammatory nature of adaptive immunity. The high amounts of Th17 cells present in the LP under normal conditions may indicate a crucial role for these cells in exhibiting tightly controlled effector functions, fighting potentially harmful bacteria likely to be encountered on a frequent basis in the intestine

(29, 30). The fact that inflammatory cytokines are produced physiologically in the intestine, further demonstrates the necessity of strong regulatory mechanisms in order to maintain intestinal homeostasis.
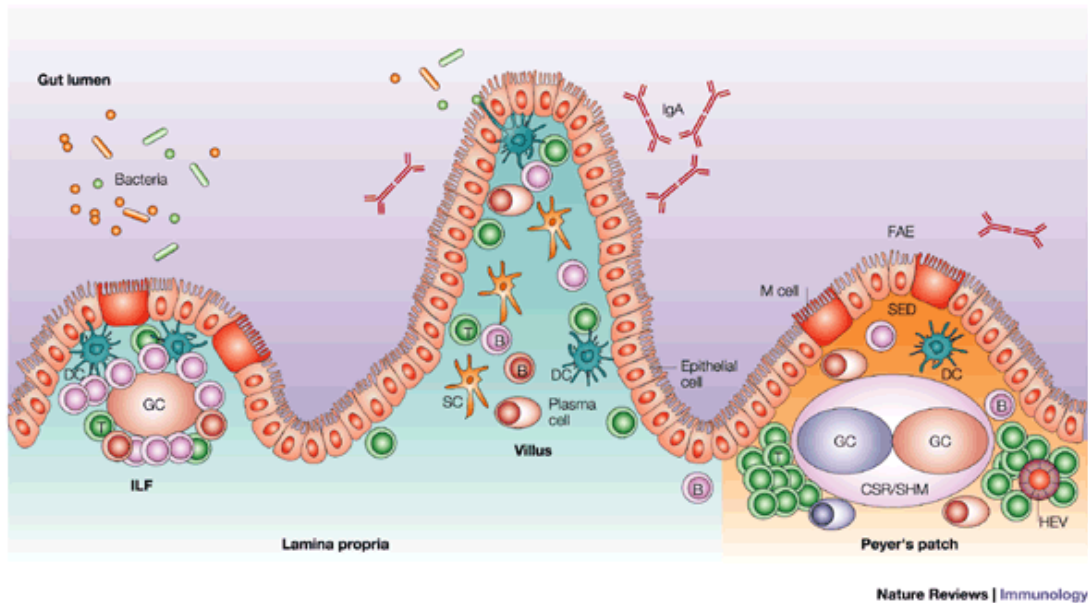
Figure 1: The gut-associated lymphoid tissue (GALT).

The intestine represents a major immune organ containing organized lymphoid structures and specialized cell types throughout the intestinal tract. The GALT includes organized lymphoid structures such as Peyer's patches (PPs), isolated lymphoid follicles (ILFs) and the mesenteric lymph nodes (MLNs), and the diffuse tissue of the epithelium and the lamina propria. PPs sample antigens or whole microorganisms through the microfold (M) cells of the follicle-associated epithelium (FAE) and the subepithelial dome (SED), which is rich in dendritic cells (DCs). PPs harbor large aggregates of B cells, which contain the germinal centers, where antibody class-switch takes place. B cells migrate into the mucosa via high endothelial venules (HEV), located around PPs. Additionally, large populations of plasma B cells, T cells and DCs can be found in the lamina propria. Some lamina propria DCs directly sample antigen from the intestinal lumen and present them to T and B cells. IgA class switching is induced upon antigenic stimulation in B cells and its secreted form is transported across the epithelium.

Adapted from: S. Fagarasan & T. Honjo, *Nature reviews immunology,* 63-72, 2003 (31)

3.2 Inflammatory bowel disease (IBD)

3.2.1 IBD: dysregulation of intestinal homeostasis

IBD is a chronic and progressive inflammatory disease of the gastrointestinal tract. Two major clinical forms of IBD can be defined: Crohn's disease (CD) and ulcerative colitis (UC) (32). As mentioned in the previous section, the mucosal immune system is constantly challenged by an immense load of microorganisms and distortion of the balanced non-inflammatory interaction between the commensal microorganisms and the intestinal immune system leads to the onset of IBD. IBD is a complex and multi-factorial disease and various alterations have been described to impact on the proper interpretation and regulation of the microenvironment. Such alterations include a number of susceptibility genes, which result in exaggerated immune responses to normal constituents of the intestinal commensal flora and environmental factors, which may predispose individuals to IBD. Both, CD and UC have a prevalence range of 10-200 cases per 100'000 individuals in North America and Europe. Highest disease incidences are found in developed and urbanized countries and in countries undergoing rapid economic development (33). The onset of IBD occurs predominantly in the second and third decade of life and the majority of affected individuals progress to a relapsing and chronic disease (34).

CD and UC show some overlapping clinical features, and in 10-15% of cases it is not possible to distinguish between the two diseases. However, although they share some clinical and pathological characteristics, they have some markedly different disease patterns. While CD resembles histopathologically a Th1-type mediated experimental colitis, UC is mediated by a Th2-like immune response. UC is restricted to the large intestine and associated with continuous mucosal inflammation, including crypt abscesses and ulcers, which typically spread from the rectum all along the large

intestine. Furthermore, mucosal inflammation in UC is characterized by a massive infiltration of lymphocytes and granulocytes into the superficial mucosal layer and a loss of goblet cells. Although various animal models of UC show a predominant Th2-mediated inflammation, there is no evidence for increased Th2-related cytokine IL-4 in human UC patients. However, higher amounts of auto-antibodies and Th2-related immunoglobulin (Ig) subclasses such as IgG1 and IgG4 as well as significantly higher amounts of Th2 cytokines IL-5 and IL-13 have been detected in UC patients (35, 36).

In contrast to UC, CD affects the whole gastrointestinal tract and is characterized by the presence of segments of normal healthy tissue between diseased regions. Although any part of the gastrointestinal tract can be affected, the terminal ileum is most commonly involved and the first mucosal lesions often appear over PPs. Pathological features of CD consist of a transmural infiltration of lymphocytes and macrophages, fissuring ulcerations, granulomas and oedemas throughout the whole intestinal wall. Formation of granulomas occurs in about 60% of patients. Inflammation in CD is typically mediated by an excessive Th1 response and is associated with an increased secretion of interferon γ (IFNγ), interleukin-12 (IL-12) and tumor necrosis factor α (TNFα) (37, 38). Unrestrained Th1 cytokine release in CD leads to the upregulation of adhesion molecules which facilitates the recruitment of more effector T cells, neutrophils and Mϕ leading to a positive amplification loop and tissue damage.

3.2.2 IBD susceptibility genes

Various epidemiological studies provided strong evidence for specific susceptibility genes associated with IBD. Studies with monozygotic and dizygotic twins strongly support that susceptibility is inherited, in particular for CD (39). To date the identification of susceptibility genes has revealed an

important role for innate and adaptive immune responses and epithelial barrier integrity in IBD (40, 41).

One of the first and most prominent susceptibility loci described, named IBD1, is located on chromosome 16. Mutations of the NOD2 gene in this region have been associated with CD (42, 43). The NOD2 gene encodes for an intracellular molecule of the NOD family that is thought to be a cytosolic receptor for the recognition of pathogenic bacterial signals. Recent reports have revealed that mutations in NOD2 negatively affect the expression of α-defensins in IECs, which may result in increased susceptibility to intestinal inflammation (44). Additionally, the transcription factor NFκB, which is activated by NOD2, has been shown to be crucial for IECs to control intestinal integrity (45). However, NOD2 gene mutations are found only in about 20% of CD patients and seem to be completely absent in UC patients, indicating that other important susceptibility genes also account for disease.

Several additional susceptibility loci for IBD have been discovered such as IBD5, interleukin-23 receptor (IL-23R) and ATG16L1, implicated in phagocytosis, innate immunity and autophagy (46, 47). The number of genes potentially involved in IBD is continuously increasing and future genetic research may focus more on phenotypes and gene-gene interactions to elucidate their disease contribution.

3.2.3 Implication of environmental factors in IBD

Beside genetic predisposition, high variation of risk among different ethnic groups living in diverse geographical locations may suggest a role of environmental factors in IBD pathogenesis (48). The striking increase in frequency of IBD over the past 50 years in industrialized countries such as North America and Europe further supports the impact of environmental

factors on the onset of IBD. Changes in the environment might affect the development of the mucosal immune system, the composition of the commensal microflora or both. The most apparent changes in industrialized countries over the last decades include an improved sanitation, a modified diet, a reduction in childhood infections as well as a reduced exposure to intestinal pathogens (49). The decline in parasitic infections in the industrialized countries is one aspect of an improved hygiene and has been associated with increased prevalence of CD. Parasites normally mount a strong Th2-type inflammatory response, which may counterbalance the Th1-type response of CD (50). Another environmental factor that has been associated with IBD is tobacco smoke. Tobacco smoking has divergent influences on the two major subtypes of IBD in such that it is protective in UC while harmful in CD (51). Further investigations on the impact of environmental factors on IBD may provide vital information for the formulation of new approaches in the treatment of IBD.
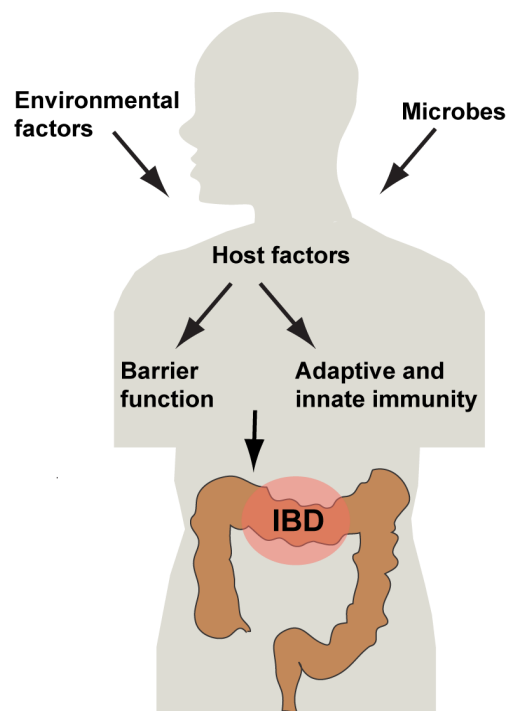
Figure 2: Patterns of IBD etiopathogenesis

IBD results from alterations in the interaction beween the commensal microorganisms and the mucosa. This can be affected by environmental factors and/or host factors, which may vary depending on the genetic inheritance. Genetic factors discovered to date affect barrier function, and adaptive and innate immunity.

Modified from: R. J. Xavier & D. K. Podolsky, *Nature 448,* 427-434, 2007 (52).

3.2.4 Therapeutic approaches to treat IBD

The development of novel therapeutic agents to treat IBD has become a very active field of research since great efforts in understanding the pathophysiology of IBD have revealed various potential new targets. Until now, the medical spectrum to treat CD and UC was mainly limited to aminosalicylates and steroids (53, 54). Application of steroids rapidly and consistently improves severe CD and UC. However, steroid treatment is ineffective in the maintenance of remission and has strong side effects, which often outweighs their benefits during long-term treatment (55, 56). Despite the fact that intestinal inflammation and associated symptoms, like abdominal

pain, diarrhea and rectal bleeding, can be diminished by current treatments there is no current cure for IBD. Current choices for therapy largely depend on disease severity, location, and the side effects and cost of the treatment. The introduction of Infliximab, an anti-TNFα medication, over a decade ago has been one of the most promising therapeutic advances in a huge spectrum of potential new drugs (57, 58). A great number of current IBD treatments undergoing pre-clinical development, target T cell differentiation, cytokine responses, adhesion molecules or intestinal repair mechanisms (Table 1).

Therapeutic approach	Agent	Target	Drug class	Disease	Phase
T cell differentiation/subsets	ABT-974	IL-12	mAB	CD	III
	Allizumab/MRA	IL-6	Receptor mAB	CD	I/II
	Daclizumab7basiliximab	CD25	mAB	UC	II
	Visilizumab	CD3	mAB	UC	I/II
Inflammatory cytokines/pathways	Certolizumab (CDP-870)	TNFa	PEG-Ab fragment	CD	III
	Adalizumab	TNFa	Small molecule	CD	III
	Fontolizumab	IFNg	Peptide	CD	II
	Semapimod	P38/JNK	Small molecule	CD	II
	RDP58	P38/JNK	Peptide	UC	II
	Rosiglitazone	PPARg	Small molecule	UC	II
Selective adhesion molecules	Alicaforsen	ICAM1	Antisense	UC	II
	Natalizumab	a4	mAB	CD	III
	MLN02	a4b7	mAB	UC	III
Innate immune stimulation	GM-CSF	Unknown	Peptide	CD	III
Intestinal repair	EGF	Unknown	Peptide	UC	III
	Growth hormone	Unknown	Peptide	CD	II
Other	Trichuris suis ova	Unknown	Helminth	CD/UC	I/II
	Probiotics/prebiotics	Balance of gut flora	N/A	CD/UC	II/III
	Autologous BM transplant	T cells?	N/A	CD	I
	Leukopheresis	Leukocytes	Device	CD/UC	III

Table 1: Novel therapeutic approaches to treat IBD

Adapted from: J. R. Podolsky DK, *Nature reviews, drug discovery,* 2006 (59)

Abbreviations: AB, antibody: mAB, monoclonal antibody; CD, Crohn's disease; EGF, epidermal growth factor; ICAM, intercellular adhesion molecule; N/A, not available; PEG, polyethylene glycol; PPAR, peroxisome proliferator-activated receptor; UC, Ulcerative colitis.

3.3 Animal models of intestinal inflammation

Although animal models of intestinal inflammation only represent a part of the highly heterogeneous characteristics of human disease, and cannot fully replace studies with patient material, they have contributed enormously to our current understanding of the pathogenesis of human IBD. Recent advances in the development of new transgenic and gene targeting technologies has opened up the possibility to more precisely study the interaction of different cell types and their soluble factors in mouse IBD models. Animal models of intestinal inflammation can be formally classified into four categories: spontaneous models, inducible models in normal mice, adoptive transfer models in immunocompromised hosts and genetically modified models (knockout mice, transgenic mice). Although the different models have a very heterogeneous origin, the great majority described so far manifests a Th1 driven mucosal inflammation.

3.3.1 Spontaneous models of colitis

As described in previous sections (3.2.2 and 3.2.3), IBD occurs in individuals that have a genetic predisposition and are presumably exposed to certain environmental factors. Animal models, which develop spontaneous colitis offer the unique possibility to define genetic susceptibility factors in more or less specified environmental compartments. A strain that spontaneously develops colitis, independently of the presence of pathogens, is C3H/HeJBir. These mice spontaneously develop colitis at about three weeks of age. Pathological features include acute and chronic lesions and ulcerations, predominantly located in the cecum and the proximal colon mucosa. CD4$^+$ T cells that react to antigens derived from the normal commensal bacterial flora have been identified to drive colitis in these mice (60).

3.3.2 Inducible models of colitis

Acute or chronic intestinal inflammation in these animal models is induced by the application of various chemical agents, leading to the disruption of the epithelial mucosal barrier. Onset of colitis is mediated by the mucosal immune system and studies in germ free mice and mice treated with antibiotics revealed, that in some inducible models the presence of commensal bacterial components is essential to promote the inflammatory response.

The most prominent models of induced colitis utilize chemical administration via the drinking water or rectal application. Commonly used chemicals include Dextran-sodium sulfate (DSS), Trinitrobenzene-sulfonic acid (TNBS) and Oxazolone (61, 62). As the description of each chemical model would exceed the scope of this thesis, only DSS will be further discussed.

Several days of feeding mice with DSS polymers via the drinking water leads to the development of an acute intestinal inflammation characterized by bloody diarrhea, ulcerations and massive granulocytic infiltration of the large intestine (63). DSS is believed to have a direct toxic effect on intestinal epithelial cells and thereby negatively affect barrier integrity. The adaptive immune system seems not to be necessary for acute disease progression, as T and B cell-deficient RAG1$^{-/-}$ or C.B-17^{scid} mice also develop severe intestinal inflammation (64, 65). Some proinflammatory cytokines seem to play a beneficial role in ameliorating acute DSS-induced colitis, as treatment with anti-IL-1 or anti-TNFα monoclonal antibodies aggravate intestinal inflammation (66, 67). Other effector cytokines in DSS-induced colitis include IL-12, IFNγ and IL-6. Neutralization of IL-12 or IFNγ in DSS-induced colitis was shown to significantly reduce intestinal inflammation (68). Additionally, mice deficient in IL-6, exhibit significantly inhibited DSS-induced inflammation

(69). Acute DSS-induced colitis is widely used to study the contribution of the innate immune system during intestinal inflammation. By contrast, administration of low doses of DSS for several cycles induces a chronic form of colitis with the participation of Mϕ and CD4$^+$ T cells. In this chronic phase of DSS-induced colitis, disease is associated with increased levels of pro-inflammatory cytokines (IL-2, IL-4 and IL-6) and shows a mixed Th1/Th2-type response (70). Combination of DSS-induced colitis with the administration of a single dose of the genotoxic colon carcinogen azoxymethane, leads to the development of inflammation-associated colorectal cancer (71). DSS-induced colitis might therefore be also a useful model to investigate the cooperation of intestinal inflammation and colon carcinogenesis.

3.3.3 Adoptive transfer models

In adoptive transfer models intestinal inflammation is induced upon transfer of certain cell types into immunocompromised mice. These models have been very useful to unravel various immunological and genetic factors contributing to disease and have provided great insight into the important role of T cells in mucosal immune regulation.

The adoptive transfer of CD4$^+$CD45RB^{high} T cells in lymphopenic animals, such as severe combined immunodeficiency (SCID) or recombination activating gene 1 (RAG1) deficient mice, leads to severe intestinal inflammation 5-8 weeks after transfer (72). Intestinal inflammation is associated with body weight loss, thickening of intestinal wall, epithelial hyperplasia and loss of goblet cells, crypt abscesses and transmural infiltration. Although adoptively transferred CD4$^+$CD45RB^{high} T cells populate the small and large intestine, inflammation is mostly limited to the colon. This phenomenon is most likely explained by the need for endogenous colonic microorganisms to provide antigenic stimulation. In the early 1990's it was

reported that transfer of recipient mice with the entire CD4$^+$ T cell population or CD4$^+$CD45RB^{low} T cells completely abrogates onset of colitis, suggesting a regulatory role of certain T cells present within these populations (73). The regulatory T cells within the CD4$^+$CD45RB^{low} T cell subset were later identified as CD25$^+$ T cells expressing Foxp3, now known as Treg cells (74, 75). These cells appear to attenuate colitis largely by virtue of their ability to secrete IL-10, as anti-IL10 antibodies are able to reverse their protective effect (76). Inflammation in this model, is thought to be driven by a Th1-type response, as neutralizing antibodies to IL-12, IFNγ or TNFα ameliorates colitis (77). Recent evidence indicates that Th17 cells are also associated with colitis in this model, but the secretion of IL-17 itself is not necessary for disease (78). By contrast production of the Th17 inducing cytoines, IL-6 and IL-23, is necessary for disease progression (79, 80). As in many other models of intestinal inflammation, bacteria-derived antigens are important for the onset of colitis.

3.3.4 Gene deficiency and transgenic models

The use of new technologies, in particular transgenic mice and gene targeting technologies account for many recent advances in the understanding of the pathophysiological mechanisms involved in IBD. Such genetically modified mice are useful to dissect specific immunoregulatory pathways and help to identify specific therapeutic strategies.

Mice with a targeted disruption in the IL-10 gene spontaneously develop colitis under conventional housing conditions (81). Presumably, the induction of spontaneous colitis in IL-10 deficient mice is due to the absence of the suppressive effects of IL-10 on cytokine production by macrophages and CD4$^+$ T cells (82). Colitis in IL-10 deficient animals is characterized by a thickening of the intestinal wall, loss of goblet cells and erosion of the

epithelial cell layer.

TNFα plays a central role in the pathogenesis of chronic intestinal inflammation in many models. Transgenic TNF^{ARE} mice, which have an impaired regulation of TNFα, spontaneously develop a chronic, relapsing inflammation in the intestine between 5 and 12 weeks of age. A deletion in the TNFα gene in these mice results in increased production of TNFα by Mϕs and other hematopoietic cells. Intestinal inflammation in TNF^{ARE} mice primarily affects the terminal ileum and less frequently the proximal colon. T and B cells are necessary to mediate colitis in TNF^{ARE} mice, since the mutation in RAG1$^{-/-}$ mice strongly attenuates disease (83).

Other gene deficiency models and transgenic models of colitis are summarized in table 2, together with all other models of intestinal inflammation.

Spontaneous	Inducible	Adoptive Transfer
C3H/HeJBir mice Cotton top tamarin Samp1/Yit mouse	Formalin/immune complexes Acetic acid Carrageeenan Indomethacin Peptidoglykan-polysaccharide Dextran sulfate sodium Radiation Cyclosporin A TNBS Oxazolon	CD45RB^{high} into SCID or RAG BMC into Cd3ϵTg26 Hsp60 specific CD8^{+} T cells into TCRβ^{-/-}
Transgenic	**Knockout**	
STAT-4 Dom. neg. N-cadherin IL-7 HSV tyrosine kinase	IL-2 IL-2Rα IL-10 CRF2-4 TGFβ TCRα Trefoil factor Mdr1a WASP TNF ARE	

Table 2: Experimental colitis models

Adapted from: S. Wirtz, *Int J of Colorectal Dis,* 15: 144-160, 2000 (84)

3.4 The Tumor Necrosis Factor (TNF) super family

Approximately thirty years ago, two molecules, lymphotoxin (LT) and tumor necrosis factor (TNF) were identified in search of new potenti al cancer treatments. Subsequent sequencing of expressed sequence tags (ESTs) of LT and TNF cDNA revealed that they were highly similar to each other (85). Having identified a strong similarity between these two molecules, research was conducted to find out if other molecules with similar functionality were also related to them. Nowadays related sequences are known as members of the condensed TNF super family of ligands (TNFSF) and receptors (TNFRSF). To-date more than forty ligands and receptors are identified as belonging to the TNF super family. These exhibit a broad cellular expression and are involved in mediating cell proliferation, survival and differentiation (86). They also have important roles in the generation of secondary lymphoid

tissue, where adaptive immune responses are most effectively initiated. A number of TNSF members are closely associated with severe human diseases, such as IBD or cancer, and thus represent interesting therapeutic targets.

TNF, LTα, LTβ and lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), along with their cognate receptors, TNF receptor 1 (TNFR1), TNF receptor 2 (TNFR2), LTβ receptor (LTβR) and Herpes virus entry mediator (HVEM), define the immediate TNF family. This group of TNFSF ligands and receptors, show a high sequence similarity and have overlapping patterns of ligand-receptor binding (87). TNFSF ligands are classically type 2 transmembrane proteins, which are either membrane bound or can be cleaved into a soluble form by metalloproteinases. Both forms are biologically active and normally self-assemble into non-covalent trimers. Receptors of the TNFSF are type 1 transmembrane glycoproteins characterized by cysteine-rich domains (CRDs) in the ligand-binding motif. Some TNFSF receptors lack adapted signaling domains, or are secreted and serve as decoy receptors or soluble regulators (86, 88).

3.4.1 HVEM and its cognate ligands

HVEM was first identified as a molecule that interacts with herpes simplex virus (HSV) through viral glycoprotein D (gD) to facilitate viral entry. HVEM is highly expressed in the lung, the kidneys and the liver, but it can also be detected at lower levels in almost all organs (89). In addition to its broad tissue distribution, HVEM is widely expressed on most hematopoietic cells, including T cells, B cells, DCs, natural killer cells (NK), monocytes and on some stromal cells (90). HVEM is expressed at relatively high levels on naïve T cells but decreases quickly following activation, and is upregulated

again at late points following activation (91).

Characteristic for members of the TNFRSF, HVEM is a type 1 transmembrane protein and contains four CRDs in the ligand-binding motif. Until recently, TNFRSF members were thought to interact exclusively with TNFSF ligands. However, HVEM has been shown to be an exception to that rule, as it has been described to interact with the TNFSF members LIGHT and LTα and in addition with the members of the Ig super family, BTLA and CD160 (89). The interaction of HVEM with each of its known cognate ligands is discussed in detail below:

i) HVEM-LIGHT. LIGHT is a type 2 transmembrane protein and assembles as a homotrimer on the cell surface. Its expression is more tightly regulated than that of HVEM as it is predominantly found in spleen and brain, with low expression on peripheral blood leukocytes, in lymph nodes and in the intestine (92). LIGHT is upregulated on activated T cells and is highly expressed by immature DC, NK cells and monocytes (93, 94). LIGHT was first identified to have a co-stimulatory role for HVEM when it was shown to promote T cell proliferation and IFNγ production, and blockade of LIGHT on DCs inhibited allogeneic T cell responses (94, 95). LIGHT-HVEM interactions have also been shown to promote activation and effector function of NK cells, neutrophils and monocytes (96, 97). Following engagement with LIGHT, HVEM recruits TNF receptor associated factors (TRAF), including TRAF2 and TRAF5. TRAF2 and TRAF5 activity leads to the activation of NFκB and c-Jun N-terminal kinase (JNK)/AP-1 pathways, which induce genes that regulate cell survival, cytokine production and proliferation (98, 99).

It is important to note that LIGHT can also act as a ligand for LTβR and has also showed binding activity for the human soluble TNF receptor DcR3 (92, 100). Although LTβR is mainly expressed on non-hematopoietic cells such as stromal cells, LIGHT-LTβR mediated signaling has recently been

shown to be involved in DC activation and expansion (101). Thus a role for LIGHT in T cell activation may occur directly via signaling through T cell expressed HVEM, or indirectly through the activation of LTβR expressed on DC (87). LTβR signaling is also crucial for the development of secondary lymphoid structures and mice deficient in either LTα or LTβR lack the PLNs, PPs and MLNs (102, 103). In contrast, mice deficient in LTβ only lack PLNs and PPs suggesting that another ligand of LTβR may contribute to the development of secondary lymphoid structures (104). LIGHT, which also binds LTβR could serve as an additional factor involved in the formation of secondary lymphoid structures. Indeed, deletion of both LTβ and LIGHT in mice led to the absence of all secondary lymphoid structures mentioned above, including the MLN (105).

ii) HVEM-LTα. LTα is a secreted, homotrimeric protein that has been shown to weakly bind HVEM *in vitro*. Similar to LIGHT, LTα may provide co-stimulatory signals to T cells by the interaction with HVEM. However, whether binding of LTα to HVEM occurs in vivo and what the exact role in the HVEM pathway is remains unclear (86).

iii) HVEM-BTLA. BTLA is a member of the Ig super family of co-stimuatory molecules and is structurally most similar to the inhibitory B7 members, including cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1). BTLA is predominantly expressed by lymphocytes and myeloid cells, with a particularly high expression on peripheral B cells and induced upon activation on T cells (106, 107). Binding of HVEM with BTLA induces the phosphorylation and association of BTLA with the T cell receptor (TCR) complex and the phosphatases SHP-1 and SHP-2 (108, 109). Although the downstream targets of SHP-1 and SHP-2 are still unknown, they are thought to dephosphorylate signaling molecules of the TCR complex and thereby attenuating T cell proliferation (109). Therefore,

BTLA likely exerts co-inhibitory functions on T cell activation and proliferation. Recent work showed, that HVEM engagement by a BTLA-Ig fusion protein leads to the activation of NFκB and enhances T cell survival following TCR stimulation *in vitro* (110).

iv) HVEM-CD160. CD160 is another Ig super family member that was recently described to interact with HVEM. CD160 is expressed on T lymphocytes, NK T cells and NK cells (111). Similar to BTLA, interaction of CD160 with HVEM functions as a negative regulator of T cell activation (112). However, the exact downstream signaling events mediated through CD160 by HVEM ligation, remain unclear.

Although each of these molecules can bind to HVEM, LIGHT and LTα bind to the CRD2 and CRD3 domains, whereas BTLA, CD160 and HSVgD bind to the CRD1 domain (112, 113). The distinct binding pattern of HVEM ligands may indicate that they compete with each other for receptor binding. This thesis concentrates primarily on the role of LIGHT-HVEM interactions during inflammation and this pathway will be discussed in more detail below.
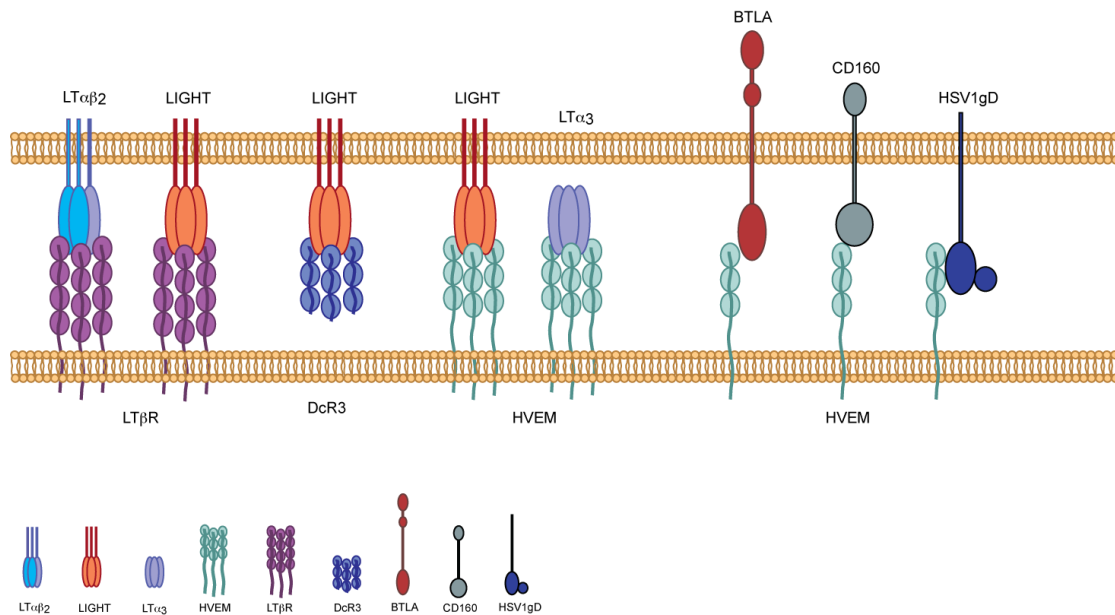
Figure 3: Scheme of the LIGHT and HVEM ligand-receptor interactions with various binding partners.

TNF ligands and receptors tend to assemble as trimers, as indicated, interacting with each other. The interaction of HVEM with BTLA, CD160 and HSVgD however, is monomeric. DcR3 only exists in humans but not in mice, as depicted in the text. The binding of $LT\alpha_3$ to HVEM has so far only been shown *in vitro* and its binding *in vivo* remains to be determined.

Modified from: M. W. Steinberg, *Seminar Immunopathology,* 31: 207-221, 2009 (114).

3.4.2 LIGHT-HVEM interactions promote inflammation.

Interaction of LIGHT with HVEM is known to promote T cell-mediated inflammatory responses by delivering co-stimulatory signals leading to the activation of T cells and subsequent production of pro-inflammatory cytokines (94, 95, 98). In vitro stimulation of lamina propria T cells with recombinant LIGHT rapidly induces IFNγ production presumably via its interaction with the co-stimulatory receptor HVEM (115). LIGHT has also been shown to increase T cell-dependent B cell proliferation and IgG and IgM secretion in human PBMCs (116). Besides a role for the LIGHT-HVEM interaction in T cell and B cell activation, LIGHT and HVEM are also crucially involved in the activation of innate immune cells. LIGHT has been shown to induce the maturation of human DC together with CD40 ligation in an *in vitro* model of DC maturation

(91). Additionally, LIGHT can induce NK cell proliferation and effector functions in a predominantly HVEM-dependent manner (117). Furthermore, LIGHT engagement of HVEM expressed on monocytes and neutrophils enhances their bactericidal activity and the production of effector cytokines (IL-8, TNF), therefore contributing to the killing of bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* (96). In an infection model with *Leishmania major,* LIGHT-HVEM interaction has been proven to be crucial for the clearance of the parasite, by promoting the induction of IL-12 and IFNγ by DCs and CD4$^+$ T cells, respectively (100). However, LIGHT deficient mice display normal immune responses following infection with *Mycobacterium tuberculosis* (118) or *influenza A* (119). A role for LIGHT-HVEM signaling in transplant tolerance was revealed, as in the absence of LIGHT mediated signals mice fail to reject MHC-mismatched cardiac allografts coinciding with decreased intragraft expression of IFNγ (120). Additionally, LIGHT has been shown to exert a prominent role in the activation of host anti-tumor responses. LIGHT stimulates inflammatory responses around the tumor stromal tissue, which leads to the infiltration and activation of T cells and subsequent destruction of the tumor (117). The engagement of LIGHT with HVEM, expressed on tumor cells, was associated with increased Fas expression on the tumor cell and therefore greatly enhances their susceptibility to apoptosis (121). In summary, LIGHT-HVEM interaction seems to be a crucial mediator of innate and adaptive cellular activation and effector function.

3.4.3 Involvement of LIGHT and HVEM interactions in intestinal inflammation

Interest in LIGHT and its binding partners as mediators of intestinal inflammation began to rise when it was observed that transgenic mice overexpressing LIGHT specifically on T cells, spontaneously develop multi-

organ inflammation, with particularly severe inflammation in the intestine. This finding suggested that constitutive or increased expression of LIGHT by T cells, dysregulates intestinal homeostasis by inducing a strong inflammatory response (122, 123). Although different LIGHT transgenic mice were generated, the overall phenotype of these mice was highly consistent with all strains exhibiting strong inflammation in the small and large intestines. Similar to other T cell mediated models of colitis, histopathological analysis revealed a massive influx of mononuclear cells, loss of goblet cells and distortion and hyperplasia of crypts in the LIGHT transgenic mice (123), (122). Interestingly, the highest proportion of T cells expressing LIGHT in the transgenic animals was found in the LP of the small and large intestines correlating with the severe inflammation observed at these sites. LIGHT has also been shown to promote the accumulation of activated T cells in the intestine by specific upregulation of gut-homing molecules such as $\alpha4\beta7$ on the activated T cells together with its binding partner, the vascular adressin MAdCAM-1, on intestinal endothelial cells (103). Further studies demonstrated that LIGHT signaling via HVEM to T cells contributes to intestinal inflammation in a model whereby LIGHT transgenic CD4$^+$CD45RB^{high} T cells are transferred into RAG deficient recipients. This has been demonstrated by the use of LIGHT transgenic and HVEM double deficient CD4$^+$CD45RB^{high} T cells, which were transferred into RAG deficient recipients. The transfer of these cells, which lack the T cell LIGHT receptor, failed to induce intestinal inflammation. In addition when LIGHT transgenic and HVEM double deficient T cells were compared with normal LIGHT transgenic T cells, they showed a decreased expression of pro-inflammatory cytokines and activation markers (124). Therefore, HVEM expression on T cells is needed for their proper expansion, activation and inflammatory response delivered by LIGHT mediated signals. Blockade of LIGHT using LTβR-Ig has also been reported to attenuate intestinal inflammation in the CD4$^+$CD45RB^{high} T cell transfer model (125) or

following TNBS or DSS administration (126, 127). However, these studies did not identify whether LTβR-Ig blockade of LIGHT attenuated disease or whether LT- LTβR interactions were involved.

Intriguingly, the LIGHT gene is contained within a region of the human chromosome 19p13.3, which was identified as a susceptibility locus for IBD (128). Additionally, LIGHT mRNA transcripts are increased in inflamed biopsies taken from IBD patients (115). Thus, LIGHT is both associated with human disease and transgenic over-expression in murine T cells drives intestinal inflammation. However, it remains unclear as to whether LIGHT plays a role in maintaining intestinal homeostasis or whether it is truly necessary for intestinal inflammation under physiological conditions. Nor is it clear whether blockade of LIGHT mediated signals would offer a feasible therapeutic for IBD.

3.5 Aims of this thesis

In recent years, LIGHT has been proposed to be crucially involved in intestinal inflammation and has been associated with human IBD. Since no clear evidence indicated that the LIGHT signaling through its receptor HVEM is promoting intestinal inflammation, we aimed to define the requirement of both TNF super family members using two distinct models of experimental colitis.

For this purpose we utilized novel HVEM or LIGHT deficient mice gifted to us by Anthony J. Coyle. We first investigated whether the absence of LIGHT or HVEM signaling impacted on the development, activation or differentiation of immune cell subsets. We next studied the role of LIGHT or HVEM on intestinal inflammation using a model of T cell-dependent colitis in mice. In our experiments naïve CD4 T cells from wildtype C57BL/6, LIGHT or HVEM deficient mice were purified and transferred into RAG1$^{-/-}$ recipients. This allowed us to specifically investigate the role of LIGHT and HVEM on the expansion, differentiation and effector function of effector T cells *in vivo.* In the second model, we addressed the role of LIGHT or HVEM signaling during intestinal inflammation mediated by innate immune cells in a model of acute dextran sulfate sodium (DSS) administration. In this model intestinal inflammation occurs independently of the presence of T- or B-lymphocytes, and is instead driven largely by pro-inflammatory cytokine secretion by innate immune cells.

3.6 References

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4 Results

4.1 Characterization of HVEM and LIGHT deficient mice

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4.2 Introduction

Members of the tumor necrosis factor (TNF) super family are crucially involved in the regulation of immune responses and maintenance of homeostasis. The recently described TNF super family member LIGHT (TNFSF14) interacts with lymphotoxin β receptor (LTβR) and Herpes virus entry mediator (HVEM) and has been shown to be necessary for cellular activation, proliferation, apoptosis and inflammatory responses [1]. HVEM shows a large tissue distribution and its expression can be detected in almost all organs [2]. HVEM is widely expressed on most hematopoietic cells, including T cells, B cells, DC, natural killer cells (NK), monocytes and on some stromal cells [3]. LIGHT expression is more tightly regulated and predominantly found in spleen, with low expression in lymph nodes and in the intestine [4]. Furthermore, expression of LIGHT and HVEM is regulated on T cells, B cells and DCs. LIGHT is upregulated upon activation of T cells, whereas HVEM expression gets downregulated. Similarly, DCs decrease expression of both LIGHT and HVEM with maturation [5-7]. These findings indicate that a strict regulation of LIGHT-HVEM interaction ensures proper and controlled initiation as well as termination of immune responses, which is crucial for immune homeostasis.

Previous studies have implicated that TNF super family members may play a role in the regulation of thymic negative selection, as many of them are critically involved in the activation and apoptosis of T cells. A recent study showed that LIGHT is involved in thymic negative selection, using TCR transgenic mice, where negative selection was induced upon administration of the antigenic peptide. Blocking of LIGHT signaling by either LTβR-Ig or HVEM-Ig significantly increased the percentage of double positive and CD8$^+$ T cells in the thymus in TCR transgenic mice. LIGHT transgenic mice, which over-express LIGHT in the thymus, have impaired thymus development and

dramatically reduced numbers of thymocytes [8]. Additionally, LIGHT has been shown to increase T cell-dependent B cell proliferation and IgG and IgM secretion in human PBMCs. However, no increase in IgA secretion was observed [9]. Thus, LIGHT is an important co-stimulatory molecule, which acts together with the CD40/CD40L system, inducing T cell-dependent B cell differentiation.

The balance between effector T cells and regulatory T (Treg) cells is essential for the control of immune homeostasis. The disruption of this balance is mainly responsible for the onset of inflammatory diseases. HVEM is expressed on Treg cells and its interaction with LIGHT was shown to support the expansion and function of Treg cells. However, in the presence of high LIGHT expression the Treg cells fail to suppress inflammatory responses in a model of T cell-mediated intestinal inflammation. Thus indicating that LIGHT can promote immune responses even in an environment where regulatory T cells are highly abundant [10].

As LIGHT and HVEM mediated signals may be crucial for the maintenance of immune homeostasis, we first wanted to assess the effect of LIGHT or HVEM gene disruption in our novel mice by investigating T and B cell development, activation and differentiation during immune homeostasis.

4.3 Materials and Methods

4.3.1 Mice

C57BL/6 mice, HVEM$^{-/-}$ [11] mice and LIGHT$^{-/-}$ [12] mice were bred and maintained under specific pathogen-free (SPF) conditions in isolated ventilated cages at Bio-Support (Zürich, Switzerland). HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were backcrossed for 9 generations to C57BL/6 background. In all experiments where mice from different genotypes were used, individual littermates were housed within the same cage or bedding from the cages of male recipients was mixed for 2-3 weeks prior to the experiment. All animal experiments were performed according to guidelines set by the State Veterinary Office of Zürich, Switzerland.

LIGHT$^{-/-}$ and HVEM$^{-/-}$ offspring were genotyped by PCR using the following primers: LIGHT primers; 5'-CAG CAG CAC ATC TTA CAG GAG-3' and 5'-TTG GAG TAC ACA TAG TAG TAA CCG-3'; NEO primers; 5'-TTC TTT TTG TCA AGA CCG ACC T-3' and 5'-CAA GCT CTT CAG CAA TAT CAC G-3'; HVEM primers; T198-F1; 5'-TGT CAG AAA ATT CAA GGT AAG CAC-3', T198-R1; 5'-CTG CCA AGG GCT ACT ATG CTC-3' and T198-IRESR; 5'-TTG CCA AAA GAC GGC AAT ATG-3'.

4.3.2 Cell isolation

Spleen, MLN, PLN and PP single cell suspensions were obtained by mechanical disruption through a 40 μM cell strainer (BD Biosciences). For the detection of cytokines, single lymphocyte suspensions were stimulated in the presence of PMA (10^{-7} M) and Ionomycin (1μg/ml) for 2h and for an additional 2h with Brefeldin A (1μg/ml). Small intestine LPL were isolated as previously described [13]. Briefly, colon tissue was cut into 0.5 cm pieces and incubated at 37°C for 30 min. in PBS containing 0.5% BSA, 2% HEPES, 1% NaPyruvate

and 10mM EDTA to remove epithelial cells. The remaining tissue further digested in complete IMDM medium containing 10% FCS and 1.5 mg Collagenase VIII (Sigma Aldrich) for 20 min. at 37°C and then smashed through a cell strainer.

4.3.3 Flow cytometry

Cells were stained with surface antibodies diluted in PBS with 0.5% BSA (Sigma Aldrich). For intracellular staining, cells were fixed in BD lysis buffer (BD Biosciences), permeabilized using 0.5% Saponin (Sigma Aldrich) in 0.5% BSA/PBS and stained with intracellular antibodies in 0.5% Saponin in 0.5% BSA/PBS. Cells were then analyzed on FACSCalibur (BD Biscience) or Cyan (Dako Cytomation) flow cytometers using FlowJo software (Tree Star). Fluorescently conjugated mAbs directed against CD4 (L3T4), CD8 (YTS156.7.7), NK1.1 (PK136), TCRβ (H57-597), CD62L (MEL-14), CD19 (6D5), CD21 (4E3), CD23 (B3B4), Foxp3 (FJK-16s), IL-17A (TC11), IFNγ (XMG1.2) were purchased from eBiosciences.

4.3.4 Detection of antibody isotypes by ELISA

Serum harvested from naïve C57BL/6, LIGHT$^{-/-}$ and HVEM$^{-/-}$ mice was analyzed for IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. Briefly, 96 well plates were coated with unlabelled goat anti-mouse antibodies to IgG1, IgG2a, IgG2b, IgG3, IgM or IgA (Southern Biotech) in ELISA coating buffer (5.88 g/l NaHCO$_3$, 3.18 g/l Na$_2$CO$_3$ (Fluka) in ddH$_2$O) overnight at 4 °C. Plates were washed with PBS/0.05% Tween®20 (Fluka) and blocked with PBS/1% BSA for 2h at RT. Serum was serially diluted in PBS/0.1% BSA starting with 1:100 dilution. Purified mouse IgG1, IgG2a, IgG2b, IgG3, IgM or IgA (Southern Biotech) were used as standarts and were serially diluted in parallel to the

samples starting at a concentration of 3 μg/ml. Samples and standarts were incubated for 2h at RT, then washed extensively and incubated with alkaline-phosphatase labeled goat anti-mouse antibodies to IgG1, IgG2a, IgG2b, IgG3, IgM or IgA (Southern Biotech) diluted in PBS/0.1% BSA. After 1h incubation at RT, plates were washed extensively and then developed using the substrate p-nitrophenyl phosphate (Sigma-Adrich). OD was measured on an ELISA reader (Bucher Biotech) at 405 nm.

4.3.5 In vitro cultures

Spleen CD4$^+$ T cells were purified using anti-CD4 micro-beads, and separated by positive selection on a magnetic column. 96 well plates were coated with 5 μg/ml of anti-CD3 over night at 4 °C and washed before cells were plated. Cells were cultured for 4 days in the presence of soluble anti-CD28 (1 μg/ml) under Th1 polarizing cytokines IL-12 (1 ng/ml) and anti-IL-4 (5 μg/ml) or under Th2 polarizing cytokine IL-4 (5 ng/ml) and anti- IL-12 (1 μg/ml) or under Th17 polarizing cytokines IL-6 (20 ng/ml) and TGFβ (5 ng/ml). At day 4 cells were harvested and analyzed by flow cytometry.

4.4 Results

4.4.1 Generation of mice genetically deficient in HVEM

To directly investigate the role of LIGHT and HVEM mediated signals in models of intestinal inflammation we used novel mice deficient in LIGHT or HVEM, which were kindly provided by Anthony J. Coyle. Mice deficient in LIGHT were generated by a targeting vector that contained exons 1-4 of LIGHT on a 10.5-kb genomic fragment of the LIGHT gene. A sequence the size of 0.8-kb around exon 1 was deleted and replaced by pMC1 neo [12] (Fig. 1A). For the generation of HVEM deficient mice homologous recombination was used to disrupt exon 3-8 and a 0.8-kb sequence around exon 3 was replaced by pMC1 neo on a 7.8-kb fragment of the HVEM gene (Fig. 2A) [11]. In both cases the targeting vector was linearized and injected into embryonic stem (ES) cells from 129 mice. ES cell clones were then screened for the correct targeted event and chimeric mice were obtained by blastocyte injection. We received offspring of LIGHT$^{-/-}$ and HVEM$^{-/-}$ mice on a mixed background and further backcrossed these mice for 9 generations before the use in our experiments. To verify gene disruption, LIGHT$^{-/-}$ and HVEM$^{-/-}$ offspring were tested by routine PCR (Fig. 1B and Fig. 2C). HVEM expression was further examined by CD4$^+$ and CD8$^+$ T cells derived from the spleen, which are known to express greater levels of surface protein than non-T cells [14]. HVEM surface protein expression was absent in CD4$^+$ and CD8$^+$ T cells isolated from HVEM$^{-/-}$ mice (Fig. 2B). Mice homozygous for the mutated genes (Fig. 1B and Fig. 2C) were normal in appearance and growth, and did not exhibit any gross defects in the organogenesis of lymphoid tissues. The generation of these mice, has since been published by Wayne W. Hancock [11, 12].

A)

B)

Figure 1. Generation of LIGHT^{-/-} mice. (A) Genomic organization of the murine LIGHT locus and the mutation induced by a targeting event. A targeting construct was generated to replace exon 1 of the LIGHT gene, containing thee initiating methionins (ATG). Exon 1 of the LIGHT gene was replaced by the neomycin resistance gene (NEO). (B) C57BL/6 (+/+), heterogeneous littermates (+/-) and LIGHT^{-/-} offspring mice (-/-) were genotyped using PCR for the LIGHT gene or the neomycin resistance gene.
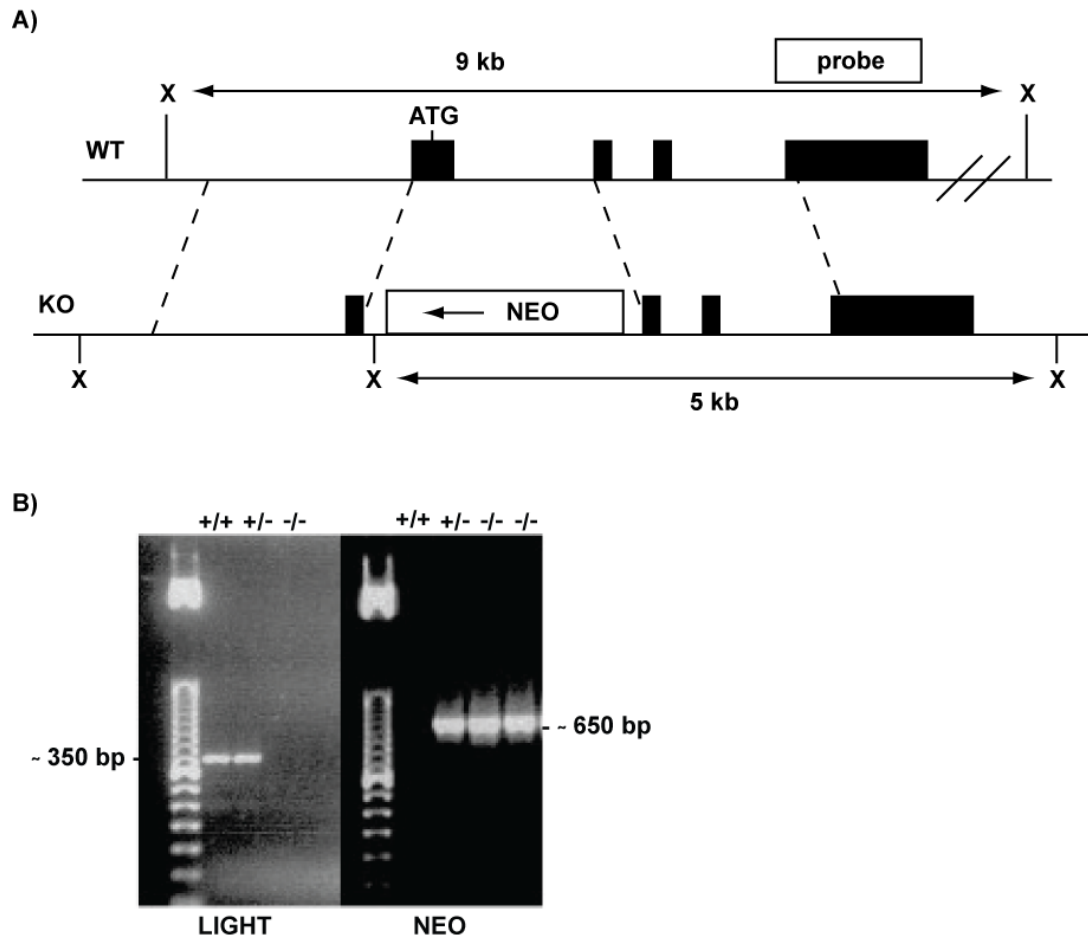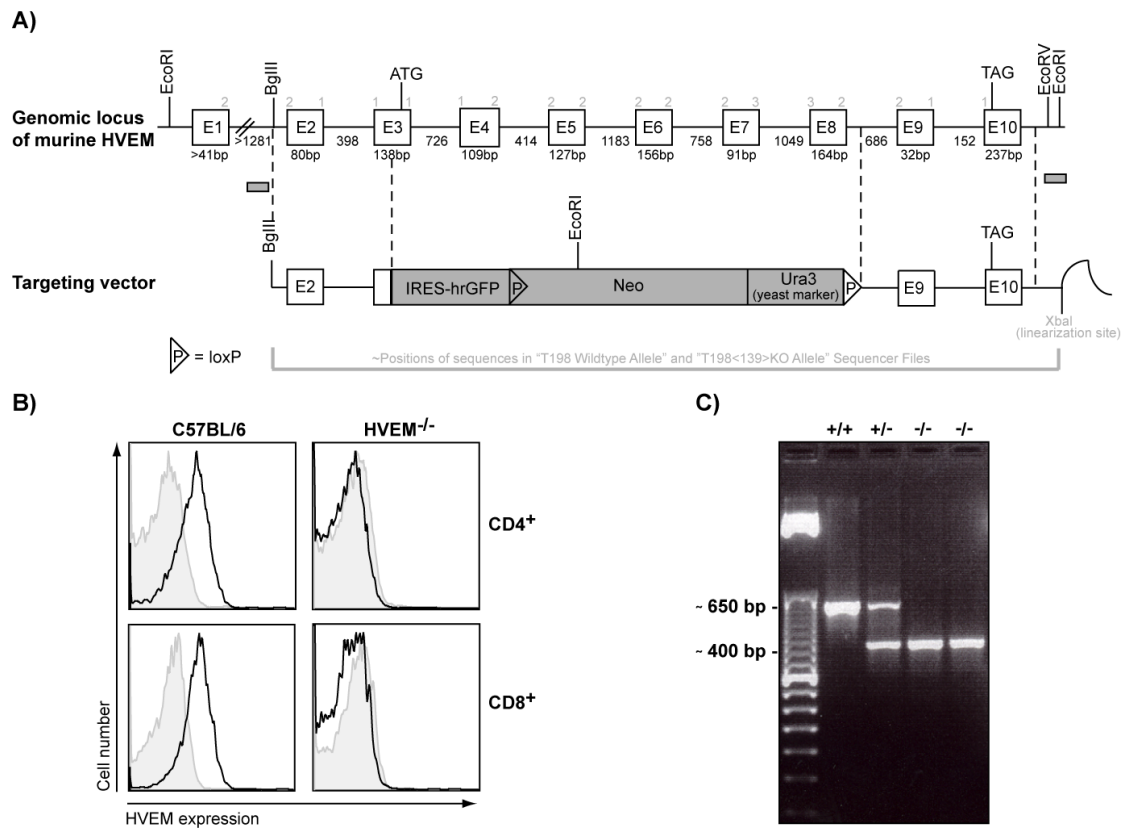
Figure 2. Generation of HVEM$^{-/-}$ mice. (A) Genomic organization of the murine HVEM locus and the mutation induced by a targeting event. Exons are shown as numbered boxes (E 1-10). An HVEM gene-targeting construct was generated to replace exon 3 with the neomycin resistance gene (NEO). (B) Whole spleen lymphocytes were stained with CD4, CD8 and HVEM antibodies and analyzed by flow cytometry. Representative histogram plots show expression of HVEM by CD4$^+$ or CD8$^+$ T cells in C57BL/6 wild type mice and generated HVEM$^{-/-}$ mice. (C) C57BL/6 (+/+), heterogeneous littermates (+/-) and HVEM$^{-/-}$ offspring mice (-/-) were genotyped using PCR.

4.4.2 LIGHT and HVEM deficiency does not grossly affect immune cell development or humoral immunity

We next investigated the impact of LIGHT or HVEM deficiency on the accumulation of mature T cells, NK cells and NKT cells in the spleen, MLN, PLN, and PP. Normal frequencies of $CD4^+$ T cells, $CD8^+$ T cells, NK cell and NKT cells were observed in naïve LIGHT and HVEM deficient mice for all organs examined (Fig. 3A-D). The activation status of T cells in $LIGHT^{-/-}$ and $HVEM^{-/-}$ mice, determined by the expression level of CD62L on $CD4^+$ and $CD8^+$ T cells, was comparable to C57BL/6 mice (Fig. 3E+F). To determine whether LIGHT or HVEM deficiency affects B cell differentiation, we additionally investigated B cell populations in the spleen, MLN, PLN and PP. Similar frequencies of $CD19^+$ cells (Fig. 4A), T1-3 B cells (Fig 4B), follicular B cells (Fig. 4C) and marginal zone B cells (Fig. 4D) were found in $LIGHT^{-/-}$ and $HVEM^{-/-}$ mice as compared to C57BL/6 mice. Furthermore, total serum antibody titers of IgG1, IgG2a, IgG2b, IgG3, IgM and IgA were comparable for all genotypes (Fig. 5A-F). These data indicate that in our LIGHT and HVEM gene deficient mice T cell and B cell differentiation is not grossly affected.
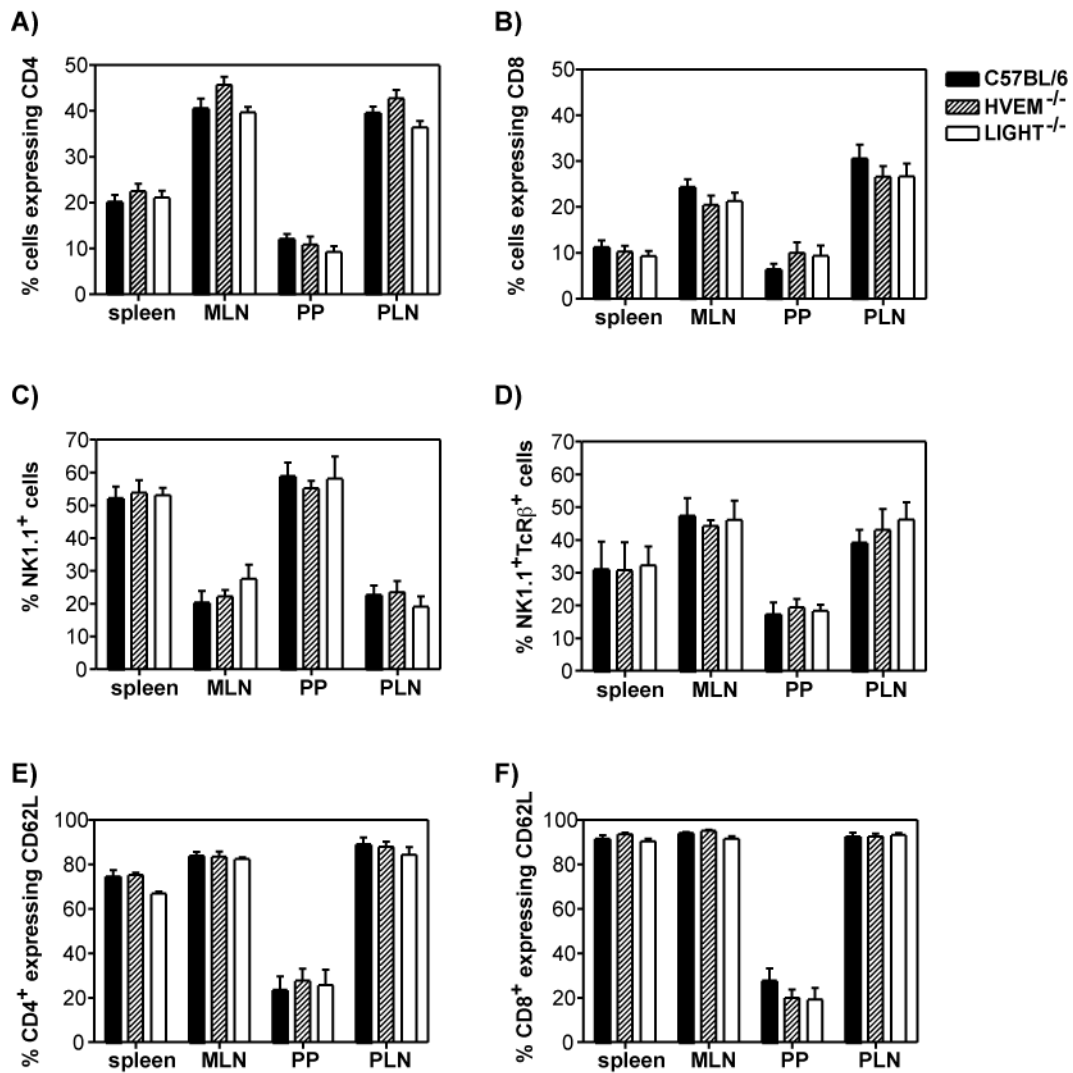
Figure 3. HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice exhibit normal frequencies of CD4$^+$, CD8$^+$, NK T cells and NK cells. Cells from spleen, MLN, Peyer's patches (PP) and peripheral lymph nodes (PLN) of age-matched naïve C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were isolated and distinct cell subsets identified by flow cytometry as (A) CD4$^+$ T cells, (B) CD8$^+$ T cells, (C) NK1.1$^+$cells, (D) NK1.1$^+$ TCRβ^+ T cells. (E) Naïve CD4$^+$ CD6L^{high} T cells or (F) naive CD8$^+$ CD62L^{high} T cells. Results represent mean surface expression ± SD (n = 7 total mice per group).
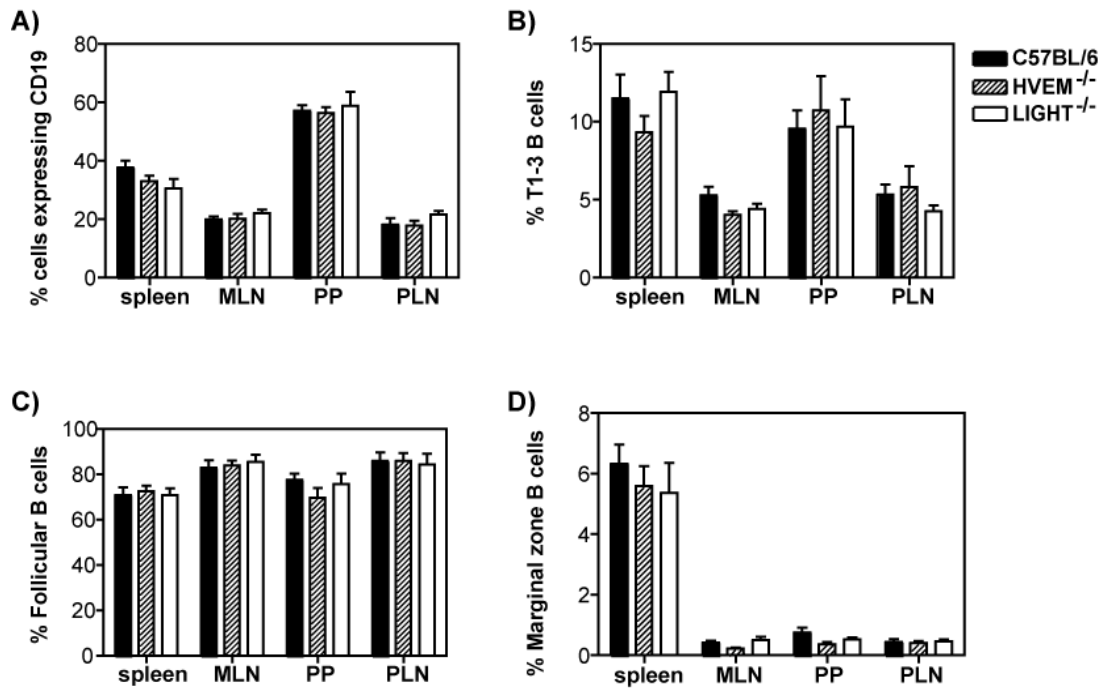
Figure 4. HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice exhibit normal proportions of transitional, follicular and marginal zone B cell subsets. Cells from spleen, MLN, Peyer's patches (PP) and peripheral lymph nodes (PLN) of age-matched naïve C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were isolated and distinct cell subsets identified by flow cytometry. (A) The proportion of total cells that are CD19$^+$ B cells. (B-D) Cells were gated for CD19 expression and analyzed according to their different levels of CD21 and CD23 surface expression; (B) Transient B cells (CD21^{low}CD23^{low}), (C) follicular B cells (CD21^{medium}CD23^{high}) and (D) marginal zone B cells (CD21^{high}CD23^{medium}). Results represent mean surface expression ± SD (n = 7 total mice per group).

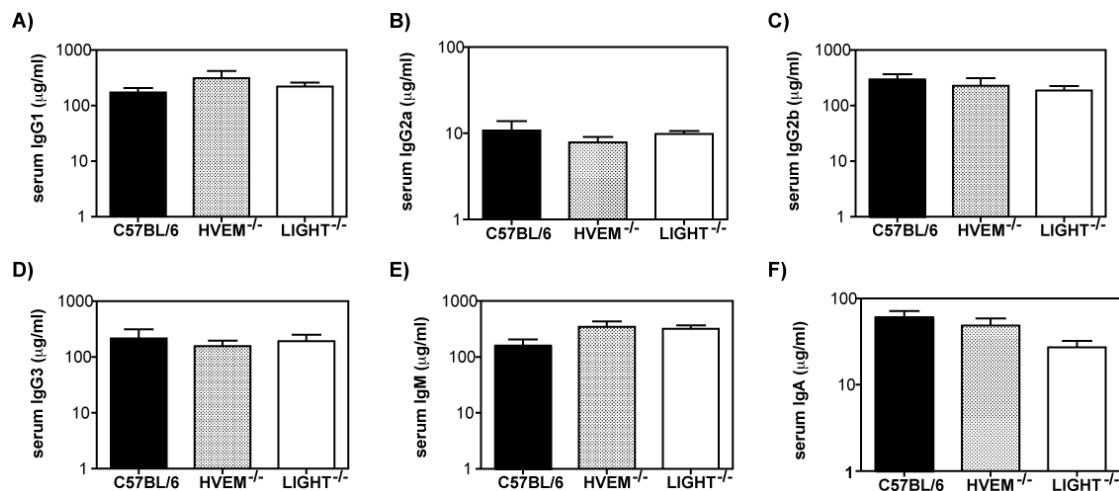**Figure 5. HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice exhibit normal serum antibody titers.** Antibody titers were assayed for the serum of age-matched naïve C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice using standard ELISA. Serum antibody titers are shown for (A) IgG1, (B) IgG2a, (C) IgG2b, (D) IgG3, (E) IgM and (F) IgA. Results represent means ± SD (n = 7 total mice per group).

4.4.3 LIGHT signaling affects T helper cell differentiation *in vivo* but not under optimal *in vitro* conditions

To investigate the contribution of LIGHT and HVEM to T cell differentiation and effector function we examined CD4$^+$ T cell subsets (Treg, Th1 and Th17) within various lymphoid tissues of naïve LIGHT and HVEM deficient mice. Analysis of the CD4$^+$ T cell compartments in spleen, PLN and MLN revealed no significant differences between the frequency of Foxp3$^+$ regulatory T cells or effector T cells producing IFNγ in LIGHT$^{-/-}$ compared to wildtype C57BL/6 mice (Fig. 6A+C). There were also no differences observed for IL-17A producing LIGHT$^{-/-}$ CD4$^+$ T cells, although the frequency of cells producing this cytokine was extremely low (Fig. 6B). In contrast, we detected a significantly increased frequency of Foxp3$^+$ CD4$^+$ T cells and a decreased frequency of IL-17A$^+$ CD4$^+$ T cells in the PP of LIGHT$^{-/-}$ mice (Fig. 6A+B). Expression of IFNγ was comparable between the two sets of mice (Fig. 6C). In contrast, HVEM$^{-/-}$ exhibited significantly increased Foxp3 expressing CD4$^+$ T cells not only in the PP but also in spleen, MLN and PLN (Fig. 7A). CD4$^+$IL-

17A$^+$ T cells in the spleen, MLN and PP of HVEM$^{-/-}$ showed similar frequencies, whereas their proportion was significantly reduced in the PLN (Fig. 7B). No differences in the various lymphoid tissues were observed for T cells producing IFNγ between C57BL/6 and HVEM$^{-/-}$ mice (Fig. 7C).

We next examined the lamina propria (LP) of the small intestine and observed an increased frequency of Foxp$^+$CD4$^+$ T cells, and a reduced frequency of IL-17A and IFNγ producing CD4$^+$ T cells in LIGHT$^{-/-}$ mice (Fig. 6 D-F). Interestingly, no differences could be seen for Foxp3$^+$ CD4$^+$ T cells, or for IL-17A and IFNγ producing CD4$^+$ T cells in HVEM$^{-/-}$ mice (Fig. 7D-F). These data may suggest a role for LIGHT stimulatory signals in regulating the homeostatic differentiation and/or maintenance of effector versus regulatory T cells within the intestine and its associated lymphoid tissues. However, further investigation is needed to evaluate whether LIGHT signaling via receptors other than HVEM, such as LTβR, may account for these effects.

We additionally investigated whether LIGHT$^{-/-}$ or HVEM$^{-/-}$ T cells have a general defect in CD4 T helper cell subset differentiation following *in vitro* stimulation in the presence of exogenous cytokines known to support Th1, Th2 or Th17 differentiation. Under saturating levels of the cytokine IL-12 and by blocking IL-4, LIGHT$^{-/-}$ and HVEM$^{-/-}$ CD4$^+$ T cells similarly expressed the characteristic Th1 cytokine IFNγ and low expression of IL-4 (Fig. 8A). Th2 cytokines IL-4, IL-5 and IL-10 were comparable between C57BL/6 and LIGHT$^{-/-}$ and HVEM$^{-/-}$ CD4$^+$ T cells following culture of cells with exogenous IL-4 and blocking IL-12 (Fig. 8C), and in contrast to the *ex vivo* data no differences in IL-17A production between LIGHT$^{-/-}$, HVEM$^{-/-}$ or C57BL/6 CD4$^+$ T cells could be noted in cultures containing IL-6 (Fig. 8B).
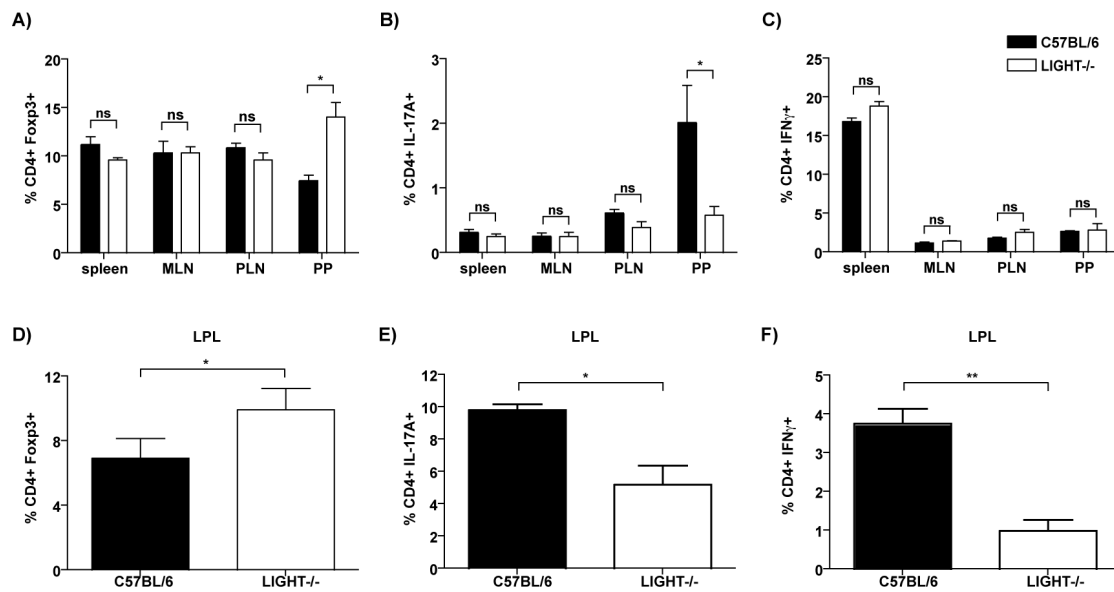
Figure 6. LIGHT$^{-/-}$ mice show altered intestinal T cell homeostasis. Whole lymphocyte suspensions were obtained from spleen, MLN, PLN and PP. LP lymphocytes were isolated as described in Materials and Methods. Lymphocyte suspensions from C57BL/6 and LIGHT$^{-/-}$ mice were either directly stained with labeled antibodies, or staining was performed after restimulation with PMA/Ionomycin. Stained whole lymphocyte suspensions were analyzed by flow cytometry to determine the frequency of (A, D) CD4$^+$Foxp3$^+$, (B, E) CD4$^+$IL-17A$^+$ and (C, F) CD4$^+$IFNγ$^+$ T cells in the indicated organs. Means ± SD are shown for one representative experiment (n = 3 total mice per group), of at least three repeats. Statistical differences between groups were calculated using an unpaired Student *t* test: $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.
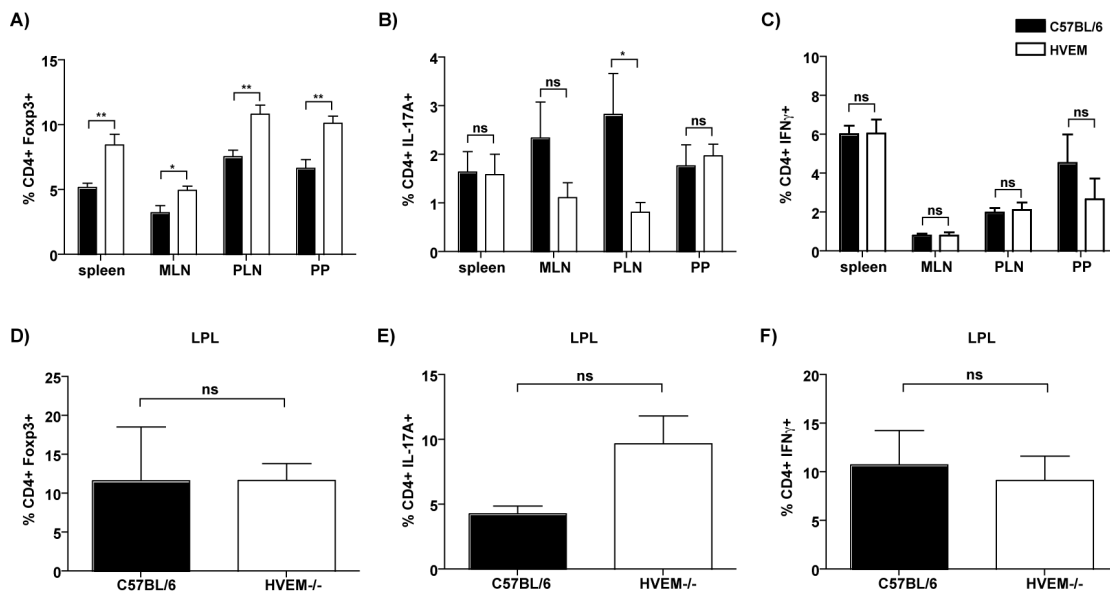
Figure 7. HVEM$^{-/-}$ mice show increased frequency of Foxp3^{+} regulatory T cells in peripheral organs. Whole lymphocyte suspensions were obtained from spleen, MLN, PLN and PP. LP lymphocytes were isolated as described in Materials and Methods. Lymphocyte suspensions from C57BL/6 and HVEM$^{-/-}$ mice were either directly stained with labeled antibodies, or staining was performed after restimulation with PMA/Ionomycin. Stained whole lymphocyte suspensions were analyzed by flow cytometry to determine the frequency of (A, D) CD4^{+}Foxp3^{+}, (B, E) CD4^{+}IL-17A^{+} and (C, F) CD4^{+}IFNγ^{+} T cells in the indicated organs. Means ± SD are shown for two representative experiments (n = 8 total mice per group). Statistical differences between groups were calculated using an unpaired Student *t* test: $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.
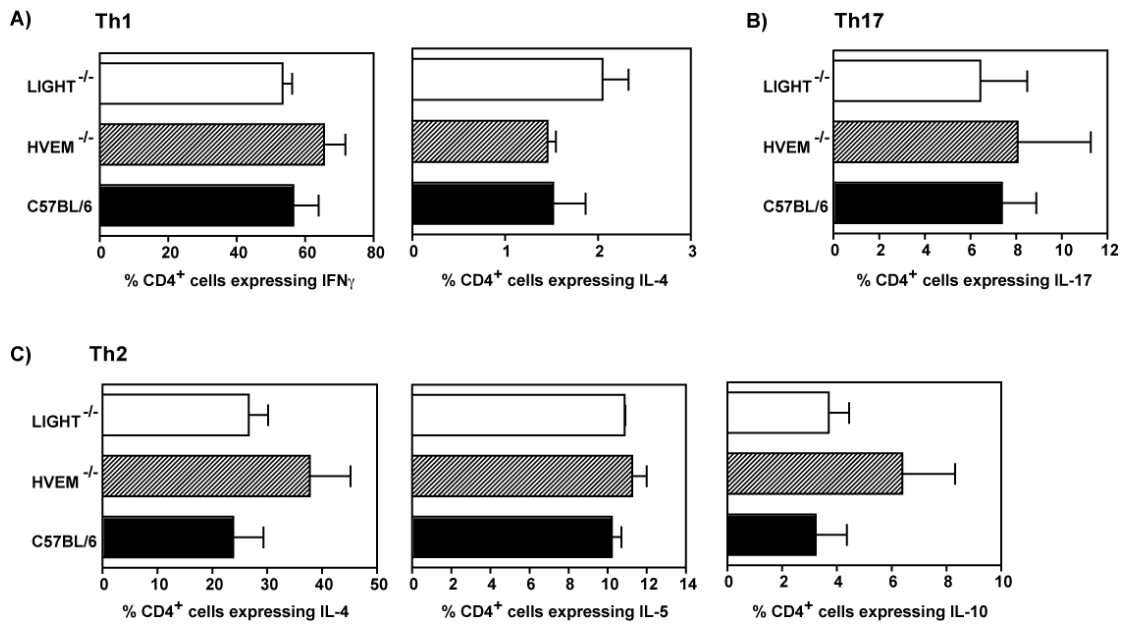
Figure 8. Similar degree of Th1, Th2 and Th17 cytokine production by HVEM^{-/-} and LIGHT^{-/-} CD4^{+} T cells under optimal polarizing conditions. MACS purified CD4+ T cells were cultured for 4 days in vitro with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml) under Th1, Th2 and Th17 polarizing conditions. (A) Percentage of IFNγ+ and IL-4+ CD4+ T cells after stimulation with polarizing cytokine IL-12 (1 ng/ml) and anti-IL-4 (5 μg/ml). (B) Percentage of IL-17A producing CD4+ T cells after IL-6 (20 ng/ml) and TGFβ (5 ng/ml) stimulation. (C) CD4+ T cells producing IL-4, IL-5 and IL-10 after stimulation with IL-4 (5 ng/ml) and blockage of IL-12. Data are shown as means ± SD of triplicate wells from one out of two independent experiments. No statistically significant differences were observed.

4.5 Discussion

Following the characterization of immune cell development and differentiation in naive mice deficient for LIGHT or HVEM-mediated signals we observed that these mice exhibit largely normal T and B cell development and antibody production. We also demonstrated that T cells from either genotype are able to differentiate normally into Th1, Th2 or Th17 cell subsets following *in vitro* stimulation using polarizing exogenous cytokines. In contrast to these *in vitro* findings, T cells isolated from the small intestine lamina propria and PPs of LIGHT deficient mice exhibited an increased ratio of Foxp3$^+$ CD4$^+$ T cells to IL-17A$^+$ CD4$^+$ T cells. Interestingly however, HVEM$^{-/-}$ mice exhibited increased Foxp3$^+$ CD4$^+$ T cells in almost all lymphoid organs except the small intestine and did not show an altered IL-17A$^+$ CD4$^+$ T cell population. Thus, the effector:Treg ratio in these two strains of mice differs indicating that their interactions with other cognate partners likely play a role in immune cell homeostasis. It should be noted however that studies of the gene impact on intestinal T cell function in naïve mice are extremely difficult and a great deal of variability was noted between experiments regarding the total numbers of IL-17A producing cells detected in the intestinal lamina propria and lymphoid organs. We made the observation that a different flora, depending on the animal facility where the mice were housed, impacts on the differentiation of the Foxp3$^+$ CD4$^+$ T cells and IL-17A$^+$ CD4$^+$ T cells in the intestine. Moving the mice from the animal facility at ETH in Zürich to the animal facility at EPF in Lausanne, affected the increased Foxp3$^+$ CD4$^+$ T cells to IL-17A$^+$ CD4$^+$ T ratio in LIGHT$^{-/-}$ mice. Even tough we still saw an increased Treg to Th17 ratio in LIGHT$^{-/-}$ mice, this was less pronounced after moving the mice to the animal facility at EPF in Lausanne. It has been previously shown, that the source and housing of mice may greatly affect the Treg:Th17 cell balance in the mucosa of the small intestine, depending on the composition of the intestinal microbiota [15].

By contrast, great care was taken to homogenize the flora of gene deficient mice and wildtype control mice prior to analysis and the differences noted between mice of different genotypes was relatively stable.

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4.7 HVEM stimulatory signals to CD4^{+} T cells promote experimental colitis

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4.8 Abstract

Stimulatory signals to the recently identified TNFRSF member HVEM have been shown to result in T cell proliferation and IFN-γ production. We investigated the role of HVEM signalling to T cells in an experimental model of T cell transfer induced colitis. HVEM$^{-/-}$ CD4$^+$CD45RB^{high} T cells expanded normally for the first few weeks following their transfer into lymphopenic hosts, but exhibited reduced proliferation and effector cytokine production at weeks 6-8 post-transfer, and failed to mediate colits. Defective expansion and effector cytokine production by HVEM$^{-/-}$ CD4$^+$ T cells could not be overcome by the provision of an inflammatory environment through the co-transfer of C57BL/6 CD4$^+$ T cells, and was mediated in part through T cell expressed LIGHT. Decreased effector function correlated with reduced expression of IL-6R and IL-23R, but not IL-21R. Reduced IL-6R surface expression was also apparent on HVEM$^{-/-}$ CD4$^+$ T cells from naïve mice and was demonstrated to play a role in LIGHT-HVEM mediated T cell proliferation *in vitro*. Taken together these data suggest that HVEM is necessary for the proper expansion and effector function of pathogenic CD4$^+$ T cells, possibly resulting from its role in maintaining IL-6R expression.

4.9 Introduction

Members of the tumor necrosis factor superfamily (TNFSF) and tumor necrosis factor receptor superfamily (TNFRSF) play a central role in the regulation of immune responses by providing signals involved in differentiation, activation, survival and homeostasis of immune cells (1). A recently described member of the TNFRSF, HVEM (Herpes virus entry mediator/TNFRSF14) can promote T cell proliferation and IFN-γ production (2, 3), and has been linked to IFN-γ production by human mucosal T cells (4). HVEM has a widespread expression, being present on most hematopoietic cells in addition to some stromal and epithelial cells (5, 6). HVEM has multiple ligands, however LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D (gD) for HVEM/TNFSF14) is thought to be the predominant ligand delivering stimulatory signals *in vivo* (7). LIGHT is expressed by activated T cells, immature DC and monocytes (8), and binds to both HVEM and lymphotoxin β receptor (LTβR) which is present on stromal cells and some hematopoietic cells including DC and monocytes (7). LIGHT-deficient mice exhibit defective T cell proliferation and activation *in vitro* (8), and fail to reject MHC-mismatched cardiac allografts coinciding with decreased intragraft expression of IFN-γ (9). However, LIGHT-deficient mice display normal immune responses following infection with *Mycobacterium tuberculosis* (10) or influenza A (11), suggesting that LIGHT may regulate some T cell responses whilst being superfluous for others.

LIGHT is contained within a region of the human chromosome 19p13.3 identified as a susceptibility locus for inflammatory bowel disease (IBD) (12), and LIGHT mRNA transcripts are over-expressed in inflamed biopsies from IBD patients (13). In an experimental model of IBD, transgenic over-expression of LIGHT on T cells resulted in a lymphoid proliferative disorder, widespread autoimmune disease and development of severe intestinal inflammation (14). Intestinal inflammation driven by transgenic over-

expression of LIGHT was found to involve signalling to both HVEM expressed by T cells and LTβR expressed by stromal cells (15). Collectively, these data implicate, but do not prove, a role for LIGHT-HVEM stimulatory interactions in promoting the expansion and function of pathogenic T cells mediating colitis.

In the current study we compared the ability of wildtype C57BL/6, HVEM^{-/-} or LIGHT^{-/-} CD4^{+}CD45RB^{high} T cells to mediate experimental colitis following their transfer into immuno-deficient RAG1^{-/-} hosts. In this model transferred CD4^{+} T cells undergo homeostatic and spontaneous proliferation within the spleen and draining lymphoid organs and these cells eventually become activated to produce Th1 and Th17 cytokines (16). Activated CD4^{+} T cells are then recruited to the large intestine where they promote an inflammatory response characterized by leukocyte infiltration, epithelial cell hyperplasia, mucin depletion from goblet cells and, in severe cases, crypt abscess formation and epithelial erosion (17). T cell transfer-induced intestinal inflammation has been shown to require intestinal bacteria (18), is dependent on production of pro-inflammatory cytokines such as IL-6 (19-21), IL-23 (22, 23), and can be prevented by co-transfer of Foxp3^{+} regulatory T cells (24). Although the exact contribution of Th1 or Th17 cells to disease remains unclear IL-17A itself is not necessary for disease (23, 25) whilst IFN-γ (26), and the Th1 cell-specific transcription factor T-bet are required (27).

In the current study we reveal a role for HVEM mediated stimulatory signalling to CD4^{+} T cells in promoting intestinal inflammation. These signals could be delivered, at least in part, through T cell expressed LIGHT and were crucial for the maintenance and inflammatory function of pathogenic intestinal T cells. We further demonstrated that attenuated inflammation in the absence of HVEM stimulatory signals correlated with a failure of CD4^{+} T cells to produce IL-17, reduced production of IFN-γ, and a reduced expression of IL-6 and IL-23 cytokine receptors.

4.10 Materials and Methods

4.10.1 Mice

C57BL/6 mice, HVEM^{-/-} (28) mice and LIGHT^{-/-} (9) mice were bred and maintained under specific pathogen-free (SPF) conditions at Bio-Support (Zürich, Switzerland). HVEM^{-/-} and LIGHT^{-/-} mice were backcrossed for 9 generations to C57BL/6 background. RAG1^{-/-} (C57BL/6) mice were purchased from the Institute for Laboratory Animal Science, University of Zürich. Congenic CD45.1-allelic C57BL/6 mice were purchased from Jackson Laboratory. All mice used in this study were 5-8 weeks old. All animal experiments were performed according to guidelines set by the State Veterinary Office of Zürich, Switzerland. In all experiments where mice from different genotypes were injected into RAG1^{-/-} recipient's efforts were made to ensure that more than one genotype was injected into individual littermates housed within the same cage or that bedding from the cages of male recipients was mixed for 2-3 weeks prior to the experiment then throughout the duration of the experiment.

4.10.2 Experimental colitis induced by CD4^{+} T cells

T cell-mediated colitis was induced by transferring 4×10^5 CD4^{+}CD25^{-} CD45RB^{high} T cells into RAG1^{-/-} mice. Cells were isolated from spleen cell preparations were labelled with anti-CD4 micro-beads and separated by positive selection on a magnetic column, according to the manufacturer's instructions (Miltenyi Biotech). Purified CD4^{+} T cells were then labelled with CD4, CD25 and CD45RB antibodies and sorted for CD4^{+}CD25^{-}CD45RB^{high} population on a FACS Vantage (BD Biosciences). Purity of CD4^{+}CD25^{-} CD45RB^{high} cells was ≥ 98%. Mice were sacrificed at 14 days, 28 days, or 6-8 weeks post transfer. The colon was removed, cut into proximal, middle and distal sections fixed in 10% formalin. All, three segments of the colon were

processed by embedding into paraffin, sectioning and staining with H&E. Those areas most affected by inflammation were further selected for histopathological grading as detailed below.

4.10.3 Histological assessment of colitis

Colonic specimens obtained as described, were scored by blind microscopic assessment of mucosal lesions. Histological scoring of colonic sections from the T cell transfer-induced colitis model was determined according to the following parameters. An estimated score was made based on first impression (score from 0-3), loss of goblet cells (score from 0-4), crypt abscesses (score from 0-3), mucosal thickness (score from 0-3), cellular infiltration (score from 0-3) and epithelial erosions (score from 0-2). The scores for each individual parameter were then added together to give a total score between 0 and 18.

4.10.4 Cell isolation and Flow cytometry

MLN single cell suspensions were obtained by mechanical disruption through a 40 μM cell strainer (BD Biosciences). Colon LPL were isolated as previously described (29). Briefly, colon tissue was cut into 0.5 cm pieces and incubated at 37°C for 30 min. in PBS containing 0.5% BSA, 2% HEPES, 1% NaPyruvate and 10mM EDTA to remove epithelial cells. The remaining tissue further digested in complete IMDM medium containing 10% FCS and 1.5 mg Collagenase VIII (Sigma Aldrich) for 20 min. at 37°C and then smashed through a cell strainer. Cells were stained with surface antibodies diluted in PBS with 0.5% BSA (Sigma Aldrich). For intracellular staining cells were fixed in BD lysis buffer (BD Biosciences), permeabilized using 0.5% Saponin (Sigma Aldrich) in 0.5% BSA/PBS and stained with intracellular antibodies in

0.5% Saponin in 0.5% BSA/PBS. For the analysis of cytokine production by intracellular staining cells were first stimulated with PMA (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) for 4 h at 37°C in IMDM medium plus 5% FCS. For the final two hours, Brefeldin A (10 μg/ml) was added to the cultures to retain cytokines in the cytoplasm. Stained cells were analyzed on FACS Calibur (BD Bioscience) or Cyan (Dako Cytomation) flow cytometers using FlowJo software (Tree Star). Fluorescently conjugated mAbs directed against CD4 (L3T4), CD25 (PC61), CD45RB (C363-16A), CD45.1 (A20), Ki-67 (MOPC-21), IL-6R (D7715A7), IL-17A (TC11), IFNγ (XMG1.2) and Foxp3 (FJK-16s) were purchased from eBiosciences.

4.10.5 Detection of cytokine mRNA expression by quantitative RT-PCR

Total RNA was isolated from MLN or colon using TRI Reagent (Molecular Research Center, Inc.) and reverse transcribed using Superscript III RT kit (Invitrogen). Transcribed cDNA was used as a template for the PCR reaction. Real-time RT-PCR was performed using Brilliant SYBR Green (Stratagene) and an iCycler (Bio-Rad Laboratories). Expression was normalized according to expression of the housekeeping gene β-Actin. Sequences of primers used: IL-6; 5'-TTC CAT CCA GTT GCC TTC TTG-3' and 5'-TCA TTT CCA CGA TTT CCC AGA G-3', IL-12p40; 5'-TAC AGT TCA GGC GCC GGA T-3' and 5'-AGA GTT AAC CTG AGG TCC GCA-3', TNFα; 5-GAA CTG GCA GAA GAG GCA CT-3' and 5'-AGG GTC TGG GCC ATA GAA CT-3', IL-21R; 5'-TCT GGA CCA TCA CCT GTG TC-3'and 5'-TTG TGG CCA GAC CTG TGT AG-3', IL23R; 5'-GCC AAG AAG ACC ATT CCC GA-3' and 5'-TCA GTG CTA CAA TCT TCT TCA GAG GAC A-3' and IL-6R; 5'-AAG AGT GAC TTC CAG GTG CC-3' and 5'-GGT ATC GAA GCT GGA ACT GC -3'.

4.10.6 In vitro cultures

Spleen CD4$^+$ spleen cells were purified using anti-CD4 micro-beads, and separated by positive selection on a magnetic column. 96 well flat bottom plates were coated with 100 ng/ml or 500 ng/ml anti-CD3 (2C11) over night at 4° C and washed with PBS before cells were plated. Purified CD4$^+$ T cells were plated out at a concentration of 2.5 × 10^5 per well in complete IMDM. Cells were stimulated for 48 h with 1:4 diluted Fc-human recombinant LIGHT (Fc-rhLIGHT) containing supernatant (between 1-10 µg/ml) prepared as described (30) in the presence or absence of blocking anti-IL-6R (CD126) at a concentration of 10 µg/ml.

4.10.7 Statistical Analysis

A two-tailed unpaired Student's *t* test with a confidence interval of 95% was performed on all data and are shown as p-values p<0.05 (*), p<0.005 (**), or p<0.0005 (***).

4.11 Results

4.11.1 HVEM stimulatory signals regulate T cell-mediated colitis.

To determine the role of HVEM-mediated signaling to CD4$^+$ T cells during intestinal inflammation we used an experimental model of colitis involving the transfer of CD4$^+$CD45RB^{high} T cells into immuno-deficient hosts. CD4$^+$CD45RB^{high} T cells were purified from the spleens of HVEM$^{-/-}$ or wildtype C57BL/6 mice and 4 x 10^5 cells injected intravenously into age and sex-matched RAG1$^{-/-}$ recipients. RAG1$^{-/-}$ recipients receiving CD4$^+$CD45RB^{high} T cells from C57BL/6 mice exhibited weight loss and intestinal inflammation commencing between day 30 and 40 after transfer (Fig. 1A-C). In contrast recipients of HVEM$^{-/-}$ CD4$^+$CD45RB^{high} T cells exhibited a clear resistance to weight loss (Fig. 1A) and reduced intestinal inflammation (Fig. 1B and C). Since T cells are known to be a major source of the HVEM stimulatory ligand LIGHT (31), we additionally investigated the role of T cell-expressed LIGHT in promoting intestinal inflammation. Transfer of CD4$^+$CD45RB^{high} T cells from LIGHT$^{-/-}$ mice into RAG1$^{-/-}$ recipients did not impact on weight loss (Fig. 1A) but did reduce intestinal inflammation (Fig. 1B and C). Attenuated intestinal inflammation observed following transfer of LIGHT$^{-/-}$ T cells was significant when compared to mice receiving C57BL/6 T cells, but was not as dramatic as that observed for mice receiving HVEM$^{-/-}$ T cells, indicating that additional sources of HVEM stimulatory ligands exist *in vivo*. Additional sources of LIGHT are likely to be present in the RAG1$^{-/-}$ recipients in the form of resident innate cells (DC, NK cells, monocytes). Alternatively, CD4$^+$ T cells may be able to receive HVEM stimulatory signals from other ligands such as LTα3.
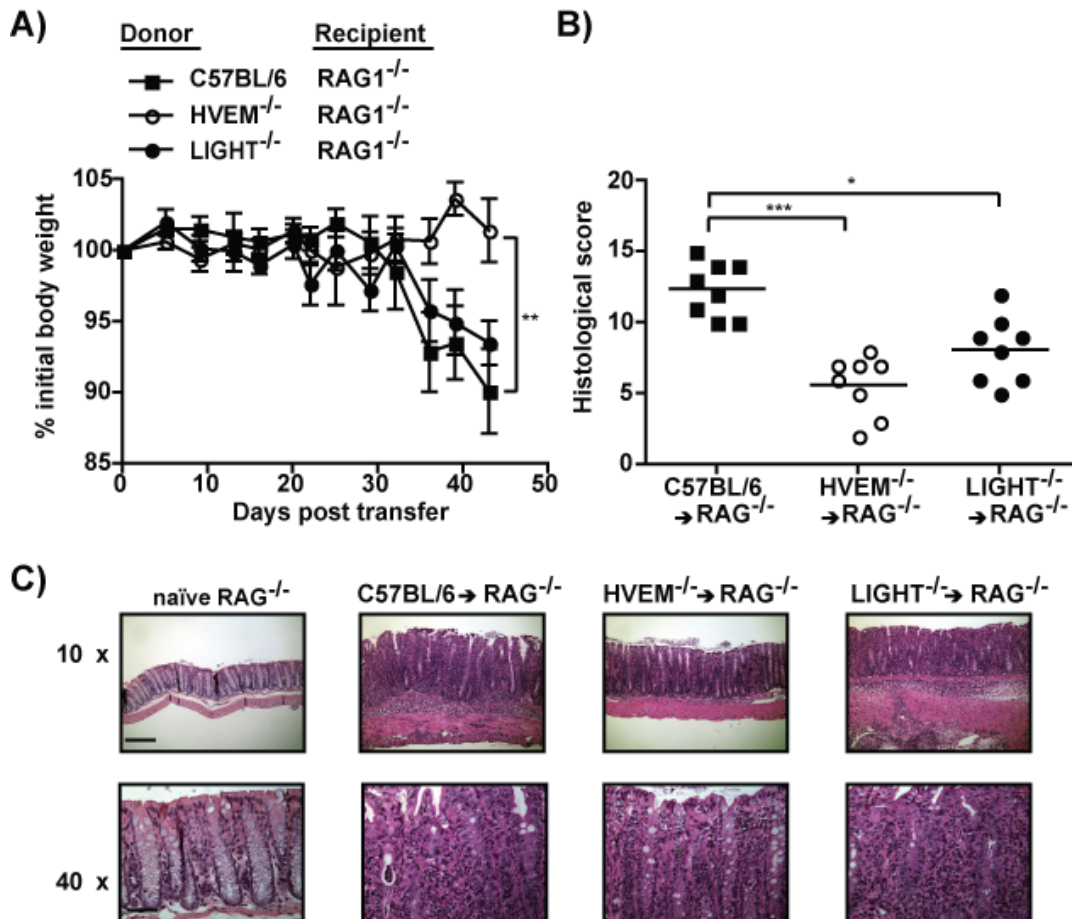
Figure 1. HVEM expression by CD4$^+$ T cells is required for T cell-mediated colitis. 4×10^5 CD4$^+$CD25$^-$CD45RB^{high} T cells from C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ mice were injected intravenously into RAG1$^{-/-}$ hosts and recipients sacrificed 6-8 weeks later. (A) Body weight was monitored regularly and is expressed as percentage change from initial body weight at day 0 for C57BL/6 (closed squares), HVEM$^{-/-}$ (open circles) or LIGHT$^{-/-}$ (closed circles) T cells transferred into RAG1$^{-/-}$ mice (n = 8 mice per group). (B) Histological scores indicating immuno-pathology were calculated as described in the Materials and Methods section and are shown for the middle part of the colon. Symbols represent individual RAG1$^{-/-}$ animals receiving either C57BL/6 (closed squares), HVEM$^{-/-}$ (open circles) or LIGHT$^{-/-}$ (closed circles) CD4$^+$CD25$^-$CD45RB^{high} T cells (n = 8 mice per group). Data from A and B represents means ± SD of two pooled experiments (n = 8 mice per group) out of four independent experiments. Statistically significant differences between groups were assessed by a two tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005. (C) Representative H&E staining of middle colon tissue-sections from control RAG1$^{-/-}$ mice, and mice receiving either C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{high} T cells. Scale bars are 10× magnification = 200μm and 40× magnification = 0.05μm.

To validate the reduced intestinal inflammation observed in mice receiving HVEM$^{-/-}$ or LIGHT$^{-/-}$ CD4^{+}CD45RB^{high} T cells we additionally investigated the production of pro-inflammatory cytokines within the MLN and colon. Mice receiving HVEM$^{-/-}$ T cells exhibited reduced levels of IL-6 and TNFα in the MLN (Fig. 2A+C) and IL-6, TNFα and IL-12p40 in the colon (Fig. 2B, D+F), but normal levels of IL-12p40 in the MLN (Fig. 2E). Mice receiving LIGHT$^{-/-}$ T cells exhibited an intermediate phenotype with significantly reduced TNFα in the MLN (Fig. 2C) and IL-6 plus IL-12p40 in the colon (Fig. 2B, D+F) but normal levels of IL-6 and IL-12p40 in the MLN and TNFα in the colon (Fig. 2A, E+D). Overall these data correlate well with the relative degrees of intestinal immuno-pathology observed in the same animals (Fig. 1) and reinforce our observations that HVEM stimulatory signals to T cells promote development of intestinal inflammation.
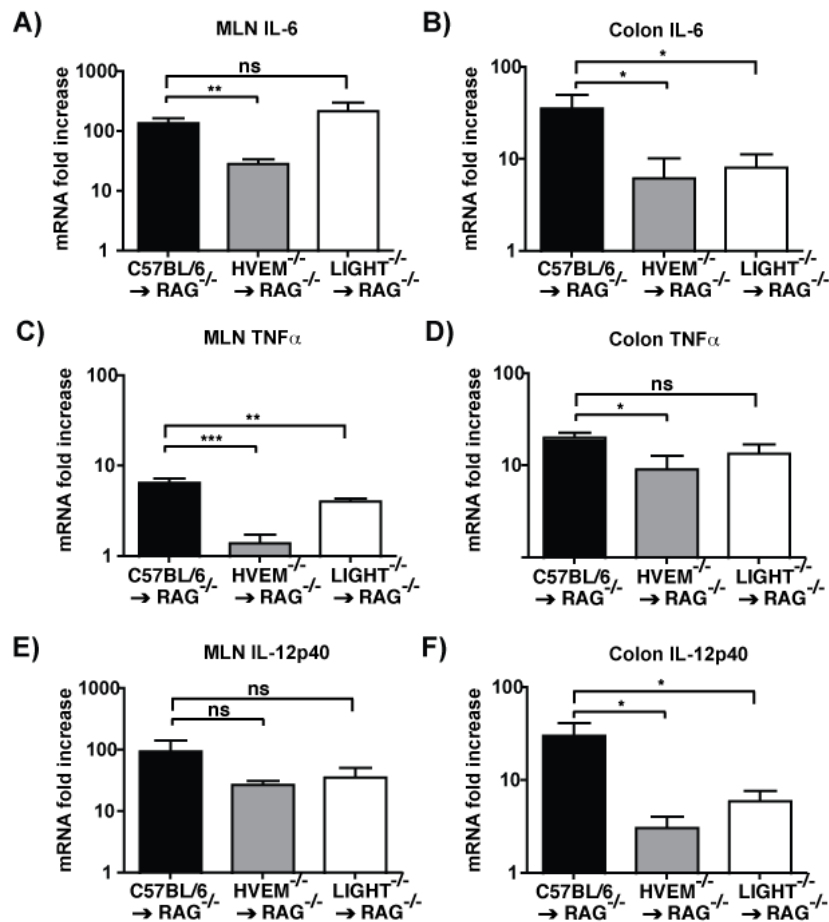
Figure 2. HVEM is required for inflammatory cytokine production. 4×10^5 CD4$^+$CD25$^-$ CD45RB^{high} T cells from C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were injected intravenously into RAG1$^{-/-}$ hosts and recipients sacrificed 6-8 weeks later. MLN mRNA expression of (A) IL-6, (C) TNFα and (E) IL-12p40 was determined in RAG1$^{-/-}$ host receiving either C57BL/6 (black bar), HVEM$^{-/-}$ (grey bar) and LIGHT$^{-/-}$ (white bar) CD4$^+$CD25$^-$CD45RB^{high} T cells. Colon mRNA expression of (B) IL-6, (D) TNFα and (F) IL-12p40 was determined in RAG1$^{-/-}$ host receiving either C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{high} T cells. For each individual sample gene expression was normalized relative to β-Actin. Values represent fold increases in mRNA expression over corresponding untreated controls. Mean $\pm$ SD is shown for two pooled experiments (n = 8 mice per group) and are representative of four independent experiments. Statistical analysis was performed using a two tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005.

We next investigated the impact of HVEM or LIGHT deficiency on the accumulation of MLN T cells and their production of inflammatory cytokines. Mice receiving C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ CD4$^+$CD45RB^{high} T cells had similar percentages of CD4$^+$ T cells in the MLN (Fig. 3A), however those mice receiving HVEM$^{-/-}$ T cells had a consequent decrease in the total number of CD4$^+$ T cells present (Fig. 3B). Both the percentage and total number of CD4$^+$ T cells producing IFN-γ or IL-17A was significantly decreased in mice receiving HVEM$^{-/-}$ T cells as compared to mice receiving C57BL/6 T cells (Fig. 3C-G). Selective deficiency of LIGHT on CD4$^+$ T cells did not impact significantly on the total number of CD4$^+$ T cells present in the MLN (Fig. 3B), or on the percentage of CD4$^+$ T cells producing IFN-γ (Fig. 3C) or IL-17A (Fig. 3E). Both parameters were slightly decreased and did not result in significantly reduced total numbers of IFN-γ^+ or IL-17A$^+$ T cells (Fig. 3D and F). These findings were verified by quantitative RT-PCR of whole MLN in showing significantly reduced IFN-γ and IL-17A mRNA expression in mice receiving T cells deficient for HVEM or LIGHT (Fig. 4A+B). Frequencies of double positive (IFN-γ^+IL-17A$^+$) T cells were slightly decreased in HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice (Fig. 5A). The number of double positive (IFN-γ^+IL-17A$^+$) T cells was significantly reduced only in RAG1$^{-/-}$ mice receiving HVEM$^{-/-}$ CD4$^+$ T cells, but not in mice receiving LIGHT$^{-/-}$ CD4$^+$ T cells (Fig. 5B). Interestingly, we observed a significantly increased frequency and total number of Foxp3$^+$ CD4$^+$ T cells following transfer of HVEM$^{-/-}$ T cells into RAG1$^{-/-}$ recipients (Fig. 6A&B). Collectively, these findings indicated that HVEM expression by CD4$^+$ T cells is required for their proper activation within the draining lymph nodes and for their effector function within the intestine during experimental colitis. In addition, these data demonstrated that HVEM-dependent CD4$^+$ T cell activation can be mediated, at least in part, by T cell-expressed LIGHT.
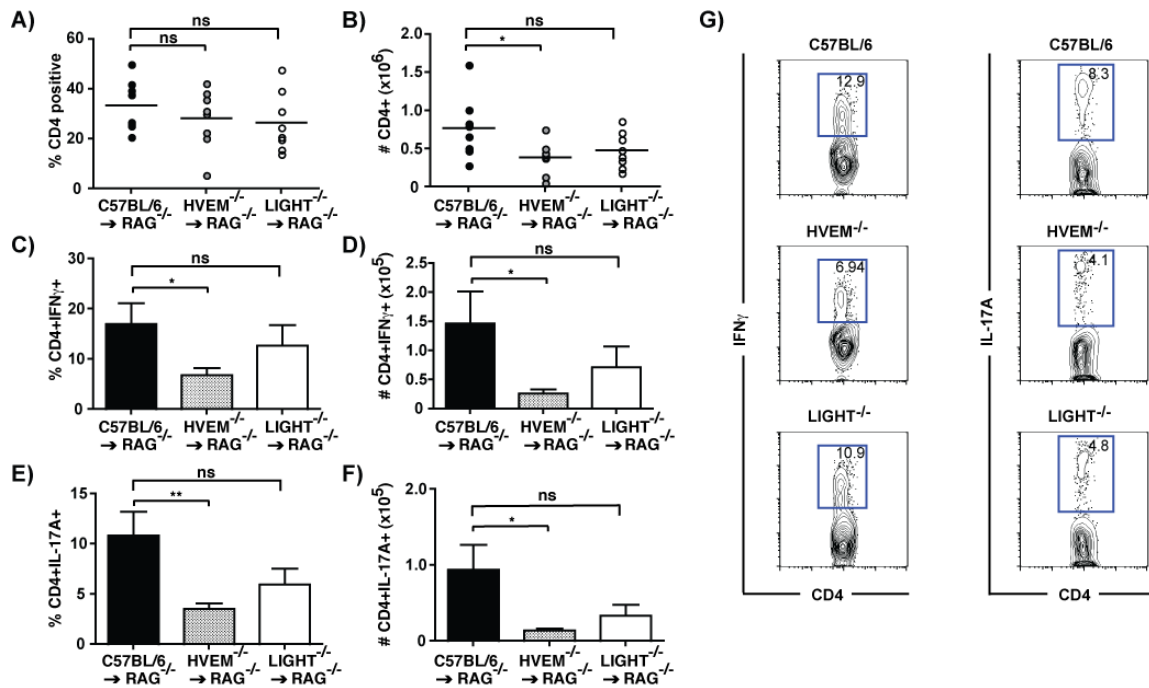
Figure 3. HVEM expression is required for the expansion and differentiation of CD4$^+$ T cells during intestinal inflammation. CD4$^+$CD25$^-$CD45RB^{high} T cells (4 × 10^5) from C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ mice were injected intravenously into RAG1$^{-/-}$ hosts, recipients sacrificed 6-8 weeks later and total MLN lymphocytes isolated. Frequency of (A) CD4$^+$, (C) CD4$^+$IFNγ^+ and (E) CD4$^+$IL-17A$^+$ T cells in C57BL/6 (closed circles and black bar), HVEM$^{-/-}$ (grey circles and squattered bar) and LIGHT$^{-/-}$ (open circles and white bar) transferred RAG1$^{-/-}$ recipients were analyzed by flow cytometry using fluorescent marker-conjugated mAbs (n = 8 mice per group). Total numbers (indicated by # symbol) of (B) CD4$^+$, (D) CD4$^+$IFNγ^+, (F) CD4$^+$IL-17A$^+$ T cells in the MLN of RAG1$^{-/-}$ mice receiving either C57BL/6 (black bar), HVEM$^{-/-}$ (striped bar) or LIGHT$^{-/-}$ (white bar) CD4$^+$CD25$^-$CD45RB^{high} T cells were calculated (n = 8 mice per group). (G) Representative FACS profiles for IFNγ and IL-17A production by C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ CD4$^+$ T cells isolated from the MLN of RAG1$^{-/-}$ recipients. Symbols and bar graphs represent means (± SD) from two pooled experiments, and are representative of four independent experiments. Statistical analysis between groups was assessed by a two tailed Student's *t* test: $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.
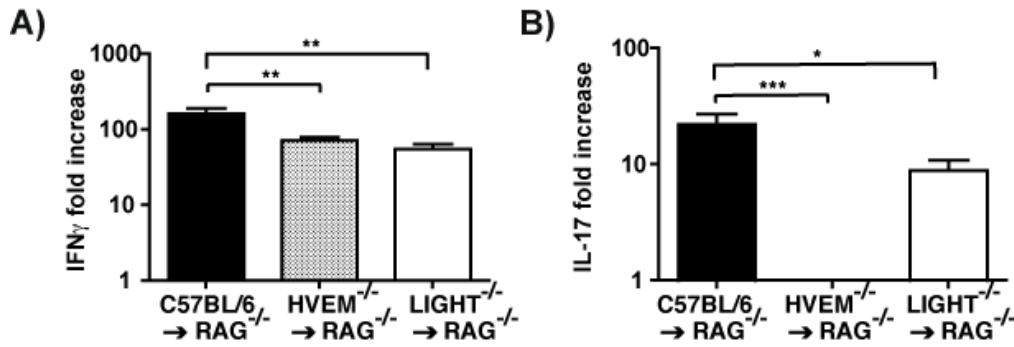
Figure 4. Significantly reduced cytokine mRNA expression in HVEM^{-/-} and LIGHT^{-/-} T cell-transferred RAG1^{-/-} mice. 4×10^5 CD4+CD25-CD45RBhight T cells from C57BL/6 and HVEM-/- mice were injected into RAG1-/- hosts and mice sacrificed 6-8 weeks later. (A) IFNγ and (B) IL-17A gene expression in C57BL/6 (black bar), HVEM-/- (squattered bar) and LIGHT-/- (white bar) transferred RAG1-/- recipients in the MLN was analyzed by quantitative RT-PCR. Each individual sample gene expression was normalized relative to β-Actin. Values represent fold increase in mRNA expression over corresponding samples from naïve RAG1-/- mice. Bar graphs represent means (± SD) from two pooled experiments (n = 8 mice per group). Statistical analysis between groups was assessed by a two tailed Student's t test: *p < 0.05, **p < 0.005, ***p < 0.0005.

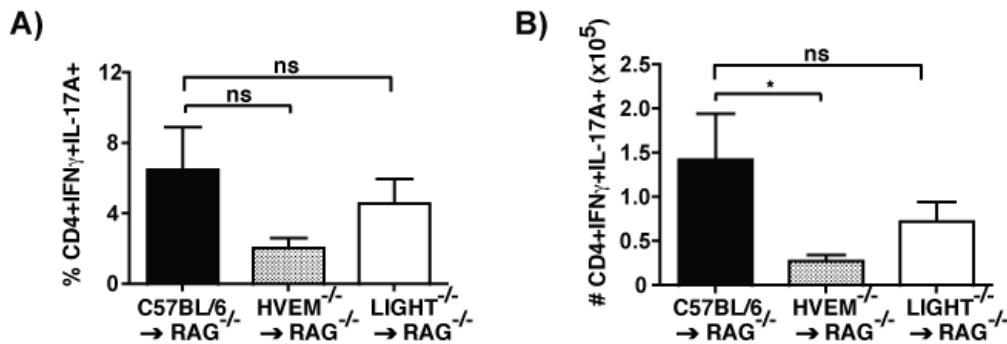**Figure 5. HVEM expression affects the differentiation of CD4^{+}IFNγ^{+}IL-17^{+} T cells during colitis.** CD4^{+}CD25^{-}CD45RB^{high} T cells (4×10^5) from C57BL/6, HVEM^{-/-} or LIGHT^{-/-} mice were injected intravenously into RAG1^{-/-} hosts, recipients sacrificed 6-8 weeks later and total MLN lymphocytes isolated. Frequency of (A) CD4^{+}IFNγ^{+} IL-17A^{+} T cells in C57BL/6 (black bar), HVEM^{-/-} (squattered bar) and LIGHT^{-/-} (white bar) transferred RAG1^{-/-} recipients were analyzed by flow cytometry. Total numbers (indicated by # symbol) of (B) CD4^{+}IFNγ^{+}IL-17A^{+} T cells in the MLN of RAG1^{-/-} mice receiving either C57BL/6 (black bar), HVEM^{-/-} (squattered bar) or LIGHT^{-/-} (white bar) CD4^{+}CD25^{-}CD45RB^{high} T cells were calculated (n = 8 mice per group). Bar graphs represent means (± SD) from two pooled experiments, and are representative of four independent experiments. Statistical analysis between groups was assessed by a two tailed Student's *t* test: [*]p < 0.05, [**]p < 0.005, [***]p < 0.0005.
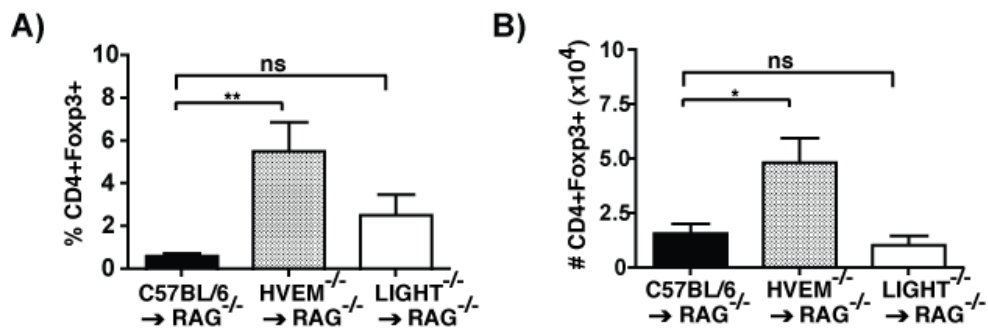
Figure 6. Induction of Foxp3^{+} regulatory T cells in HVEM^{-/-} transferred RAG1^{-/-} recipient mice. 4×10^5 CD4^{+}CD25^{-}CD45RB[hight] T cells from C57BL/6, HVEM^{-/-} and LIGHT^{-/-} mice were injected intravenously into RAG1^{-/-} hosts and mice sacrificed 6-8 weeks later. (A) Percentage of regulatory T cells expressing Foxp3 in the MLN of CD4^{+}CD25^{-}CD45RB[hight] T cell transferred mice (n = 8 mice per group). (B) Total numbers of CD4^{+}Foxp3^{+} T cells were calculated for the MLN of RAG1^{-/-} hosts. Data represent means ± SD of 8 individual mice per group from two experiments and representative of four independent experiments. Statistically significant differences between groups were assessed by a two tailed Student's *t* test: [*]p < 0.05, [**]p < 0.005, [***]p < 0.0005.

4.11.2 Attenuated CD4$^+$ T cell expansion and cytokine production in the absence of HVEM stimulatory signals cannot be overcome by inflammatory conditions.

We hypothesized that the decreased numbers of HVEM$^{-/-}$ CD4$^+$ T cells, and their inability to produce normal levels of inflammatory cytokines, may result from inadequate T cell-mediated DC activation and/or reduced production of pro-inflammatory cytokines. To address this we performed a co-transfer of equal numbers of HVEM$^{-/-}$ and congenic C57BL/6 CD4$^+$CD45RB^{high} T cells into RAG1$^{-/-}$ recipients. Mice receiving both sets of T cells developed severe colitis similar to that observed in mice receiving C57BL/6 T cells alone. However, both the percentage and total number of HVEM$^{-/-}$ CD4$^+$ T cells was markedly reduced compared to their C57BL/6 counterparts (Fig. 7A and B). HVEM deficiency also resulted in reduced percentages and total numbers of IFN-γ^+ or IL-17A$^+$ cells (Fig. 7C-G). Similar reductions in the percentage of CD4$^+$ T cells and the production of IL-17A were observed for lymphocytes isolated from the colon lamina propria (Fig. 8A-C). No inducible Foxp3 expressing Treg cells were detected upon co-transfer of HVEM$^{-/-}$ and C57BL/6 CD4$^+$CD45RB^{high} T cells. Thus, in contrast to our original hypothesis the co-transfer of C57BL/6 T cells allowed for the full development of inflammatory C57BL/6 T cells capable of initiating intestinal inflammation, but these cells acted in a competitive manner with HVEM$^{-/-}$ T cells to further reduce their expansion.
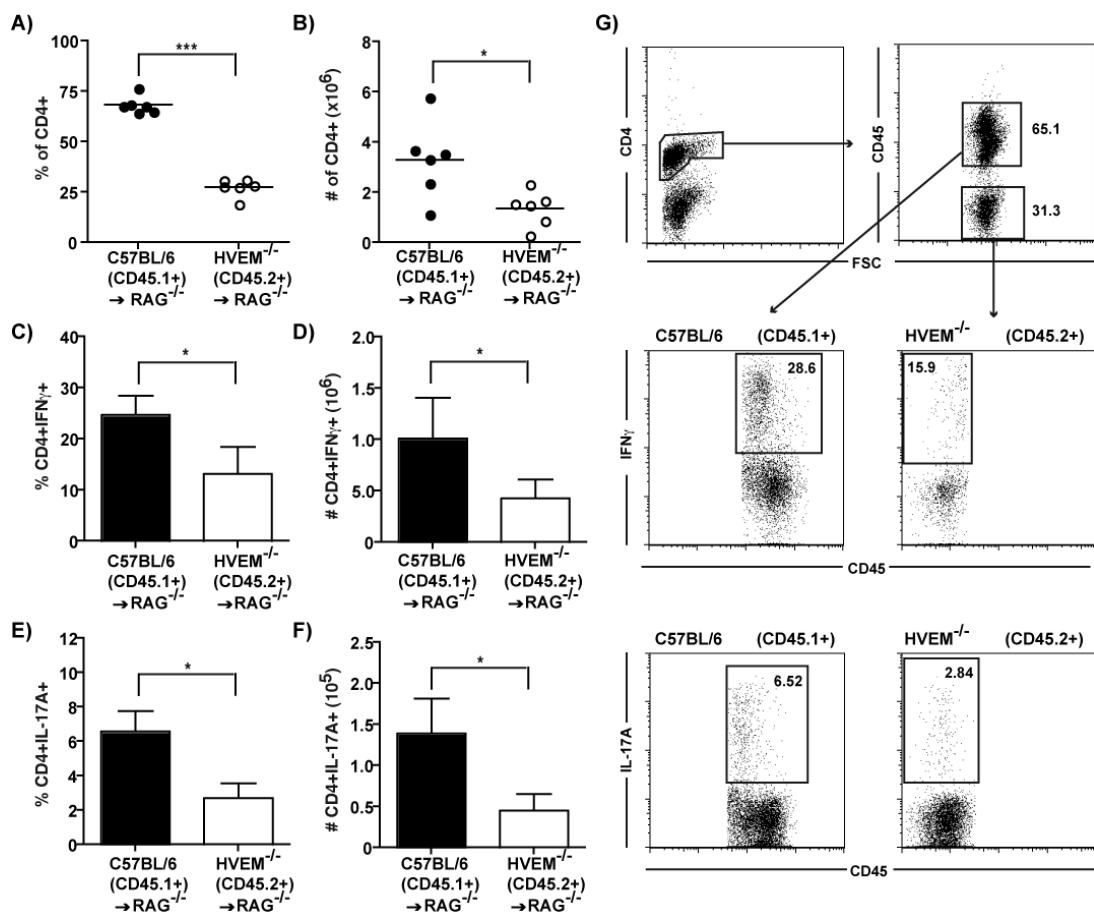
Figure 7. Attenuated HVEM$^{-/-}$ CD4$^+$ T cell expansion and cytokine production cannot be overcome by the presence of C57BL/6 CD4$^+$ T cells. Purified 4 × 10^5 CD4$^+$CD25$^-$ CD45RB^{hight} T cells from congenic C57BL/6 (CD45.1$^+$) and HVEM$^{-/-}$ (CD45.2$^+$) mice were injected together at a 1:1 ratio into RAG1$^{-/-}$ recipients and mice were sacrificed 6-8 weeks later. Whole MLN cell suspensions from RAG1$^{-/-}$ recipients were analyzed by flow cytometry and percentages of C57BL/6 (closed circles and black bar) and HVEM$^{-/-}$ (open circles and white bar) (A) CD4$^+$, (C) CD4$^+$IFNγ$^+$ and (E) CD4$^+$IL-17A$^+$ T cell lymphocytes assessed (n = 6 mice per group). Total numbers (indicated by # symbol) of C57BL/6 (closed circles and black bar) or HVEM$^{-/-}$ (open circles and white bar) for (B) CD4$^+$, (D) CD4$^+$IFNγ$^+$ and (F) CD4$^+$IL-17A$^+$ T cells were calculated (n = 6 mice per group). (G) Flow cytometry plots showing gating strategy to distinguish C57BL/6 (CD45.1$^+$) from HVEM$^{-/-}$ (CD45.2$^+$) MLN CD4$^+$ T cells recovered from RAG1$^{-/-}$ recipients and representative plots of CD4$^+$ T cell IFNγ and IL-17A cytokine staining. Symbols and bar graphs represent means ± SD from two pooled experiments and are representative of three independent experiments. Statistical analysis between groups were calculated using the two tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005.
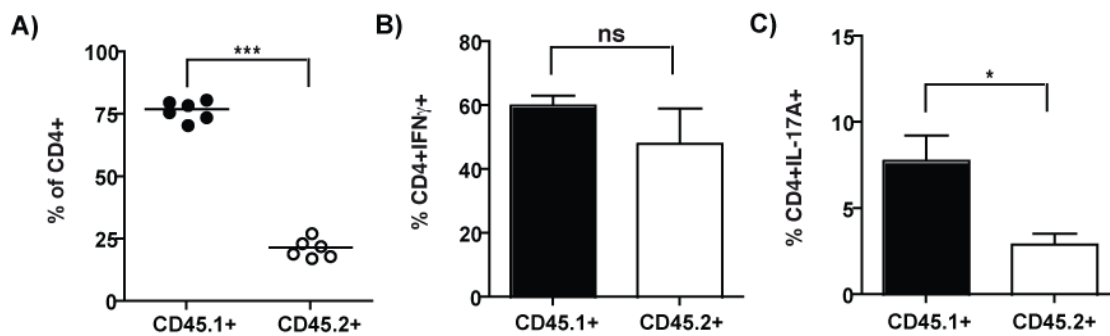
Figure 8. Attenuated HVEM^{-/-} CD4⁺ T cell expansion and cytokine production in the colon cannot be overcome by the presence of C57BL/6 CD4⁺ T cells. Purified 4×10^5 CD4⁺CD25⁻CD45RB^{hight} T cells from congenic C57BL/6 (CD45.1⁺) and HVEM^{-/-} (CD45.2⁺) mice were injected together at a 1:1 ratio into RAG1^{-/-} mice and recipients sacrificed 6-8 weeks later. Colon LPL from RAG1^{-/-} recipients were analyzed by flow cytometry and percentages of C57BL/6 (closed circles and black bar) and HVEM^{-/-} (open circles and white bar) (A) CD4⁺, (C) CD4⁺IFNγ⁺ and (E) CD4⁺IL-17A⁺ T cell lymphocytes assessed (n = 6 mice per group). Data represent means ± SD of 6 individual mice per group from two pooled experiments. Statistical analysis between groups (n = 6 mice per group) was performed by a two tailed Student's *t* test: [*]p < 0.05, ^{**}p < 0.005, ^{***}p < 0.0005.

4.11.3 HVEM^{-/-} CD4⁺ T cells exhibit normal expansion at early time-points following their transfer into lymphopenic hosts.

Our earlier findings indicated that HVEM deficiency on CD4⁺ T cells results in an inherent defect in the ability of these cells to expand and produce inflammatory cytokines. We next set out to determine whether this defect occurred due to a failure to undergo homeostatic and/or spontaneous expansion following their transfer into lymphopenic hosts, or whether it was related to a defect in Th cell differentiation. For this purpose a time-course experiment was performed whereby RAG1^{-/-} recipients receiving HVEM^{-/-} CD4⁺ T cells together with congenic wildtype C57BL/6 CD4⁺ T cells were sacrificed at day 14, 28 and 50 following transfer. The percentage and total numbers of CD4⁺ T cells was then determined and the fraction of CD4+ T cells expressing proliferation marker Ki-67 examined.

No differences in the percentage or total number of HVEM$^{-/-}$ versus wildtype CD4^{+} T cells present in the draining MLN (Fig. 9A and B) or colon (Fig. 10A and B) were observed at day 14 following transfer. The fraction of CD4^{+} T cells expressing Ki-67 was also similar for HVEM$^{-/-}$ and C57BL/6 cells (Fig. 9C and Fig. 10C). At day 28 following transfer the total numbers of HVEM$^{-/-}$ CD4^{+} T cells, and the percentage of these cells expressing Ki-67 were not significantly different in the MLN (Fig. 9D and F) or colon (Fig. 10D and F), but a significant reduction was noted for the percentage of CD4^{+} HVEM$^{-/-}$ T cells relative to C57BL/6 cells in both organs (Fig. 9E and Fig. 10E). In keeping with the data presented in Fig. 7, both the percentage and total number of HVEM$^{-/-}$ T cells was reduced compared to wildtype cells by day 50 post-transfer (Fig. 9G and H, Fig. 10G and H). This correlated with a decreased fraction of HVEM$^{-/-}$ CD4^{+} T cells expressing the proliferation marker Ki-67 (Fig. 9I&10I), and attenuated production of IFN-γ and IL-17A (Fig. 7). No significant production of IFN-γ or IL-17A was noted for either C57BL/6 or HVEM$^{-/-}$ T cells time-points earlier than day 50.

Taken together these data indicate that HVEM deficiency does not alter the ability of CD4^{+} T cells to undergo an initial expansion following their transfer into lymphopenic hosts, but are instead required to maintain T cell proliferation at later time-points and to promote the differentiation of effector cells producing IFN-γ or IL-17A.
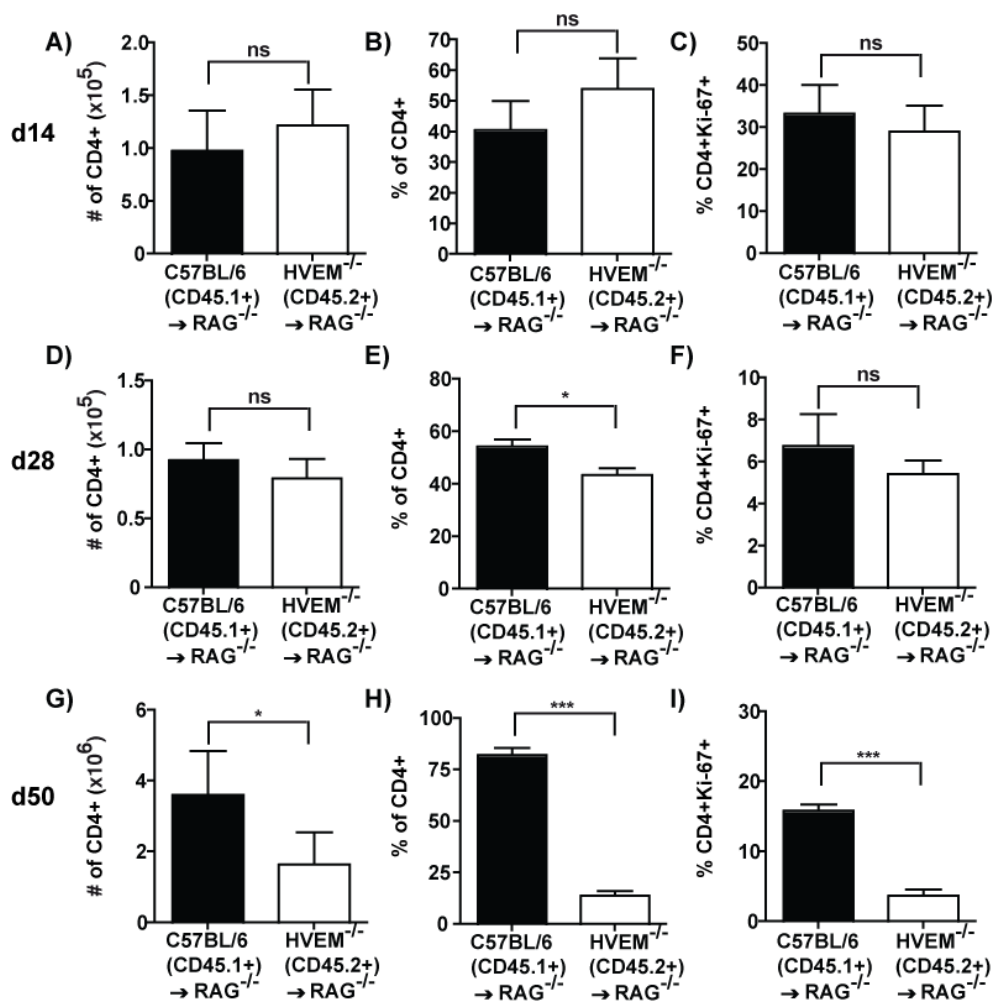
Figure 9. HVEM expression is required for the prolonged expansion of CD4⁺ T cells and differentiation of effector cells. 4×10^5 CD4⁺CD25⁻CD45RB^hight T cells from congenic C57BL/6 (CD45.1⁺) and HVEM^{-/-} (CD45.2⁺) mice were injected together at a 1:1 ratio into RAG1^{-/-} recipients and mice were sacrificed at the indicated time points following transfer. MLN lymphocyte suspensions were counted and the total number (indicated by # symbol) of transferred C57BL/6 (CD45.1⁺, black bar) or HVEM^{-/-} (CD45.2⁺, white bar) CD4⁺ T cells determined. CD4⁺ T cell numbers at (A) day 14, (D) day 28 and (G) day 50 post transfer into RAG1^{-/-} recipient mice are shown. Frequencies of C57BL/6 (CD45.1⁺, black bar) or HVEM^{-/-} (CD45.2⁺, white bar) CD4⁺ T cells were assessed by flow cytometry at (B) day 14, (E) day 28 and (H) day 50 after injection into RAG1^{-/-} mice. Expression of the proliferation marker Ki-67 by C57BL/6 (CD45.1⁺, black bar) or HVEM^{-/-} (CD45.2⁺, white bar) CD4⁺ T cells was analyzed by flow cytometry at (C) day 14, (F) day 28 and (I) day 50 after injection. Data represent means ± SD of two pooled experiments (n = 6 mice per group) and are representative of three independent experiments. Statistically significant differences between groups (n = 6 mice per group) were assessed by a two tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005.

Figure 10. HVEM expression is required for the expansion of CD4$^+$ T cells in the colon during intestinal inflammation. 4×10^5 CD4$^+$CD25$^-$CD45RB^{hight} T cells from congenic C57BL/6 (CD45.1$^+$) and HVEM$^{-/-}$ (CD45.2$^+$) mice were injected together at a 1:1 ratio into RAG1$^{-/-}$ recipients and mice sacrificed at the indicated time points after transfer. Colon lamina propria suspensions were counted and the total number (indicated by # symbol) of transferred C57BL/6 (CD45.1$^+$, black bar) or HVEM$^{-/-}$ (CD45.2$^+$, white bar) CD4$^+$ T cells calculated. CD4$^+$ T cell number at (A) day 14, (D) day 28 and (G) day 50 post transfer into RAG1$^{-/-}$ recipient mice. Frequencies of C57BL/6 (CD45.1$^+$, black bar) or HVEM$^{-/-}$ (CD45.2$^+$, white bar) CD4$^+$ T cells were assessed by flow cytometry at (B) day 14, (E) day 28 and (H) day 50 after injection into RAG1$^{-/-}$ mice. Expression of the proliferation marker Ki-67 by C57BL/6 (CD45.1$^+$, black bar) or HVEM$^{-/-}$ (CD45.2$^+$, white bar) CD4$^+$ T cells was analyzed by flow cytometry at (C) day 14, (F) day 28 and (I) day 50 after injection. Data represent means $\pm$ SD of two pooled experiments (n = 6 mice per group) and are representative of three independent experiments. Statistically significant differences between groups (n = 6 mice per group) were assessed by a two tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005.
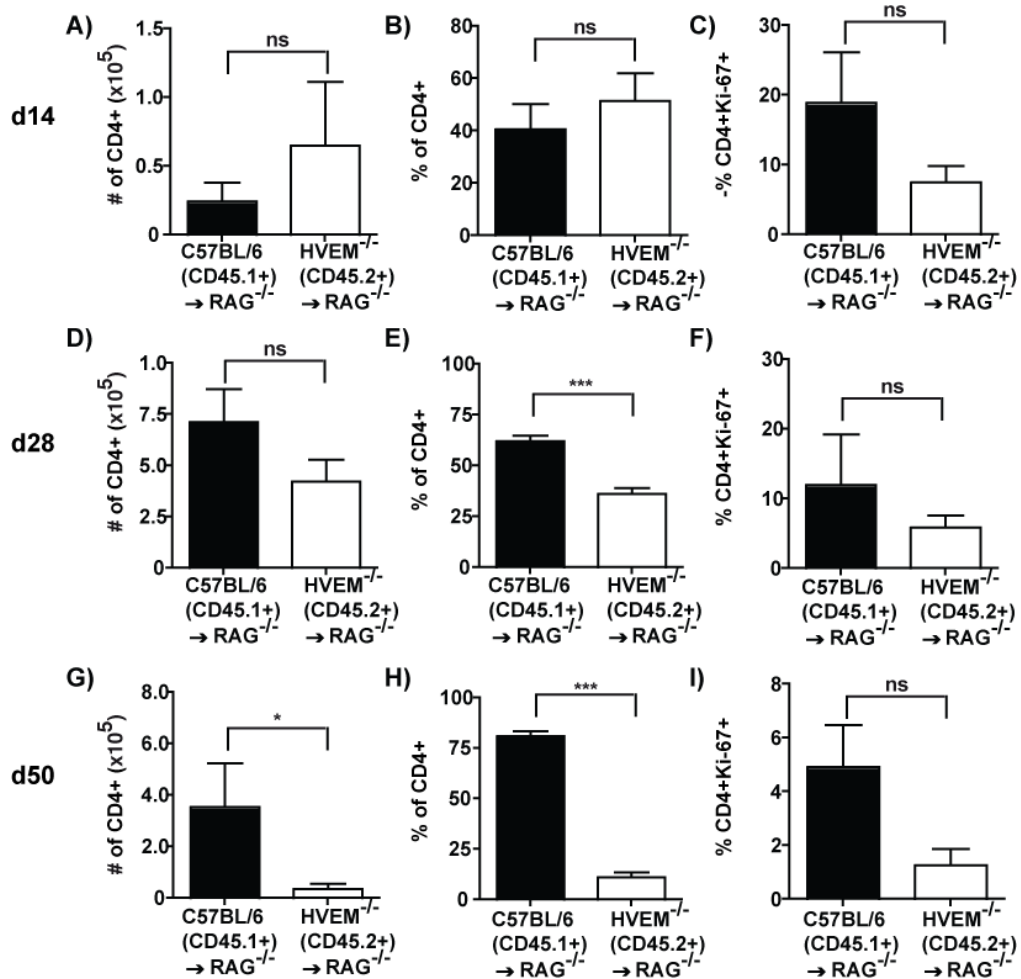
4.11.4 HVEM$^{-/-}$ CD4$^+$ T cells exhibit altered cytokine receptor expression

Our earlier data indicated that HVEM stimulatory signals largely function to promote the differentiation of effector T cells following their transfer into lymphopenic mice. The competitive advantage of wildtype C57BL/6 cells over HVEM$^{-/-}$ CD4$^+$ T cells in this setting further indicated that HVEM may function to render CD4$^+$ T cells responsive to cytokines present in limiting amounts in the surrounding microenvironment. To investigate this we examined the impact of HVEM on the expression of receptors for IL-6, IL-21 and IL-23, which are known to regulate the survival and differentiation of IL-17 producing T cells (32). Mice receiving C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ CD4$^+$CD45RB^{high} T cells alone exhibited comparable expression of the T cell activation markers CD44 and CD62L (Fig. 11A) and normal levels of IL-21R mRNA in the MLN (Fig. 12A). In contrast MLN from mice receiving HVEM$^{-/-}$ or LIGHT$^{-/-}$ T cells both showed a marked reduction in mRNA expression of IL-23R (Fig. 11B) and an almost complete abrogation of IL-6R gene expression in the MLN (Fig. 12C). Importantly, the percentage of CD4$^+$ T cells were comparable in all samples used for RNA analysis indicating that there was a selective loss in IL-6R and IL-23R expression on these cells (Fig. 3A). IL-23R and IL-6R mRNA expression were also reduced in the colon of mice receiving HVEM$^{-/-}$ T cells (Fig. 13A+B). Collectively, these data indicate that HVEM signaling is required for optimal IL-6R and IL-23R expression by CD4$^+$ T cells. However it is not clear from these experiments whether reduced IL-6R and IL-23R expression by HVEM$^{-/-}$ T cells *in vivo* simply correlates with reduced inflammation or whether it contributes to the failure of these cells to differentiate into pathogenic cells.
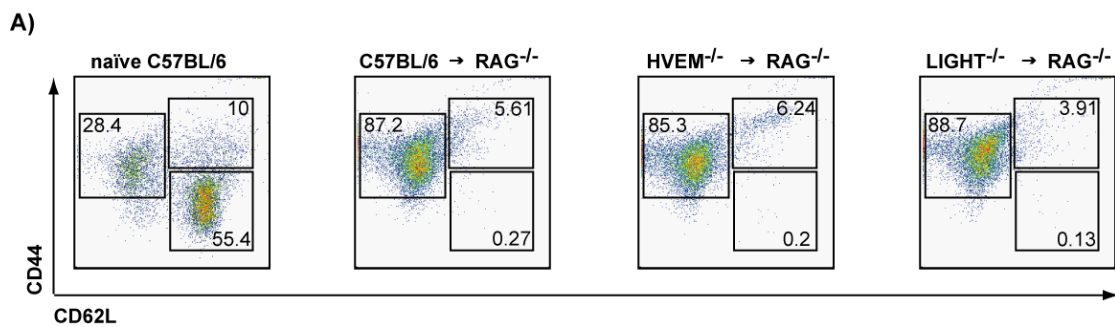
A)

Figure 11. Comparable activation of CD4$^+$ T cells in transferred RAG1$^{-/-}$ mice. 4×10^5 CD4$^+$CD25$^-$CD45RB^{high} T cells from C57BL/6, HVEM$^{-/-}$ or LIGHT$^{-/-}$ mice were injected intravenously into RAG1$^{-/-}$ hosts and mice sacrificed 6-8 weeks later. (A) MLN lymphocytes from C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{high} T cell transferred mice and from naïve control C57BL/6 mice were isolated and stained for CD44 and CD62L expression and analyzed by flow cytometry.
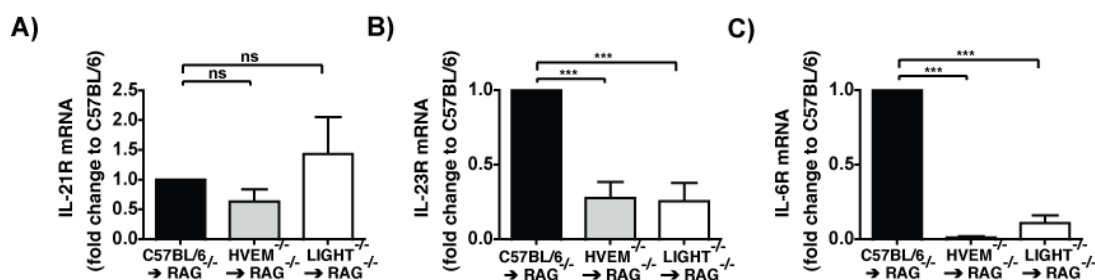
Figure 12. HVEM$^{-/-}$ CD4$^+$ T cells exhibit altered cytokine receptor expression. Total RNA was isolated from MLN of RAG1$^{-/-}$ mice transferred with C57BL/6 (black bar), HVEM$^{-/-}$ (striped bar) or LIGHT$^{-/-}$ (white bar) CD4$^+$CD25$^-$CD45RB^{high} T cells 6-8 weeks after transfer. Cytokine receptor expression was analyzed by quantitative real-time PCR and relative mRNA expression for (A) IL-21R, (B) IL23R and (C) IL-6R shown. Data are expressed as fold change of recipients receiving gene deficient compared to C57BL/6 T cells. Data represent means ± SD of 8 individual mice per group from two experiments and representative of four independent experiments. *p < 0.05, **p < 0.005, ***p < 0.0005.

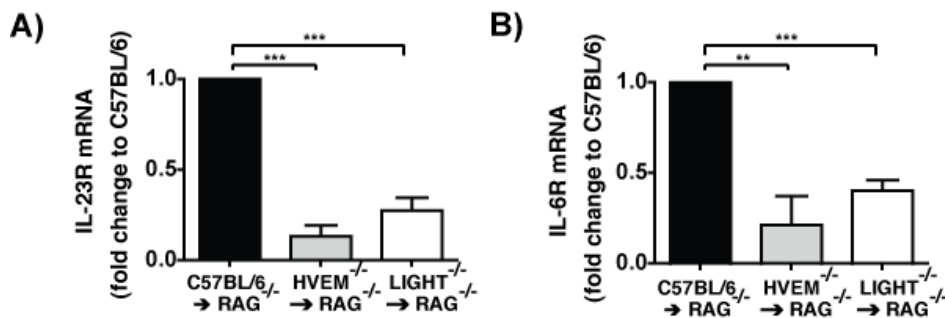**Figure 13. HVEM$^{-/-}$ CD4$^+$ T cell-transferred mice exhibit altered cytokine receptor expression in the colon.** Total RNA was isolated from colon of RAG1$^{-/-}$ mice transferred with C57BL/6 (black bar), HVEM$^{-/-}$ (striped bar) or LIGHT$^{-/-}$ (white bar) CD4$^+$CD25$^-$CD45RB^{high} T cells 6-8 weeks after transfer. Cytokine receptor expression was analyzed by quantitative real-time PCR and relative mRNA expression for (A) IL23R and (B) IL-6R shown. Data are expressed as fold change of recipients receiving gene deficient compared to C57BL/6 T cells. Data represent means ± SD of 8 individual mice per group from two experiments and representative of four independent experiments. *p < 0.05, **p < 0.005, ***p < 0.0005.

4.11.5 HVEM is required for optimal IL-6R surface expression by naïve CD4$^+$ T cells

We next performed flow cytometry to examine expression of the IL-6R α subunit (CD126) by CD4$^+$ T cells. FACS analysis showed that HVEM$^{-/-}$ CD4$^+$ T cells exhibited significantly reduced surface IL-6R expression as compared to C57BL/6 T cells following their co-transfer into RAG1$^{-/-}$ hosts (Fig. 14A). As IL-6R surface expression has been reported to be largely restricted to naïve cells (33), we also examined CD4$^+$ T cells present in the spleen or MLN of naïve mice. The percentage of naive CD44^{low}CD62L^{high} naïve CD4$^+$ T cells staining positively for IL-6R was significantly reduced in the MLN, but not the spleen, of naïve HVEM$^{-/-}$ mice compared to naïve C57BL/6 mice (Fig. 15A+B). In keeping with previous reports (33) little or no IL-6R surface expression was observed for CD44^{high}CD62L^{low} effector CD4$^+$ T cells from either strain. Thus a reduced IL-6 responsiveness of HVEM$^{-/-}$ cells present in the draining MLN of colitic mice may account for the inability of

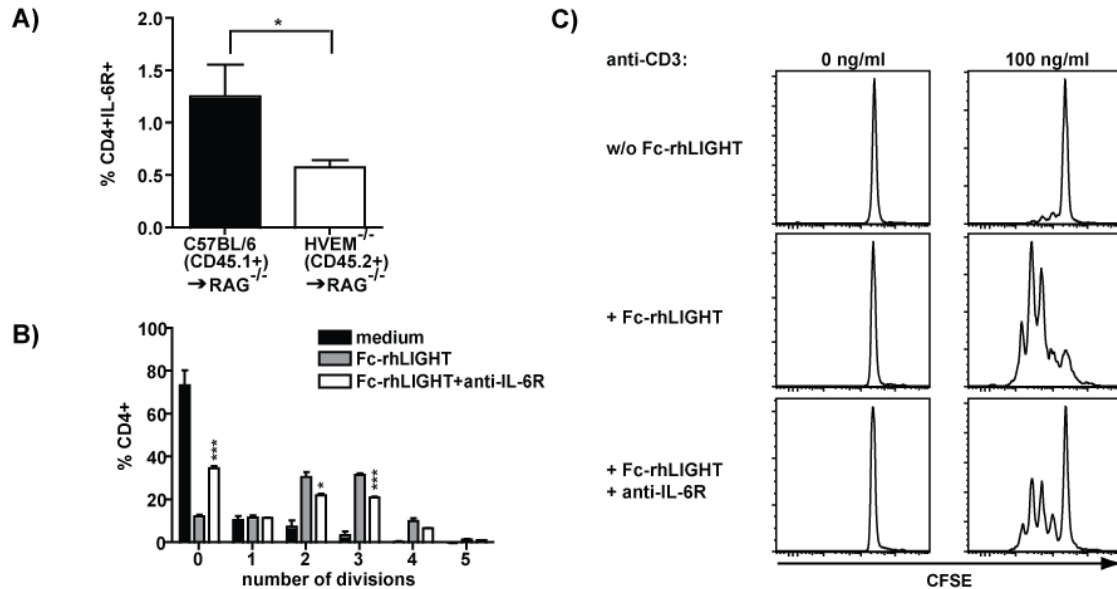these cells to differentiate into IFN-γ or IL-17A producing effector cells.

Figure 14. HVEM^{-/-} CD4⁺ T cells exhibit altered cytokine receptor expression. (A) Flow cytometric analysis of IL-6Rα (CD126) expression following the co-transfer of C57BL/6 (black bar) and HVEM-/- (white bar) CD4+ T cells in the MLN of RAG1-/- mice at 6-8 weeks post transfer. Data represent means ± SD of one experiment (n = 4 mice per group). (B) In vitro CFSE dilution of purified C57BL/6 CD4+ T cells following stimulation ± plate bound anti-CD3 (100 ng/ml) plus (grey bar) minus (black bar) 1:4 diluted Fc-rhLIGHT supernatant (≈ 1-10 μg/ml) in the presence (white bar) or absence (grey bar) of blocking anti-IL6R (5 μg/ml) monoclonal antibody. Data presented are from triplicates of one experiment showing means ± SD, and are representative of three independent experiments. Statistical differences between Fc-rhLIGHT ± anti-IL-6R stimulated cell divisions (determined by CFSE dilution) were calculated using a two tailed Student's t test: *p < 0.05, **p < 0.005, ***p < 0.0005. (C) Representative FACS plots of CD4+ T cell following in vitro culture ± anti-CD3 (100 ng/ml) supplemented with either 1:4 diluted Fc-rhLIGHT alone or Fc-rhLIGHT in the presence of anti-IL-6R (5 μg/ml).
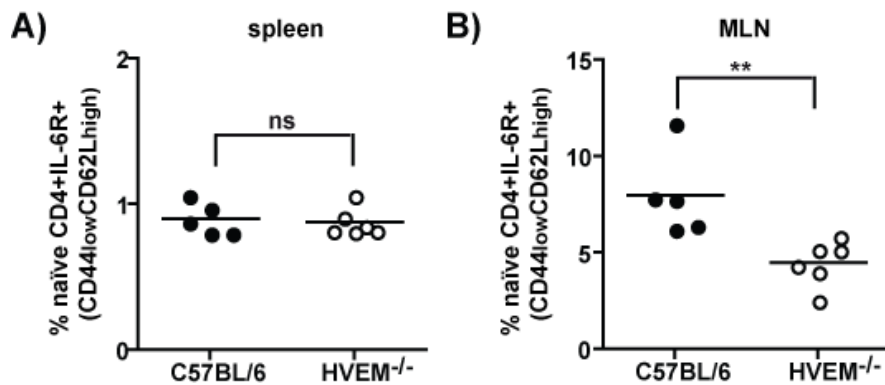
Figure 15: Reduced IL-6R expression by naïve HVEM$^{-/-}$ CD4$^+$ T cells. Whole cell suspensions obtained from spleen and MLN of naïve C57BL/6 and HVEM$^{-/-}$ mice were stained with fluorescence labeled anti-CD44, anti-CD62L and anti-IL-6R (CD126) monoclonal antibodies and analyzed by flow cytometry. (A) Frequency of naïve IL-6R expressing CD4$^+$CD44^{low}CD62L^{high} T cells in the spleen of C57BL/6 (closed circles) and HVEM$^{-/-}$ (open circles). (B) Percentage of C57BL/6 and HVEM$^{-/-}$ naïve CD4$^+$CD44^{low}CD62L^{high} MLN T cells expressing IL-6R. Data represent means ± SD of one experiment (n = 5-6 mice per group) and are representative of two independent experiments. Statistically significant differences between groups were assessed by a two tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005.

We next investigated whether the ability of recombinant LIGHT to support T cell proliferation could be reversed by addition of an antagonistic anti-IL-6R to the culture. Recombinant LIGHT promoted CD4$^+$ T cell proliferation *in vitro* (Fig. 14B and C) and this required CD4$^+$ T cell expressed HVEM, as recombinant LIGHT did not further enhance anti-CD3-induced proliferation in HVEM$^{-/-}$ CD4$^+$ T cells (Fig. 16A+B). Addition of anti-IL-6R to the culture resulted in a modest but reproducible inhibition of LIGHT-induced T cell proliferation indicating that LIGHT-HVEM signals promote T cell expansion through mechanisms partially involving IL-6-IL-6R signaling (Fig. 14B and C).
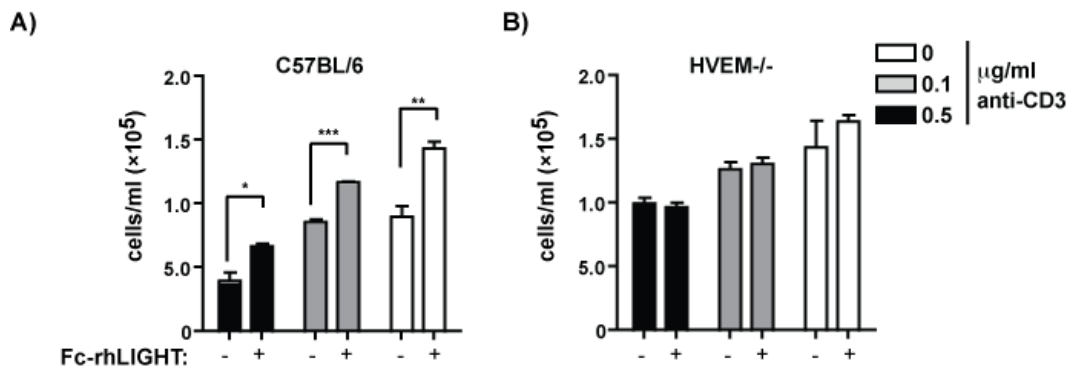
Figure 16: CD4+ T cell-expressed HVEM is required for LIGHT promoted T cell proliferation *in vitro*. Purified C57BL/6 and HVEM-/- spleen-derived CD4+ T cells were labelled with CFSE and cultured 48h in complete medium, 0.5 μg/ml or 1 μg/ml plate-bound anti-CD3. A) Total cell counts of C57BL/6 CD4+ T cells after 48h (n=3 mice per group). B) Total cells/ml of HVEM-/- CD4+ T cells. Data represent plots from one out of three independent experiments (n = 3 per group). Statistically significant differences between groups were assessed by a two tailed Student's *t* test: *p < 0.05, **p < 0.005.

4.12 Discussion

In the current study we identified an essential role for HVEM-mediated stimulatory signals to CD4^{+} T cells in promoting experimental colitis. HVEM deficiency did not alter the ability of CD4^{+} T cells to undergo expansion during the first few weeks following their transfer into lymphopenic hosts, but rather functioned to maintain their expansion at late time-points and to promote their differentiation into pathogenic Th1 and Th17 cells. This is in part mediated through T cell-derived LIGHT and mainly effects T cell expansion and differentaition at late-timepoints.

To determine why HVEM deficiency results in a failure of CD4^{+} T cells to differentiate into pathogenic T cells capable of eliciting intestinal inflammation we investigated their expression of IL-21R, IL-23R and IL-6R. HVEM deficiency, and to a lesser extent LIGHT deficiency, was observed to impact on IL-23R and IL-6R, but not IL-21R mRNA expression in the draining MLN and colon of colitic mice. Reduced surface expression of IL-6R on CD4^{+} T cells was confirmed by flow cytometry using an anti-IL-6R antibody and was also evident on CD44^{low}CD62L^{high} CD4^{+} cells recovered from the MLN of naïve HVEM^{-/-} mice. Interestingly no differences in IL-6R expression were noted for naïve cells present in the spleen of HVEM^{-/-} or C57BL/6 mice, and the percentage of CD4^{+} T cells expressing IL-6R was reduced in this organ compared to the MLN. This observation may indicate that the local microenvironment in the MLN plays a role in maintaining IL-6R expression, however the exact mechanisms by which this could occur remain unknown. IL-6R signalling was further demonstrated to play an important role in mediating LIGHT-HVEM induced T cell proliferation. Collectively, our data indicate that HVEM stimulatory signals play an important role in regulating IL-6R expression and IL-6 induced T cell expansion.

An inability of HVEM^{-/-} CD4^{+} T cells to respond to IL-6 could potentially

explain the inability of these cells to promote intestinal inflammation *in vivo* since IL-6 is known to play an important role in promoting colitis, both in chemically induced models (34) and following T cell transfer (19, 35). In T cell transfer models IL-6 appears to be derived from dendritic cells (36) and from pathogenic T cells (37). A complete absence of IL-6 results in reduced numbers of CD4^{+} T cells and diminished production of IL-17A, whilst its impact on IFN-γ production is less clear and differs between reports (20, 21). IL-6 signaling to T cells can be mediated by classical signaling following binding of soluble IL-6 to the IL-6R, which promotes its association with gp130 (CD130), or via trans signaling in which soluble IL-6R and IL-6 bind to gp130 as a ligand-recptor complex (38). Naïve T cells typically express the highest degree of IL-6R and this is lost following TCR stimulation then regained on memory T cells (33). Recently, classical IL-6 signaling was demonstrated to be required for Th17 differentiation, whilst trans IL-6 signaling functioned to maintain the cytokine profile of activated Th17 cells (39). Our own data indicates that HVEM signaling to T cells may impact on IL-6R surface expression and consequently on T cell expansion and Th17 differentiation *in vivo*. However, why defects in T cell expansion are only evident at late time-points following the transfer of HVEM^{-/-} T cells into RAG1^{-/-} hosts remains unclear as Feng *et al.* (36) recently reported that IL-6 cytokine production was crucial for the early spontaneous proliferation of T cells transferred into lymphopenic hosts. Further experiments investigating the role of IL-6R surface expression - as opposed to IL-6 cytokine production - will be necessary to clarify the exact relationship between HVEM signaling, IL-6R expression and intestinal inflammation.

Increasing evidence indicates an important role for IL-23 in promoting intestinal inflammation (40), and IL-23 is known to contribute to the maintenance of Th17 cells (32). Recently (23), attenuated colitis in IL-23^{-/-} RAG1^{-/-} recipients was shown to require the induction of Foxp3^{+} T cells, which

acted to inhibit the effector function of Foxp3⁻ T cells. Thus IL-23 appears to function to prevent intestinal inflammation largely through its ability to suppress the conversion of naive T cells into regulatory cells. Although we observed increased frequencies of Foxp3⁺ cells following the single transfer of HVEM⁻ᐟ⁻ T cells into RAG1⁻ᐟ⁻ recipients, this was not evident during co-transfer experiments even though HVEM⁻ᐟ⁻ T cells failed to differentiate into Th1 or Th17 effector cells in this setting. These observations suggested that HVEM did not impact on the frequency of Foxp3⁺ T cells directly but that numbers of regulatory T cells are instead associated with an absence of inflammation. They also indicated that decreased responsiveness of HVEM⁻ᐟ⁻ T cells to IL-23 is unlikely to explain defective effector cell differentiation. Instead, reduced IL-23R expression may simply reflect a reduced number of differentiated Th17 cells in the absence of HVEM.

Both IL-6 and IL-23 are recognized to contribute to a variety of other inflammatory diseases including EAE (41, 42) and arthritis (43, 44). No doubt this lies in part with their ability to promote the development and maintenance of pathogenic Th17 cells (45, 46). Although the relevance of HVEM-mediated stimulatory signals in these disease models remains to be ascertained, treatment of NOD mice with HVEM-Ig has been reported to attenuate the development of autoimmune diabetes (47). However, it is important to note that HVEM can also act as ligand for the Ig-family molecules B and T lymphocyte attenuator (BTLA) and CD160 to deliver inhibitory signals (48). Steinberg *et al.* (49) recently identified a predominant role for HVEM-BTLA interactions in preventing intestinal inflammation, and mice lacking HVEM exhibit increased susceptibility to experimental EAE (14), presumably resulting from abrogated BTLA signalling since BTLA deficiency results in a similar phenotype (50). BTLA⁻ᐟ⁻ mice also produce auto-antibodies and develop spontaneous autoimmune hepatitis-like disease (51). Thus, whilst HVEM-mediated co-stimulation of CD4⁺ T cells may play a role in promoting

the development of pathogenic T cells, blockade of HVEM could potentially disrupt both positive and negative regulatory pathways and thus complicate disease outcome. Therapeutic blockade of LIGHT itself may therefore offer an interesting and safer strategy for the treatment of IBD.

4.12.1 Acknowledgements

4.13 References

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4.14 HVEM regulates the activation of innate cells during intestinal inflammation

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4.15 Abstract

Inflammatory bowel disease (IBD) is a chronic disease in which abnormal immune responses against commensal bacteria results in inflammation of the intestinal wall. Multiple cell types and signals underlie this intestinal inflammation and an improved understanding of how this process is regulated is key to the development of improved therapeutics. Stimulatory interactions between the recently identified TNF superfamily members, TNFSF14 (LIGHT) and Herpesvirus entry mediator (HVEM), have been associated with IBD, however studies providing conclusive evidence of their involvement are lacking. We have used mice genetically deficient in LIGHT or HVEM in an experimental system that models in which the activation of innate immune cells promotes intestinal inflammation. Our data demonstrates that stimulatory HVEM interactions are required for the full activation of innate cells and consequent intestinal inflammation and indicates that these are likely to be derived in part through LIGHT.

4.16 Introduction

Inflammatory bowel disease (IBD) is a heterogeneous disorder, including Crohn´s disease (CD) and ulcerative colitis (UC), which afflict approximately 0.1% of the Western population (1, 2). The etiology of both CD and UC is still unclear, however recent evidence implicates a role of aberrant immune responses directed against intestinal bacteria in driving disease (reviewed in (3)). In CD immune pathogenesis is associated with increased production of pro-inflammatory cytokines and a Th1 cell bias (4-6). Most animal models of IBD are characterized by increased Th1 cytokine production and therefore reflect human CD. These models reproduce distinct components of the immune responses associated with IBD, and the information provided from their use has undoubtedly led to significant progress in the understanding of disease pathology and the development of current therapeutic interventions.

TNF is an important contributor to intestinal inflammation, and its blockade with anti-TNF antibodies effectively diminishes inflammation in IBD patients. LIGHT and Herpes virus entry mediator (HVEM) comprise a ligand-receptor pair of the TNF super family and several lines of evidence indicate LIGHT as a crucial mediator of intestinal inflammation (7, 8), as mentioned earlier in the section 3.4.3.

We subjected mice deficient for LIGHT or HVEM to Dextran sulfate sodium (DSS)-induced colitis and investigated the impact of gene deficiency on diarrhea, ulcerations and cellular infiltration of the colon. Our studies showed that the HVEM-mediated stimulatory signals were an essential component of innate immune cell activation, pro-inflammatory cytokine production and intestinal pathology. Therefore, HVEM stimulatory signals may play a fundamental role in IBD by promoting the activation of innate components of the immune responses.

4.17 Materials and Methods

4.17.1 Mice

C57BL/6 mice, HVEM$^{-/-}$ (9) mice and LIGHT$^{-/-}$ (10) mice were bred and maintained under specific pathogen-free (SPF) conditions in isolated ventilated cages at Bio-Support (Zürich, Switzerland). All animal experiments were performed using mice aged between 6 and 8 weeks old and completed accordingly to guidelines set by the State Veterinary Office of Zürich, Switzerland. Mice from different genotypes were housed within the same cage or bedding from the cages of male recipients was mixed for at least 2-3 weeks prior to all the experiments.

4.17.2 Generation of bone marrow chimeras

To generate chimeric mice, C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were lethally irradiated with 950 rad one day before reconstitution with intravenously injected 2×10^6 bone marrow-derived cells. Irradiated C57BL/6 mice were reconstituted with either C57BL/6 or HVEM$^{-/-}$ bone marrow-derived cells. Irradiated HVEM$^{-/-}$ mice were reconstituted with C57BL/6 bone marrow-derived cells. All reconstituted mice were maintained under SPF conditions for at least 8 weeks before experiments. 2-3 weeks prior to the experiment mice from different genotypes were either co-housed in the same cage, or bedding was mixed.

4.17.3 DSS-induced experimental colitis

Acute colitis was induced in age-matched C57BL/6, HVEM$^{-/-}$, LIGHT$^{-/-}$ and RAG1$^{-/-}$ mice, by oral administration of Dextran sulfate sodium (DSS) (MP Biomedicals) at a concentration of 5% (w/v) in drinking water for 4 days. Age-matched C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice receiving normal drinking water

served as controls. Mice were evaluated daily for changes in body weight or the development of clinical symptoms. Six days after the induction of colitis mice were sacrificed by CO_2 inhalation, the abdominal cavity was exposed and the entire colon was removed from the cecum to the anus. Thereafter the colon was cut into proximal, middle and distal sections and a small piece from each section removed for RNA isolation. Additional proximal, middle and distal sections of colon were immediately fixed in buffered 10% formalin for latter histological analysis. In all instances all three sections of the colon were first analyzed by histological means and the area most afflicted by disease chosen for further analysis.

4.17.4 Assessment of the clinical activity score during DSS-induced colitis

Assessment of body weight, stool consistency and the presence of occult/gross blood by a guaiac test (Hemoccult Sensa; Beckman Coulter) were determined at the day of sacrifice for all mice. Colitis was quantified with a clinical score, as described by Cooper et al. (11), using the parameters of weight loss, stool consistency and fecal blood. Briefly, weight loss was considered as negligible (0 points), 1-5% (1 point), 5-10% (2 points), 10-15% (3 points) or ≥15% (necessitating sacrifice and given 4 points). Stool character was characterized as normal (0 points), soft with well-formed pellets (2 points), or diarrhea (4 points). For occult blood, scores were given as an absence of blood (0 points), a positive hemoccult score (2 points) or gross bleeding (4 points). The scores for each individual parameter were then added together to give a total score between 0 and 12.

4.17.5 Histological assessment of colitis

Colonic specimens obtained as described were fixed in formalin for at least 24 hours, embedded into paraffin, and cut into 4-5 μm sections. Sections were then stained with hematoxylin and eosin (H&E) for blind microscopic assessment of mucosal lesions. Histological scoring for DSS colon sections was performed, with slight modifications, as previously described by Schenk et al. (12). Briefly, for inflammation scores were given as rare inflammatory cells in the lamina propria (0 points), increased numbers of lymphocytes and granulocytes in the lamina propria (1 point), confluence of inflammatory cells extending into the submucosa (2 points), or transmural extension of the infiltrate (3 points). For crypt damage scores reflected intact crypts (0 points), loss of every third crypt (1 point), loss of two out of three crypts (2 points), complete crypt loss (3 points), or change of epithelial surface with epithelial erosion (4 points). For evaluation of the confluence of epithelial erosion scores reflected an absence of epithelial erosion (0 points), 1-2 foci of epithelial erosion (1 point), 3-4 foci (2 points), or confluent epithelial erosion (3 points). The scores for each individual parameter were then added together to give a total score between 0 and 10.

4.17.6 Detection of cytokine mRNA expression by quantitative RT-PCR

Quantitative RT-PCR was performed using cDNA isolated from colonic tissue samples. cDNA was prepared from total RNA isolated using TRI Reagent (Molecular Research Center, Inc.), treated with DNase (Invitrogen) to avoid genomic DNA contamination, and reverse transcribed using the Superscript III RT kit (Invitrogen). Transcribed cDNA was used as a template for the PCR reaction. Real time RT-PCR was performed using Brilliant SYBR Green (Stratagene) and an iCycler (Bio-Rad Laboratories). Expression was normalized according to expression of the housekeeping gene β-Actin.

Sequences of the primers used: β-Actin; 5'-CTT TTC ACG GTT GGC CTT AG-3' and 5'-CCC TGA AGT ACC CCA TTG AAC-3', CCL3; 5'-AGA TTC CAC GCC AAT TCA TC-3' and 5'-CCC AGG TCT CTT TGG AGT CA-3', CCL4; 5'-TTC TGT GCT CCA GGG TTC TC-3' and 5'-AGC AAA GAC TGC TGG TCT CA-3', CCL5; 5'- CAA TCT TGC AGT CGT GTT TG-3′ and 5′-AGA ATC AAG AAA CCC TCT ATC-3', IL-6; 5'-TTC CAT CCA GTT GCC TTC TTG-3' and 5'-TCA TTT CCA CGA TTT CCC AGA G-3', IFNγ; 5'-GCT CTG AGA CAA TGA ACG CTA C-3' and 5'-TTC TAG GCT TTC AAT GAC TGT GC-3', TNFα; 5-GAA CTG GCA GAA GAG GCA CT-3' and 5'-AGG GTC TGG GCC ATA GAA CT-3', CXCL9, 5'-GCA AAA GTG AGC TCC AGA AGG-3' and 5'-AGC TTC CCA GAT CAC AGA GG-3'.

4.17.7 Statistical Analysis

For all data shown significant differences between groups were calculated using an unpaired two-tailed Student's *t*-test with the confidence interval set at 95%. Statistically significant differences are indicated as $p < 0.05$ (*), $p < 0.005$ (**), or $p < 0.0005$ (***).

4.18 Results

4.18.1 HVEM is required for DSS-induced colitis.

To investigate the role of LIGHT or HVEM signaling during intestinal inflammation mediated by innate immune cells, we examined the response of $HVEM^{-/-}$ or $LIGHT^{-/-}$ mice to DSS-induced intestinal damage. In this model, administration of DSS in the drinking water results in weight loss, intestinal epithelial cell damage and immune-mediated colonic inflammation. As expected wild type C57BL/6 mice exhibited severe weight loss and intestinal inflammation following acute DSS administration (Fig. 1A). In contrast $HVEM^{-/-}$ mice exhibited significantly reduced weight loss and reduced rectal bleeding following DSS treatment (Figure 1A and B). In addition, $HVEM^{-/-}$ mice showed attenuated intestinal immuno-pathology as determined by histological analysis of leukocyte infiltration, crypt destruction and epithelial erosion within the colon (Figure 1C+D). $LIGHT^{-/-}$ mice exhibited an intermediate phenotype indicating that the absence of stimulatory LIGHT-HVEM interactions was at least partially responsible for the observed resistance of $HVEM^{-/-}$ mice to DSS-induced intestinal inflammation (Fig. 1A-D). Importantly, the absence of inflammation in $HVEM^{-/-}$ mice was not due to a delayed response, as mice did not exhibit weight loss even at late time points following DSS administration.
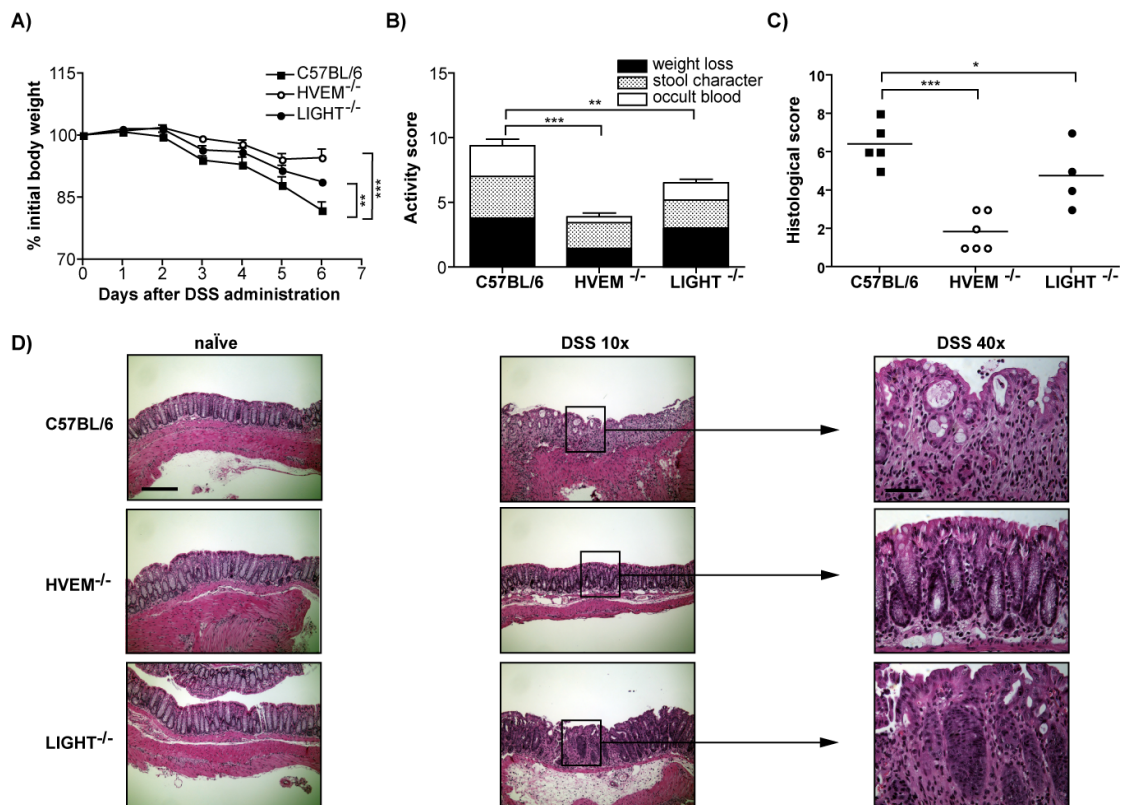
Figure 1. HVEM$^{-/-}$ mice are resistant to DSS-induced colitis. C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were given 5% DSS in the drinking water for 4 days, then returned to normal drinking water and sacrificed at day 6. (A) Body weight was monitored daily and is expressed as percentage change from initial body weight at day 0. (B) Clinical activity scores were assessed at the time of sacrifice by a combination of total weight loss, stool character and occult blood. Data in A and B represent the mean ± SD for two independent experiments (n = 8-10 total mice per group). (C) Histological scores indicating immuno-pathology were calculated as described in the Materials and Methods and are shown for the distal part of the colon of individual mice. Symbols show individual animals from two independent experiments (n = 8-10 mice per group). (D) Representative H&E staining of distal colon tissue sections from control and DSS-treated mice. Scale bars are 10× magnification=200 μm and 40× magnification=0.05 μm. Statistical differences between groups were calculated using a two-tailed Student's *t* test: *p < 0.05, **p < 0.005, ***p < 0.0005.

4.18.2 Chemically induced intestinal pro-inflammatory cytokine and chemokine production is attenuated in the absence of HVEM.

To further assess the impact of HVEM on innate immunity during DSS-induced intestinal inflammation we analyzed the production of chemokines and pro-inflammatory cytokines within the intestine. For this purpose we chose to study those cytokines and chemokines induced following DSS administration, which are produced largely by innate immune cells. DSS-induced production of CCL3 (MIP-1α) (Fig. 2A), CCL4 (MIP-1β) (Fig. 2B), IL-6 (Fig. 2F), CXCL9 (Fig. 2G), CCL5 (RANTES) (Fig. 2C), IFNγ (Fig. 2D) and TNFα (Fig. 2E) was severely attenuated in the absence of HVEM signaling (Fig. 2A-G). These cytokines and chemokines are largely produced by local DC, monocytes and stromal cells, NK cells and/or T cells and their attenuation in the absence of HVEM indicates that HVEM is required for both innate and adaptive immune cell activation in this model. In contrast, the absence of LIGHT had only a minor impact on chemokine expression and did not significantly alter pro-inflammatory cytokine production (Fig. 2A-G).

These data suggested that HVEM could signal directly to innate immune cells within the intestine to promote the production of pro-inflammatory cytokines and chemokines following DSS administration. The intermediate phenotype of LIGHT$^{-/-}$ mice - in terms of chemokine and cytokine expression, weight loss and intestinal immuno-pathology - indicated that LIGHT only partially accounts for HVEM-mediated stimulatory signals in our model and raises the possibility that additional HVEM-stimulatory ligands contribute to intestinal inflammation *in vivo.*
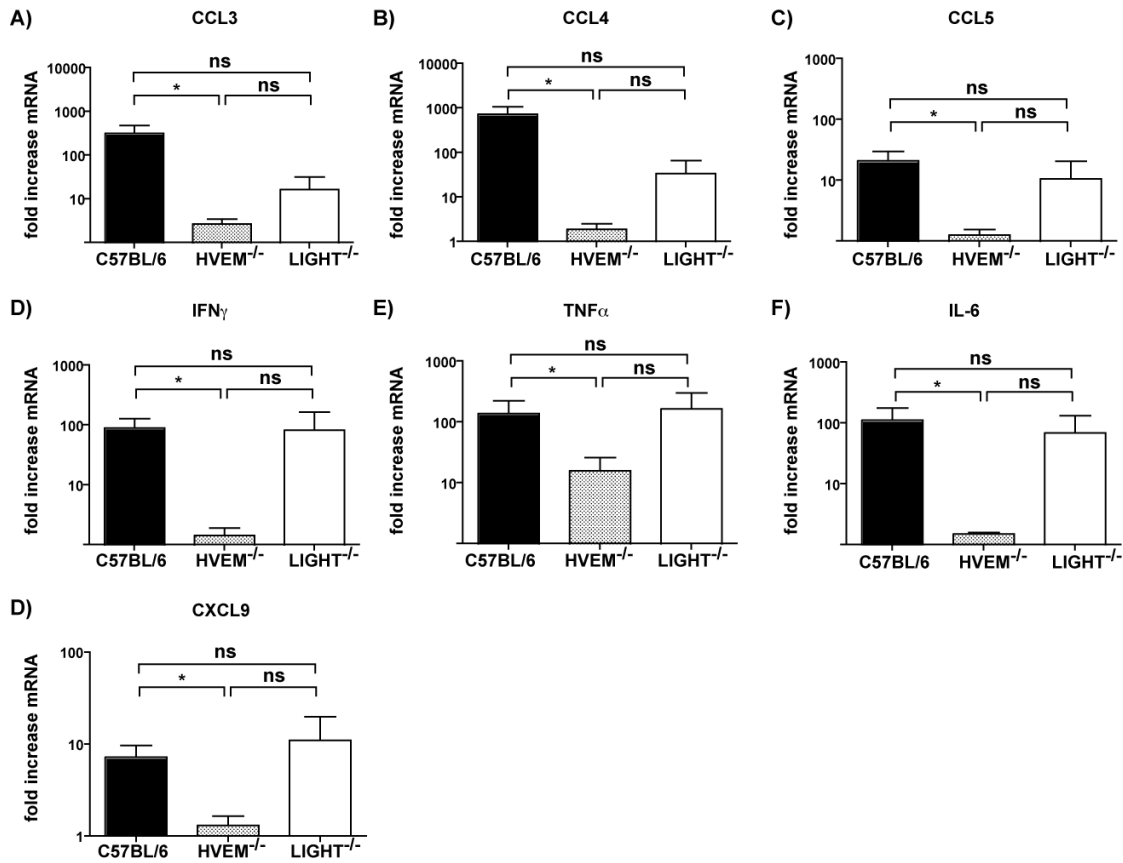
Figure 2. HVEM is required for DSS-induced pro-inflammatory cytokine and chemokine production. C57BL/6, HVEM$^{-/-}$ and LIGHT$^{-/-}$ mice were given 5% DSS in the drinking water for 4 days, then returned to normal drinking water. At day 6 mice were sacrificed and the colon removed for RNA isolation. (A) CCL3, (B) CCL4, (C) CCL5, (D) IFNγ, (E) TNFα, (F) IL-6 and (G) CXCL9 gene expression in the distal colon was analyzed by quantitative RT-PCR. For each individual sample gene expression was normalized relative to β-Actin. Values represent fold increases in mRNA expression over corresponding untreated controls. Means ± SD are shown for two independent experiments (n = 8-10 total mice per group). Statistical differences between groups were calculated using an unpaired Student t test: *p < 0.05, **p < 0.005, ***p < 0.0005.

4.18.3 Cells of the adaptive immune system are not required for acute DSS-induced intestinal inflammation

Although DSS-induced intestinal inflammation can lead to the activation and recruitment of T cells, previous reports have indicated that neither T cells, B cells or NK cells are required for intestinal inflammation (13, 14). To confirm these findings and further prove that our studies indicated a defect in innate immune cell activation in the absence of HVEM we subjected RAG1$^{-/-}$ mice to the DSS-induced colitis model using the same parameters as in Figure 1. In line with previous reports we observed comparable weight loss, diarrhea and rectal bleeding in DSS-treated C57BL/6 and T- and B cell-deficient RAG1$^{-/-}$ mice (Fig. 3A+B). Both groups exhibited moderate to severe colitis with inflammatory cell infiltration extending into the submucosal layer, erosion of the surface epithelial layer and subtotal crypt loss (Figure 3C+D). Thus, the disease reduction observed in the absence of HVEM but not in the absence of recombinase activating gene-1 therefore indicated that HVEM is necessary to promote the activation of innate immune responses sufficient for disease induction.
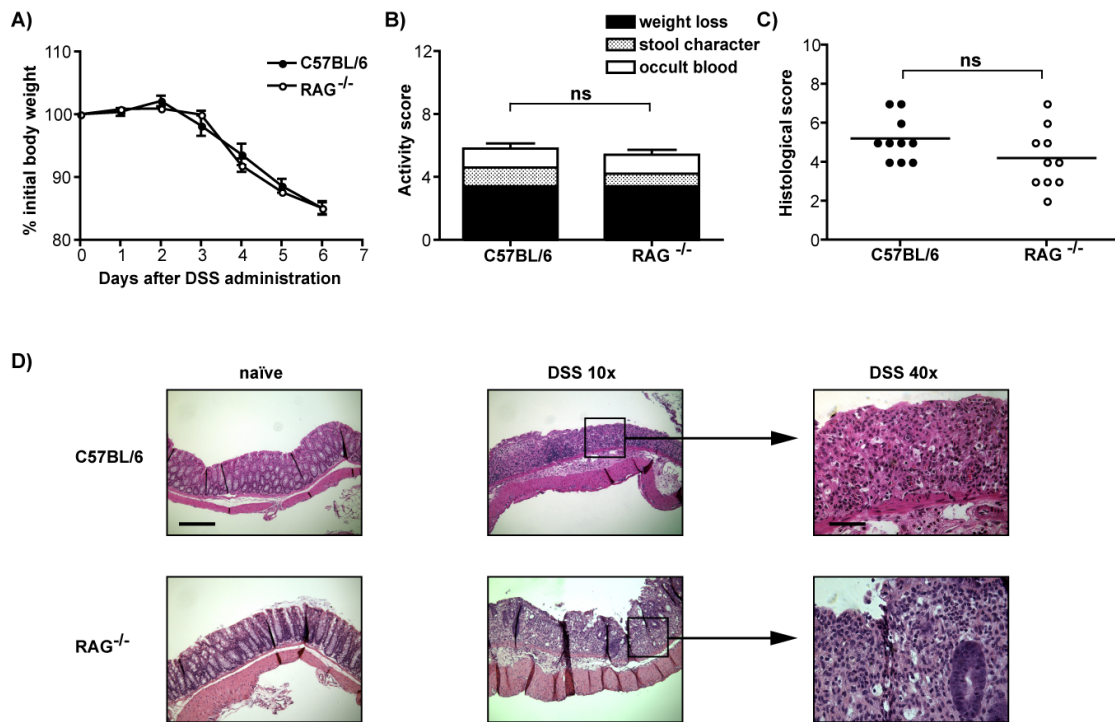
Figure 3. Comparable disease in C57BL/6 and RAG1$^{-/-}$ mice following DSS administration. C57BL/6 and RAG$^{-/-}$ mice were given 5% DSS in the drinking water for 4 days, then returned to normal drinking water and sacrificed at day 6. (A) Body weight was monitored daily and is expressed as percentage change from initial body weight at day 0. (B) Clinical activity scores were assessed at the time of sacrifice by a combination of total weight loss, stool character and occult blood. Data in A and B represent the mean ± SD for two independent experiments (n = 10 total mice per group). (C) Histological scores indicating immuno-pathology were calculated as described in the Materials and Methods and are shown for the distal part of the colon of individual mice. Symbols show individual animals from two independent experiments (n = 10 mice per group). (D) Representative H&E staining of distal colon tissue sections from control and DSS-treated mice. Scale bars are 10× magnification=200 μm and 40× magnification=0.05 μm. No statistical differences were observed between groups as determined using an unpaired Student *t* test.

4.18.4 HVEM expression by stromal cells is partly required for DSS-induced colitis

HVEM is not only expressed on most hematopoietic cells, but also on some stromal and epithelial cells (15). Therefore, we next wanted to investigate the contribution of stromal and epithelial cell expressed HVEM in DSS-induced intestinal inflammation. In order to directly study the impact of non-hematopoietic versus hematopoietic-expressed HVEM on DSS-induced colitis, we generated chimeric mice. C57BL/6 mice were either reconstituted with C57BL/6 or HVEM$^{-/-}$ bone marrow-derived cells and HVEM$^{-/-}$ mice were reconstituted with C57BL/6 bone marrow-derived cells. This resulted in mixed chimeric mice, where HVEM signaling is restricted to either the hematopoietic or non-hematopoietic cellular compartment. Intestinal inflammation in the chimeric mice was then induced by the administration of DSS in the drinking water. As expected, C57BL/6 mice reconstituted with C57BL/6 bone marrow-derived cells exhibited severe intestinal inflammation characterized by substantial weight loss, diarrhea and occult blood in the stool (Fig. 4A+B). Although not significant, HVEM$^{-/-}$ mice reconstituted with C57BL/6 bone marrow-derived cells showed reduced intestinal inflammation, diarrhea and rectal bleeding following treatment with DSS (Fig. 4A-C). Similarly, a slight reduction in intestinal inflammation was also seen in C57BL/6 mice reconstituted with HVEM$^{-/-}$ bone marrow-derived cells (Fig. 4A-C). These results suggest that both stromal and hematopoietic HVEM expression contributed to DSS-induced intestinal inflammation. However, these results will need to be confirmed by further experiments and the HVEM-HVEM controls included.
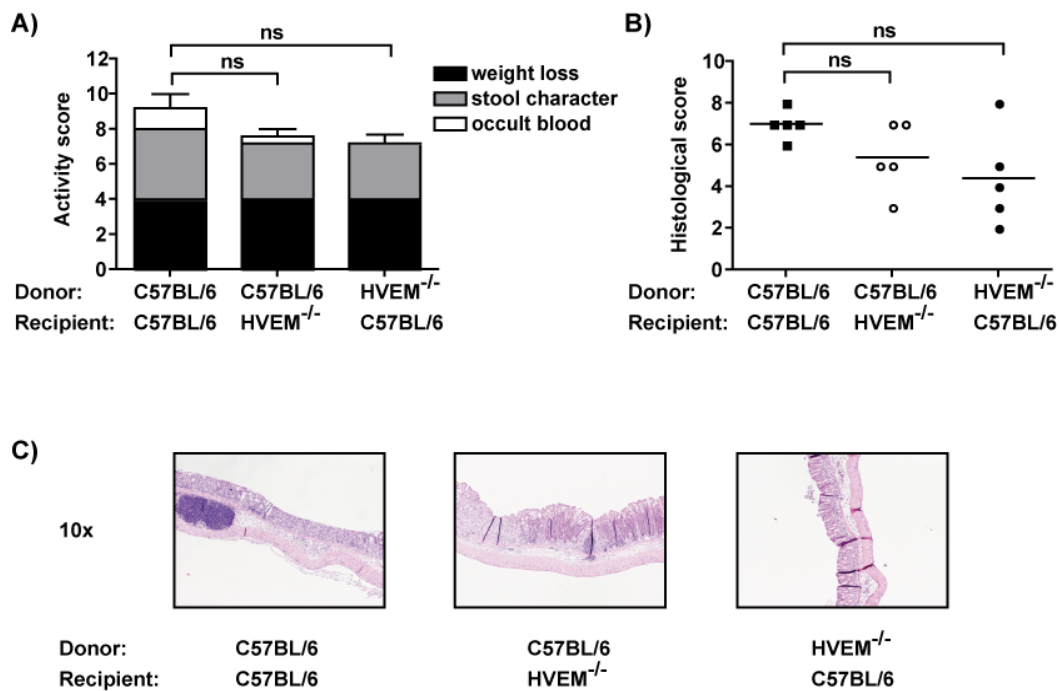
Figure 4. Partial contribution to intestinal inflammation of non-hematopoietic expressed HVEM. Chimeric mice were given 5% DSS in the drinking water for 4 days, then returned to normal drinking water and sacrificed at day 6. (A) Clinical activity scores were assessed at the time of sacrifice by a combination of total weight loss, stool character and occult blood. Data in A and B represent the mean ± SD for two independent experiments (n = 10 total mice per group). (B) Histological scores indicating immuno-pathology were calculated as described in the Materials and Methods and are shown for the distal part of the colon of individual mice. (C) Representative H&E staining of distal colon tissue sections from DSS-treated mice. Scale bars are 10× magnification = 200 µm Symbols show individual animals from one independent experiments out of two (n = 5 mice per group). No statistical differences were observed between groups as determined using an unpaired Student *t* test.

4.19 Discussion

LIGHT-HVEM interactions have been previously implicated in IBD but the therapeutic potential of targeting this stimulatory pathway remains unclear. In the previous chapter we assessed a role for these molecules in promoting T cell activation during intestinal inflammation and in this chapter we utilized a model of acute DSS induced intestinal inflammation to address the role of innate immune cell activation. In this model, administration of DSS via the drinking water results in damage to the intestinal epithelium that in turn allows infiltration of intestinal bacteria into the underlying tissue resulting in acute inflammatory events mediated largely by innate immune cells. DSS-induced acute intestinal inflammation was attenuated in HVEM$^{-/-}$ mice, and partially reduced in mice genetically deficient for LIGHT. Reduced weight loss, rectal bleeding and immuno-pathology in HVEM$^{-/-}$ mice were associated with diminished levels of pro-inflammatory cytokines, possibly resulting from reduced activation of intestinal APCs. Additionally, partial reduction in intestinal inflammation was observed in chimeric mice, which either lack HVEM expression on the hematopoietic or the non-hematopoietic cellular compartment. Thus stimulatory signals delivered through HVEM, to innate immune cells, as well as to stromal and/or epithelial cells are necessary for the development of experimental intestinal inflammation.

Although LIGHT is a known stimulatory ligand for HVEM, LIGHT$^{-/-}$ mice only exhibited a partial defect in DSS-mediated intestinal inflammation. These data indicate that alternative stimulatory ligands for HVEM must exist in vivo. LTα3 is produced by activated T-, B- and NK-cells and has been reported to bind to HVEM in vitro (16). Although not formally proven, it was postulated that LTα3 delivers stimulatory signals to HVEM based on the finding that both LIGHT and LTα3 bind to the CRD2 and CRD3 regions of HVEM (17). Paradoxically, LTα-deficient mice are reported to exhibit increased disease

severity following DSS administration suggestive of a regulatory role for LTα in this model (10). However, it should be kept in mind that delineating a clear role for LTα3-HVEM interactions *in vivo* is difficult as LTα forms a heterodimer with LTβ which acts to stimulate stromal cell-expressed LTβ receptor (LTβR), an interaction that is essential for lymphoid organogenesis and organization (reviewed in (18)). Accordingly, LTα deficient mice exhibit defective lymph node development (19, 20), and the absence of Peyer′s patches and mesenteric lymph nodes in these mice complicates accurate investigation of the impact of LTα3-HVEM interactions on intestinal inflammation.

In summary, we have demonstrated that HVEM-mediated signals play an essential role in the development of intestinal inflammation. However, the direct impact of HVEM signals on cells of the innate immune system, such as DC or monocytes remains to be further elucidated.

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5   General discussion

The aim of this thesis was to investigate how LIGHT- and HVEM-mediated stimulatory signals regulate intestinal immune responses during homeostasis or inflammation. We first investigated whether LIGHT or HVEM gene deficiency impacts on the development, activation or differentiation of T and B cells under homeostatic conditions. Secondly, we assessed the role of LIGHT or HVEM stimulatory signals in the induction and maintenance of innate and adaptive immune responses during intestinal inflammation.

To investigate the role of LIGHT or HVEM in intestinal inflammation we used two mouse models replicating distinct components of human IBD. These studies have allowed us to reveal that HVEM-mediated co-stimulatory signals, and to a lesser extent LIGHT-mediated co-stimulatory signals, to both innate immune cells and CD4+ T cells formed an essential component of immune cell activation, proliferation, pro-inflammatory cytokine production and intestinal pathology.

These findings are in keeping with previous observations where treatment of mice with an inhibitory LTβ receptor (LTβR)-Ig fusion protein - postulated to block LIGHT-mediated signaling through both HVEM and LTβR - can attenuate $CD4^+CD45RB^{high}$ T cell-induced colitis in SCID mice, bone marrow-transplanted tg26 colitis models and hapten- or dextran sodium sulphate (DSS)-induced colitis (1).

Although similar effects on intestinal inflammation were seen for both $LIGHT^{-/-}$ and $HVEM^{-/-}$ mice, one caveat of our studies is that the independent impact of these genes cannot be clearly attributed to the interaction of LIGHT with HVEM, as other ligand-receptor interactions need to be taken into account (Figure 1). Although in both cases gene absence resulted in attenuation of disease it is important to remember that LIGHT can also

mediate stimulatory signals via LTβR. Indeed, a previous study by Wang et al. demonstrated, that LIGHT-mediated intestinal inflammation in a T cell-dependent model of colitis depends on both, HVEM and LTβR (2). LIGHT$^{-/-}$ mice of course lack both LIGHT- LTβR signaling and LIGHT-HVEM signalling, thus we cannot completely exclude a role for LTβR-mediated signaling in the abrogation of the intestinal inflammation following T cell transfer or DSS administration. By comparison, HVEM$^{-/-}$ mice lack not only HVEM-LIGHT interactions but also HVEM-BTLA and HVEM-CD160 interactions. LIGHT is well known to deliver stimulatory signals through HVEM making our observations of attenuated intestinal inflammation in the absence HVEM signaling likely to reflect a lack of LIGHT-HVEM intereactions. However, it was recently reported that BTLA binding to HVEM has been provide survival signals to T cells via HVEM mediated intracellular signaling pathways (3). This finding complicates our own conclusions and indicates that predominant inflammatory role observed or HVEM-mediated signaling in our experiments may reflect an absence of LIGHT-HVEM or BTLA-HVEM interactions.

In spite of these caveats our experiments do conclusively show that the absence of HVEM leads to an attenutation of intestinal inflammation indicative that HVEM-mediated stimulatory signals play a predominant role in disease progression. HVEM signaling to both CD4$^+$ T cells and innate immune cells was determined to be important for intestinal inflammation. HVEM-mediated signals to T cells were important for their survival and development into effector cells with colitogenic potential and for IL-17 production. This may be in part due to the role of HVEM in maintaining the IL-6R expression on naive HVEM$^{-/-}$ T cells. We also found a role for LIGHT mediated signaling in promoting IL-6 dependent T cell proliferation supporting the idea that the HVEM-mediated stimulatory signals to T cells were mediated by LIGHT in our model. HVEM-mediated stimulatory signals to innate immune cells were important for full inflammation following DSS administration and these signals

were likely to be delivered in part through LIGHT as LIGHT deficiency resulted in an intermediate phenotype. However, stimulatory ligands other than LIGHT must exist. As discussed in the results part 2, such ligands may include BTLA or LTα3.

Although we noted attenuated intestinal inflammation in the absence of HVEM, it is important to keep in mind that HVEM can also act as an inhibitory ligand and that therapeutic targeting of HVEM may interfere with both stimulatory and regulatory pathways. HVEM signaling to BTLA (4) and CD160 (5) can result in an inhibition of T cell responses (4-6). Indeed, inhibitory signals mediated by HVEM have been postulated to explain observations in HVEM^{-/-} mice of increased mortality during ConA-mediated autoimmune hepatitis (7), and increased susceptibility to MOG peptide-induced (EAE) (7).

During the course of this thesis Steinberg et al. demonstrated a critical role for HVEM expressed by stromal cells in preventing the onset of intestinal inflammation (8). In contrast to our results, Steinberg et al. did not observe an increased weight loss in RAG^{-/-} mice transferred with either HVEM or LIGHT deficient T cells. However, similar to our observations, they also saw a clear reduction in the histopathological score in the colon of these mice. These data indicate that even though they did not see any gross difference in weight loss, the absence of HVEM or LIGHT on the T cells diminished intestinal inflammation. Another important point is that the weight loss in this model does not always correlate with intestinal inflammation and may rather account for the systemic disease. We performed a more extensive analysis of the T cell response in the intestine and draining MLN and observed a more profound role for HVEM in promoting intestinal inflammation. Intriguingly, their study revealed an accelerated intestinal inflammation when HVEM expression was specifically lacking on radioresistant cells in the RAG^{-/-} recipients. This indicated that HVEM expression by stromal cells plays a predominantly anti-

inflammatory role during intestintal inflammation. This anti-inflammatory role of HVEM was further shown to be mediated through the inhibitory signaling via T cell expressed BTLA. Contrasting our results, they conclude that HVEM has a dominant inhibitory role in intestinal inflammation. A possible explanation for the apparent discrepancies between this studies and our own is that BTLA-HVEM and CD160-HVEM inhibitory signals may function largely to switch-off immune responses, whilst HVEM stimulatory signals are required in some instances to turn-on immune responses. Support for this hypothesis comes from the finding that expression levels of BTLA and CD160 are increased on T cells following their activation (9).

In summary, we have demonstrated that HVEM-mediated stimulatory signals play a non-redundant role in the development of intestinal inflammation. We show that HVEM is required for the activation of both the innate and adaptive arms of the immune response, and conclude that HVEM mediated signaling predominantly leads to the promotion of intestinal inflammation.
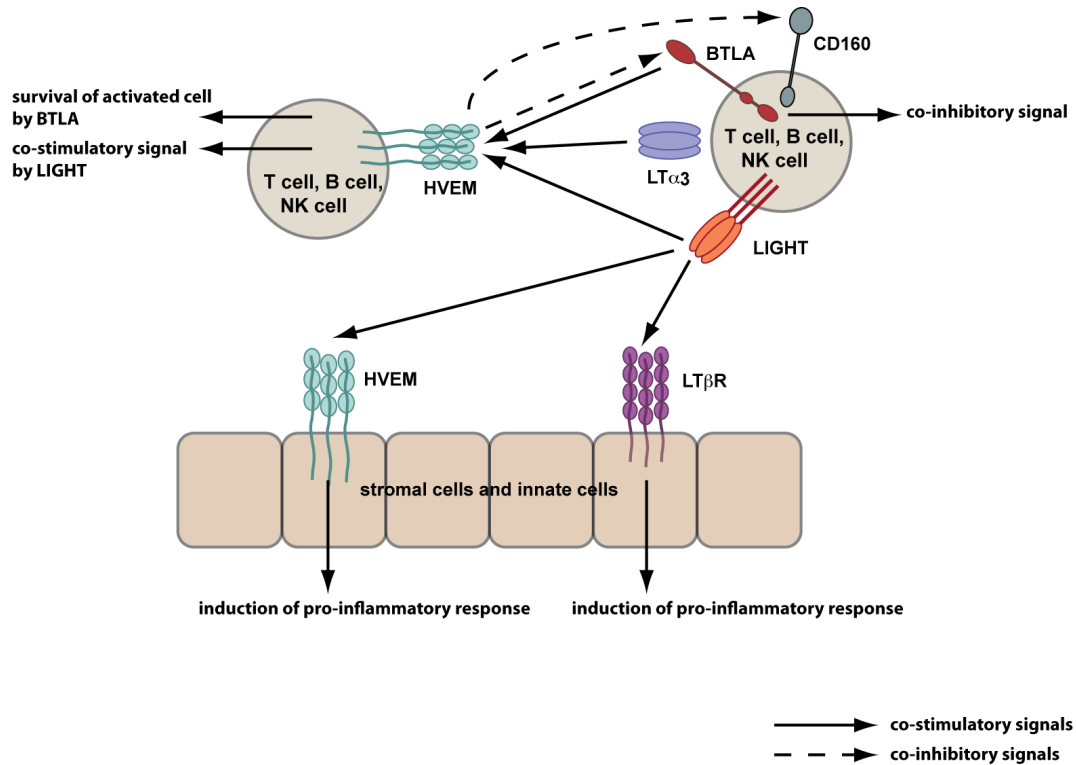
Figure 1: Hypothetical roles of HVEM and its cognate interaction partners during intestinal inflammation. LIGHT, BTLA and CD160 all bind to HVEM. Signals mediated via HVEM following binding of LIGHT or BTLA can induce stimulatory signals that potentiate survival and inflammatory responses (solid line) and are important for the initiation of inflammatory responses. Inhibitory signals (dotted line) mediated through BTLA or CD160 following their ligation to HVEM function to down-regulate inflammation at late time-points during immune responses. LIGHT can also promote inflammatory responses through binding of an alternative ligand, LTβR, present on stromal cells and some DC.

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6 Appendix

6.1 Abbreviations

BTLA	B- and T- lymphocyte attenuator
CD	Crohn's disease
CRD	Cysteine-rich domain
CTLA-4	Cytotoxic T- lymphocyte antigen-4
DC	Dendritic cell
DSS	Dextran-sodium sulfate
EST	Expressed sequence tag
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphoid tissue
gD	glycoprotein D
GIT	Gastrointestinal tract
HVEM	Herpes virus entry mediator
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFNγ	Interferon γ
IL	Interleukin
ILF	Isolated lymphoid follicles

LP	Lamina propria
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
JNK	c-Jun N-terminal kinase
LIGHT	lymphotoxin-like, exhibits inducible expression and competes with herpes simplex glycoprotein D for HVEM, a receptor expressed by T- lymphocytes
LN	Lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTβR	Lymphotoxin β receptor
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
Mϕ	Macrophage
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization protein
PAMP	Pathogen-associated molecular patterns
PD-1	Programmed death-1
PP	Peyer's patches
PRR	Pattern recognition receptor

RAG	Recombinase activating gene
SCID	Severe combined immunodeficiency
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNBS	Trinitrobenzene-sulfonic acid
TNF	Tumor necrosis factor
TNFSF	Tumor necrosis factor super family
TRAF	TNF receptor-associated factor
Treg	Regulatory T cell
UC	Ulcerative colitis

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