Cytomegalovirus modulates acute colitis and impairs the resolution of inflammatory processes

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Summary

Human cytomegalovirus (HCMV) is an opportunistic pathogen that is ubiquitously present in the human population and implicated in numerous inflammatory processes within the body. The virus is a leading cause of morbidity and mortality in immunocompromised individuals, causing severe end organ diseases such as HCMV colitis. In patients with inflammatory bowel disease (IBD), the prevalence of active intestinal HCMV replication is significantly increased and associated with exacerbated disease progression. However, the underlying pathomechanism by which an intestinal HCMV manifestation affects both the intestine under homeostatic conditions and the inflammatory processes during an acute colitis remains to be elucidated. The present study aims to investigate to which extent a CMV infection affects the intestinal milieu.

In immunocompetent BALB/c mice a mouse CMV (MCMV) infection established a robust viral replication in the colonic tissue. Consequently, a mild pro-inflammatory milieu was induced mediated by alterations in the secretion of TNFα, IFNγ and granzyme B. Furthermore, the viral replication induced apoptosis in the intestinal epithelium, and thereby, compromised the barrier integrity and disrupted the homeostatic conditions in the colon. To further assess the consequences of the viral replication on acute intestinal inflammation, mice were infected during the course of dextran sodium sulfate (DSS)-induced colitis. Consistent with the findings in IBD patients, the viral infection aggravated the inflammatory processes during acute colitis and impaired the subsequent resolution of intestinal inflammation compared to uninfected colitis mice. During the recovery phase, the MCMV infection induced the accumulation of pro-inflammatory innate and adaptive immune cells in the colonic lamina propria. Notably, the presence of cytotoxic $CD8^+$ T cells contributed to the intestinal inflammatory processes. The depletion of these pro-inflammatory T cells resulted in a tremendous amelioration of colitis and enhancement of intestinal inflammatory resolution. Furthermore, the compromised recovery phenotype of the infected mice was further mediated by the functional impairment of the intestinal stem cells (ISCs), which were demonstrated to be susceptible to MCMV. Subsequent RNA sequencing analysis of infected ISCs indicated a wide array of differently expressed genes mediating severe metabolic changes for the multipotent stem cells. Moreover, the infected ISCs exhibited a significantly increased expression of chemokines, thereby orchestrating the demonstrated accumulation of the pro-inflammatory immune cells in the gut. Finally, vaccination with the ΔM27-MCMV live-attenuated virus demonstrated robust protection against a challenge MCMV infection during acute colitis, emphasising the urgent need for the development of a potent human CMV vaccine in the context of IBD. In conclusion, this study provides new insight into the pathomechanism by which CMV affects the inflammatory processes in the gut during acute colitis.

Zusammenfassung

Das humane Cytomegalievirus (HCMV) ist ein opportunistischer Erreger, der in der menschlichen Bevölkerung ubiquitär vorkommt und an zahlreichen Entzündungsprozessen im Körper beteiligt ist. Bei Personen mit einer geschwächten Immunabwehr führt das Virus zu einer hohen Morbidität und Mortalität und verursacht schwere Endorganerkrankungen, wie HCMV-Kolitis. Bei Patienten mit chronisch-entzündlichen Darmerkrankungen (CED) ist die Prävalenz der intestinalen HCMV-Replikation signifikant erhöht und ist zudem mit einer Verschlechterung des Krankheitsverlaufs assoziiert. Der zugrundeliegende Pathomechanismus, durch den eine intestinale HCMV-Manifestation sowohl den Darm unter homöostatischen Bedingungen als auch die Entzündungsprozesse während einer akuten Kolitis beeinflusst, ist bislang ungeklärt. Ziel der vorliegenden Arbeit ist es zu untersuchen, inwiefern eine CMV Infektion das intestinale Milieu beeinflussen kann.

In immunkompetenten BALB/c Mäusen führte eine Infektion mit murinem CMV (MCMV) zu einer robusten Virusreplikation im Kolongewebe. Infolgedessen wurde ein leichtes pro-inflammatorisches Milieu induziert, das durch Veränderungen in der Sekretion von TNFα, IFNγ und Granzym B vermittelt wurde. Darüber hinaus induzierte die virale Replikation Apoptose in den Darmepithelzellen, wodurch die Integrität der Darmbarriere beeinträchtigt und die homöostatischen Bedingungen im Kolon gestört wurden. Um die Auswirkungen der Infektion auf eine akute Darmentzündung zu untersuchen, wurden Mäuse im Verlauf einer durch Dextransulfat-Natrium ausgelösten Kolitis infiziert. In Übereinstimmung mit den Beobachtungen bei CED-Patienten, wurden die Entzündungsprozesse während der akuten Kolitis durch die virale Infektion verstärkt und das anschließende Abklingen der entzündlichen Prozesse im Vergleich zu nicht infizierten Mäusen mit Kolitis beeinträchtigt. In der Erholungsphase wurde eine Akkumulation von entzündungsfördernden, angeborenen und adaptiven Immunzellen in der Lamina propria des Kolons durch die MCMV-Infektion beobachtet. Die Anwesenheit zytotoxischer $CD8^+$ T-Zellen trug maßgeblich zu den entzündlichen Prozessen im Darm bei, während eine Depletion dieser Zellen zu einer signifikanten Verbesserung der Kolitis und einer verbesserten Auflösung der Entzündung im Darm führte. Darüber hinaus wurde die beeinträchtigte Genesung der infizierten Mäuse durch eine beeinträchtigte Funktionalität der intestinalen Stammzellen (ISCs) vermittelt, die sich als anfällig für MCMV erwiesen. Eine anschließende RNA-Sequenzierung infizierter ISCs zeigte eine Vielzahl unterschiedlich exprimierter Gene, die schwerwiegende metabolische Veränderungen der multipotenten Stammzellen zur Folge haben können. Darüber hinaus wurde eine signifikant erhöhte Expression von Chemokinen in den infizierten intestinalen Stammzellen beobachtet, was die nachgewiesene Anhäufung von entzündungsfördernden Immunzellen im Darm induziert haben könnte. Zudem konnte eine Impfung mit dem

abgeschwächten Lebendvirus ΔM27-MCMV einen robusten Schutz gegen eine MCMV-Infektion während der akuten Kolitis induzieren. Dies verdeutlicht die dringende Notwendigkeit der Entwicklung eines wirksamen humanen CMV-Impfstoffs im Zusammenhang mit IBD. Zusammenfassend hat diese Studie neue Einblicke in den Pathomechanismus ermöglicht, durch den CMV die Entzündungsprozesse im Darm während einer akuten Kolitis beeinflusst.

1. Introduction

1.1. Anatomy of the intestinal tract

The intestinal tract fulfils several important functions: the digestion of the ingested diet, absorption of nutrient and water as well as the protection against external insults, as it is permanently confronted with challenges from the environment. The human intestinal tract shapes a surface of estimated of >30m^2 [1] in form of a continuous tube, that can be divided in the upper and lower gastrointestinal tract (GI). The upper GI is composed of the oral cavity, oesophagus, stomach & the small intestine (SI), while the lower GI contains the large intestine (colon), rectum and anus. The different compartments each fulfil distinct physiological functions [2].

The SI and colon consist of a smooth muscle tube, assembled from different layers (Fig. 1). Three sub-layers shape the innermost mucosa: the epithelium encompassing the gut lumen, surrounded by the lamina propria and an inner muscle layer (*muscularis mucosa*). The mucosal epithelium is adjusted to the regional function of the GI such as the secretion of mucus and enzymes to aid in digestion or protection of the mucosa. The lamina propria consists of connective tissue, important for transport of ingested nutrients and comprises a large part of the intestinal immune system. The submucosa comprises blood and lymphatic vessels and nerves, followed by *muscularis propria*, a layer of circular and longitudinal muscles important for the peristalsis of the intestinal tract. The smooth muscle peristalsis is responsible for the movement of the luminal contents, ensures optimal contact time to the epithelium for digestion and absorption. The outermost layer of the gut is the serosa, a fibrous layer [2].

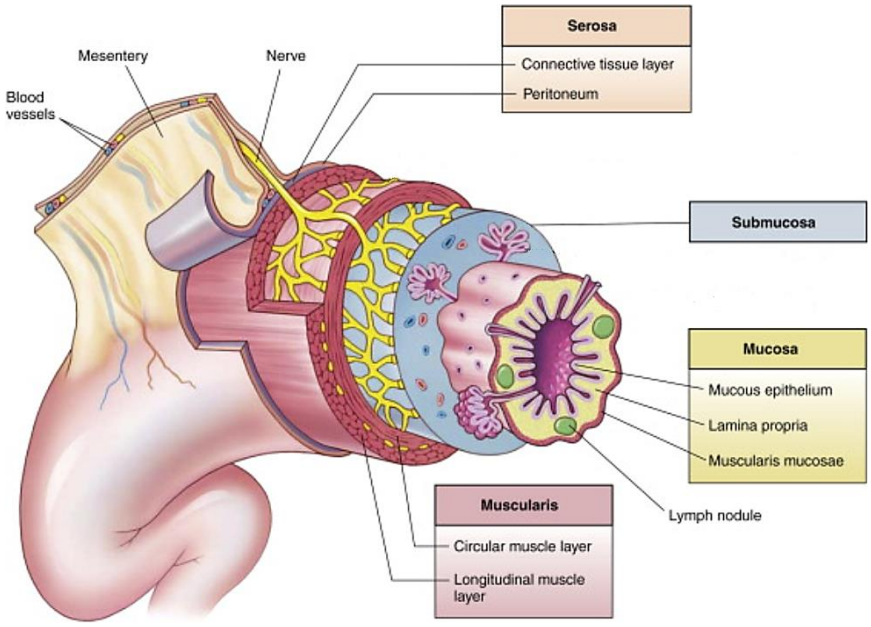

Fig. 1: The different layers of the gastrointestinal tract. The wall of the GI is assembled from innermost mucosa, the submucosa, the *muscularis propria* and the outermost serosa (adapted from [2]).

Nutrient absorption is an adjusted and fine-tuned process, that allows nutritional components to pass but at the same time blocks and protects against potentially harmful material like pathogens and toxins [3-5]. Responsible for this is the intestinal barrier, which is composed of the epithelial barrier, the overlying mucus layer and the underlying mucosal immune system.

1.2. Intestinal epithelial barrier

The intestinal epithelium is a physical barrier that regulates the interaction between luminal contents, immune cells and the underlying tissue. It consists of a continuous single layer of cells, which stretches from the outlet of the stomach to the anus. The intestinal epithelial cells (IECs) are connected by intercellular junctions, also termed as apical junctional complex (AJC) at the lateral membrane of the cells. The AJC is formed by tight junctions, adherens junctions and desmosomes [6]. Tight junctions are transmembrane proteins like claudins and occludin, which form pores to modulate ion transport [7], scaffolding proteins like Zonula Occludens family proteins (ZO-1, -2, and -3), which connect transmembrane proteins and regulatory proteins [8]. Adherens junctions and desmosomes supply adhesive forces for cell-cell interactions by components such as cadherin proteins, linker proteins and intermediate filaments [9-11]. The AJC does not lead to the formation of an absolute barrier, but rather to a selective permeability, which regulates the exchange of molecules via two defined routes dependent on size and charge. The pore pathway has a high capacity and is responsible for the passage of large quantities. It induces a charge-selective passage of small ions and uncharged molecules. The second leak pathway has a low capacity, inducing the flux of larger ions and molecules independent of their charge. The permeability is fine-tuned and varies along the intestinal tract due to variations in the composition of the AJC [12]. The loss of AJC functionality and apoptosis of epithelial cell is induced in several diseases, leading to the loss of local barrier function and therefore enable an unrestricted pathway, where luminal contents can cross the barrier in an uncontrolled manner [11].

Further, the IECs are organized in millions of crypt and villus units. Villus are finger-like protrusion of the barrier that extend into the lumen, while crypts are invaginations formed into the epithelium. The crypt and villus formation improves the absorptive capacity of the intestinal tract by massively increasing the surface area [5]. The structure of the epithelial surface is adjusted to the regional function of the GI, as the absorptive capacity but also the contact time of the luminal content varies. In the SI and especially the duodenum, the longest villi can be found, while the length of the villi decrease continuously along the intestinal tract. In the colon no villi are formed, here the epithelial layer constitutes only of crypt units [13].

To minimize epithelial damage and the risk of infections, which would further endanger the integrity of the epithelial barrier, the epithelium is continually renewed. Therefore, the intestinal

tract is a highly dynamic structure. The lifetime of IECs is approximately 3-5 days [14]. Presupposing the ongoing exchange of cells are the intestinal stem cells (ISCs), long-lived, multipotent cells at the bottom of the intestinal crypts (Fig. 2). The ISCs are responsible for the self-renewal and homeostatic epithelial turnover and can be identified by the expression the leucine-rich repeat–containing G protein–coupled receptor 5 (Lgr5) on the cell surface. Lgr5 is mechanistically responsible for binding its ligands R-spondin1-4 proteins, to induce the canalisation of the stem cell self-renewal [15, 16]. ISCs undergo asymmetric division to generate progenitor cells, also termed as transit-amplifying (TA) cells [14]. TA cells rapidly proliferate and differentiate into mature IECs, which migrate upwards the crypt/villus-axis. The only exception are paneth cells in the SI, which stay at the bottom of the crypts. Mature IECs undergo apoptosis after their short lifetime, shedding finally into the intestinal lumen [17].

The microenvironment of the ISCs is defined as the intestinal niche, directly affecting their fate and maintaining the optimal stemness. The surrounding cells, such as the subepithelial mesenchymal and hematopoietic cells, neurons and attached epithelial cells are important to keep balance between epithelial turnover and neoplastic overgrowth. Important modulatory factors of the ISCs are the pro-epidermal growth factor (pro-EGF), Wnt, R-spondin and Notch ligands [18, 19]. TA cells can differentiate into six different epithelial cell types (Fig. 2). The absorptive cell lineage comprises the enterocytes, important for the nutrient uptake along the intestinal tract and the microfold (M) cells, responsible for antigen-sampling and which will be introduced more detailed in the following paragraphs. The secretory cell lineage encompasses goblet cells, responsible for the mucus secretion, paneth cells, important for the host defence against microbes, enteroendocrine cells and tuft cells. The secretory cells support the intestinal barrier function via the secretion of mucus, hormones and antimicrobial peptides (AMPs) [19]. The ratio of different cell types is highly balanced, important to maintain homeostasis and adapted to the functions of the intestinal compartment [14].

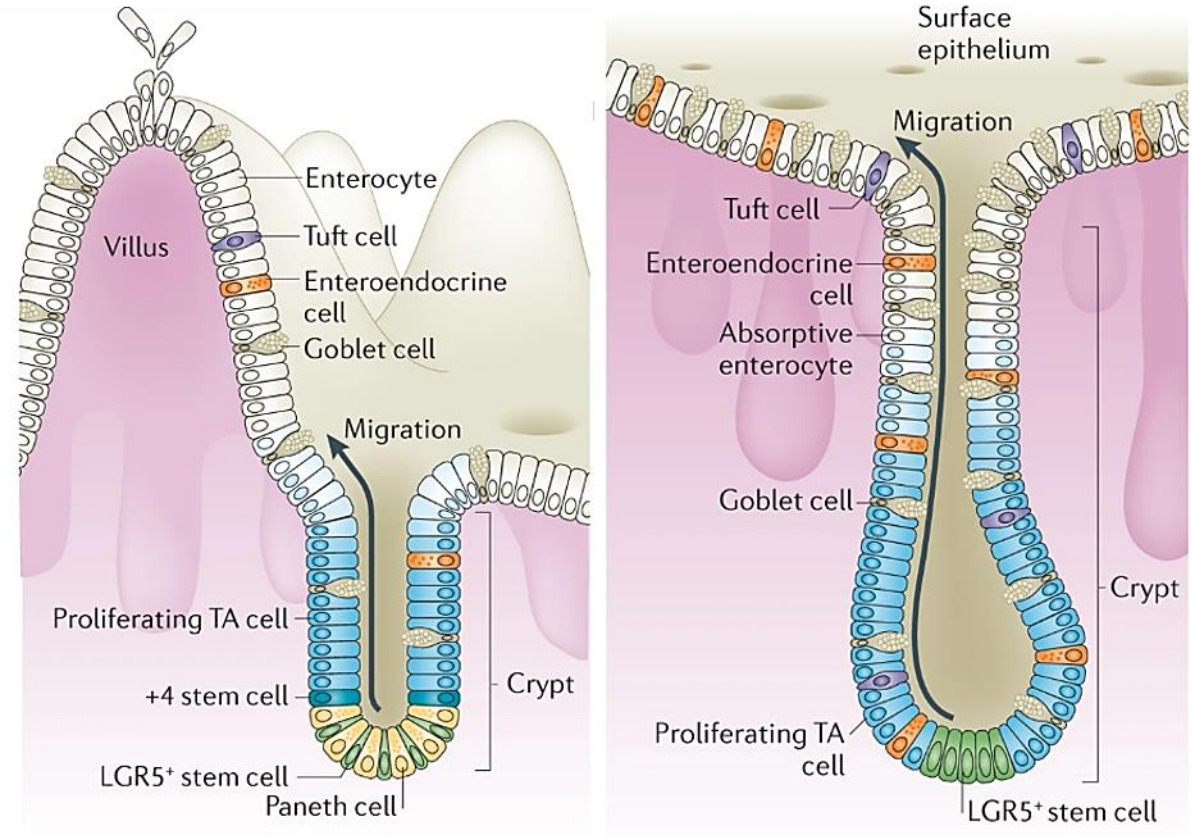

Fig. 2: Intestinal stem cells (ISCs)-mediated self-renewal of the intestinal epithelium. In the small intestine (SI, **left**) and the colon (**right**) ISCs undergo asymmetric divisions to generate progenitor transit-amplifying (TA) cells. TA cells can differentiate into absorptive epithelial cells (enterocytes) and secretory epithelial cells (goblet cells, paneth cells (only in the SI), enteroendocrine cells and tuft cells). Mature intestinal epithelial cells (IECs), except paneth cells, migrate upwards the crypt/villus-axis. Apoptotic IECs shedd into the intestinal lumen (Figure adapted from [20]).

While paneth cells can only be found in the SI [21], the frequency of e.g. goblet cells increases in the colon, where they constitute 25% of all epithelial cells in comparison to its occurrence of 10% in the SI. Goblet cells are responsible for the secretion of glycosylated proteins, the mucins, building up the mucus layer, which is protectively covering the epithelial cells. In the colon, the generated mucus layer is thicker compared to the one in the SI [22]. The colonic mucus-layer can be dived into the inner dense layer, which functions as a physical barrier and further contains AMPs and immunoglobulin A (IgA) to keep commensal and pathogenic bacteria in distance to the epithelium, and a looser layer, which serves as habitat for the commensal bacteria [22-24]. AMPs exert antimicrobial activities. In the SI they are produced by epithelial and Paneth cells and contribute to the innate host defence [25]. Beside the distinct function of the different epithelial cell types, the epithelium can orchestrate intestinal immune cells by the secretion of immunomodulatory factors such as cytokines and chemokines [26].

1.3. Intestinal immune system

The intestinal tract is a mucosal surface harbouring the largest number of immune cells of any tissue in the human body. Immune cells are derived from haematopoietic stem cells. They differentiate in the bone marrow or thymus into progenitor cells of the myeloid or lymphoid lineage. Common myeloid progenitors can further develop via precursor cells of the granulocyte lineage into innate immune cells like monocytes, macrophages, mast cells and granulocytes. Common lymphoid progenitor give rise to mature lymphocytes like innate lymphoid cells (ILCs) including natural killer (NK) cells, but also T and B and NK-T cells of the innate or adaptive immunity [27]. The intestinal tract is continually exposed to a variety of antigens and immune stimuli like commensal and pathogenic bacteria, viruses and fungi as well as dietary constituents. The intestinal immune system is fine-tuned and highly dynamic to adapt to and function within those constantly changing conditions. Activation of pro-inflammatory processes in response to pathogens and the tolerance of symbiotic microbiota and food antigens need to be strongly balanced [28].

The majority of immunological processes is taking place in the intestinal mucosa, which is further organized in different mucosal lymphoid structures. It can be classified physically but also by its function into inductive and effector sites. The inductive sites comprise the gut-associated lymphoid tissue (GALT), either clustered as Peyer's patches (PPs) in the small intestine or isolated lymphoid follicles (ILFs) along the whole intestinal tract and the intestinal draining mesenteric lymph nodes (mLN). PPs mainly localize in the distal ileum of the SI and are lymphoid organized structure, containing adaptive immune cells organized in B-cell follicles surrounded by T-cell areas as well as innate immune cells [29]. ILFs are smaller than PPs but with a similar function and structure, while mLN are encapsulated and connected to the afferent lymphatics. mLN filter lymph from the intestine and are the largest lymph nodes in the human body [30]. The inductive sites are important for initial priming and differentiation of adaptive immune cells. The effector sites encompass the lamina propria and the intestinal epithelium. The lamina propria is a loosely packed connective tissue, on one side important as the scaffolding for the formation of the villus and crypt structures and on the other side the area, where the majority of intestinal immune cells, including cells of the innate and adaptive immunity, are localized [21, 31, 32]. The innate and adaptive immunity are described in more detail in the following sections and summarized in Fig. 3.

1.3.1. Innate immunity

The innate immunity is evolutionary highly conserved. It forms the first line of defence against invading pathogens and harmful molecules to prevent infection and maintain homeostasis [33]. It comprises the recognition of foreign antigens, restriction of the spread of pathogens and their

elimination and the initiation and activation of the more specific adaptive immune response, if the innate immune response is not sufficient to eliminate the threat [34]. Elements of the innate immunity include the chemical and physical barriers induced by the intestinal epithelium and the overlying mucus layer, humoral effector mechanisms such as the AMPs, the complement system and cytokines as well as cellular responses [35]. If a pathogen overcomes the first barriers, innate immune cells have to induce an immediate non-specific immune response. The recognition of conserved pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), important danger signals is an essential function of the innate immune system. PAMPs are exogenous components, essential and highly conserved in microorganisms including polysaccharides like lipopolysaccharides (LPS), lipids and nucleotides, while DAMPs include endogenous molecules which are released during inflammation and tissue damage like e.g. mitochondrial deoxyribonucleic acid (DNA) [36]. They are recognized by pattern recognition receptors (PRRs), expressed by a broad variety of cells of the innate immunity including hematopoietic cells like granulocytes, macrophages, dendritic cells (DCs) and NK cells as well as non-hematopoietic cells like epithelial, endothelial cells and fibroblasts of mucosal tissues [37]. Binding induces immunoprotective effects such as the induction of inflammation and immune responses to infection [38].

Several receptor types have been identified as PRRs including different subfamilies such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs) [36]. TLRs are type-I transmembrane glycoproteins. In the humans, 10 different TLRs have been discovered so far. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface and important for the recognition of microbial derived membrane components. TLR3, 6, 7, 8 and 9 are exclusively expressed in intracellular vesicles like endosomes and responsible for the recognition of nucleic acids of microorganisms [39]. NLRs and RLRs are located in the cytoplasm and recognize nucleic acids and cell wall components of intracellular pathogens like viruses but also bacteria and parasites, which are able to escape the phagosome by entering the cytoplasm of the host cell [38]. After the recognition of pathogens via PRR-binding, innate immune cells are activated and can induce the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-6 and IL-1, chemokines and other pro-inflammatory factors and can implement the direct killing of a pathogen [38].

Phagocytes such as monocytes, macrophages and neutrophils are important constituents of the killing capability of the innate immunity. Upon activation, phagocytes are able to engulf particles, microbes or even infected cells, which have been marked e.g. by immunoglobulins. The process is entitled as phagocytosis [27]. In the phagosome killing is induced by the release of reactive oxygen species (ROS) and phospholipases to induce sterilization, followed by the

degradation via myeloperoxidase (MPO), lysosomes and the acid conditions in the phagosome [40]. Additionally to the phagocytosis, macrophages have the ability to produce different cytokines. Macrophages can have a pro-inflammatory or immunomodulatory phenotype, dependent on the activation signals during the maturation and differentiation of the immature precursor cells. Cytokines such as interferon (IFN)γ and TNFα as well as activation signals like PRR-binding induce the differentiation into pro-inflammatory macrophages capable to produce high amounts of IFNγ, IL-6, IL-12, and TNF. On the other hand, IL-4, IL-10, and IL-13 induce an immunomodulatory macrophage phenotype capable to produce e.g. IL-10 and transforming growth factor (TGF)-β with anti-inflammatory and tissue regeneration properties [41, 42].

Further, the release of cytotoxic granules by granulocytes is another fast response against pathogens by the innate immunity. Upon activation granulocytes can induce the degranulation process to release their content of the granules into the extracellular matrix and directly target pathogens [43]. Three different cell types of granulocytes can be distinguished by nuclear morphology, size, granule content, and their function. Neutrophils are the smallest granulocytes with a granule-loaded cytoplasm, able to phagocytose and kill pathogens intracellularly in the phagosomes via ROS and proteases as well as to immobilize and kill extracellular pathogens in neutrophil extracellular traps (NETs). NETs are mediated by the release of netlike structures of DNA coated with histones, elastase and MPOs [44]. Eosinophils contain granules in the cytoplasm packed with specialized and cytotoxic enzymes like eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). They are known to be important during the defence against parasitic infections but also enhanced during allergic reactions [45]. The third type of granulocytes are the basophils, containing histamine and other preformed mediators in their granules and are important in the host defence against helminths [46].

Another important function of innate immunity is the antigen presentation by antigen presenting cells (APCs) to adaptive immune cells, bridging innate to the adaptive immunity. Classical APCs of the innate immune system are DCs and to a lower extent macrophages [47], which are present in inductive as well as effector sites. Due to the expression of PRRs and receptors for apoptotic cells as well as their phagocytic function, APCs are important for the early recognition of pathogens and the uptake and sampling of antigens. DCs are able to extend their dendrites through the epithelial cell tight junctions into the lumen, to directly sample antigens via phagocytosis and receptor-mediated endocytosis [48]. Further M cells, specialized epithelial cells of the follicle-associated epithelium [49] as well as enterocytes and goblet cells can passage antigens through the epithelium to the underlying APCs in inductive and effector sites [50]. Antigens are then further processed in the APCs and the resulting peptides can be presented by major-histocompatibility-complex (MHC) molecules on the

surface of mature APCs to adaptive immune cells to induce priming of naïve T cells and their differentiation. Other immune cells such as mast cells, basophils and eosinophils can function as atypical APCs by an inducible expression of MHC molecules [47].

Another group of innate immune cells comprise the ILCs, enriched at mucosal barriers and rapidly activated by cytokines and growth factors [51]. ILCs can have diverse functions including promoting fast host defence against pathogens, regulating immune responses against microbiota and orchestrating early tissue repair in the intestine [52]. They have a classic lymphoid cell morphology but lack the expression of typical adaptive T and B lymphocyte cell-surface markers such as T and B cell receptors. ILCs can be classified into cytotoxic ILCs, NK cells, and non-cytotoxic ILC1, ILC2 and ILC3 [53, 54]. Non-cytotoxic ILCs share cytokine profiles with the common adaptive T helper (Th) cells. Cytotoxic NK cells are able to induce granzyme- and perforin-mediated cytolysis during immunity to viral and bacterial infections [52, 55].

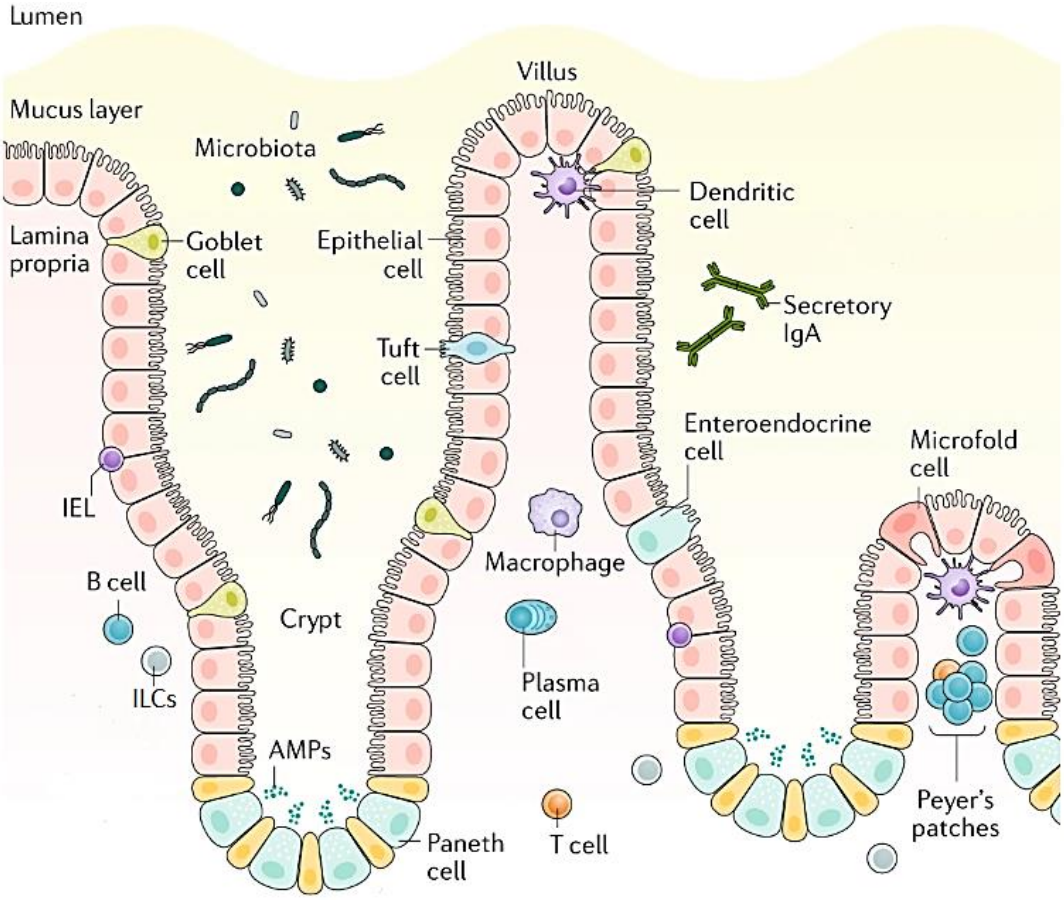

Fig. 3: Anatomy of the gut immune system. The intestinal epithelial barrier is covered by a dense mucus layer and separates the lumen from the lamina propria. The intestinal lumen and the mucus offer habitat to the microbiota. Mucus-producing goblet cells, AMPs-producing paneth cells, enteroendocrine cells, tuft cells and Mircofold (M) cells are part of the epithelium. Innate and adaptive immune cells are located in the lamina propria and the Peyer's patches. Plasma cells secrete Immunoglobulin A (IgA) into the mucus layer. Intestinal epithelial lymphocytes (IEL) are attached to the epithelium (adapted from [56]).

1.3.2. Adaptive immunity

The adaptive immunity encompasses specialized immune defence mechanisms developed late in evolution. It can be characterized by two fundamental features: specific antigen recognition and the development of a immunological memory by the production of long-lived immune cells [57]. The innate immune system possesses the capacity to sense a range of pathogens. However, the scope of PAMPs that it is capable of recognizing is still restricted and some pathogens evolved mechanism to avoid recognition by the PRRs. The adaptive immune systems enable a fine-tuned repertoire of antigen-specific receptors to initiate a broad and targeted immune response [58]. T and B lymphocytes form the adaptive immunity by inducing antigen-specific cellular and humoral immune responses, enabled through antigen-specific receptors expressed on the cell surface of B and T cells. B cells express immunoglobulins (Igs) such as the B cell receptor (BCR) or its secreted form: the antibodies, recognizing unprocessed antigens in their native form and responsible for the humoral immune response. T cells express the T cell receptor (TCR) recognizing processed antigens presented on MHC molecules to induce a cellular immune response [27, 59].

The production and secretion of antibodies, mediated by B cells and the differentiated plasma cells, are the central element of the adaptive humoral immune response. B cells arise in the bone marrow [58] and further develop in secondary lymphoid organs such as the spleen or the intestinal GALT and mLN [60], where they can differentiate into mature follicular B cells upon encountering a strong BCR signal [61]. Follicular B cells recirculate in the lymphatic system until cognate antigen encounter. They either differentiate into short-lived low-affinity IgM secreting plasma cells or mature with T cell help in a germinal centre under affinity maturation and Ig class switching into memory B cells or long-lived plasma cells. Germinal centre derived B cells are able to secrete IgG, IgA or IgE with a higher binding affinity [58, 62, 63].

T cells can be distinguished by their function and expression of TCR co-receptor molecules into immunomodulatory cluster of differentiation (CD)4^+ T cells and cytotoxic $CD8^+$ T cells. $CD4^+$ T cells are important for regulated specific immune responses. Their functions range from activation of innate immune cells, B and cytotoxic T cells to the suppression of inflammatory immune reactions. To perform their immune-specific effector functions, naïve $CD4^+$ T cells are deployed to secondary lymphoid organs [64] where they get activated and differentiate by the recognition of three distinct signals. The first signal is the binding of the TCR and the co-receptor CD4 to its specific antigen derived peptides presented on MHC class II complex on professional APCs. In case of naïve T cell activation, DCs are considered to be most potent activators [65]. Second, co-stimulatory signals of the DCs such as the binding of CD28 on T cells to its DC ligands CD80 and CD86 is needed for final activation [66]. $CD4^+$ T cells undergo further differentiation into $CD4^+$ T cell effector subtypes stimulated by the

surrounding cytokine milieu of the microenvironment, the third signal, and orchestrated by the expression of a master transcription factor (Fig. 4). Comparable to the ILC subsets of the innate immunity, differentiated $CD4^+$ T cells mediate their immunomodulatory functions by the secretion of cytokines [67]. Important for Th1 differentiation are the cytokines IL-12, secreted by activated APCs and IFNγ, produced e.g. by NK cells in response to IL-12 [68] and its transcriptional master regulator T-box transcription factor (T-bet) [69]. Th1 cells mediate immune responses against viruses and intracellular pathogens via the secretion of IFNγ and TNFα, which further activates macrophages by induction of ROS and phagocytic properties [67, 70]. Th2 differentiation is mediated by IL-2 and IL-4 and its master transcription factor GATA-binding protein (GATA3) [71]. Th2 T cells produce cytokines such as IL-4, IL-5 and IL-13, important for humoral immune response by induction of B cell activation and differentiation and further induce recruitment of eosinophils and mast cells e.g. during helminth infections [67]. Th17 differentiate in the presence of L-6, IL-23, and TGF-β and mediated by the transcription factor retinoic acid receptor-related orphan receptor gamma-T (RORγt) [72, 73]. They are able to secrete IL-17A and IL-22, which are important for the immune response to extracellular bacteria and fungi. IL-17A induces the secretion of chemokines via epithelial cells to promote neutrophil recruitment, and both cytokines together are known to induce AMP expression by IECs to strengthen the barrier function to pathogenic bacteria [67, 74, 75].

Regulatory T cells (Tregs) can be classified into natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs develop in the thymus, while iTreg development is induced from naïve T cells in the secondary lymphoid organs in the presence of IL-10 and TGF-β. These cells express their respective master transcription factor forkhead box P3 (FOXP3) upon stimulation [67, 76]. Tregs maintain immunologic tolerance to foreign and self antigens, and enable in the intestinal tract e.g. the tolerance of commensal microbiota [77]. Further, Tregs induce anti-inflammatory conditions after pathogen clearance, to prevent pathology by an overshooting immune response [78]. They secrete anti-inflammatory cytokines such as IL-10, IL-35 and TGF-β [67, 79].

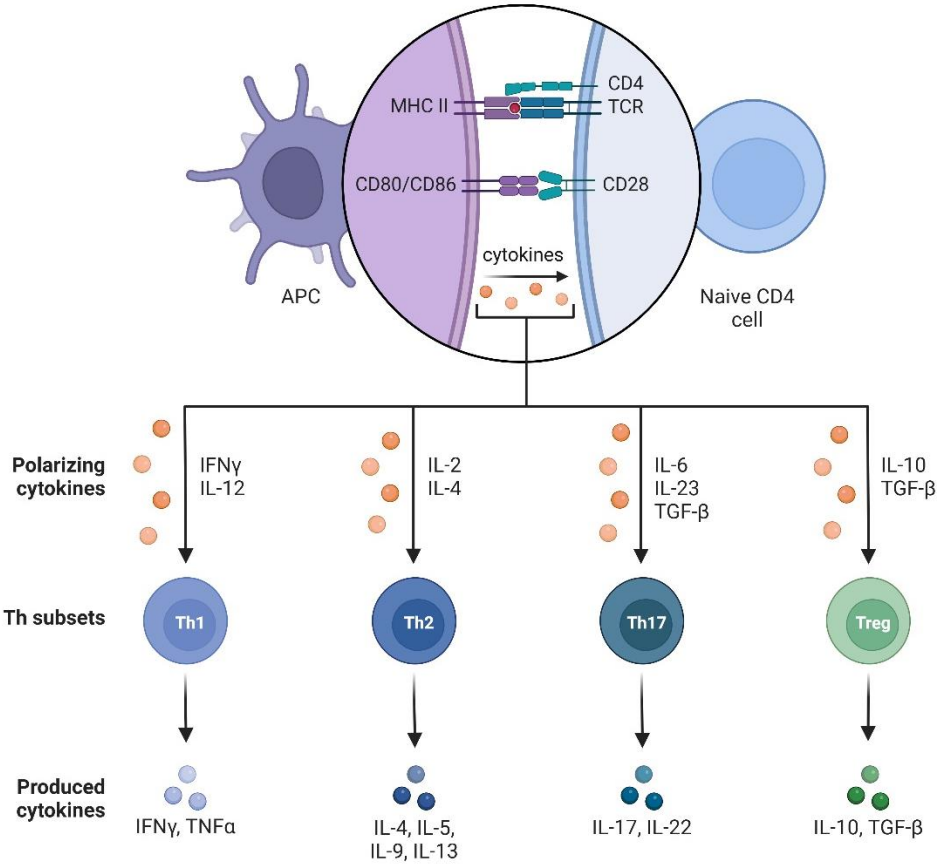

Fig. 4: CD4⁺ T cell activation and differentiation into T helper (Th) subsets. Naïve CD4⁺ T cells get activated upon binding their specific peptide presented on MHC class II complex on APCs via TCR and CD4 co-receptor binding. Co-stimulatory signals such as CD28 binding to its ligand CD80/CD86 is needed. The surrounding cytokine milieu is important for determining the T helper (Th) cell differentiation into Th1, Th2, Th17 and Treg cells (adapted from 'T cell activation and differentiation' from BioRender.com/biorender-templates, 2025).

Naïve CD8⁺ T cells recognize their target antigens presented on MHC class I complex on APCs via the TCR and the co-receptor CD8 in the secondary lymphoid organs. MHC class I molecules present peptides from antigens, which are degraded inside the proteasome of the cell [80]. Further, co-stimulatory receptors such as CD28 and CD40 are involved during the activation process of CD8⁺ T cells [66, 81]. For the differentiation into effector cells, cytokines such as IL-2, IL-12 and IL-15 are needed, which are produced by T helper cells and innate immune cells [82, 83]. CD8⁺ effector cells migrate into the periphery such as the effector sites of the intestinal tract, where they are able to massively expand upon secondary antigen-encounter, to generate a high number of effector cells. MHC class I molecules are expressed on almost all nucleated cells of the human body and thus CD8⁺ T cells are able to recognize their specific target antigen at many different locations and fulfil their effector functions [82]. Cytotoxic CD8⁺ T cells are important for the clearance of intracellular pathogens. They are able to rapidly kill infected cells via three distinct mechanisms. Upon TCR stimulation, the CD8⁺ T cell can release perforin and granzymes into the intracellular space, where perforin forms pores

into the membrane of the target cell so that the cytotoxic granzymes can enter the cytosol and initiate apoptosis [84]. Further, surrounding target cells can be affected via the release of the cytokines TNFα and IFNγ. Binding of TNFα to the TNF receptor (TNFR) on the target cell induces apoptosis through the caspase cascade. IFNγ can induce the upregulation of MHC class I and Fas receptors (CD95). Both, the Fas receptor and the TNFR belong to the death receptors and are part of the third pathway. By binding of the Fas ligand expressed on the cell surface of the $CD8^+$ T cell to the Fas receptor, apoptosis is induced in the target cell [85].

Activated lymphocytes migrate via chemotaxis from the inductive sites such as the mLN or the PPs into the lamina propria of the intestinal tract. The process of chemotaxis is mediated by adhesion molecules and chemokines, which are expressed or released at the effector sites, and the corresponding ligands and chemokine receptors that are expressed on the activated lymphocytes [86]. The majority of $CD4^+$ and $CD8^+$ T cells remain in the lamina propria, while a small fraction also continues migrating into the epithelium of the intestinal tract [87], to form IELs. IELs are in direct contact to the epithelial cells, mediated by the interaction between CD103 on lymphocytes and E-cadherin expressed on the basolateral side of epithelial cells [88]. Antigen-experienced T cells are part of the first line of defence of the intestinal immune system against invading pathogens, as they have the proximity to gut antigens, but also important to maintain gut homeostasis [28, 89]. The lamina propria lymphocytes (LPLs) include antigen-experienced $CD4^+$, $CD8^+$ T cells and plasma cells [21, 90] and form the largest amount of adaptive lymphocytes in the human body. Together with the broad variety of innate immune cells, they form a dense network important for the prevention of pathogen entry and spread across the intestinal mucosa [28].

1.4. Intestinal inflammation

The dynamic crosstalk and complex interaction between the intestinal microbiota, the epithelial barrier and the gut immune system comprising the innate and adaptive immunity is fundamental for the maintenance of homeostasis in the intestinal tract. In healthy individuals, the homeostatic conditions are not only essential for the tolerance of food antigens and the commensal microbiome, but also offer a protective immunity to a plethora of pathogens. Perturbations or even the breakdown of the complex pathways of intestinal homeostasis can trigger aberrant inflammatory responses, which may extend to chronic inflammation and severe pathologies found in chronic diseases such as the inflammatory bowel disease (IBD) [91].

1.4.1. Inflammatory bowel disease

IBD is characterized by a chronic relapsing inflammation of the intestinal tract, whereby phases of acute inflammatory flare-ups and asymptomatic remission alternate. IBD encompasses two

clinically important forms: Crohn's disease (CD) and ulcerative colitis (UC) [92]. CD induces a transmural inflammation, which can affect any part of the gastrointestinal tract, but is most commonly located in the terminal ileum, cecum, perianal area and the colon. UC induces mucosal inflammations, limited to mucosa and submucosa of the rectum and the colon [93]. Clinical manifestations of UC and CD patients include symptoms of abdominal pain, diarrhoea, bloody stool, weight loss and fever. Major complications such as abscesses, fistulas and strictures can occur in CD and cryptitis and crypt abscesses in UC patients [94], dramatically affecting the patient's quality of life.

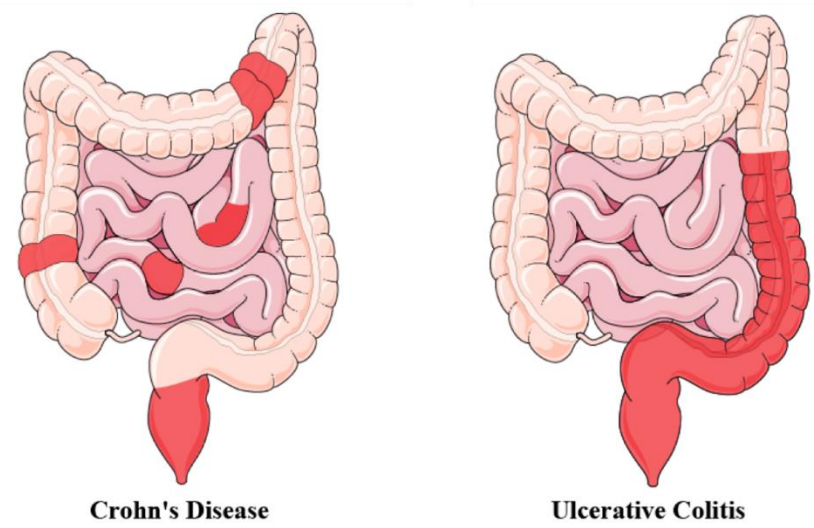

Fig. 5: The two clinically forms of IBD. Crohn's disease (**left**) is characterized by a transmural inflammation present in any part of the gastrointestinal tract. Ulcerative colitis (**right**) induces mucosal inflammations of the rectum and the colon. The red areas indicate intestinal inflammation (adapted from [95]).

IBD is a chronic, incurable disease that affects both sexes and is typically diagnosed early in life, especially in the age group of people between 20 and 30 years, with a second peak in the elderly population [96]. In the middle of the 20th century the highest prevalence was reported in Europe and North America. However, over the last years IBD rapidly emerges also in developing nations in Asia, South America and the Middle East, indicating an epidemiological relation to the westernized lifestyle and progress of industrialisation. Thus, nowadays IBD has risen to a global health challenge with continuously rising prevalence on every continent [97]. A global study considering more than 195 countries between 1990 and 2017 reported a constantly rising prevalence of IBD of 84.3 per 100.000 persons globally in 2017, while in western Europe the prevalence was higher with up to 136.6 per 100,000 persons [98]. With a large number of new cases diagnosed each year in combination with an aging global society, IBD is expected to exponentially rise over the next decade, emerging not only to a global health threat but also to an imminent overburdening of the healthcare systems [99]. Despite the large number of newly developed treatment options, still half of the IBD patients are non-responders

to therapy due to the variety of this disease. Thus, there is an urgent need for novel treatment opportunities for optimal medical support of individual patients, aiming for personalized medicine as a future goal [100, 101]. However, this requires further investigations of the pathogenesis of IBD.

The precise etiology of IBD remains unclear. A complex interaction between genetic predisposition, abnormalities in the immune system, environmental impact and dysregulation of the gut microbiome are considered to be involved (Fig. 6). The genetic study made the most rapid progress due to technical advances in DNA analysis and sequencing as well as the establishment of accessible databases in the last years [102]. More than 200 susceptible gene loci for IBD have been identified so far, with a partial overlap between CD and UC [103]. *NOD2* mutations were the first gene loci associated with the susceptibility for CD, occurring nowadays in one-third of CD patients [104]. The wild type NOD2 is an essential intracellular bacterial sensor, involved in the immune response against pathogens as well as in the tolerance of commensal microbiota [105]. Further, also defects in the function of IL-10 [106] as well as defects in pathways of the epithelial barrier function, innate and adaptive immunity and metabolism of cellular homeostasis are associated with CD and/ or UC [107, 108]. Still only a small proportion, estimated 20 to 25% of all IBD patients, are ascribed to mutations in the identified gene loci. A phenomenon termed “genetic vacuum”, which cannot explain the complete pathogenesis of IBD. Nowadays, gene to gene, gene to pathway and gene to environment interactions are more in focus of the genetic studies of IBD [109].

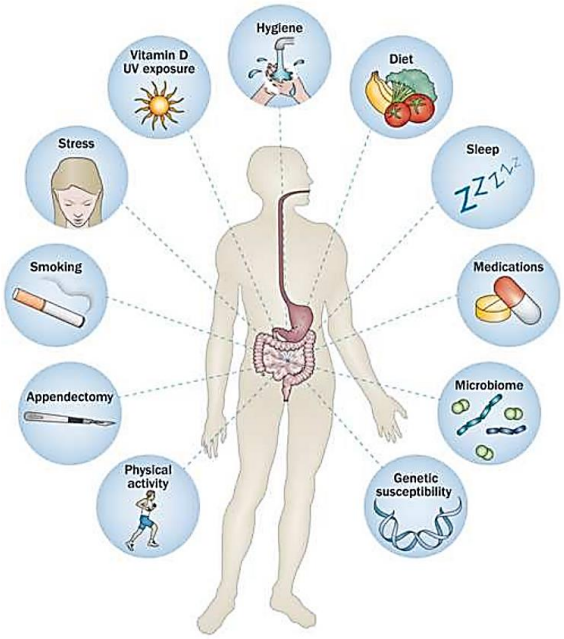

Fig. 6: Etiology of IBD. IBD is governed by a complex interplay of genetics, abnormalities in the immunity, environmental factors and changes in the microbiome (adapted from [110]).

Environmental risk factors for IBD include drugs, diet and social stress. In meta-analysis, smoking is considered to significantly increase the risk for CD and lower the response to medical therapy, while smoking at the same time lowered the risk for UC [111]. The underlying mechanisms are not completely understood, but alterations in innate and adaptive immune response of the two major forms of IBD seem to play an important role [112]. Also, vitamin D deficiency [113, 114] as well as air pollution is considered to have negative implications. The rising incidence of IBD in developing countries goes in line with the development of industrialization [115], as high levels of NO_2 and SO_2 increase the risk of UC and CD [116]. Fat and sugar-rich food exacerbates the course of CD by promoting intestinal inflammation, underlining the effect of westernized life style on IBD development [117].

Immunological, dysregulated innate and adaptive immune pathways contribute to the chronic intestinal inflammation in IBD patients. Genetic mutations and environmental factors contribute to the impairment of the epithelial barrier function. Defective AMP production as well as impairment of the AJC complex of the IECs are known, leading to an increased barrier permeability of a plethora of antigens [118, 119]. These are in turn recognized by PRRs on innate immune cells, leading to a release of pro-inflammatory factors such as cytokines and chemokines, triggering the accumulation and activation of innate and adaptive immune cells in the gut. Consequently, increased secretion of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β have been reported in IBD patients [120]. Neutrophils are recruited as first line of defence to the damaged barrier. The increased neutrophil activity with a concomitant NET and ROS release are associated with further impaired barrier functions and epithelial damage [121]. Further, the phagocytic activity and secretion of cytokines of intestinal macrophages is increased in IBD patients, triggering dramatic inflammation and promoting fibrosis in IBD patients [91]. Due to changes in the resident intestinal ILC subsets [122] and different alterations in the adaptive immunity, CD and UC are characterized by distinct Th immune responses. Increased intestinal IL-18 and IL-12 levels trigger a Th1-driven immune response in CD [123, 124], resulting in higher IFNγ and IL-2 levels [125]. UC on the other hand is dominated by a Th2-driven immune response, indicated by an overexpression of IL-5 and IL-13 in the intestine [126]. Furthermore, the increased IL-17A levels in CD and UC patients indicates also an involvement of the Th17 immune response in the development of IBD pathogenesis .[127]

In addition, dysregulation of the intestinal microbiome such as a reduced biodiversity and a certain instability of the faecal microbiome in IBD patients were identified as causal factors of IBD development [128, 129]. The early intake of antibiotics can lead to paediatric IBD due to its negative effects on the development of the commensal flora in infants [130]. Furthermore, the alterations of the intestinal immune responses in IBD increase the susceptibility to potential

infectious triggers. Bacterial infections by *Mycobacterium avium* [131], *Salmonella* or *Campylobacter* [132] as well as viral infections for example by cytomegalovirus (CMV) [133] are associated with more severe outcomes for IBD patients and interfere in the development of the disease.

1.4.2. Animal models of IBD

To investigate the pathogenesis of diseases such as IBD, animal models are valuable and indispensable tools [134]. More than 50 different animal models were developed in the past years in the context of IBD, enabling an advanced knowledge and new insights into the pathogenesis of intestinal inflammation. The current models can be divided by mode of induction: genetically induced models as well as inducible models such bacterial or chemically induced models. Each model demonstrates different aspects of IBD and advances the understanding of pathogenesis, but it must be considered that none reflects all patterns of the human disease [135].

1.4.2.1. Dextran sulfate sodium-induced colitis

The dextran sodium sulfate (DSS)-induced colitis model is a widely used chemical model to initiate intestinal inflammation e.g. in mice and was first reported by Okayasuo *et al.* in 1990 [136]. DSS is water-soluble and can therefore be administered orally via the drinking water. It is a negatively charged sulphated polysaccharide with a variable molecular weight (MW), ranging between 5 and 1400 kDa [137]. The different MW of DSS induces deviating pathologies, dependent on the ability of the chemical to penetrate the intestinal mucus layer [138, 139]. In the lumen of the colon, DSS forms nano-lipocomplexes with medium chain length fatty acids (MCFAs) produced by the colonic microbiota. The formed nanovesicles are able to pass the mucus layer, directly interact with the IECs and induce channels within the cell membrane. Larger molecules like pathogenic proteins can pass, cause damage to the epithelial monolayer and initiate the downregulation of tight junction proteins such as ZO-1. Due to the impairment of the barrier integrity, further intestinal content such as commensal bacteria, pathogens, toxins and antigens can pass the barrier and reach the lamina propria, where an intestinal inflammation occurs. During the early phase of DSS-treatment, Th1-cytokines mediate the influx of inflammatory macrophages and neutrophils, inducing a noxious effect in the distal part of the colon [140-142]. Ongoing inflammation triggers further Th1/2 responses, which also negatively interfere with colonic healing [143].

The clinical observations in DSS-treated mice are comparable to human colitis. Mice exhibit body weight loss, diarrhoea, and occult blood in the faeces. Histologically, DSS induces the loss of basal crypts, mucin depletion, increased apoptosis of IECs and an imbalance in apoptosis and proliferation [144]. Due to the limitation of the intestinal inflammation to the colon

and rectum, DSS is considered a relevant model especially for UC rather than the CD research [145, 146]. By variation of MW, concentration, duration, frequency and animal models, acute, chronic or relapsing colitis models can be induced [136].

1.5. Cytomegalovirus

The Human Cytomegalovirus (HCMV), also referred as human betaherpesvirus 5, is part of the *Beta-Herpesvirinae* subfamily within the *Herpesviridae* family and highly discussed in the context with IBD [133]. HCMV is a pathogen that exclusively infects humans and is ubiquitously distributed. HCMV seroprevalence is considered to range between 40 and 95% of adult populations, depending on geographical origin, ethnicity, sex and socioeconomic status. The highest infection rates are present in South America, Africa, and Asia and the lowest in Western Europe and United States of America [147]. HCMV, like all CMVs are enveloped viruses with a double-stranded viral DNA genome. The envelope, the tegument and a capsid of icosahedral symmetry structure the virion. The HCMV genome includes over 230 genes encoding more than 50 membrane proteins and 25 membrane glycoproteins embedded in the virion envelope [148].

HCMV is naturally transmitted via close contact to secretions such as saliva, genital secretions and breast milk or biological fluids like urine and blood of an infected individual. Further, it can be transmitted to the foetus *in utero* during pregnancy in case of a primary infection of the mother. HCMV infections are considered to be the most common viral congenital infection, responsible for the induction of birth defects or other long-term health problems of the new born [149]. Haematopoietic and solid-organ transplantation are another risk of infection. Breakthrough HCMV infections remain a major complication post transplantation, affecting allograft function and increase the patient's morbidity and mortality significantly [150]. During primary infection, HCMV can infect several organs. The broad organ tropism indicates the ability of the virus to replicate in several different cell types. So far endothelial and epithelial cells, fibroblasts, smooth muscle cells and immune cells such as monocytes, macrophages and dendritic cells have been identified as target cells of HCMV [151].

The viral entry into the target cell is highly dynamic due to its ability to attach to various host cell receptors for virus-cell-interactions. The primary attachment occurs via the glycoprotein complex of the virion envelope, containing the glycoproteins gB and gH/gL as well as further co-factor proteins [152, 153]. Depending on the target cell, a cell-type specific complex is formed on the virion. For the infection of fibroblasts, HCMV only requires gH, gL and gO for the direct fusion with the target plasma membrane, while in epithelial and endothelial cells the cell entry is achieved via macropinocytosis and endosome fusion with gH, gL and UL128-131 [154, 155]. Further target cell receptors are essential for the attachment. It is known, that

HCMV is able to bind glycosaminoglycans like heparan sulphate, integrins, epidermal growth factor receptors (EGF-R) and neuropilin-2 [156-160], but further HCMV receptors appear likely to mediate entry into other cell types [156]. After reaching the cytosol, HCMV enters the cell nucleus, to induce the viral gene expression and virus assembly of new virions in three phases. The transcription of immediate early (IE) genes encode transcription factors to induce early (E) genes coding for proteins involved especially in the replication of viral genes like the viral DNA polymerase (pUL54). The expression of structural late (L) genes follows, encoding for the capsid, envelope and tegument proteins. In the final phase, the viral genome is encapsulated and new virions are released via budding for the transmission to new target cells. Either free virus particles are released to the extracellular space or can directly attach to a neighbouring cell [161].

Due to the co-evolution of the CMVs and the immune systems of different hosts, the CMV has developed several immune evasion mechanisms. The HCMV encodes counter mechanisms against various immune responses. The arsenal of immunomodulatory functions enables the establishment of lifelong infections by hiding from the host immune system and the potential of a reinfection of the host with an already established immune response to HCMV. The immune evasion mechanisms directly target antigen presentation by MHC class I and class II complexes, antagonize innate immune mechanisms such as the IFN-mediated antiviral response and the viral production of cytokine mimicry to disturb paracrine functions of the immune system [162-164].

1.5.1. HCMV-related end organ diseases

The impact of HCMV induced disease in human is diverse and dependent on the host immune response. An infection of healthy individuals is typically asymptomatic or causes a mild illness like mononucleosis-like syndrome [165], while infection of immune compromised individuals such as human immunodeficiency viruses (HIV)-infected individuals or patients with immunosuppressive therapy induce CMV-related severe disease progression [166]. Therefore, individuals with an HCMV infection identified e.g. via diagnostic approaches and individuals with an HCMV disease with manifestations of clinical symptoms have to be differentiated [167]. Such as other members of *Herpesviridae* family, after resolution of an acute infection HCMV establishes a lifelong latent infection [167]. Latent HCMV genomes can be found in many tissues such as the salivary glands and the digestive tract, and especially the colon is one of the major sites for latency and reactivation. Viral inclusions have been detected in fibroblasts, epithelial, endothelial and smooth muscle cells [168]. HCMV reactivation is mainly induced in situations of impaired immunity or stress.

HCMV disease, such as retinitis, gastrointestinal ulceration, hepatitis or pneumonitis occurs most likely during reactivation of latent HCMV in an immunocompromised host (Fig. 7). HCMV colitis is rather uncommon in immunocompetent hosts, but still might occur during primary infection [169], while in immunocompromised hosts it is the second most common manifestation of end-organ disease after HCMV retinitis [167, 170, 171]. Clinical symptoms are weight loss, abdominal pain, watery diarrhoea, haematochezia, fatigue and fever, very difficult to distinguish from typical symptoms of other chronic intestinal diseases such as IBD [172].

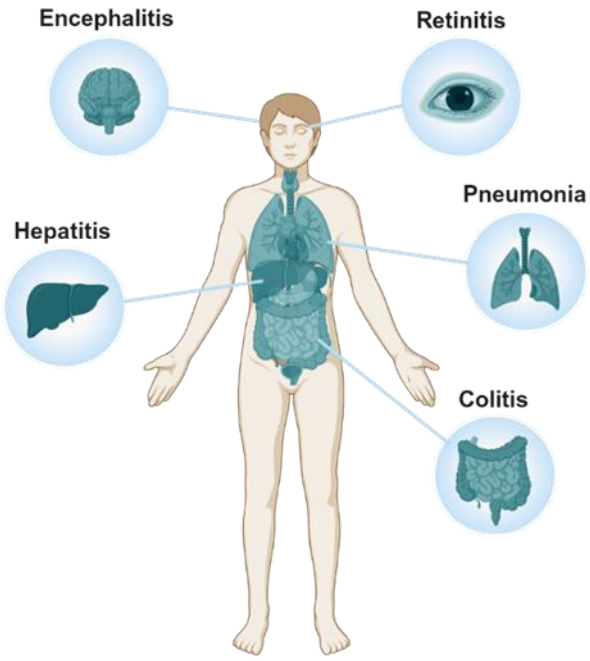

Fig. 7: CMV-related end organ diseases. In immunocompromised individuals a primary infection or reactivation of latent HCMV can lead to end organ diseases like HCMV hepatitis, encephalitis, retinitis, pneumonia and colitis (created with BioRender.com).

1.5.2. CMV infections in IBD

The first association between HCMV and IBD was described in a case report by Powell *et al.* in 1961, where a cytomegalic inclusion disease was described in a UC patient [173]. Since then, mutual influence of both diseases are highly discussed. Nowadays, meta-analysis such as the one of Ya-li LV *et al.* indicate, that HCMV infections occur 7-8 times more often in IBD patients compared to healthy controls [174]. Further, IBD patients have a higher prevalence of HCMV colitis compared to non-IBD patients. Here especially UC patients show a higher association compared to CD and healthy individuals [175]. HCMV colitis is estimated to occur in 0.53 to 4% in IBD patients overall [176, 177], while the true incidence might even be higher, as HCMV diagnostic is not routinely performed in patients with IBD inflammatory flare-ups [177]. The diagnosis of HCMV in IBD is challenging, as clinical or endoscopic symptoms

coincide between IBD and HCMV colitis. Current guidelines recommend the detection of HCMV markers in multiple fresh tissue biopsies from inflamed intestinal areas via immunohistochemistry (IHC) and quantitative polymerase chain reaction (qPCR), as HCMV markers are hard to detect in healthy tissue [178, 179]. Unfortunately, these methods are not applied uniformly in clinics all over the world.

From a clinical point of view, IBD patients with HCMV disease require more often surgical treatment and have a higher mortality rate compared to patients negative for HCMV [180]. Also the treatment efficacy seems to be directly impaired by HCMV, as steroid-refractory colitis is more often triggered in UC patients resulting in a worse disease prognosis [181, 182]. In line with this, the prevalence of HCMV colitis in severe, steroid-refractory disease is estimated to be 36% and therefore much higher in comparison with the overall prevalence of HCMV in IBD patients [181]. The significant increase in mortality in CD and UC patients with HCMV colitis is the result of multi organ failure and shock, leading to an increase of intensive care unit treatment along with increased hospitalization costs from an economic point of view [177].

Nevertheless, the impact of HCMV infection on the development and severity of IBD is still not generally accepted. The reciprocal influence is recently discussed in various studies and reviews, but the pathophysiological mechanisms are largely unknown. Due to the lack of promising murine models and the limited availability of human specimens from IBD patients with acute HCMV infection, detailed investigations are required.

1.6. Aim of this study

Although IBD was initially considered as a health burden of the developed world, the large number of new cases diagnosed each year in the developing nations as a result of industrialisation and the imposition of Western lifestyles is turning the chronic disease into a global health threat. The distinctive chronic inflammation of the intestinal tract in combination with immunosuppressive treatments makes IBD patients particularly susceptible to challenging infections. HCMV, as an opportunistic pathogen is ubiquitously present in the global human population and involved in many inflammatory processes, causing various clinical complications. Inducing a lifelong latent infection, HCMV frequently reactivates during episodes of stress or impaired immunity. Consequently, HCMV is a major cause of morbidity in immunocompromised patients as consequence of severe end organ diseases such as HCMV hepatitis, encephalitis and colitis. The prevalence of HCMV infections in IBD patients, particularly in UC patients, is significantly higher compared to non-IBD patients. However, the consequences of the higher HCMV occurrence are still highly debated in the research field of IBD. The challenging and inconsistent diagnosis of HCMV colitis in IBD patients complicates the definition of a uniform clinical picture. It is now widely accepted that the recurrent reactivation of HCMV during periods of IBD inflammatory flare-ups can exacerbate intestinal inflammation and consequently worsen the disease manifestation of the patient. While clinical statistics, such as the enhanced requirement of surgical interventions and the increase of steroid-refractory cases underscore this association, but the pathophysiological mechanisms by which these two diseases impact each other remain largely elusive.

The aim of the present study is to investigate the consequences of CMV manifestation in the intestinal tract under homeostatic and inflammatory conditions. More specifically, intestinal target cells of CMV replication as well as the induced pathological effects were studied. The interplay between CMV-induced immune responses and DSS-induced acute intestinal inflammation was of particular interest. The results of this study contribute to the comprehensive understanding of the pathomechanism that might enable the development of novel therapeutic strategies for IBD patients with HCMV-associated disease in the future.

2. Material & Methods

2.1. Materials

In the ensuing sections and tables, a detailed enumeration provides all experimental animals, viral strains, consumables, reagents, technical equipment and software utilized in the present study.

2.1.1. Experimental animals and husbandry

All experimental mice were bred and housed under specific pathogen-free (SPF) conditions at the central laboratory animal facility of the University Hospital Essen. Mice were kept under a 12 h light and 12 h dark cycle at 23°C in in individually ventilated (ICV2) cages with food and water ad libitum. For the experiments, female and male mice were used at an age of ≥ 8 weeks. All experiments were conducted in accordance with the German Animal Welfare Act and approved by the Office of nature, environment and consumer protection (LANUV) of North Rhine-Westphalia, Germany.

2.1.1.1. BALB/c mice

BALB/cOlaHsd mice were obtained from Inotiv, Inc (Horst, Netherlands) at an age of 6 – 8 weeks.

2.1.1.2. Lgr5-Reporter mice

Lgr5-2A-EGFP-2A-CreERT2 mice (Lgr5-Reporter mice) were obtained from the Yilmaz lab, Koch Institute, Massachusetts Institute of Technology (MIT), Cambridge, United States of America (USA). Lgr5-Reporter mice were taken over into the breeding of the central laboratory animal facility of the University Hospital Essen. The mice are on C57BL/6J background and express the reporter enhanced green fluorescent protein (eGFP) in the Lgr5+ ISCs located at the bottom of the crypts of the small intestine and the colon. The P2A-EGFP-T2A-CreERT2 cassette is inserted at the 5′ end of the endogenous Lgr5 gene [183].

2.1.2. Cell culture

The MCMV titrations were performed using murine embryonic fibroblasts (MEFs) cultured in DMEM / FCS Media. The MEFs were kindly provided by the research group Trilling, Institute for The Research on HIV and AIDS-associated Diseases, University of Duisburg-Essen, Essen, Germany.

2.1.3. Mouse Cytomegalovirus strains

All used MCMV strains were kindly provided by the research group Trilling, Institute for The Research on HIV and AIDS-associated Diseases, University of Duisburg-Essen, Essen, Germany

Tab. 1: MCMV strains

Strains	**Source**
wt-MCMV	Reconstituted from the MCMV-BAC described in Jordan *et al.* [184]
MCMV:*mCherry*	Initially described in Trilling *et al.* [185]
ΔM27 MCMV	Initially described in Trilling *et al.* [186]

2.1.4. Consumables

Tab. 2: Consumables

Consumables	**Manufacturer**
Acella 50 slides	Anvajo GmbH, Dresden, Germany
Cell strainer (40, 70, 100 µm)	Falcon, Durham, USA
Chromatography paper (3 x 100 cm)	Whatman GmbH, GE Healthcare Life Sciences, Dassel, Germany
Culture plate (6-, 12-, 24-, 96-well)	Greiner BioOne, Frickenhausen, Germany
Disposable syringe (2, 10 ml)	BD Biosciences, Heidelberg, Germany
Histology cassettes	Kartell Spa, Noviglio, Italy
Micro reaction tubes (0.6 mL)	Biozyme Scientific GmbH, Oldendorf, Germany
Micro reaction tubes (1.5, 2 mL)	Sarstedt, Nümbrecht, Germany
Needle (26G x 13 mm)	BD Biosciences, Heidelberg, Germany
Opti-Seal Optical sealing sheet	BIOplastics, Landgraaf, Netherlands
Pipettes (5, 10, 25 mL)	Greiner BioOne, Frickenhausen, Germany
Pipettes tips (10, 200, 1000 µL)	Sarstedt, Nümbrecht, Germany

Round bottom plates (96-well)	Greiner BioOne, Frickenhausen, Germany
Syringe (1 mL, 2 mL, 10 mL)	BD Biosciences, Heidelberg, Germany
Syringe Filcons (30 µm)	BD Biosciences, Heidelberg, Germany
Tubes (15, 50 mL)	Greiner BioOne, Frickenhausen, Germany
Tubes round bottom (12 x75 mm) FACS-tubes	Falcon, Durham, USA

2.1.5. Chemicals

Tab. 3: Chemicals

Chemicals	**Manufacturer**
Brefeldin A (BFA)	Sigma-Aldrich, St. Louis, USA
Collagenase IV	Sigma-Aldrich, St. Louis, USA
Dextran sulfate sodium – MW, 36-50 kDa (DSS)	MP Biomedicals, Eschwege, Germany
Disodium hydrogen phosphate dihydrate [Na_2HPO_4 x 2 H_2O]	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol, absolute	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethyleneglycoltetraacetic acid (EGTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
FACS Clean Solution	BD Biosciences, Heidelberg, Germany
FACS Flow Sheath Fluid	BD Biosciences, Heidelberg, Germany
FACS Rinse Solution	BD Biosciences, Heidelberg, Germany
Fetal calf serum (FCS)	Sigma-Aldrich, St. Louis, USA
Fixable viability dye eFluor780 (FvD)	eBioscience, San Diego, USA
Heparin-sodium-25000-ratiopharm©	Merckle GmbH, Blaubeuren, Germany
IGEPAL CA-630 (NP-40)	Sigma-Aldrich, St. Louis, USA
Ionomycin calcium salt	Sigma-Aldrich, St. Louis, USA

Magnesium chloride [$MgCl_2$]	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Matrigel Growth Factor Reduced Basement Membrane Matrix	Corning, Bedford, USA
Methyl cellulose	Sigma-Aldrich, St. Louis, USA
Paraformaldehyde (PFA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Penicillin	Sigma-Aldrich, St. Louis, USA
Penicillin-Streptomycin (10.000 U/mL)	Gibco, Life Technologies, Carlsbad, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, USA
Potassium chloride [KCl]	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium dihydrogen phosphate [KH_2PO_4]	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium bicarbonate [$NaHCO_3$]	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride [NaCl]	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Streptomycin	Sigma-Aldrich, St. Louis, USA
Y-27632	STEMCELL Technologies, Vancouver, Canada
β-Mercaptoethanol	Promega, Mannheim, Germany

2.1.6. Buffer and Media

Tab. 4: Buffers

Buffers	**Ingredients / Manufacturer**
FACS buffer	PBS buffer 2 % (v/v) FCS 2 mM EDTA
PBS buffer	Demineralized water 8 g/L Sodium chloride 0.2 g/L Potassium chloride 1.44 g/L Disodium hydrogen phosphate dihydrate 0.2 g/L Potassium dihydrogen phosphate
PBS / EDTA	PBS buffer 3 mM EDTA
TripleLE Express	Gibco, Life Technologies, Carlsbad, USA

Tab. 5: Media

Media	**Ingredients / Manufacturer**
DMEM	Dulbecco's modified eagle medium (DMEM) + 4.5 g/L D-Glucose + Pyruvate Gibco, Life Technologies, Carlsbad, USA
DMEM / FCS	Dulbecco's modified eagle medium (DMEM) + 4.5 g/L D-Glucose + Pyruvate 10% v/v FCS 100 µg/mL Streptomycin 100 U/mL Penicillin
IMDM	Iscove’s modified Dulbecco’s medium (IMDM) + GlutaMax I + 25 mM HEPES Gibco, Life Technologies, Carlsbad, USA

IMDM complete	IMDM + GlutaMax I + 25mM HEPES 10% v/v FCS 100 µg/mL Streptomycin 100 U/mL Penicillin 25 µM ß-Mercaptoethanol
MEM (10X)	Minimum Essential Medium (MEM) 10X + Earl's Salt Gibco, Life Technologies, Carlsbad, USA
Methyl cellulose solution	19,55 g/L Methyl cellulose in ddH_2O 5% v/v FCS 55 g/L $NaHCO_3$ 100 µg/mL Streptomycin 100 U/mL Penicillin 10% MEM (10X)
Organoid Growth Medium	IntestiCult™ organoid growth medium (Mouse), STEMCELL Technologies, Vancouver, Canada 100 µg/mL Streptomycin 100 U/mL Penicillin 1 µL/mL Y-27632
RPMI medium	Roswell Park Memorial Institute (RPMI) 1640 medium + GlutaMax I + 25 mM HEPES Gibco, Life Technologies, Carlsbad, USA
RPMI / FCS	RPMI 1640 + GlutaMax I + 25 mM HEPES 20 % v/v FCS
RPMI / FCS / EGTA / $MgCl_2$	RPMI 1640 + GlutaMax I + 25 mM HEPES 1 % v/v FCS 1 mM EGTA 1.5 mM Magnesium chloride

2.1.7. Kits

Tab. 6: Kits

Kits	Manufacturer
DNeasy® Blood and Tissue Kit	Qiagen, Hilden, Germany
FoxP3/Transcription Factor Staining Buffer Set	eBioscience, San Diego, USA
Luminex Discovery Assay Mouse Multi-Analyte	R&D Systems, Minneapolis, USA
NEB Next Single Cell / Low Input RNA Library Prep Kit	New England Biolabs GmbH, Frankfurt, Germany
NovaSeq 6000 S1 Reagent Kit (100 cycles)	Illumina, San Diego, USA

2.1.8. Antibodies

Tab. 7: FACS antibodies

Epitope	Fluorochrome	Clone	Manufacturer
CCR2	FITC	SA203G11	BioLegend, San Diego, USA
CCR5	PerCP-Cy5.5	HM-CCR5	BioLegend, San Diego, USA
CCR6	eFluor660	sirx6	Invitrogen, Karlsruhe, Germany
CD3	FITC	17A2	BioLegend, San Diego, USA
CD4	PB	RM4-5	BD Biosciences, Heidelberg, Germany
CD8	PB PE PE-Cy7	53-6.7 53-6.7 53-6.7	BD Biosciences, Heidelberg, Germany BD Biosciences, Heidelberg, Germany eBioscience, San Diego, USA
CD11b	PerCP-Cy5.5	M1/70	BD Biosciences, Heidelberg, Germany
CD11c	FITC	HL3	BD Biosciences, Heidelberg, Germany
CD19	FITC	1D3	BD Biosciences, Heidelberg, Germany
CD25	PE	PC61	BD Biosciences, Heidelberg, Germany

CD31	PE	390	BioLegend, San Diego, USA
CD45	PerCP-Cy5.5 PE	30-F11 30-F11	BioLegend, San Diego, USA BioLegend, San Diego, USA
CD49b	APC	DX5	BioLegend, San Diego, USA
CD69	APC FITC	H1.2F3 H1.2F3	BioLegend, San Diego, USA eBioscience, San Diego, USA
CD326	APC	G8.8	eBioscience, San Diego, USA
CXCR3	PE	CXCR3-173	eBioscience, San Diego, USA
CXCR6	PE-Cy7	SA051D1	BioLegend, San Diego, USA
FoxP3	FITC	FJK-16s	eBioscience, San Diego, USA
F4/80	PE	BM8	eBioscience, San Diego, USA
GrzmB	APC	GB12	Invitrogen, Karlsruhe, Germany
IFNγ	FITC	XMG1.2	BD Biosciences, Heidelberg, Germany
Ki-67	PE-Cy7	SolA15	eBioscience, San Diego, USA
Ly6G	APC	1A8	BioLegend, San Diego, USA
SiglecF	PE-Cy7	1RNM44N	Invitrogen, Karlsruhe, Germany
Ter-119	PE	TER-119	BioLegend, San Diego, USA
H-2Db MCMV M45 Tetramer	PE	Sequence: HGIRNASFI	MBL International Corporation, Woburn, USA

Tab. 8: Depleting antibodies used for *in vivo* experiments

Antibodies	Clone	Manufacturer
*InVivo*MAb anti-mouse CD8α	YTS 169.4	BioXCell, New Hampshire, USA
IgG2b isotype control	LTF-2	BioXCell, New Hampshire, USA

2.1.9. Primers

Tab. 9: Primers used for qPCR

Gene	Primer sequence	Annealing temperature
MCMV *M45*	GAAGATCCGCATGTCCTTCAG AATCCGTCCAACATCTTGTCG	55°C
Rps9 (mus musculus)	CTGGACGAGGGCAAGATGAAGC TGACGTTGGCGGATGAGCACA	55°C

2.1.10. Equipment

Tab. 10: Equipment

Equipment	Manufacturer
7500 Fast Real-Time PCR System	Thermo Scientific, Darmstadt, Germany
APEXVIEW APX100 microscope ORCA-Fusion Digital camera C14440	Evident Scientific, Tokyo, Japan Hamamatsu Photonics K.K., Hamamatsu, Japan
BD FACSAria IIIu cell sorter	BD Biosciences, Heidelberg, Germany
Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Centrifuge MULTIFUGE 3SR+	Thermo Fisher Scientific, Waltham, USA
Flow cytometer BD LSRII	BD Biosciences, Heidelberg, Germany
Fluidlab R-300	Anvajo GmbH, Dresden, Germany
Heracell 150i CO2-Incubator	Thermo Scientific, Darmstadt, Germany

Heraeus Multifuge 3SR+	Thermo Scientific, Darmstadt, Germany
Illumina NovaSeq 6000	Illumina, San Diego, USA
Luminex MAGPIX Analyzer	Luminex Corporation, Austin, Texas, USA
Magnetic stirrer Variomag	Thermo Scientific, Darmstadt, Germany
Microscope Binokular Axiovert Z1	Carl Zeiss Microscopy GmbH, Jena, Germany
MilliQ water purification system	Merck KGaA, Darmstadt, Germany
NanoDrop 1000 spectrophotometer	PeqLab, Erlangen, Germany
Neubauer counting chamber	Superior, Marienfeld, Germany
Vortexer D-6013	Neo Lab, Heidelberg, Germany
Water bath	GFL, Burgwedel, Germany
Work Bench MSC-Advantage	Thermo Scientific, Darmstadt, Germany

2.1.11. Software and Scripts

Tab. 11: Software

Software	**Supplier or Source**
7500 Fast System Software v2.3	Thermo Scientific, Darmstadt, Germany
Olympus cellSens APEX 100	Evident Scientific, Tokyo, Japan
BD FACS Diva Software v8.0.1	BD Biosciences, Heidelberg, Germany
Endnote 21	PDF Tron Systems Inc., Vancouver, Canada
FastQC	By Simon Andrews, Babraham Bioinformatics
Fiji ImageJ v1.52i	By Rasband WS, U. S. National Institutes of Health; Bethesda, USA
Graph Pad Prism v7.05	GraphPad Software, La Jolla, USA
Luminex xPONENT Software v4.2	Luminex Corporation, Austin, USA
Microsoft Office 2016	Microsoft Corporation, Redmond, USA

NanoDrop Software ND-1000 v3.7.1	Thermo Scientific, Darmstadt, Germany
Python v3.12.3	Python Software Foundation, Wilmington, USA
R Software v4.3.3	R Foundation for Statistical Computing, Vienna, Austria

Tab. 12: Scripts

Scripts / Packages	**Supplier or Source**
AnnotationDbi v1.64.1 clusterProfiler v4.10.1 openxlsx2 v1.13 org.Mm.eg.db 3.18.0 readxl v1.4.3	R Foundation for Statistical Computing, Vienna, Austria
edgeR Bioconductor package	Bioconductor – open source software for bioinformatics https://www.bioconductor.org/
FastQC tool	Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data [187]. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
matplotlib.pyplot v3.9.2	Python Software Foundation, Wilmington, USA
Spliced Transcripts Alignment to a Reference (STAR) aligner	Dobin, A., et al., STAR: ultrafast universal RNA-seq aligner [188].

2.2. Methods

2.2.1. Animal procedures

2.2.1.1. MCMV infection

MCMV infections were performed with WT MCMV or MCMV:*mCherry*. For all *in vivo* infections, mice were infected intraperitoneally (i.p.) with 2 x 10^5 plaque forming units (PFU) MCMV in a final volume of 100 µl PBS per mouse, controls were injected i.p. with 100 µl PBS.

2.2.1.2. MCMV infection during DSS-induced acute colitis

Acute colitis was induced in BALB/c mice by the administration of 3% DSS within the drinking water for seven consecutive days. Afterwards, the mice received normal drinking water for seven consecutive days, to induce intestinal regeneration. MCMV-infection was additionally applied at day 7 of the DSS-induced colitis model. The disease activity index (DAI), compromising the parameters of changes in body weight, stool consistency and rectal bleeding, was monitored on a daily basis. The composition of the DAI score is shown in Tab. 13.

Tab. 13: Disease activity index (DAI)

Score	**0**	**1**	**2**	**3**	**4**
Proportional body weight loss to the initial weight	none	1 - 5 %	6 – 10 %	11 – 20 %	> 20 %
Stool consistency	normal	loose stool	very loose stool	-	watery diarrhoea
Rectal bleeding	no bleeding	-	slight bleeding	-	gross bleeding

2.2.1.3. Depletion of $CD8^+$ T cells

To induce the systemically depletion of $CD8^+$ T cells *in vivo*, BALB/c mice were injected i.p. with 200µg anti-mouse CD8α (clone YTS169.4, BioXCell) in a final volume of 200µl / mouse on day 6, 9 and 12 of the DSS-MCMV-induced colitis model.

2.2.1.4. Vaccination with ΔM27 MCMV

For the vaccination experiments ΔM27 MCMV, a live attenuated virus lacking the STAT2-antagonist [189], was used. BALB/c mice were vaccinated i.p. with ΔM27 MCMV and 5 weeks after the vaccination the DSS-MCMV-induced colitis model was applied.

2.2.1.5. Macroscopic analysis and tissue biopsy sampling

Colon, salivary glands, spleen and liver were collected. To remove luminal content the colons were rinsed with 10 ml ice-cold PBS. Fat and connective tissue were removed and the colon length and weight were determined.

In order to determine the viral load, biopsies of 10 – 20 mg per collected organ were sampled. Specifically, colon biopsy was excised from the rectal end, salivary gland biopsy from the submaxillary gland, liver biopsy from the median lobe and the spleen biopsy from the centre of the organ. Biopsies were snap frozen in liquid nitrogen and stored at -80°C until DNA extractions were performed (see chapter 2.2.3.1).

To examine cytokine secretion in the colon, further explants of 10 – 20 µg from the rectal end of the colons were incubated in 300 µl IMDM complete for 6 h at 37°C. Medium was collected, centrifuged at 6800 g for 10 min, supernatants were collected and frozen at -80°C until the determination of cytokine levels by Luminex technology (see chapter 2.2.3.3). Remaining colonic tissue was either used for histological analysis (see chapter 2.2.1.6) or lamina propria lymphocyte isolations (see chapter 2.2.2.2) or crypt isolations (see chapter 2.2.2.7).

For virus titrations (see chapter 2.2.2.1), total organs were snap frozen in liquid nitrogen and stored at -80°C until further processing.

2.2.1.6. Histology

To determine the degree of inflammation within the colonic tissue, histopathological analysis were conducted. The colons were embedded in form of a swiss role in histology cassettes between two filter papers and fixed in 4 % paraformaldehyde (PFA). Fixed samples were send for sectioning, staining and scoring to Prof. Dr. Robert Klopfleisch (Freie Universität Berlin, Institute of Veterinary Pathology, Berlin, Germany). The scoring and imaging was performed in a blinded manner. The colons were sectioned into 4 – 5 µm thick slices and stained with haematoxylin and eosin (H&E). Tissue sections were analysed via microscopy for immune cell infiltration of the lamina propria and tela submucosa, epithelial defects, crypt hyperplasia, goblet cell loss and crypt abscesses. Each aspect was scored from 0 (not present) to 3 (severe) for the proximal, middle and distal part of the colon. The histopathological score was determined as the sum of the scores (maximum score = 45).

Caspase 3 (Casp3)-staining of the colon sections was performed as described in Mabrok *et al.* [190]. Images were dearrayed by colour deconvolution and pseudo colouring using Fiji ImageJ, to depict the nucleus/DNA (blue) and Casp3 (red) in different channels. In the merged images $Casp3^+$ cells were identified (purple).

2.2.2. Cell biological procedures

2.2.2.1. Virus titration

MCMV titers were determined by standard plaque titration as described in Brune *et al.* [191]. Briefly, primary MEFs were seeded on 48-well tissue culture plates in DMEM / FCS media until

cells were subconfluent. Snap frozen organs were homogenized, serial diluted and added to the MEF-culture (performed as triplicates). To increase the sensitivity of the assay, centrifugal enhancement (900 g, 30 min) was conducted followed by an incubation of 1 h at 37°C. Afterwards, medium was removed from the MEFs, viscous Methylcellulose solution added and the cells incubated for further 5 days at 37°C. Finally, the virus plaques were counted microscopically and the PFU/g tissue was calculated.

2.2.2.2. Colonic lamina propria lymphocyte isolation

For the isolation of lamina propria lymphocytes (LPL), the colons were opened longitudinally and cut into 1 cm pieces. The pieces were washed twice by an incubation in 40 ml PBS / EDTA solution for 10 min at 37°C with continuous stirring at 280 rpm. Loose epithelial cells and residual EDTA were removed by two further incubations in 20 mL RPMI / FCS / EGTA / $MgCl_2$ medium for 10 min at 37°C with stirring at 280 rpm. The tissue pieces were then vigorously vortexed for 15 seconds in 10 ml RPMI / FCS / EGTA / $MgCl_2$, washed with PBS to remove residual EGTA and then chopped into small pieces. The tissue was digested in 30 ml RPMI / FCS medium containing 100 U / ml collagenase IV for 1 h at 37°C with stirring at 280 rpm. To further disintegrated the minced tissue, the suspension was raised and lowered 10 constitutive times in a syringe at the middle and end of the 1 h incubation. The cell suspension was then filtered through a 40 µm cell strainer, spun down at 450 g for 15 min and the cell pellet resuspended in IMDM complete. Finally, the cells were filtered through a 30 µm cell strainer to eliminate tissue remains and stored on ice for further processing.

2.2.2.3. Flow cytometry

Flow cytometry was used to identify and analyse the phenotype of single cells in a cell suspension. Colonic LPL cell suspensions and cultured ISCs (see chapter 2.2.2.8) were analysed in this study using the antibodies listed in Tab. 7. Measurement was conducted on the flow cytometer BD LSR II and evaluated using the BD FACSDiva Software.

2.2.2.4. Staining of surface markers

Staining of the cell surface proteins of the analysed single cell suspensions were initiated by transferring 100 – 200 µl of the cell suspension into a 96-well round bottom plate. To remove the IMDM complete media, cells were centrifuged at 300 g for 5 min at 4°C and washed with PBS. For staining, antibodies and fixable viability dye (FvD) were diluted properly to the working concentrations and incubated together with the cell suspensions for 10 min at 4°C in the dark. Staining was stopped by the addition of PBS and the residual antibodies were removed by centrifugation at 300 g for 5 min at 4°C. The surface stained cells were either analysed by flow cytometry (in FACS buffer) or subsequently used for intracellular staining.

2.2.2.5. Staining of intracellular markers

For the analysis of intracellular markers such as FoxP3, Ki-67 or granzyme B (GrzmB), the FoxP3/Transcription Factor Staining Buffer Set was used following the manufacturer's instructions. After surface staining, cells were treated with the fixation / permeabilization solution for 1 h at 4°C in the dark. To remove the fixation / permeabilization solution, cells were centrifuged at 300 g for 5 min at 4°C and washed with permeabilization buffer. For intracellular staining, cells were incubated for 30 min at 4°C in the dark with the according antibodies diluted in permeabilization buffer. To remove the residual antibodies, permeabilization buffer was added and the cells were centrifuged at 300 g for 5 min at 4°C. The cell pellets were resuspended in FACS buffer and used for flow cytometry analysis.

For the staining of the cytokine IFNγ, freshly isolated cell suspensions from the LPL protocol were incubated in 10 ng / mL phorbol 12-myristate 13-acetate (PMA), 1 µg / mL ionomycin and 5 µg / mL brefeldin A (BFA) for 4 h at 37°C. Following, cell surface staining was conducted (see chapter 2.2.2.4) and cells were subsequently fixed in 2% PFA for 15 min at 4°C in the dark. Cells were then washed with FACS buffer, centrifuged at 300 g for 5 min at 4°C and incubated in 0,1 % IGEPAL in H_2O for 4 min in the dark to induce permeabilization. After an additional centrifugation and washing step with FACS buffer, cells were stained intracellular for IFNγ by adding the antibodies diluted in FACS buffer for 30 min at 4°C in the dark. Residual antibodies were removed by the addition of FACS buffer followed by a centrifugation at 300 g for 5 min at 4°C. For flow cytometry analysis cells were resuspended in FACS buffer.

2.2.2.6. Fluorescence-activated cell sorting

Fluorescence-activated cell sorting of ISCs from epithelial single cell suspensions or ISC *in vitro* cultures (see chapter 2.2.2.7 and 2.2.2.8) was conducted on the BD FACSAria IIIu cell sorter. Single cell suspensions were stained for the cell surface markers CD31, CD45, TER119 (lineage negative / Lin^- markers) and CD326 (Epcam, expressed on ISCs). Cells were suspended in FACS buffer, filtered through a 40 µm cell strainer and cell sorting was performed. $Lgr5^+$ ISCs were identified as Lin^- $Epcam^+$ GFP^+ cells, MCMV:*mCherry* infected ISCs were identified additionally by the mCherry (excitation max. 587 nm, emission max. 610 nm) signal.

2.2.2.7. Crypt isolation, organoid culture and intestinal stem cells

To assess the functionality of the ISCs and to identify the target cells of intestinal MCMV infection, crypts were isolated from the colonic tissue. Therefore, colons were rinsed twice with ice-cold PBS to remove luminal content. Fat and connective tissues were removed, the colons opened longitudinally and cut into two pieces. Tissue pieces were incubated in 50 ml PBS

containing 5mM EDTA for 15 min at 37°C. To remove the PBS / EDTA solution, tissue pieces were washed with ice-cold PBS. To detach the crypts from the colon tissue, 15 ml ice-cold PBS was added three consecutive times to the tissue pieces, the tubes were shaken vigorously for 20 seconds and the PBS fractions containing the intestinal crypts were sampled in a new tube. Subsequently, the crypts were centrifuged at 300 g for 5 min at 4°C, resuspended in IMDM complete media and stored on ice for further processing.

To identify the MCMV target cells in the colon, crypts of MCMV:*mCherry* infected Lgr5^{+}-reporter mice were isolated at 5 dpi and subsequently analysed by fluorescence microscopy (see chapter 2.2.3.5).

For fluorescence-activated cell sorting, isolated crypts from Lgr5^{+}-reporter mice were dissociated into single cells by incubation in 1 ml TrypLE-Express for 2 min at 37°C. Enzymatic dissociation was stopped by the addition of 14 ml IMDM complete media and subsequent centrifugation at 300 g for 5 min at 4°C. Single cells were resuspended in FACS buffer and cell surface markers were stained for fluorescence-activated cell sorting as described in the previous chapter (see chapter 2.2.2.6).

To evaluate the functionality of the ISCs, crypts were isolated from mice of the DSS-induced acute colitis model. Crypts were counted under the microscope and then embedded in 40 µl of IMDM complete : Matrigel (1:2 ratio, v/v) on 24-well culture plates. After polymerization (15 min at 37°C), 500 µl of organoid growth medium was added. To examine the enteroid forming efficacy, the exact number of crypts after plating and the enteroids formed 24 h after cultivation at 37°C were counted. The enteroid survival and morphology was assessed microscopically 6 days after plating. In between, enteroids were provided every second day with fresh medium.

2.2.2.8. Cultivation and *in vitro* infection of intestinal stem cells

For the *in vitro* MCMV infection of ISCs, stem cells from Lgr5^{+}-reporter mice were sorted into organoid growth medium. For cultivation, 30 µl of IMDM complete : Matrigel (1:2 ratio, v/v) was added in 48-well culture plates and polymerization was induced for 10 min at 37°C. Then, a defined number of ISCs were added on top of the semi-polymerized Matrigel and polymerization was continued for further 5 min at 37°C. For infection, 200 µl organoid growth medium containing a defined multiplicity of infection (MOI) of 1, 10 and 100 was added and the plate was cultured for 24 h at 37°C. As control, ISCs were cultured in 200 µl organoid growth medium only.

The frequency of infected ISCs was analysed by flow cytometry or fluorescence microscopy via the detection of the mCherry signal. For the RNA sequencing experiments

(see chapter 2.2.3.4), MCMV-infected and uninfected ISCs were sorted directly into lysis buffer of the NEB Next Single Cell / Low Input RNA Library Prep Kit.

2.2.3. Molecular biological procedures

2.2.3.1. DNA isolation

The isolation of DNA from snap frozen organ biopsies was performed using the DNeasy® Blood and Tissue Kit following the manufacturer's protocol. In brief, tissue biopsies of 10 – 20 mg were cut and then enzymatically digested by proteinase K in ATL buffer. The solution containing the DNA was mixed with ethanol, transferred onto DNeasy Mini spin columns followed by two washing steps. DNA was eluted with AE buffer and the DNA concentration and purity was analysed with the NanoDrop spectrophotometer and the ND-1000 software. DNA samples were stored at -80°C until further analysis.

2.2.3.2. Real-time quantitative PCR

To quantify the abundance of the MCMV gene *M55* in relation to the mouse reference gene *Rps9* within the DNA samples, real-time quantitative polymerase chain reaction (qPCR) was performed. Therefore, the Maxima SYBR Green/ROX qPCR Master Mix 2x and the 7500 Fast Real Time PCR System was used according to the manufacturer's protocol. The composition of the master mix is shown in Tab. 14 and the program for the qPCR in Tab. 15. The DNA samples were used to generate a standard series for the relative quantification of the gene abundance, normalized to the reference gene as shown in the following formula:

$$Relative\ abundance = \frac{mean\ quantity\ of\ the\ target\ gene}{mean\ quantity\ of\ the\ reference\ gene}$$

To determine changes between different treatment groups, the fold change was calculated as in the following formula:

$$Fold\ change = \frac{realtive\ abundance\ of\ each\ sample}{mean\ abundance\ of\ the\ control\ group}$$

Tab. 14: Master mix for qPCR

volume	components
1 µl	DNA
4 µl	water
5 µl	Primer mix
10 µl	Maxima SYBR Green/ROX qPCR Master Mix

Tab. 15: Program for qPCR

phases	steps	temperature	time	cycles
denaturation phase	initial denaturation	95°C	10 min	1
cycling phase	denaturation	95°C	15 seconds	45
	annealing	55°C	30 seconds	
	extension	72°C	30 seconds	
melt curve phase	melt	60 – 90°C (continuously rising)	1 min per degree	1

2.2.3.3. Cytokine detection via Luminex technology

The detection of secreted cytokines of colon explants was performed using Luminex technology and a customized kit from R&D systems according to the manufacturer's protocol. The kit enabled the detection of the cytokines TNFα, IFNγ, granzyme B, IL-6, IL-1β, IL-10 and the chemokine CXCL10. The assay was performed on the Luminex 200 system and evaluated with the Luminex xPONENT software.

2.2.3.4. RNA sequencing

The RNA sequencing library was generated from cell lysate of *in vitro* MCMV infected and untreated ISCs using the NEB Next Single Cell / Low Input RNA Library Prep Kit (New England BioLabs) according to the manufacturer's protocol. Libraries were sequenced on Illumina NovaSeq 6000 using NovaSeq 6000 S1 Reagent Kit (100 cycles, paired end run). Library preparation, sequencing and data processing was kindly performed by Prof. Dr. Robert Geffers (Helmholtz centre for Infection Research (HZI), Braunschweig, Germany). Quality report was

generated by FASTQC tool and data trimming performed using fastq-mcf. Trimmed reads were aligned to the reference genome (GRCm39 *Mus musculus*) using the open source read aligner STAR.

Analysis of differential gene expression (DEG) was performed using the generalized linear models (glm)-based statistical method of the edgeR Bioconductor package. Figures were generated using Python and R software and gene ontology (GO) analysis was performed using the R software with the packages indicated in Tab. 12, supported by Dr. Erik Lange, University hospital Essen, Institute for Medical Microbiology, Essen, Germany.

2.2.3.5. Fluorescence microscopy

Fluorescence microscopy was performed on an APEXVIEW APX100 microscope using a ORCA-Fusion Digital camera C14440 camera on Olympus cellSens APEX 100 software. Images were taken using a 10x / 20x / 40x objective (Olympus). The Olympus cellSens APEX 100 software was used to process the fluorescence images.

2.2.4. Statistics

For statistical analysis, the GraphPad Prism version 7.05 was used. To analyse whether the data are normal distributed, D'Agostino-Pearson and Shapiro-Wilk normality tests were applied. Normal distributed data were examined for statistical significances using unpaired t test and not normally distributed data by Mann-Whitney test. Further, one-way and two-way ANOVA followed by Dunn's multiple comparison test or Tukey's multiple comparison test were performed. Statistical analysis of the RNA sequencing data was performed as described in chapter 2.2.3.4. Statistical significances were defined with *P < 0.05; **P < 0.01, ***P < 0.001 and ****P < 0.0001.

3. Results

3.1. Impact of Cytomegalovirus infection on the intestinal tract

HCMV is an opportunistic pathogen that can infect multiple cell types in different organs. Consequently, the virus is implicated in many inflammatory processes [192]. The mutual influence of the HCMV infection and inflammatory conditions in IBD has been widely discussed in recent decades. Previous studies have demonstrated an increased prevalence of active HCMV replication in the colon of IBD patients [193], however, further research is required to determine the underlying pathophysiologic mechanisms. Therefore, in this study intestinal CMV replication was investigated using the MCMV infection in models of intestinal homeostasis and inflammation.

3.2. MCMV replication in the colonic tissue

To address the potential effects of a CMV infection on intestinal homeostasis, a MCMV infection model was established in immunocompetent mice. Naïve BALB/c mice were infected with MCMV i.p. and viral loads were measured in certain organs and at various time points. As previously reported, a robust MCMV replication has been documented in organs such as the spleen, the liver, and the salivary glands in immunocompetent mice [194]. As expected, this study also revealed elevated viral titers in the spleen and liver at 2 and 5 days post-infection (dpi), with a decline observed at 7 dpi (Fig. 8 A and B). At later time points, high viral titers were detected in the salivary glands, starting to increase at 5 dpi and further rising at later time points (Fig. 8D). Of particular interest, the viral replication in the colon was comparable to the levels observed in spleen and liver. In contrast to the viral kinetics observed in these organs, MCMV was detected with a delay in the colonic tissue, peaking at 5 and 7 dpi (Fig. 8 C).

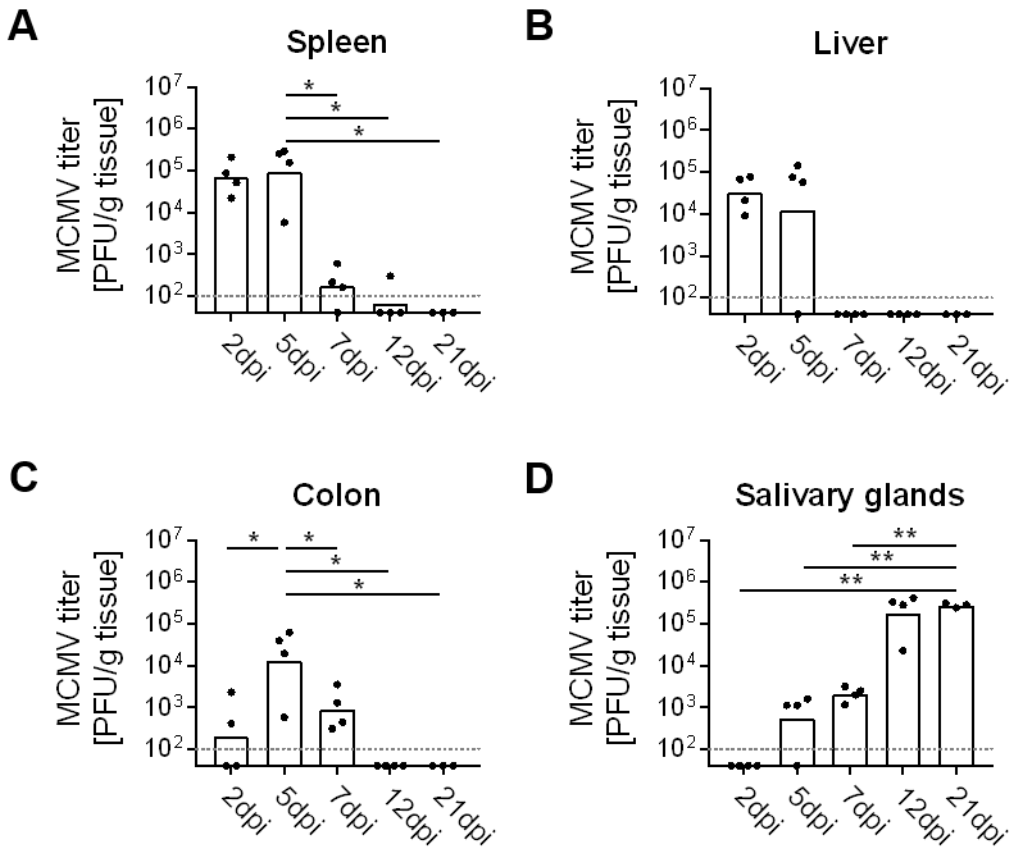

Fig. 8: MCMV infection induces a robust viral replication in the colon. BALB/c mice were i.p. infected with MCMV ($2x10^5$ PFU). Samples were taken at indicated time points and viral loads were determined (A) in spleen, (B) liver, (C) colon, and (D) salivary glands. Data from one experiment (n = 4 mice per group) are shown as geometric mean, titrations were conducted as quadruplicates and dots show viral titers of individual mice. Dotted line indicates detection limit. Statistical analysis were performed using One-way ANOVA followed by Tukey's multiple comparison test. *P < 0.05; **P < 0.01. Results have already been published in [169].

Current guidelines for the diagnosis of HCMV infections in IBD patients recommend the quantification of HCMV markers in fresh tissue biopsies via qPCR. Consequently, a detection of the MCMV load via qPCR was further established in this study. The abundance of the MCMV gene *M55*, which encodes the envelope glycoprotein B (gB) [195], was thus quantified in relative abundance to the housekeeping gene *Rps9* from the host in the DNA isolated from fresh tissue biopsies of infected mice. The qPCR detection method showed comparable viral kinetics to the standard plaque titration assay shown in Fig. 8. High viral loads were detected at early time points in spleen and liver, peaking at 2 and 5 dpi (Fig. 9 A and B), while colonic MCMV replication was detectable with a delay at 5 and 7 dpi (Fig. 9 C). Furthermore, MCMV was detectable within the salivary glands over the course of the infection. Despite the fact, that the quantification via qPCR is less sensitive at certain time points in comparison to the titration experiments, MCMV occurrence was still detectable. Due to the comparatively limited quantity

of tissue material required for detection via qPCR, this method is applied in the ensuing experiments.

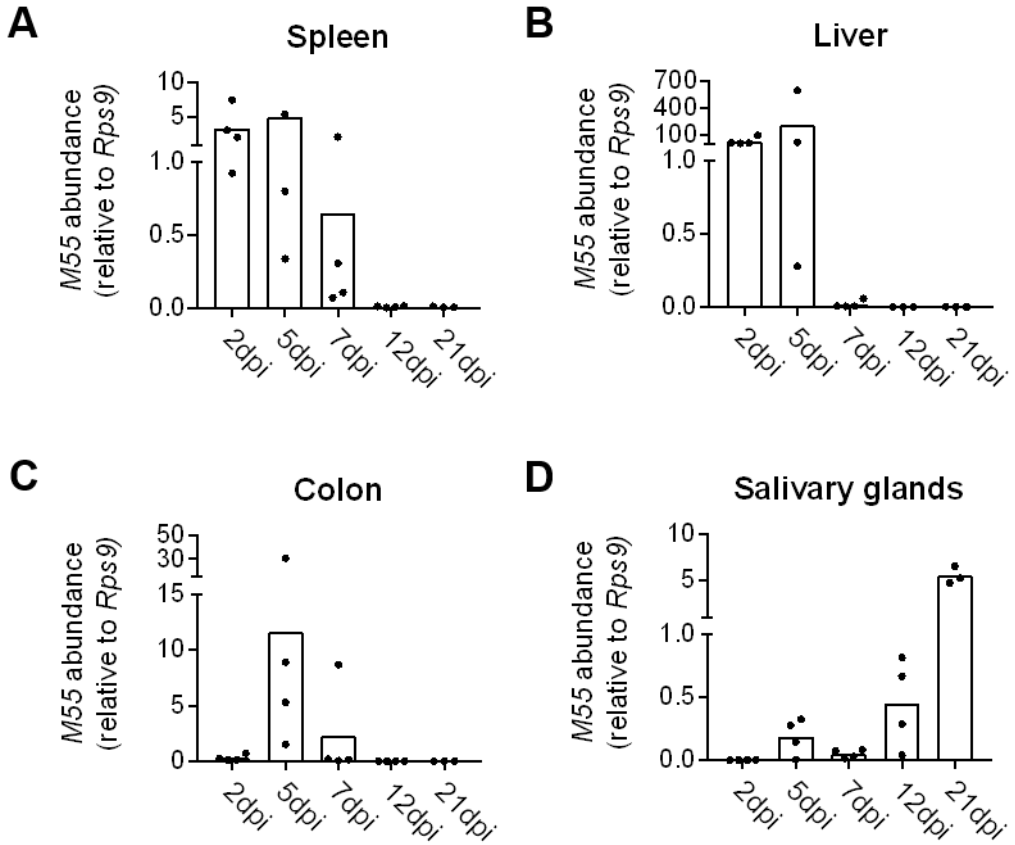

Fig. 9: Detection of MCMV replication by quantitative PCR. BALB/c mice were i.p. infected with MCMV ($2x10^5$ PFU). Biopsies from (A) spleen, (B) liver, (C) colon and (D) salivary glands were taken at indicated time points. DNA was extracted and the abundance of the viral gene *M55* relative to *Rps9* was quantified by qPCR. Data shown from one experiment (n = 4 mice per group).

3.3. Colonic MCMV replication induces mild intestinal inflammation

The MCMV infection model, which demonstrated a robust replication in colonic tissue, was utilised to investigate whether elevated colonic MCMV titers induce pathological alterations in the colon. At 5 and 7 dpi, during the peak of MCMV replication within the colon, an increased secretion of the pro-inflammatory cytokines TNFα and IFNγ and the cytotoxic granule molecule granzyme B (GrzmB) was induced (Fig. 10 A - C). Furthermore, the determination of cleaved caspase 3 (Casp3) by IHC staining of colonic tissue sections indicated an increased level of apoptosis in the colonic epithelium. Representative images demonstrate that the amount of $Casp3^+$ cells was enhanced in the colonic tissue of MCMV-infected mice at 5 dpi. In combination with the findings presented in Le-Trilling and Ebel *et al.*, it has been demonstrated that MCMV replication in the colon results in the disruption of the intestinal barrier and the induction of mild inflammation in an immunocompetent host. In addition to the induction of pro-

inflammatory cytokines and apoptosis in the intestinal epithelium, intestinal MCMV infection has been shown to result in a short-term disruption of the microbial composition, changes in the colonic architecture, and consequently, a disturbance of intestinal homeostasis [169].

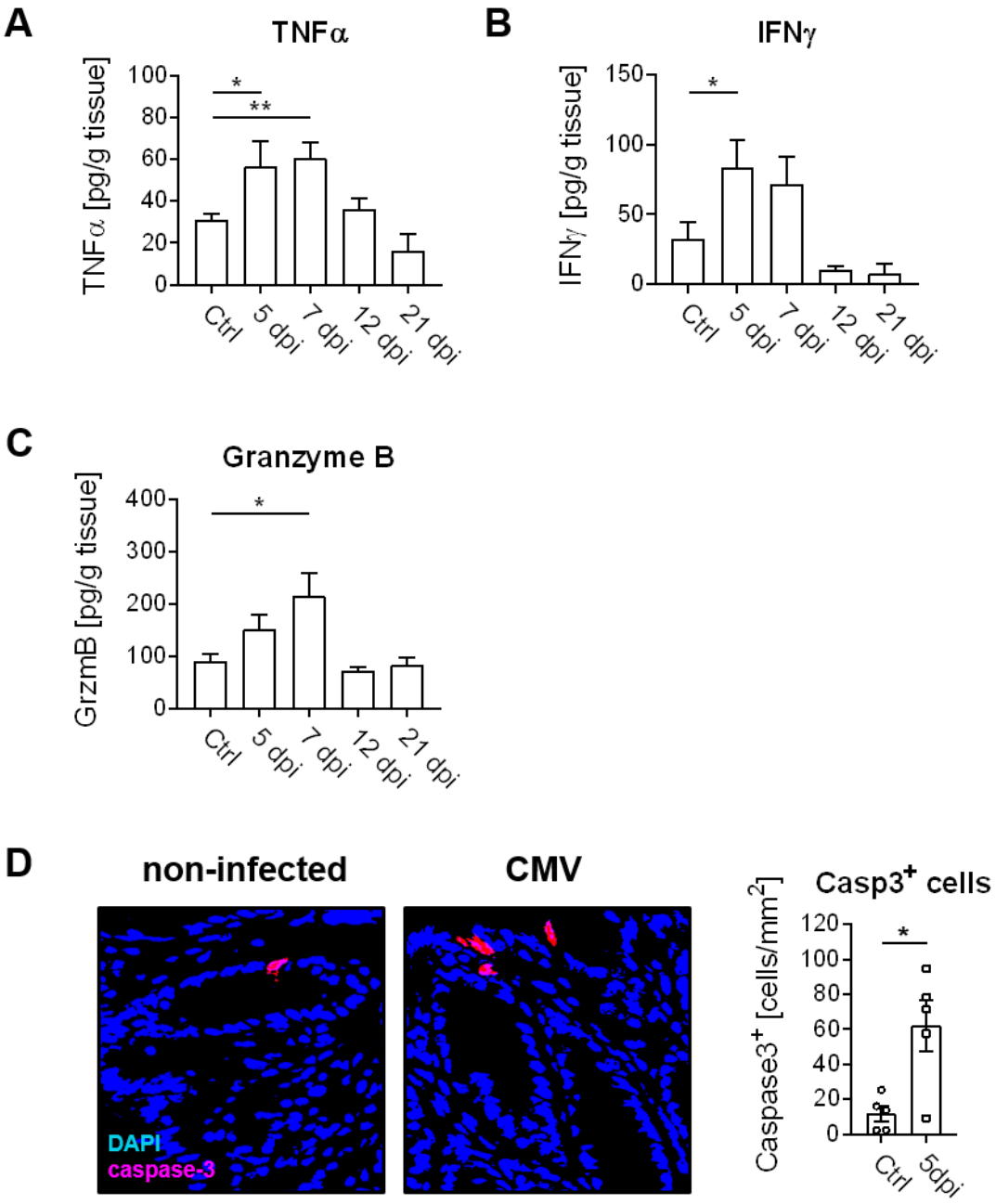

Fig. 10: MCMV replication induces mild intestinal inflammation. BALB/c mice were i.p. infected with MCMV (2x10^5 PFU). Colonic explants were taken at indicated time points and cultured *in vitro*, secretion of (A) TNFα, (B) IFNγ and (C) granzyme B (GrzmB) were analysed in the supernatants by Luminex technology (n = 3 - 40). (D) Representative images of immunohistochemistry (IHC) stained colonic tissue sections from non-infected and MCMV-infected mice 5 dpi (n = 5 mice per group). Nucleus/DNA (hematoxylin) and caspase 3 (3,3'-diaminobenzidine (DAB)) were stained. Color deconvolution and pseudo coloring was performed on images to depict nucleus/DNA in blue, caspase3 (Casp3) in red and merged resulting in purple Casp3$^+$ cells. All data are presented as mean ± SEM. Statistical analysis were performed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test (A, B, C) or unpaired t test (D). *P < 0.05; **P < 0.01. Results have already been published in [169].

3.4. MCMV infection exacerbates intestinal inflammation in DSS colitis

The mild intestinal inflammation induced by MCMV in immunocompetent hosts has the potential to predispose in individuals already suffering from colitis to an even stronger inflammatory response, thereby exacerbating their condition. To investigate this, colitis was induced in naïve BALB/c mice by the administration of DSS in the drinking water for 7 consecutive days followed by 7 days of normal drinking water. Additionally, one group of DSS-treated mice was concomitantly infected with MCMV at day 7 (Fig. 11 A). Mice that received during the howl experimental setup normal drinking water and that were either PBS-injected (Ctrl) or MCMV-infected (CMV) at day 7 were used as reference groups.

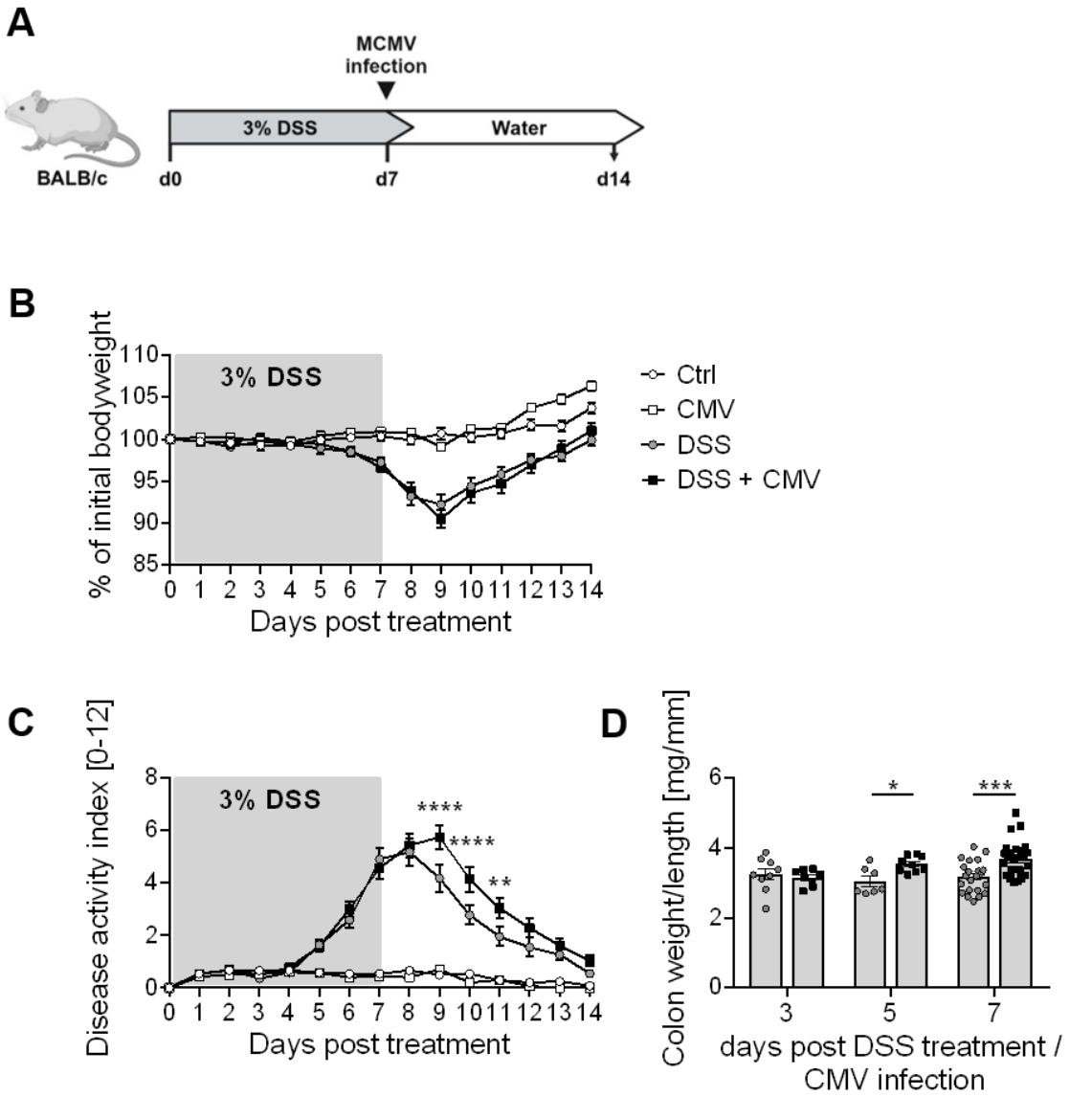

Fig. 11: MCMV infection extends intestinal inflammation of DSS-induced colitis. (A) BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment (n = 25 – 30 mice per group). Daily monitoring of (B) proportional body weight changes to the initial weight and (C) disease activity index.

(D) Colon weight to length ratio of non-infected and infected mice at indicated time points post DSS treatment. All data are presented as mean ± SEM. Statistical analysis were performed using two-way ANOVA followed by Tukey's multiple comparison test (B, C) or Mann-Whitney test (D). Significances in (B) and (C) are shown between DSS- and DSS + CMV-groups. *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.

Severity of disease was monitored daily via body weight changes and the disease activity index (DAI). The DSS-treated mice developed an acute intestinal inflammation with the peak at day 9 post treatment, indicated by a pronounced body weight loss and a high DAI. While there was no difference in the degree of body weight loss in only DSS-treated mice and mice additionally infected with MCMV (Fig. 11 B), the DAI indicated an increased and prolonged intestinal inflammation during the peak of colitis due to MCMV infection. Further, the recovery until day 14 was impaired in the infected mice (Fig. 11 C). MCMV-only infected mice did not exhibit body weight loss or DAI. The prolonged intestinal inflammation was supported by an increase of the colon weight to length ratio at 5 and 7 days post MCMV infection in comparison to DSS-only treated mice. The ratio can be used as a first indicator for an inflamed colonic tissue (Fig. 11 D).

Histological analysis were implemented for detailed investigations of the colonic pathology. In line with the previous results, an increased histopathological damage could be observed in the MCMV infected DSS-treated mice in comparison to the control groups (Fig. 12 A). The infection resulted in increased colonic hyperplasia, goblet cell loss, epithelial defects and immune cell infiltration during the recovery from DSS colitis. The histopathological effects and especially the changes in the colonic architecture induced by the MCMV infection during DSS colitis are shown in representative images of Fig. 12 B. Given that the mice had already entered the recovery phase of DSS-induced colitis by day 14, the histological results support the evidence that MCMV has a negative impact on the regenerative capacity of the intestine, as they still showed high levels of inflammation in the colon.

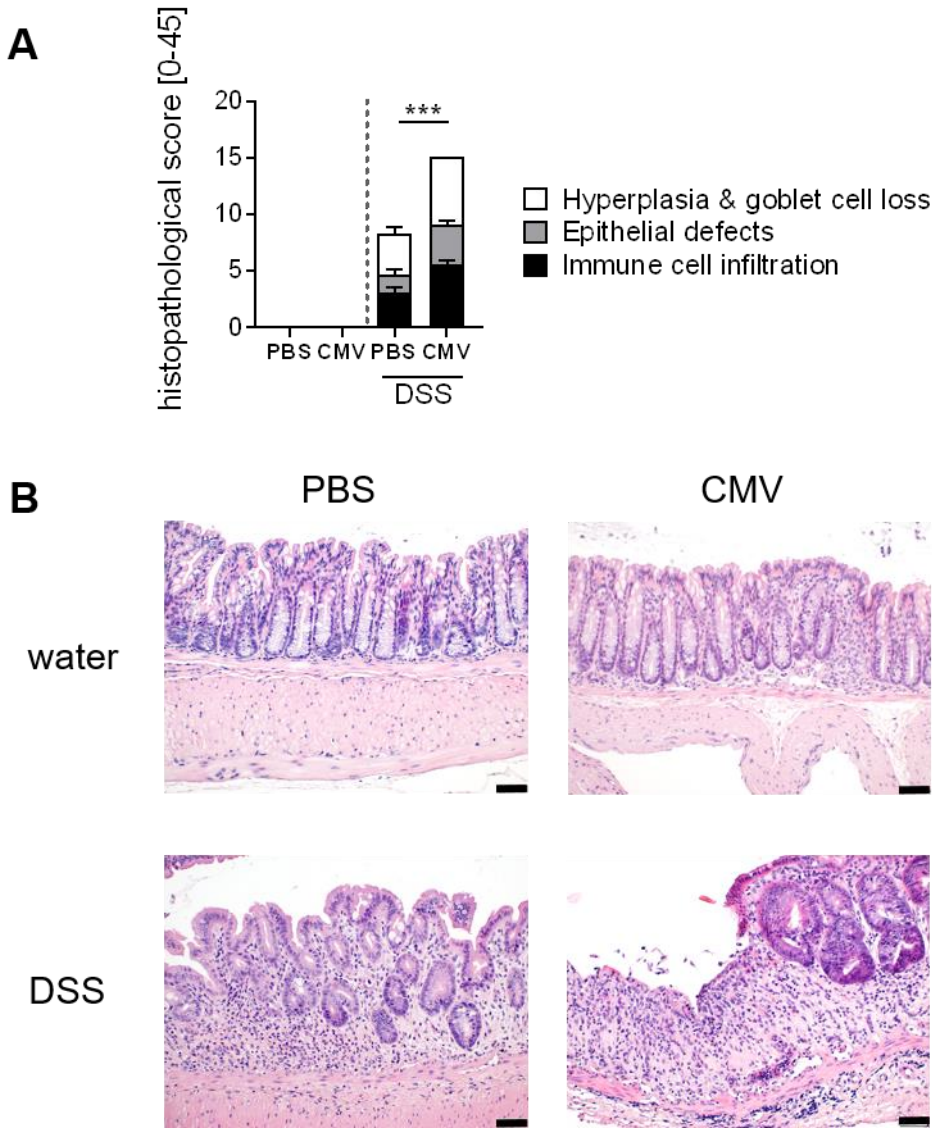

Fig. 12: MCMV infection impairs intestinal regeneration post DSS colitis. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. (A) Histopathological score, including hyperplasia and goblet cell loss, epithelial effects and immune cell infiltration was determined at day 14 (n = 10 mice per group). (B) Representative images of H&E stained colon sections. Black scale bar = 50 µm. Data are presented as mean ± SEM. Statistical analysis were performed using unpaired t test (A). ***P < 0.001.

3.5. DSS-induced inflammation promotes the establishment of colonic MCMV infection

To determine whether the enhanced pathology observed in MCMV-infected DSS colitis mice was caused by elevated MCMV levels in the colon, viral loads were quantified via qPCR. The DSS-induced intestinal inflammation may have influenced the establishment of the MCMV infection in the colon and, moreover, in other distinct organs. Indeed, intestinal inflammation reduced in tendencies viral loads in the spleen and the liver 7 days post MCMV infection, while

the viral load in the colon and the salivary glands was apparently not affected (Fig. 13 A). However, the inflammatory processes in the colon at early time points in turn proved to be beneficial for the establishment of infection in the colon. While MCMV-only infected mice exhibited robust viral replication only 5 or 7 dpi, DSS colitis mice displayed a robust MCMV infection as early as 3 dpi (Fig. 13 B). The early MCMV accumulation in the colon might have influenced the course of infection in other organs, such as the spleen and liver.

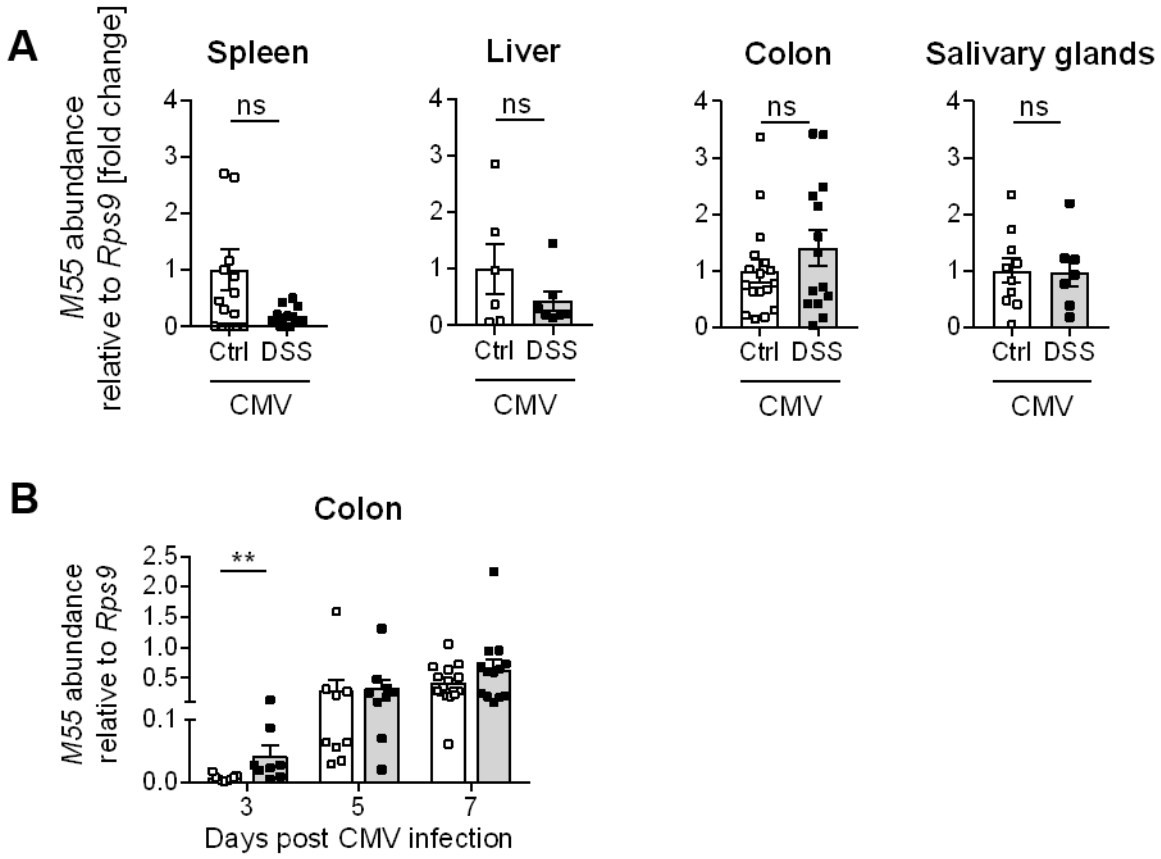

Fig. 13: Intestinal inflammation accelerates the establishment of colonic MCMV infection. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were i.p. infected with MCMV ($2x10^5$ PFU) at day 7 post DSS treatment. (A) Biopsies from spleen, liver, colon and salivary glands were taken at 7 days post infection. DNA was extracted and the abundance of the viral gene *M55* relative to *Rps9* was quantified by qPCR. The fold change between untreated and DSS treated mice is shown. (B) Abundance of the viral gene *M55* relative to *Rps9* in the colon at indicated time points after the infection. Data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney tests. Ns = not significant, **P < 0.01.

3.6. Colonic MCMV infection enhances the pro-inflammatory milieu in DSS colitis mice

To further investigate the MCMV-mediated impairment of intestinal regeneration after DSS colitis, the persistent inflammation was examined in detail at the end o the experiment at day 14. The MCMV-infected DSS colitis mice exhibited altered cytokine profiles compared to the DSS-only mice with higher levels of the pro-inflammatory cytokine TNFα and in tendency, more IL-1β (Fig. 14 A). In addition, levels of the immunomodulatory cytokine IL-10 also tended to be elevated in colon tissue of MCMV-infected DSS-treated mice (Fig. 14 B).

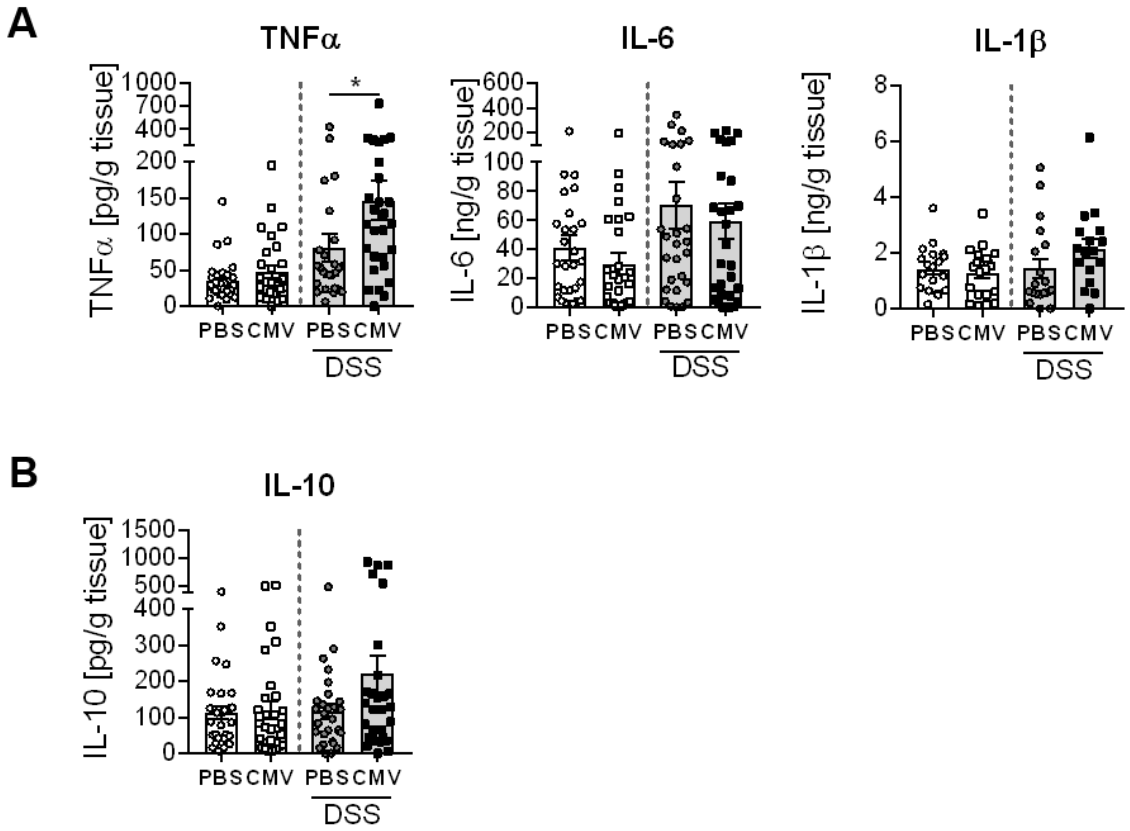

Fig. 14: MCMV replication induces altered colonic cytokine profiles during the recovery from DSS-induced colitis. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment (n = 25 – 30 mice per group). Supernatants of *in vitro* cultured colonic explants were analysed for the secretion of (A) the pro-inflammatory cytokines TNFα, IL-6 and IL-1β and (B) the anti-inflammatory cytokine IL-10 using Luminex technology at day 14. All data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney tests. *P < 0.05.

As the altered cytokine profile suggests modifications of the intestinal immune response, both the innate and adaptive immune cell frequencies were assessed. Accordingly, a significant expansion of macrophages, NK cells, neutrophils and eosinophils was observed in the colon of MCMV-infected mice during the recovery from DSS colitis compared to uninfected DSS colitis mice. The MCMV infection of healthy mice significantly elevated the levels of macrophages, while the other innate immune cells were increased only moderately and to a lower extent than in mice with DSS-induced colitis upon infection (Fig. 15 A - D).

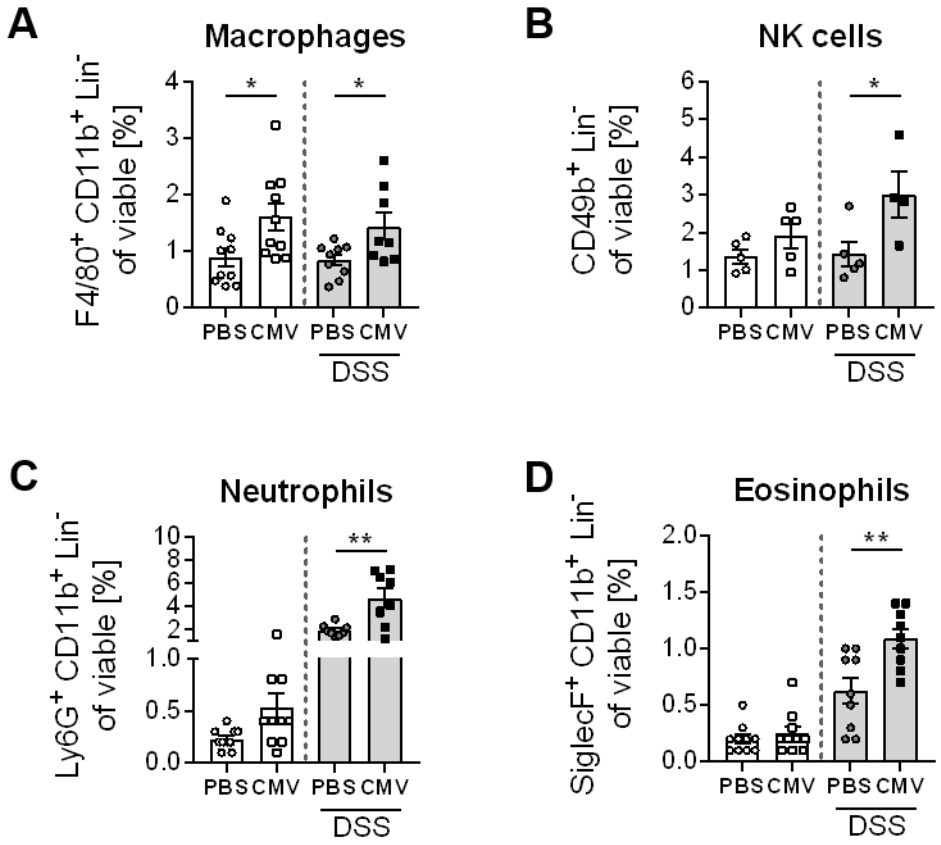

Fig. 15: MCMV infection induces accumulation of innate immune cells in the colon during the recovery from DSS-induced colitis. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. Colonic lamina propria immune cells were isolated at day 14 and frequencies were analysed by flow cytometry (n = 5 – 10 mice per group). (A) Macrophages were defined as viable $F4/80^+$ $CD11b^+$ Lin^- cells, (B) NK cells were defined as viable $CD49b^+$ Lin^- cells, (C) neutrophils were defined as viable $Ly6G^+$ $CD11b^+$ Lin^- cells and (D) eosinophils were defined as viable $SiglecF^+$ $CD11b^+$ Lin^- cells. Lin = Lineage markers (CD3, CD19, CD11c for A - D and SiglecF for A, B, C). All data are presented as mean ± SEM. Statistical analysis were performed using unpaired t tests. *P < 0.05, **P < 0.01.

Additionally, changes in the composition of the adaptive immune cells in the lamina propria of the colon were identified. Moderate changes in the frequencies of conventional $CD4^+$ ($cCD4^+$) T cells and B cells and a significant increase of Tregs were observed in infected DSS colitis mice in comparison to the control groups (Fig. 16 A, C, D). Most strikingly, the colonic MCMV replication induced a significant accumulation of conventional $CD8^+$ ($cCD8^+$) T cells in comparison to uninfected mice. The strongest elevation in frequencies was found in MCMV-infected DSS-treated mice (Fig. 16 B).

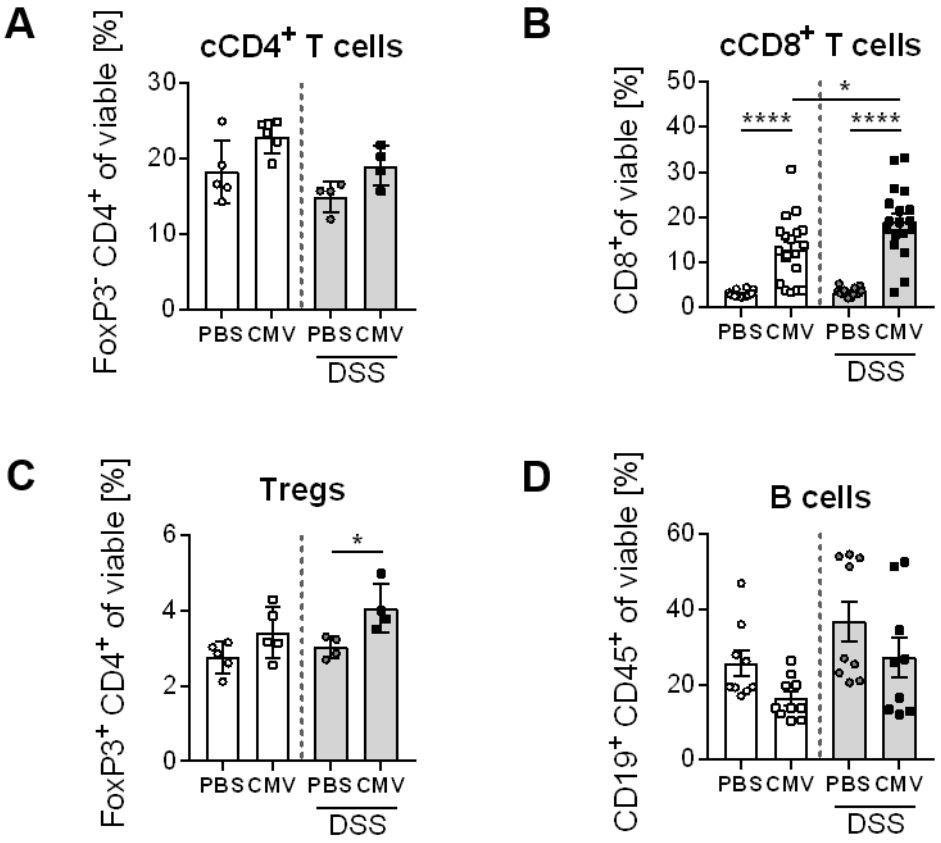

Fig. 16: MCMV replication induces accumulation of $CD8^+$ T cells in the colon during recovery from DSS-induced colitis. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. Colonic lamina propria lymphocytes were isolated at day 14 and frequencies were analysed by flow cytometry (n = 5 – 20 mice per group). (A) Conventional (c) $CD4^+$ T cells were defined as viable $CD4^+$ $FoxP3^-$ cells, (B) $cCD8^+$ T cells were defined as viable $CD8^+$ cells, (C) regulatory T cells (Tregs) were defined as viable $CD4^+$ $FoxP3^+$ cells and (D) B cells were defined as viable $CD19^+$ $CD45^+$ cells. All data are presented as mean ± SEM. Statistical analysis were performed using unpaired t tests. *P < 0.05; ****P < 0.0001.

3.7. MCMV infection induces the accumulation of cytotoxic CD8⁺ T cells in the colon during the recovery of DSS colitis

The strong accumulation of CD8⁺ T cells observed in the colon in response to MCMV infection indicates a direct involvement of adaptive immunity in the aggravated pathology post DSS colitis. To investigate this point, the CD8⁺ T cells were characterized by analysing the expression of activation markers, proliferation markers and the production of effector molecules (Fig. 17).

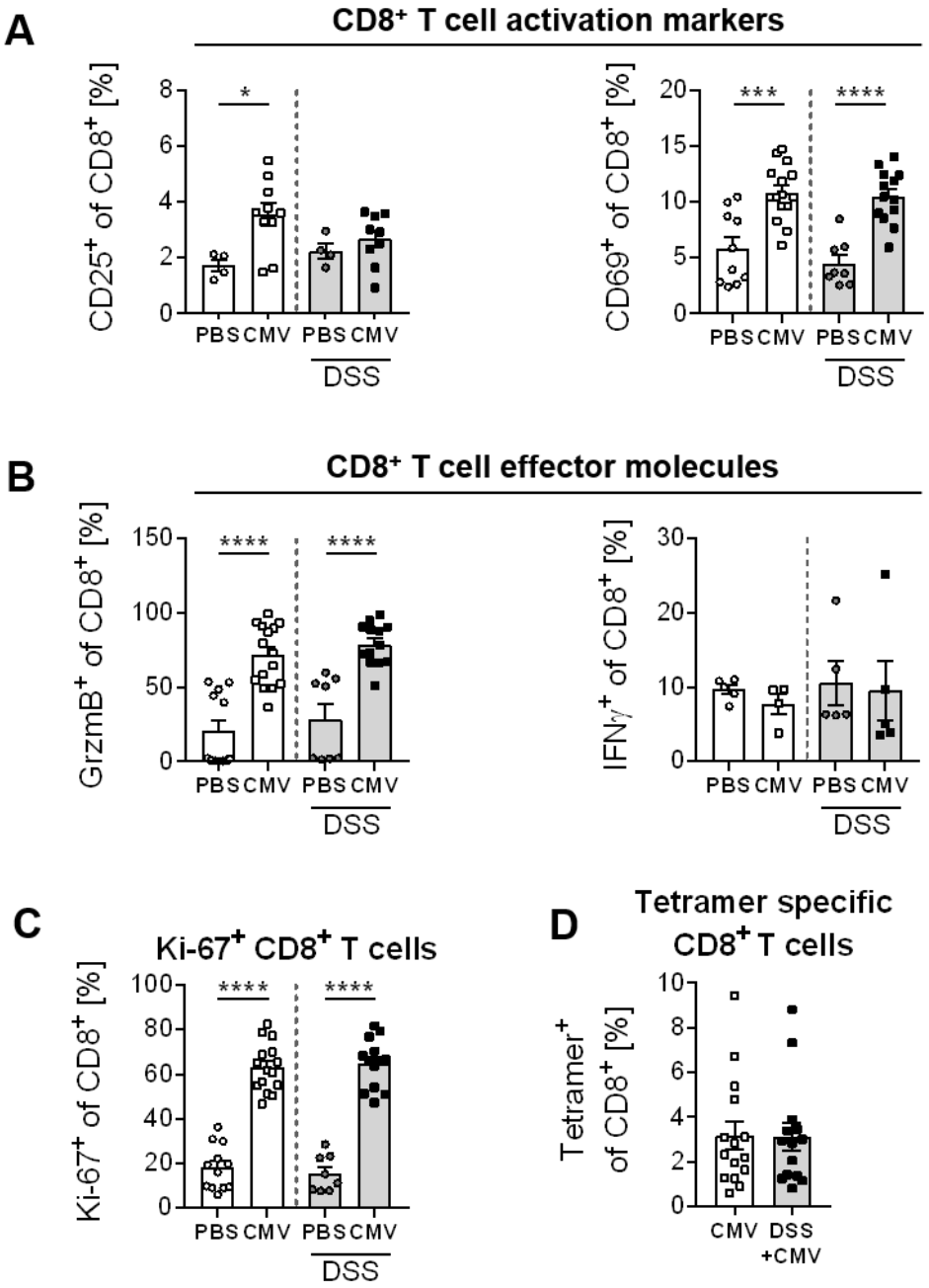

Fig. 17: MCMV infection enhances the activation and cytotoxicity of CD8⁺ T cells in the colon during recovery of DSS-induced colitis. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of

normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. Colonic lamina propria lymphocytes were isolated at day 14 and frequencies were analysed by flow cytometry (n = 5 – 15 mice per group). (A) Expression of activation markers CD25 and CD69, (B) production of effector molecules granzyme B (GrzmB) and IFNγ and (C) expression of proliferation marker Ki-67 was analyzed on viable $CD8^+$ cells. (D) MCMV tetramer specific $CD8^+$ T cells. All data are presented as mean ± SEM. Statistical analysis were performed using unpaired t tests. *P < 0.05; ***P < 0.001, ****P < 0.0001.

Accordingly, $CD8^+$ T cells of infected mice showed a higher expression of the early activation marker CD69, accompanied by an enhanced production of the cytotoxic granule molecule granzyme B and an up-regulation of the proliferation marker Ki-67 (Fig. 17 A-C).

Furthermore, it was determined whether the inflammatory state of DSS colitis might impact the proportion of MCMV-specific $CD8^+$ T cells and thus explain the strongest accumulation of the adaptive immune cells during the recovery of DSS-induced colitis. To this end, a labelled MHC-tetramer was utilised, which directly bound to the respective TCR against the MCMV antigen M45. However, the frequency of MCMV tetramer-specific $CD8^+$ T cells remained unaltered (Fig. 17 D), suggesting an overall influx of cytotoxic $CD8^+$ T cells into the colon triggered by the MCMV infection.

3.8. MCMV-induced exacerbated pathology during DSS colitis is mediated by cytotoxic $CD8^+$ T cells

To confirm that the enhanced pathology of DSS colitis after MCMV-infection is mediated by cytotoxic $CD8^+$ T cells, a specific depletion of $CD8^+$ T cells via i.p. administration of a CD8-depleting antibody was subsequently applied in the model (Fig. 18 A). Depletion was induced one day prior to MCMV infection and was confirmed in the blood at day 14 of the experimental setup (data not shown). Consequently, $CD8^+$ T cell-depleted and MCMV-infected mice exhibited an ameliorated DAI during the course of DSS-induced colitis (Fig. 18 C), accompanied by a decrease incolon weight to length ratio at day 14 of the experimental model (Fig. 18 D). Both disease parameters were alleviated to the level of only DSS-treated mice, indicating that the cytotoxic $CD8^+$ T cells play an important role in the intestinal pathology.

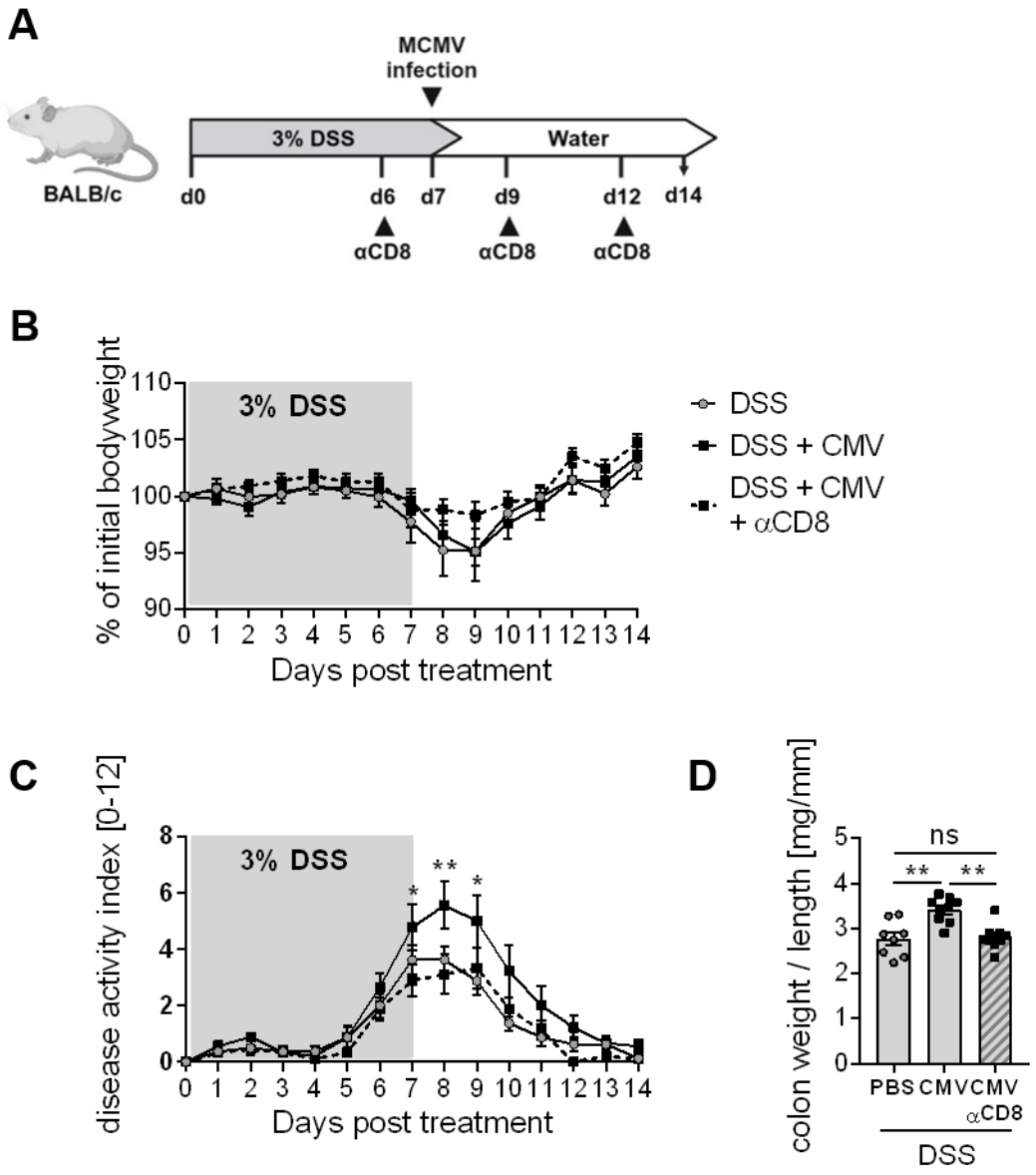

Fig. 18: CD8⁺ T cell-depletion alleviates the exacerbating impact of the MCMV infection on the course of DSS-induced colitis. (A) BALB/c mice were treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. CD8⁺-depleting antibody was i.p. injected to one group at indicated time points (n = 10 mice per group). Daily monitoring of (B) proportional body weight changes to the initial weight and (C) disease activity index. (D) Colon weight to length ratio was examined at day 14. All data are presented as mean ± SEM. Statistical analysis were performed using two-way ANOVA followed by Tukey's multiple comparison test (B, C) or One-way ANOVA followed by Tukey's multiple comparison test (D). Significances in (C) are shown between DSS+ CMV and DSS + CMV + αCD8-groups. *P < 0.05; **P < 0.01.

This was further underlined by the histological evaluation as in colonic tissue sections of CD8⁺ T cell-depleted mice the extent of crypt hyperplasia, goblet cell loss, epithelial effects and immune cell infiltration was significantly reduced in comparison to MCMV-infected DSS colitis mice with a pronounced CD8⁺ T cell response (Fig. 19 A and B).

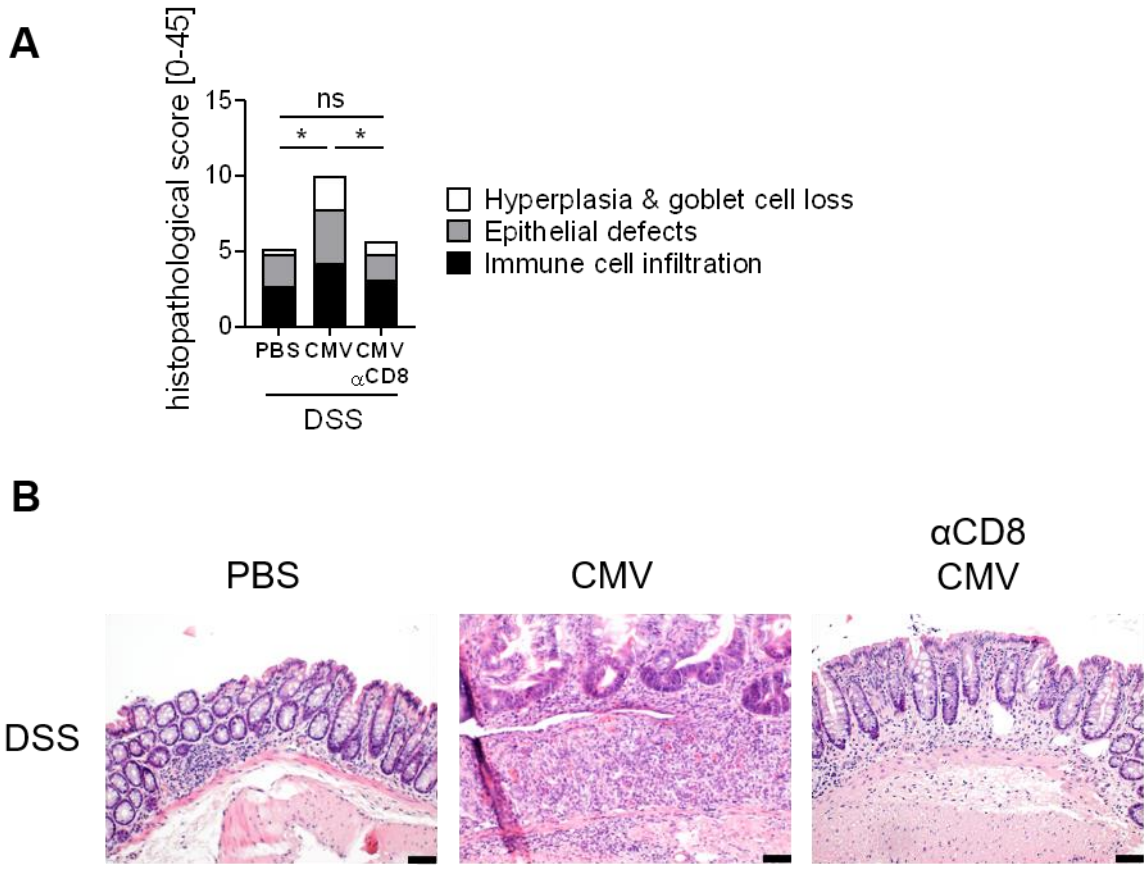

Fig. 19: CD8⁺ T cell depletion favours intestinal regeneration post MCMV-DSS-induced colitis. BALB/c mice were treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV (2x10^5 PFU) or PBS injected at day 7 post DSS treatment. CD8⁺-depleting antibody was i.p. injected to one group at indicated time points. (A) Histopathological score, including hyperplasia and goblet cell loss, epithelial effects and immune cell infiltration was determined at day 14 (n = 10 mice per group). (B) Representative images of H&E stained colon sections. Black scale bar = 50 µm. Data are presented as mean ± SEM. Statistical analysis were performed using unpaired t tests (A). ns = not significant, *P < 0.05.

To investigate, whether the depletion of CD8⁺ T cells has an effect on the systemic MCMV dissemination and virus control, the viral load was analysed via qPCR in distinct organs (Fig. 20 A). Accordingly, 7 days post MCMV infection the viral load was significantly enhanced in spleen and liver in CD8⁺-depleted mice. Thus, the CD8⁺ T cells seem to play an essential role for the anti-MCMV immune response in these organs. In the colon and the salivary glands the viral load was unaltered. The accumulation of CD8⁺ T cells in the colon upon MCMV infection suggests a certain role for these cells in the antiviral immune response. Interestingly, the depletion of CD8⁺ T cells did not lead to an uncontrolled viral replication in the colonic tissue. Consequently, other immune cells are likely to be responsible for viral control in the gut.

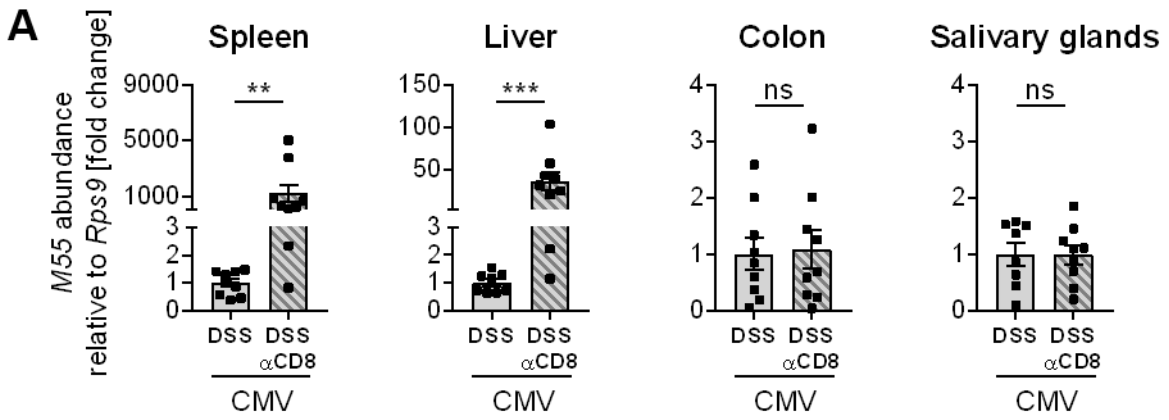

Fig. 20: CD8⁺ T cell depletion enhances MCMV replication in the spleen and liver, but not in the colon. BALB/c mice were treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. CD8⁺-depleting antibody was i.p. injected to one group at indicated time points. (A) Biopsies from spleen, liver, colon and salivary glands were taken at 7 days post infection. DNA was extracted and the abundance of the viral gene *M55* relative to *Rps9* was detected by qPCR. The fold change between untreated and mice treated with CD8⁺-depleting antibody is shown. Data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney test. ns = not significant, **P < 0.01, ***P < 0.001.

In conclusion, the enhanced pathology in CD8⁺ T cell-depleted mice during the MCMV-DSS colitis model lends further support to the theory that the accumulation of cytotoxic CD8⁺ T cells in the colon is responsible for the extensive pathological damage observed and contributes to the impairment of intestinal regeneration from colitis.

3.9. MCMV infection alters the colonic regenerative capacity

In addition to the immunological aspects that have been observed in the MCMV-mediated exacerbated phenotype in the DSS colitis model, defects of the intestinal barrier could be a contributing factor to the impaired recovery of these mice. The regenerative capacity of the intestinal tract is highly dependent on the functionality of intestinal stem cells (ISCs). ISCs are located at the bottom of the crypts of the intestine and responsible for the self-renewal, epithelial turnover and repair of damaged intestinal mucosa [196]. Intestine-derived organoids are a faithful *in vitro* model to study the functionality of ISCs and the regenerating intestinal epithelium [197]. In this study, potential defects in the regenerative capacity of the colon were initially determined by the isolation of colonic crypts from mice of the MCMV- and DSS-induced colitis model (Fig. 11 A) at day 12 post DSS treatment and during the peak (5 dpi) of colonic MCMV infection. Colonic crypts were plated for the formation of organoids and subsequently evaluated.

The absolute number of intact colonic crypts isolated from MCMV-infected and DSS-treated mice was significantly decreased in comparison to healthy control mice. The lowest number was isolated from MCMV-infected DSS colitis mice (Fig. 21 A). The enteroid forming efficiency was subsequently determined by quantifying the intact organoids formed from a defined number of intact crypts. Interestingly, the isolated crypts from MCMV-infected DSS colitis mice showed again weakest efficiency (Fig. 21 B), indicating the strongest restriction in the functionality of ISCs. While the overall survival of the formed organoids was not altered (Fig. 21 C), the enteroid morphology was severely altered in the MCMV infected and DSS-treated groups (Fig. 21 D).

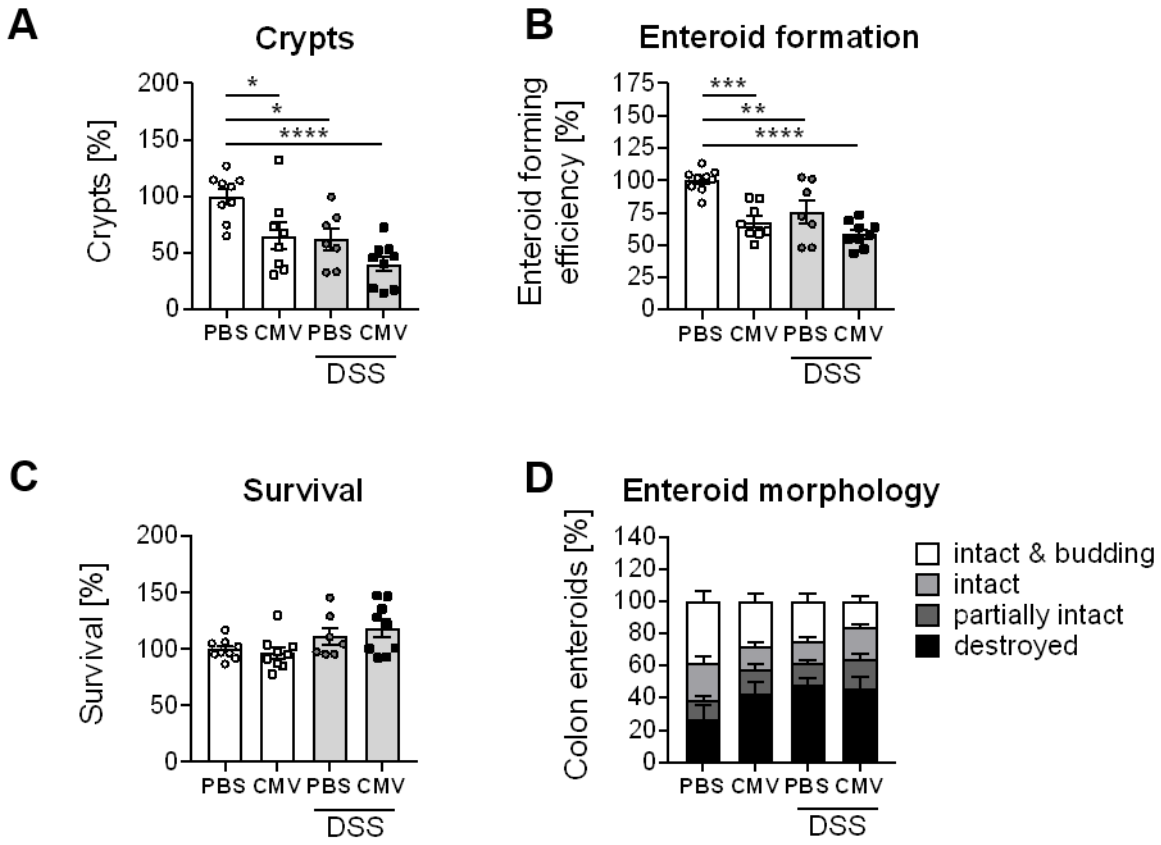

Fig. 21: MCMV replication during DSS-induced colitis impairs the *in vitro* enteroid forming efficacy. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 5 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. Colonic crypts were isolated at day 12 (n = 9 mice per group). (A) Relative numbers of isolated crypts (the mean of the PBS-group was set as 100%), (B) the relative enteroid forming efficacy (the mean of the PBS-group was set as 100%), (C) the relative enteroid survival (the mean of the PBS-group was set as 100%) and (D) the enteroid morphology are shown. Results from 3 independent experiments (3 mice per experiment per group) are shown and each sample was examined in quadruplicates. All data are presented as mean ± SEM. Statistical analysis were performed using one-way ANOVA followed by Tukey's multiple comparison test . *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.

The representative images in Fig. 22 illustrate the physiological changes of the organoids generated from MCMV-infected and/or DSS-treated mice. The lowest number of intact and budding organoids, alongside the highest proportion of destroyed organoids, was observed in mice infected with MCMV and suffering from DSS-induced colitis (Fig. 21 D and Fig. 22 A). Altogether, the results suggest that MCMV infection directly affected the functionality of colonic ISCs, which might have caused the impaired recovery of the DSS colitis.

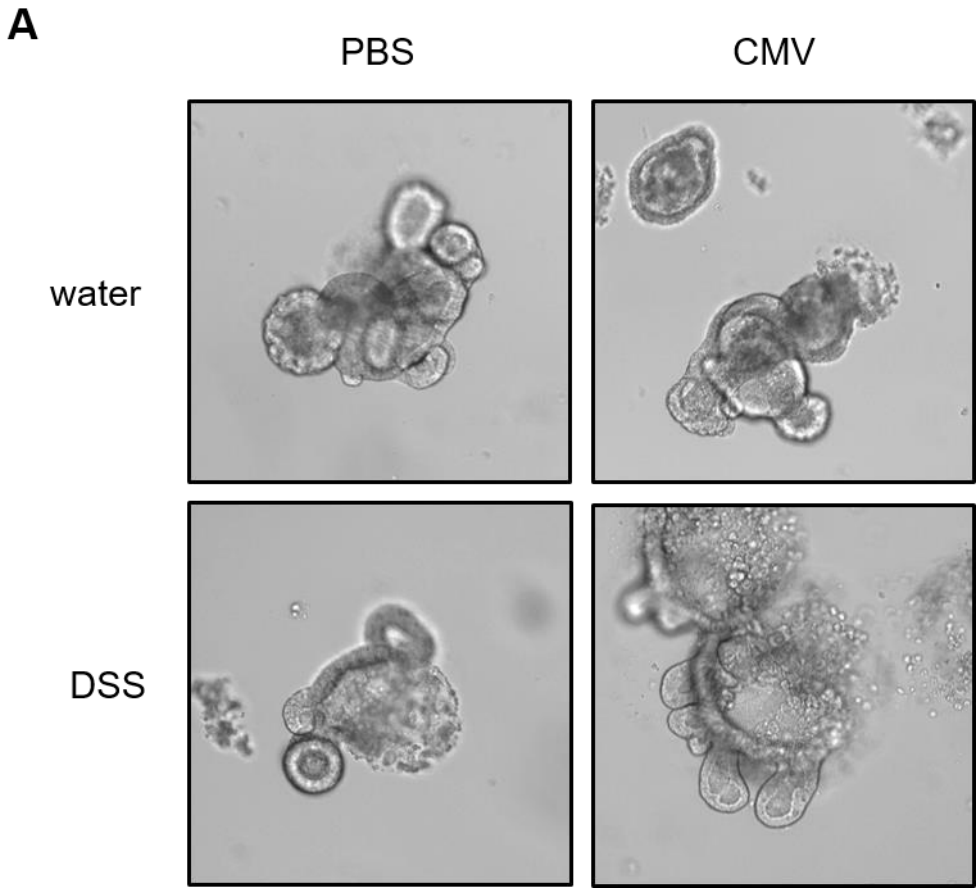

Fig. 22: MCMV replication and DSS-induced colitis impair the *in vitro* enteroid morphology. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 5 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. Colonic crypts were isolated at day 12 (n = 9 mice per group). (A) Representative images from enteroids generated from mice, that were untreated, treated with 3% DSS and/or i.p. infected with MCMV or injected with PBS *in vivo* are shown.

3.10. Colonic intestinal stem cells are susceptible to MCMV infections

In order to ascertain the provenance of the restricted functionality of the ISCs, it was investigated whether they constitute a direct target for MCMV infection in the colon. To address this issue, *Lgr5-EGFP*-reporter mice, that express the fluorescence protein GFP in the $Lgr5^+$ ISCs of the small and large intestine [183], were infected i.p. with MCMV:*mCherry* [185]. Colonic crypts were isolated 5 dpi and examined via immunofluorescence (IF) microscopy (Fig.

23 A). By the detection of the GFP- and mCherry-signal, ISCs and MCMV-infected cells were identified in the colonic crypts. MCMV-infected cells were found to be localised in different regions of the crypts. Importantly, infected ISCs were also detected as double positive for GFP and mCherry signals (Fig. 23 B).

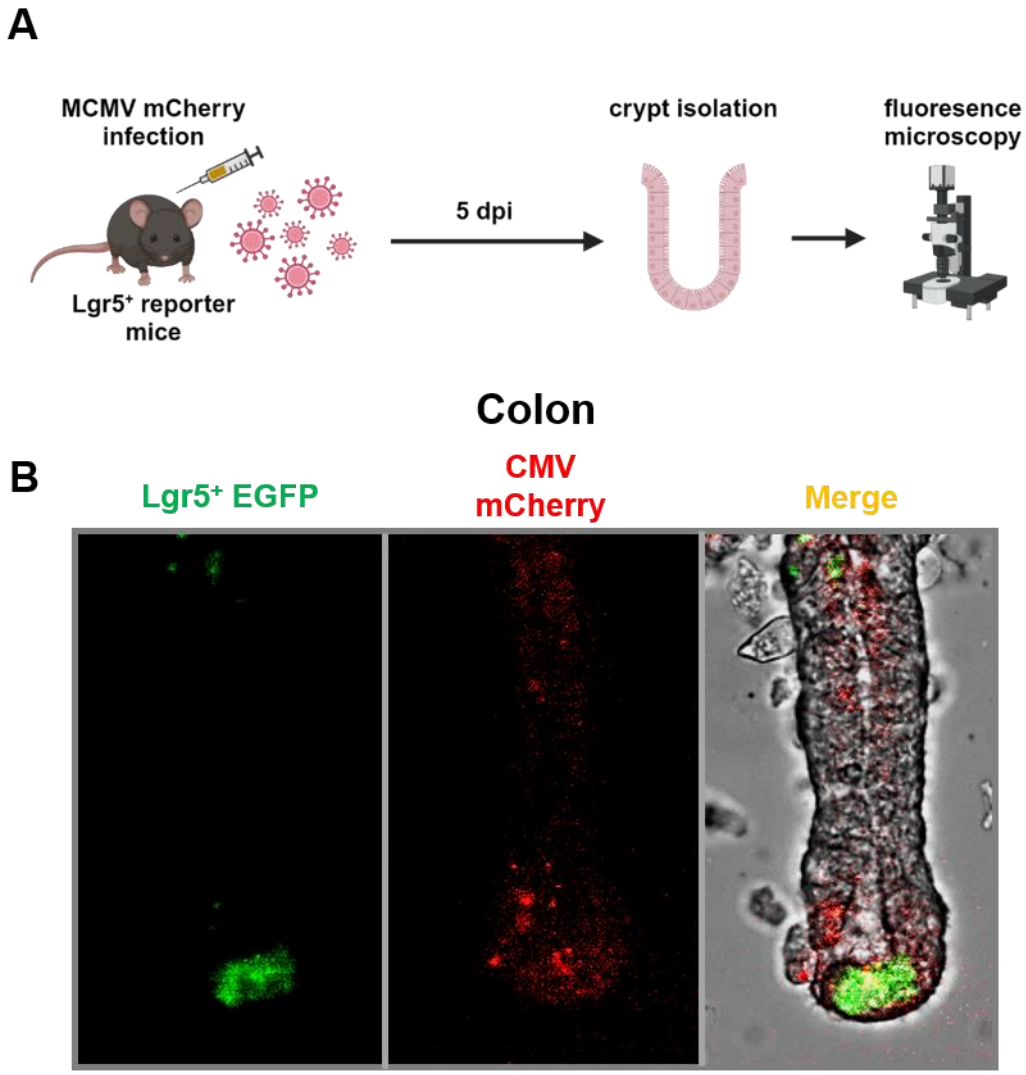

Fig. 23: Colonic intestinal stem cells are a direct target of MCMV infection. (A) Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-EGFP reporter mice were i.p. infected with MCMV:*mCherry* (2x10^5 PFU). 5 days post infection (5dpi) colonic crypts were isolated and investigated under the fluorescence microscope. (B) Representative images of EGFP fluorescence signal from Lgr5$^+$ intestinal stem cells (ISCs; green), mCherry fluorescence signal of MCMV infected cells (red) and merged fluorescence signals resulting in yellow MCMV infected Lgr5$^+$ ISCs in colonic crypts.

In order to gain further insights into the cellular consequences of MCMV infection on ISCs, mRNA sequencing was performed. Colonic crypts from *Lgr5-EGFP*-reporter mice were isolated, dissociated into single cells and ISCs were FACS-sorted. The ISCs were further plated *in vitro* and infected with increasing multiplicity of infection (MOI) of MCMV:*mCherry* for 24 h (Fig. 24 A). The identification of MCMV-infected ISCs was conducted through the

utilisation of IF microscopy and FACS analysis. The viability of the ISCs remained unaltered 24 hours following the infection (Fig. 24 B and C). The mRNA sequencing was performed on uninfected and MCMV-infected ISCs 24 h after *in vitro* cultivation and additional FACS sorting.

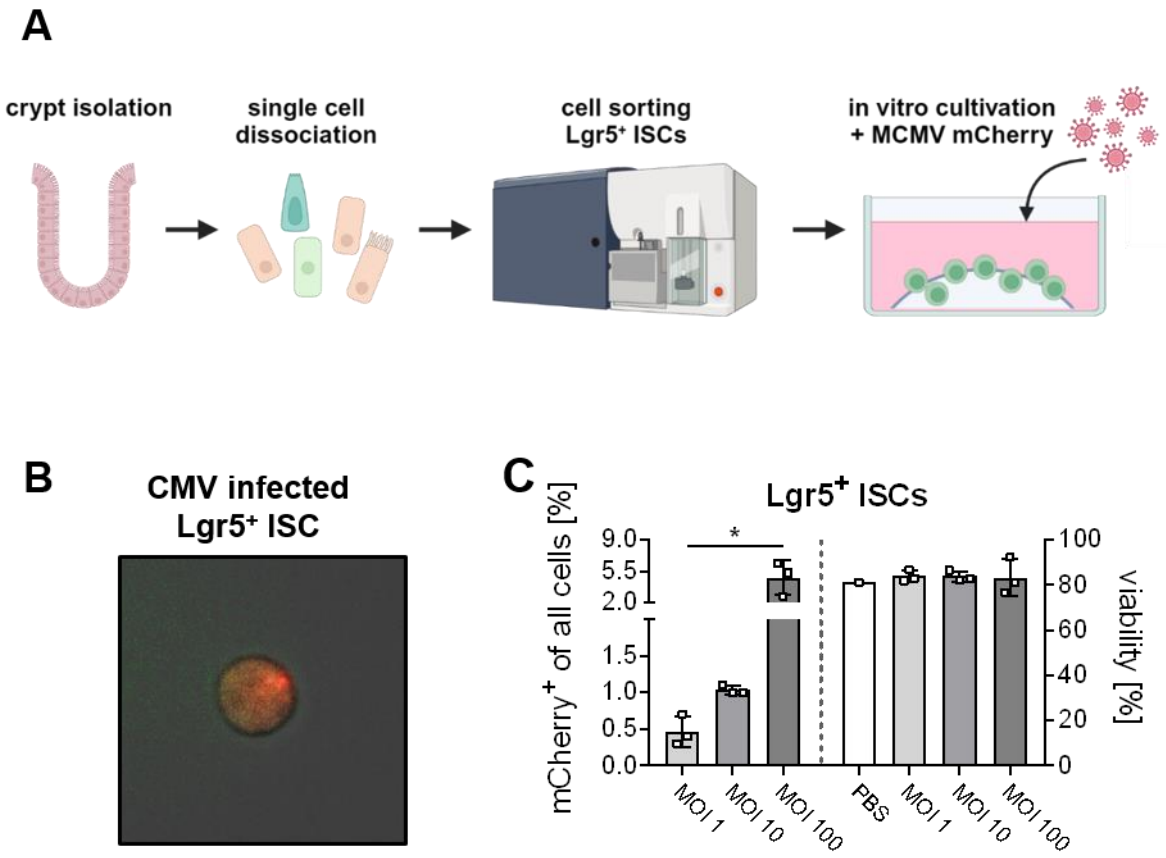

Fig. 24: *In vitro* MCMV infection of colonic Lgr5⁺ ISCs. (A) Colonic crypts from Lgr5-EGFP reporter mice were isolated, dissociated into single cells and Lgr5⁺ ISCs were FACS-sorted. Lgr5⁺ ISCs were cultivated *in vitro* on Matrigel in culturing medium containing defined multiplicity of infection (MOI) of MCMV:*mCherry* for 1 day. (B) Representative fluorescence microscope image of an *in vitro* MCMV infected Lgr5⁺ ISCs. (C) Frequencies of infected Lgr5⁺ ISCs were analysed by FACS and viability was determined 1 dpi. Data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney test. *P < 0.05.

3.11. MCMV infection induces the upregulation of chemokine signalling in ISCs

A total of 28,378 differentially expressed genes (DEGs) were detected in comparison of untreated and MCMV-infected ISCs. In consideration of the substantial number of DEGs, the present study concentrates on the most significantly DEGs induced by MCMV infection. Intriguingly, among the top 15 genes that have been shown to be upregulated in the ISCs upon infection, the four chemokine-encoding genes *Ccl5, Ccl20*, *Cxcl10* and *Ccl2* are present (Fig. 25 A). These genes indicate a strong induction of chemotaxis of immune cells towards the infected ISCs.

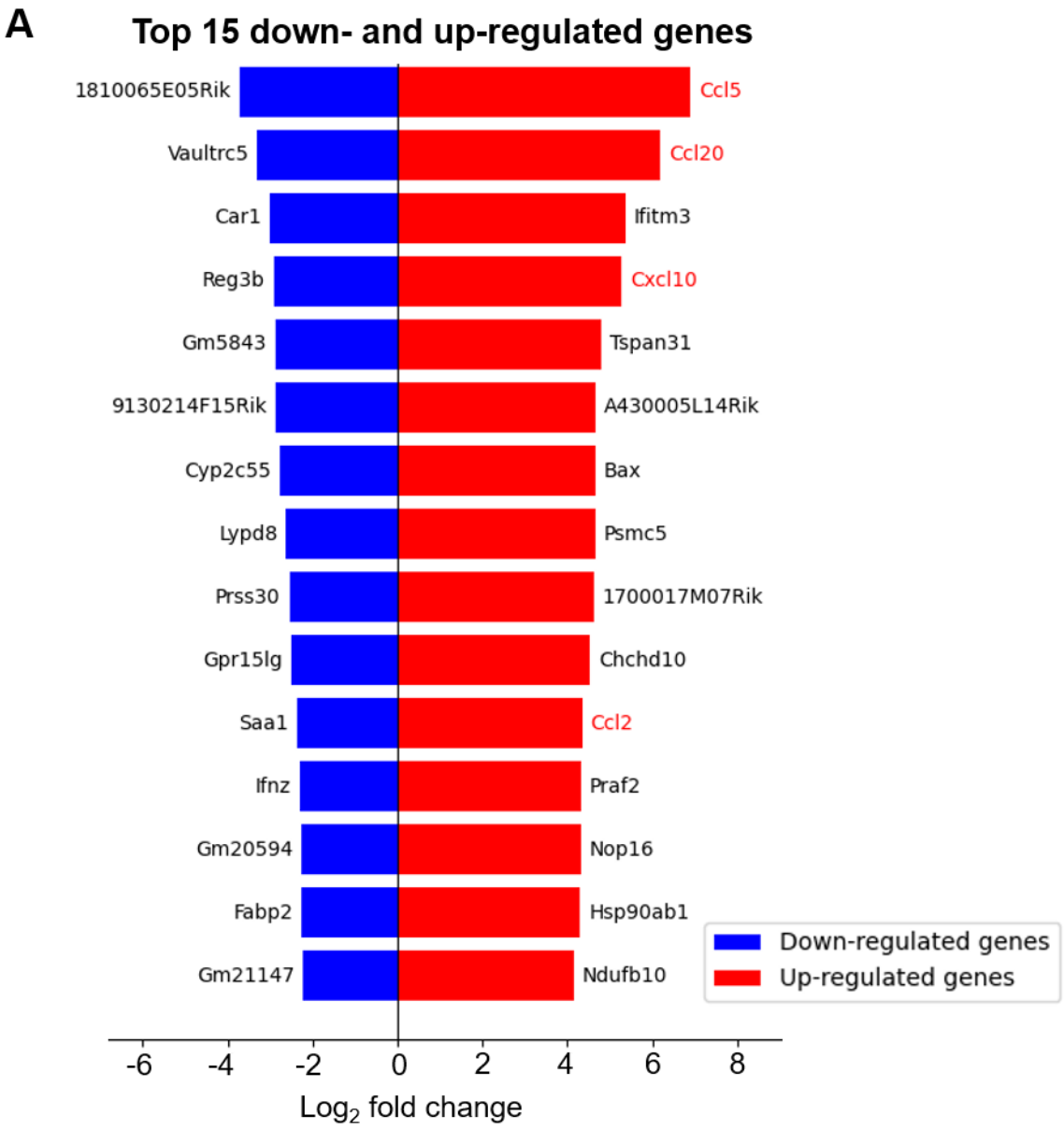

Fig. 25: RNA sequencing reveals altered gene expression in MCMV-infected ISCs. Colonic crypt-derived ISCs from *Lgr5-EGFP*-reporter mice were cultivated on matrigel and half were additionally infected with MCMV:*mCherry in vitro* for 24 h. Untreated and MCMV-infected ISCs were subjected to mRNA sequencing analysis. A total of 28378 differentially expressed genes (DEGs) were detected. (A) The top 15 down- and up-regulated genes of ISCs upon MCMV-infection are depicted, categorised from the log2 fold change. Genes encoding for chemokines are highlighted in red.

Further analysis of biological processes (BP) that were found to be upregulated by the MCMV infection serves to further corroborate this hypothesis. Gene ontology (GO) BP analysis of the top 100 upregulated genes indicated a strong induction of cytokine-mediated signalling pathways, cellular response to chemokines as well as the induction of chemotaxis of immune cells such as lymphocytes, monocytes and granulocytes or more specifically neutrophils and T cells in the ISCs mediated by the MCMV infection (Fig. 26 A). Interestingly, this goes well in

line with the accumulation of innate immune cells as well as the $CD8^+$ T cells detected in the MCMV-infected DSS colitis mice.

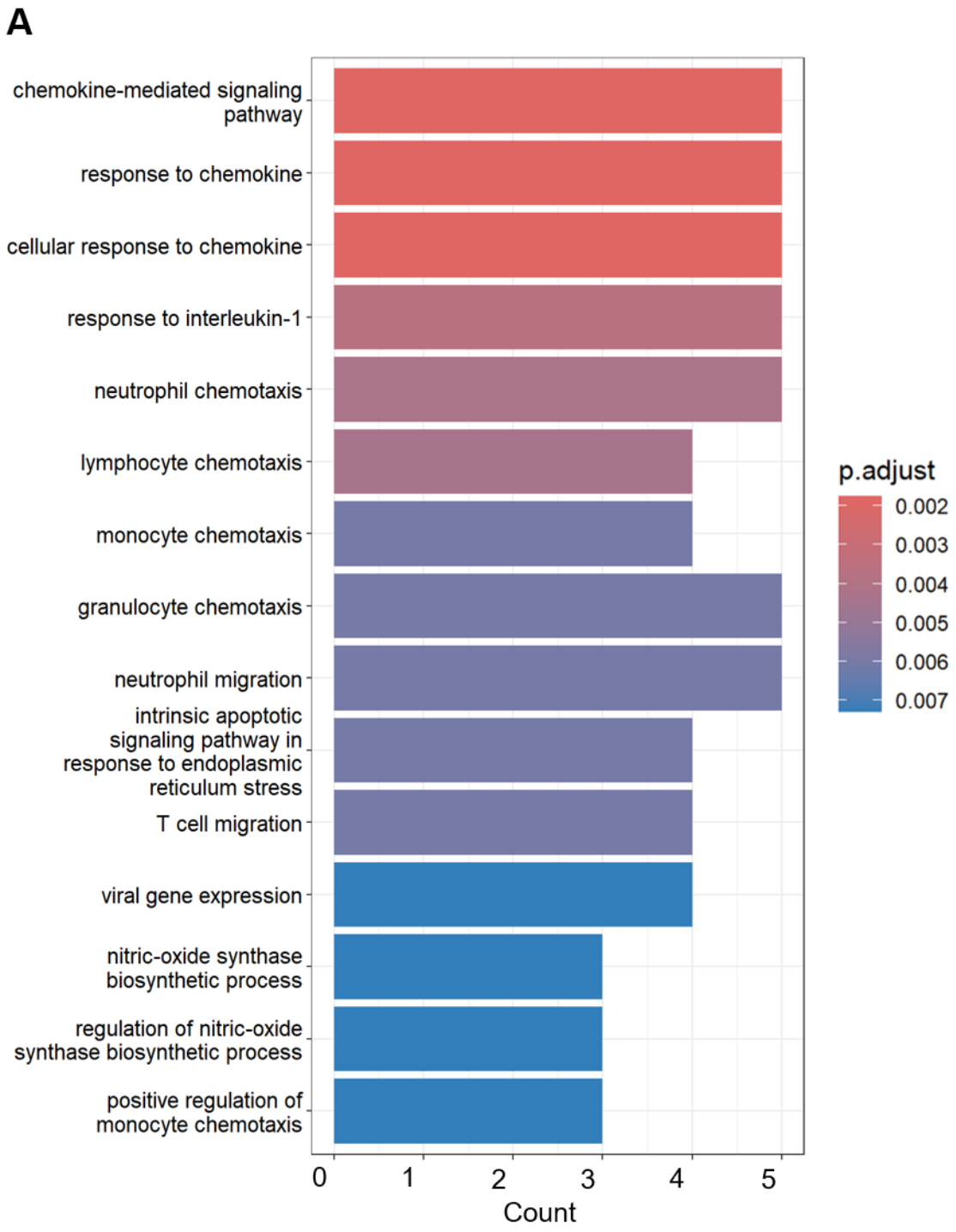

Fig. 26: Gene ontology analysis of biological processes induced by the MCMV infection of ISCs. Colonic crypt-derived ISCs from *Lgr5-EGFP*-reporter mice were cultivated on Matrigel and half were additionally infected with MCMV:*mCherry in vitro* for 24 h. Untreated and MCMV-infected ISCs were subjected to mRNA sequencing analysis. (A) Significantly altered biological processes (BP) from the Gene Ontology (GO) analysis performed on the top 100 upregulated genes found in the RNA-sequencing are presented. The y-axis illustrates the BP pathway terms and the x-axis denotes the

absolute count of genes of the top 100 allocated to the BP. The hue of the bars symbolizes the adjusted P values.

Given that the MCMV-induced severe pathology in the DSS colitis mice was mediated by a strong accumulation of cytotoxic CD8$^+$ T cells and that GO analyses predicted MCMV-induced chemotaxis of T cells towards infected ISCs, the DEGs were subsequently examined for chemokines, which are known to interfere with the migration of CD8$^+$ T cells. Accordingly, the expression of the CC chemokines CCL2, CCL5 and CCL20 as well as the expression of the CXC chemokines CXCL10 and CXCL16 were found to be elevated in infected ISCs (Fig. 27 A and B).

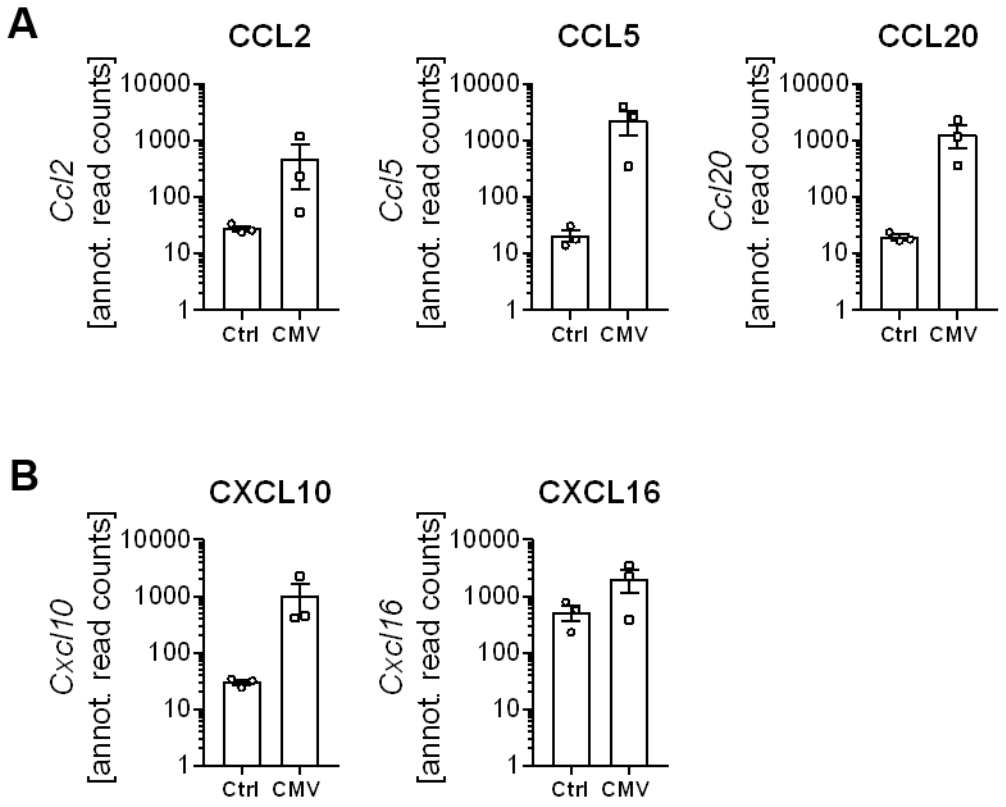

Fig. 27: MCMV-infected ISCs upregulate the expression of chemokines affecting CD8$^+$ T cell migration. Colonic crypt-derived ISCs from Lgr5-EGFP-reporter mice were cultivated on Matrigel and half were additionally infected with MCMV:*mCherry in vitro* for 24 h. Untreated and MCMV-infected ISCs were subjected to mRNA sequencing analysis. (A) CC chemokines *Ccl2, Ccl5* and *Ccl20* and (B) CXC chemokines *Cxcl10* and *Cxcl16*. Shown are the normalized read counts of the DEGs.

3.12. CXC chemokine receptors mediate accumulation of cytotoxic CD8$^+$ T cells in MCMV-infected DSS colitis mice

Chemokines play a crucial role in the migration of immune cells, such as CD8$^+$ T cells, towards sites of inflammation. To elucidate the chemokines and their corresponding receptors that might be responsible for the MCMV-mediated accumulation of CD8$^+$ T cells in the inflamed

colon during the recovery from DSS-induced colitis, $CD8^+$ T-cells were screened for the expression of various chemokine receptors.

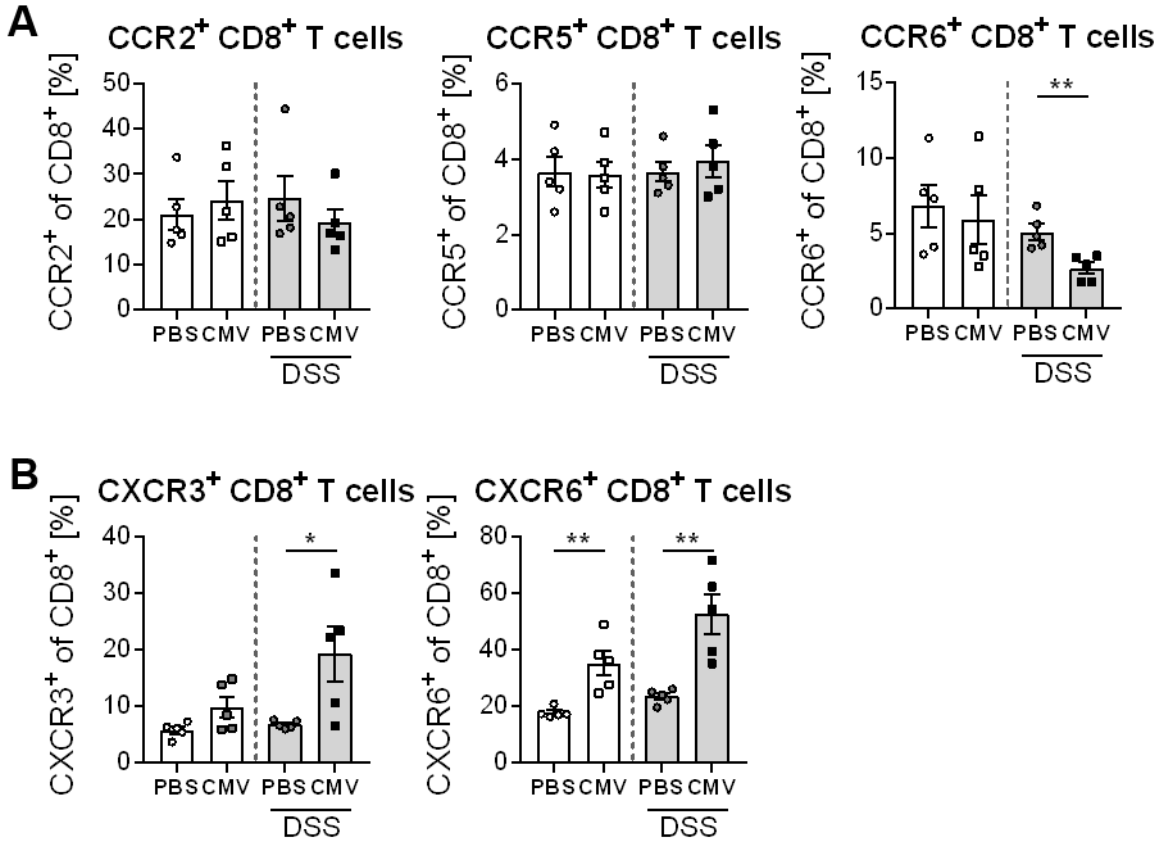

Fig. 28: CXCR3 and CXCR6 upregulation mediate the MCMV-induced accumulation of $CD8^+$ T cells in the colon during recovery from DSS-induced colitis. BALB/c mice were left untreated or treated with 3% DSS in the drinking water for 7 consecutive days to induce acute intestinal inflammation, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment. Colonic lamina propria lymphocytes were isolated at day 14 and frequencies were analysed by flow cytometry (n = 5 mice per group). (A) Expression of CC chemokine receptors CCR2, CCR5 and CCR6 and (B) expression of CXC chemokine receptors CXCR3 and CXCR6 were analyzed on viable $CD8^+$ cells. All data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney tests. *P < 0.05; **P < 0.01.

Interestingly, CC chemokine receptors and CXC chemokine receptors showed different expression profiles on accumulated cytotoxic $CD8^+$ T cells in the colon. The expression levels of CC chemokine receptors (CCR2 and CCR5) remained unaltered, while CCR6 expression was significantly reduced (Fig. 28 A). In contrast, the CXC chemokine receptors CXCR3 and CXCR6 exhibited significantly increased expression levels on $CD8^+$ T cells (Fig. 28 B).

In summary, MCMV is able to infect several cell types of the intestinal epithelium, including $Lgr5^+$ ISCs. Infected ISCs strongly upregulate the expression of genes encoding for chemokines such as CXCL10 and CXCL16. During the recovery from DSS-induced colitis, cytotoxic $CD8^+$ T cells accumulate in the colon following MCMV infection, inducing pathological

defects and impairing the intestinal regeneration. The cytotoxic $CD8^+$ T cells express the CXC chemokine receptors CXCR3 and CXCR6. The CXCL10 – CXCR3 and/or the CXCL16 – CXCR6 mediated chemotaxis may cause the accumulation of the $CD8^+$ T cells in the colon.

3.13. ΔM27-MCMV vaccination protects against the MCMV-exacerbated pathology during DSS colitis

In summary, the present study provides further evidence that an active CMV infection affects the inflammatory processes during acute colitis. The development of a HCMV vaccine is considered a preventative treatment option for HCMV-seronegative IBD patients. Notwithstanding the progress that have been made in the vaccine research, a vaccine is still not available. Live-attenuated CMVs are promising candidates in the vaccine research filed. Le-Trilling *et al.* have recently demonstrated, that the live-attenuated ΔM27 MCMV mutant, lacking the STAT2-antagonist, induces a potent immune protection against challenge infections by MCMV [189]. To determine, whether this vaccine can induce protection against the MCMV-mediated pathology in the DSS colitis model, BALB/c mice were vaccinated with ΔM27-MCMV 5 weeks prior initial DSS-treatment and 6 weeks prior to the challenge WT-MCMV infection (Fig. 29 A).

At first, the abundance of viral DNA was analysed in the colonic tissue 7 day post the WT-MCMV infection, to address whether the vaccination provided a protective immunity to WT-MCMV replication in the gut. Consistent, the ΔM27-MCMV-vaccination significantly reduced the viral load in the colon after the challenge infection (Fig. 29 B). To further determine the effects on DSS-colitis, common pathological markers were examined. Interestingly, vaccinated mice displayed significant less body weight loss during the course of DSS-induced colitis and a lower DAI during the peak of acute intestinal inflammation. In addition, a reduced DAI during the subsequent recovery phase compared to non-vaccinated mice after challenge infection was observed (Fig. 29 C and D). The improved intestinal regeneration and decreased viral load is also reflected in the reduced colon weight to length ratio of the vaccinated mice (Fig. 29 E).

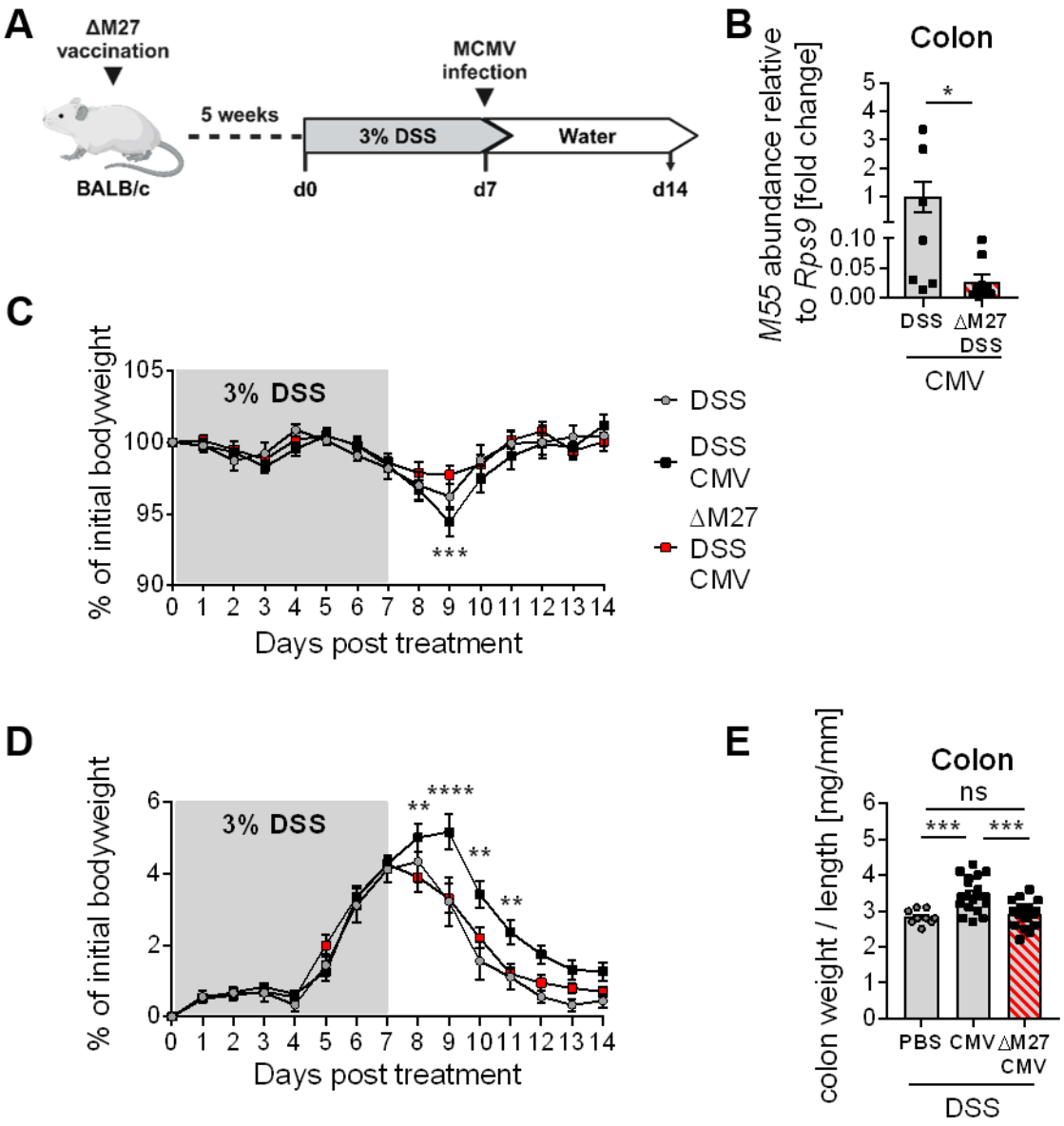

Fig. 29: ΔM27-MCMV vaccination protects against the exacerbating impact of the MCMV infection on the course of DSS-induced colitis. (A) BALB/c mice were vaccinated with ΔM27-MCMV 5 weeks prior to the treatment with 3% DSS in the drinking water for 7 consecutive days, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment and 6 weeks post vaccination (n = 20 mice per group). (B) Biopsy from colon was taken at 7 dpi. DNA was extracted and the abundance of the viral gene *M55* relative to *Rps9* was detected by qPCR. The fold change between unvaccinated and mice vaccinated with ΔM27-MCMV is shown. Daily monitoring of (C) proportional body weight changes to the initial weight and (D) disease activity index. (E) Colon weight to length ratio was examined at day 14 post DSS treatment. All data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney test (B), two-way ANOVA followed by Tukey's multiple comparison test (C and D) or One-way ANOVA followed by Tukey's multiple comparison test (E). Significances in (C) and (D) are shown between DSS + CMV- and ΔM27 + DSS + CMV-groups. ns = not significant, *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.

The beneficial effect of vaccination on intestinal regeneration was further supported by the results of the histological evaluation. In colon tissue sections from ΔM27-MCMV vaccinated

mice, significantly less crypt hyperplasia, goblet cell loss, epithelial effects and immune cell infiltration were observed compared to unvaccinated mice with or without challenge MCMV infection (Fig. 30 A and B).

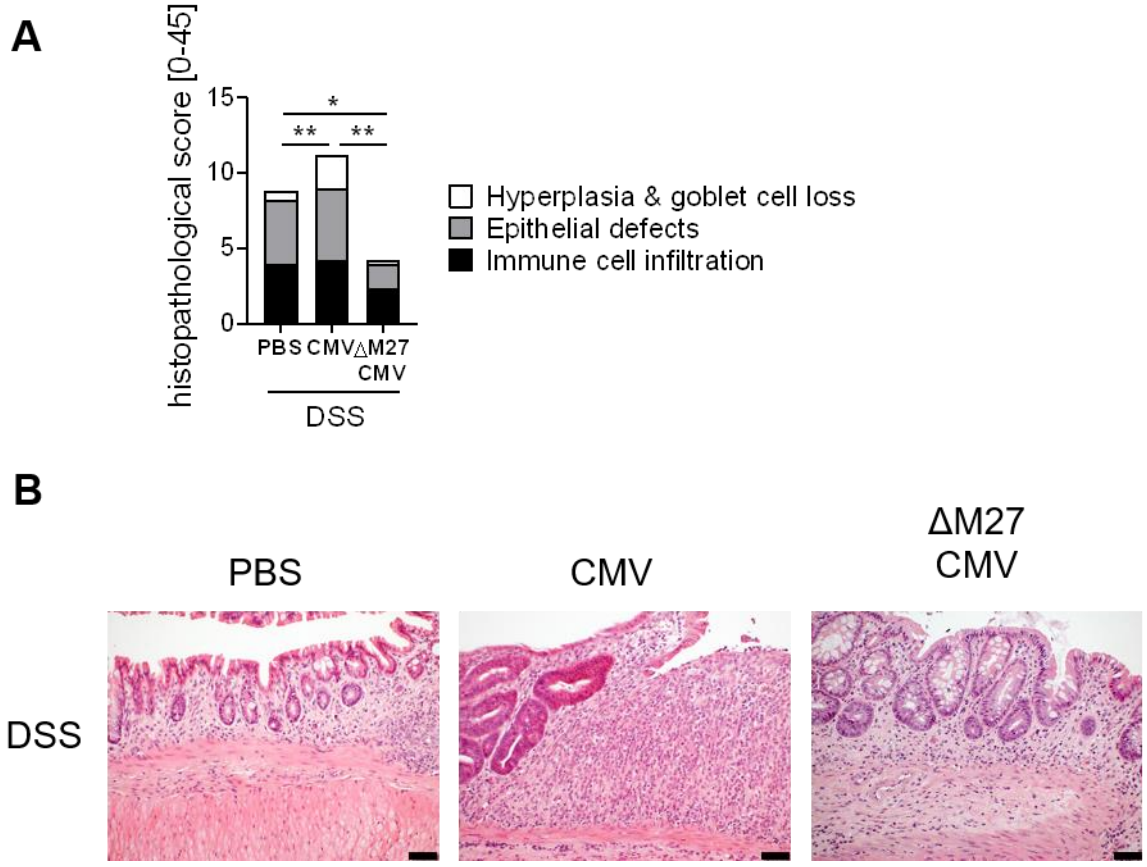

Fig. 30: ΔM27-MCMV vaccination protects against the CMV-induced impairment of intestinal regeneration post DSS-induced colitis. BALB/c mice were vaccinated with ΔM27-MCMV 5 weeks prior to the treatment with 3% DSS in the drinking water for 7 consecutive days, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment and 6 weeks post vaccination. (A) Histopathological score, including hyperplasia & goblet cell loss, epithelial effects and immune cell infiltration was determined at day 14 (n = 5 mice per group). (B) Representative images of H&E stained colon sections. Black scale bar = 50 µm. Data are presented as mean ± SEM. Statistical analysis were performed using unpaired t tests (A). *P < 0.05; **P < 0.01.

Consistent with these results, the MCMV-mediated colonic accumulation of cytotoxic $CD8^+$ T cells was significantly reduced in the vaccinated mice (Fig. 31 A). In contrast, the proportion of MCMV-specific $CD8^+$ T cells was significantly increased following ΔM27-MCMV vaccination, which could account for the reduced viral load during challenge (Fig. 31 B). Furthermore, levels of the chemoattractant CXCL10 were significantly reduced and the secretion of the pro-inflammatory cytokine TNFα tended to decrease in the colon (Fig. 31 C).

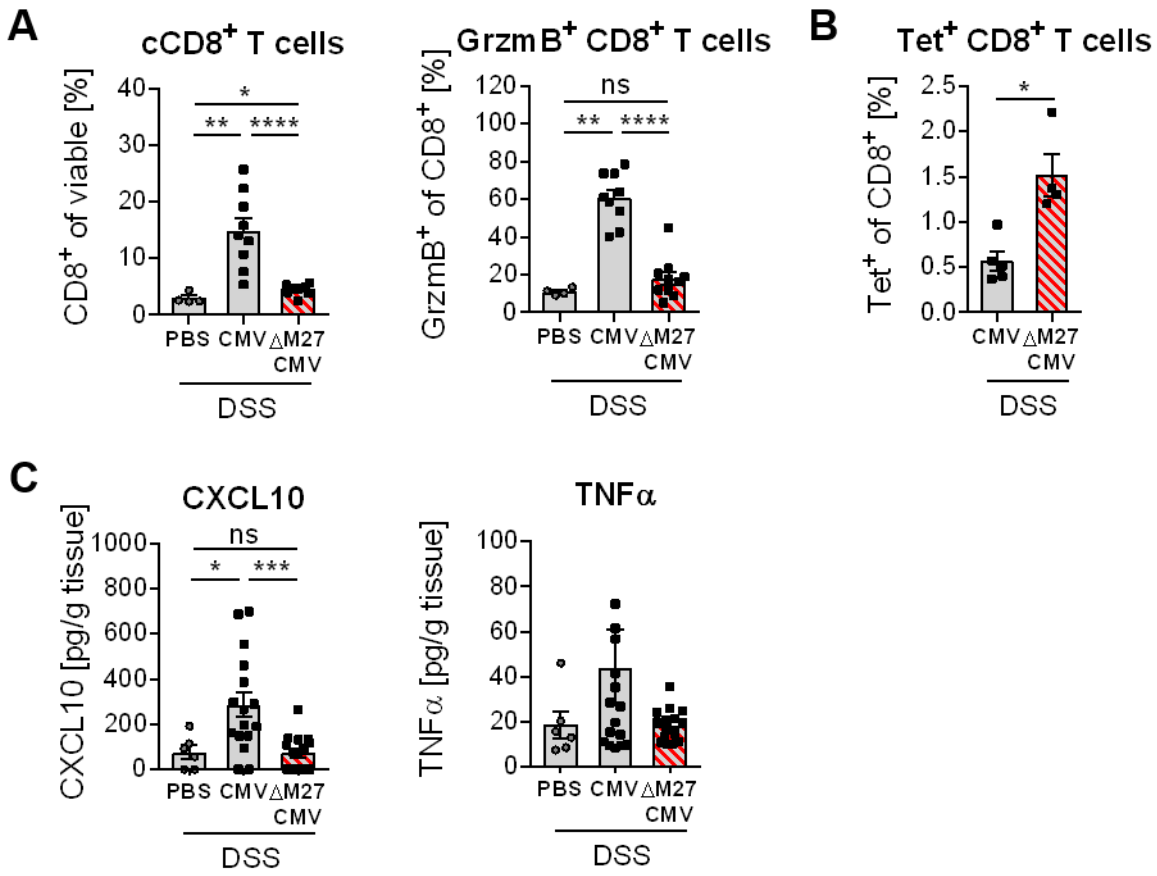

Fig. 31: ΔM27-MCMV vaccination diminish the CMV-induced accumulation of cytotoxic CD8⁺ T cells in the colon during recovery from DSS-induced colitis. BALB/c mice were vaccinated with ΔM27-MCMV 5 weeks prior to the treatment with 3% DSS in the drinking water for 7 consecutive days, followed by 7 days of normal drinking water. Mice were either i.p. infected with MCMV ($2x10^5$ PFU) or PBS injected at day 7 post DSS treatment and 6 weeks post vaccination. Colonic lamina propria lymphocytes were isolated at day 14 post DSS-treatment and frequencies were analysed by flow cytometry (n = 4-10 mice per group). (A) cCD8+ T cells and the expression of GrzmB on was analyzed on viable CD8⁺ cells. (B) MCMV tetramer specific CD8⁺ T cells. (C) Supernatants of *in vitro* cultured colonic explants were analysed for the secretion of the pro-inflammatory cytokine TNFα and the chemokine CXCL10. All data are presented as mean ± SEM. Statistical analysis were performed using Mann-Whitney tests. ns = not significant, *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.

In summary, the live attenuated ΔM27-MCMV mutant vaccine provided strong protection against WT MCMV infection in DSS colitis mice. It completely prevented the pathology-enhancing effects of colonic MCMV replication, improving both colitis progression and subsequent intestinal regeneration. The vaccine also reduced the accumulation of cytotoxic CD8⁺ T cells and viral replication in the colon, significantly improving outcomes.

3.14. Antiviral treatment improves the outcome of an IBD patient with HCMV colitis

So far, no adequate HCMV vaccine is available on the market as a preventive treatment option for IBD patients. In addition, inconsistent diagnosis of primary infections or HCMV reactivation

events during inflammatory flare-ups of IBD are considered to have serious long-term consequences for the patients [198]. Therefore, early diagnosis is crucial and offers the opportunity of HCMV antiviral treatment.

In the present study, the efficacy of antivirals were demonstrated in an ulcerative colitis patient diagnosed with HCMV colitis. Pathological features of HCMV colitis in ulcerative colitis are indicated in the endoscopic images of Fig. 32 A. Strikingly, antiviral treatment significantly improved the outcome of the ulcerative colitis patient (Fig. 32 B).

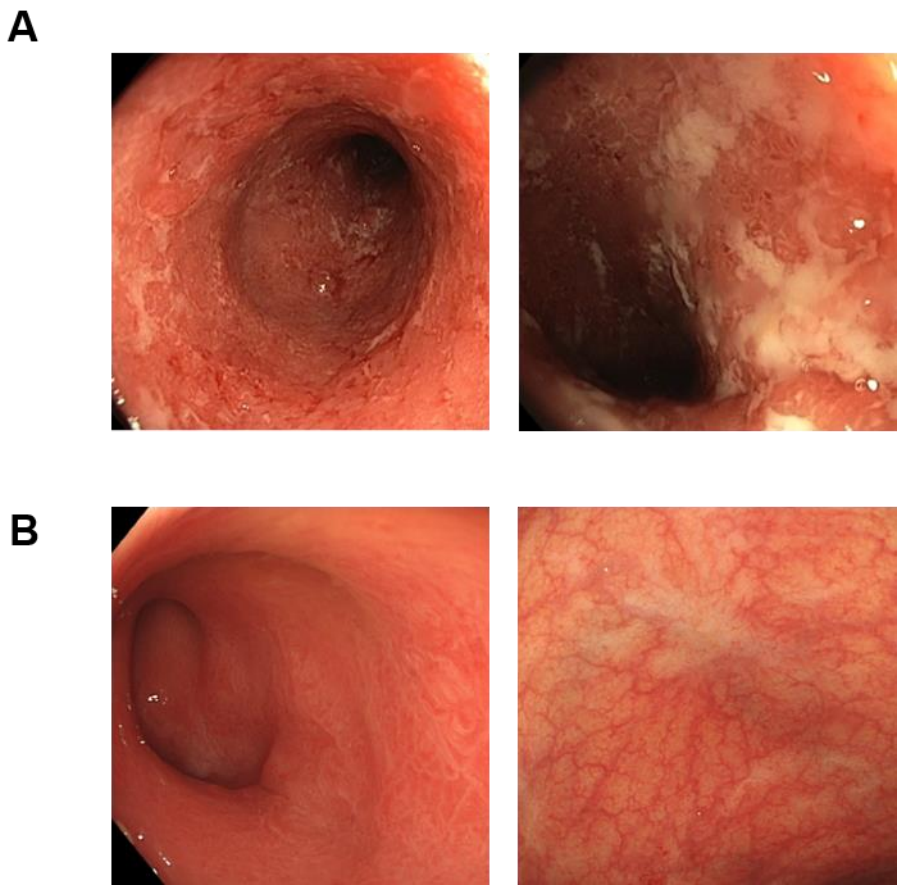

Fig. 32: HCMV colitis induced pathology of an IBD patient improved by antiviral treatment. Endoscopic features of HCMV colitis in a UC patient (A) before and (B) after the treatment with antivirals are reflected in representative endoscopic images.

Overall, this study offers new insights into how cytomegalovirus infection impacts acute intestinal inflammation. A strong $CD8^+$ T cell response in the colon and the susceptibility of intestinal stem cells to CMV infection hinder inflammation resolution and regeneration. The findings from CMV vaccination in mice suggest promising preventive strategies for HCMV-related pathology in IBD patients and highlight the importance of developing a human CMV vaccine, particularly for HCMV-seronegative IBD patients.

4. Discussion

The impact of cytomegalovirus on the intestinal tract has received considerable attention in CMV research over the past decades. The role of the opportunistic pathogen in chronic intestinal inflammation, which is of particular relevance in IBD, has been subject of debate. While intestinal HCMV replication is well documented [133, 199], the consequences for the intestine both under homeostatic conditions and during acute and chronic inflammatory processes remain to be elucidated. HCMV is considered as an exacerbating factor for the severity of IBD, as the prevalence of active HCMV infection in the colon is significantly increased in IBD patients [193] and viral infections are associated with more severe disease manifestations [200]. A comprehensive understanding of the mutual influence of both diseases could potentially lead to novel therapeutic approaches, especially for HCMV-seropositive IBD patients who do not respond to conventional treatments. The present study investigated the pathological effects of MCMV infection on intestinal homeostasis and acute inflammation in a DSS-induced colitis mouse model. Particular interest was the modulation of the immune response and the direct target cells of MCMV replication in the intestinal tract.

In the present study, the consequences of MCMV infection on gut homeostasis were first assessed. A robust MCMV replication was detected in the colonic tissue of immunocompetent BALB/c mice, accompanied by the induction of a mild inflammatory cytokine milieu and an increase of apoptotic epithelial cells within the intestinal barrier (Fig. 8 – 10). These results alongside supplementary findings are included in the study from Le-Trilling and Ebel *et al.*. Particularly, detrimental effects on the homeostatic conditions of the gut were demonstrated, as the viral replication affected further the microbial composition and disrupted the intestinal epithelial barrier integrity of the BALB/c mice [169]. Intestinal homeostasis relies on a complex interaction between the luminal microbiota, the intestinal epithelium and the underlying host immune system. The integrity of the epithelial barrier offers a physical barrier and protects against invading pathogens and harmful environmental compounds [201]. Disruption of intestinal homeostasis, as induced by the MCMV infection, can lead to extensive inflammation and the development of diseases.

In humans, the clinical manifestations of HCMV in the intestinal tract can progress to the end-organ disease HCMV colitis, which is common in immunocompromised individuals such as organ transplant recipients, HIV-infected individuals, and IBD patients. Clinical manifestations include gastrointestinal bleeding, abdominal pain and fever. In immunocompetent hosts, HCMV colitis is less frequent, but several descriptive case series reported end-organ damage associated with primary HCMV infection among immunocompetent individuals [202-204]. For example, the meta-analysis from Galiatsatos *et al.* identified 44 HCMV colitis patients who

were neither immune deficient nor taking immunosuppressive medications. Especially in elderly male patients, HCMV disease was accompanied with an increase of surgical interventions and worsened survival [205]. Because of the high incidence in the elderly and the more severe consequences in this population, it is critical to determine the extent to which these patients should be considered immunocompetent [206]. Additionally, comorbidities emerging in later life in combination with the intake of medications may obscure the clinical picture [205, 207].

Nevertheless, HCMV colitis is also commonly detected in younger patients inducing generally mild disease progression [205], consistent with the mild intestinal inflammation observed in MCMV-infected immunocompetent mice in the present study and the study of Le-Trilling and Ebel *et al*.. An inflammatory milieu was detected during the peak of MCMV infection in the colonic tissue, characterised by an increased secretion of the pro-inflammatory cytokines IFNγ, TNFα, granzyme B (Fig. 10) and IL-6 [169]. Each cytokine has its own potential to affect the intestinal barrier and increase the intestinal permeability, but especially the interplay of cytokines can promote intestinal barrier permeability. For IFNγ and TNFα it was shown, that high levels of IFNγ induce the upregulation of the TNF receptors 1 and 2 (TNFR1/2) on the basolateral surface of epithelial cells, while the binding of TNFα to TNFRs mediate the disruption of the barrier via dysregulation of the tight junction structure [208, 209]. Furthermore, IL-6 has a negative impact on the integrity of the intestinal barrier [210], while increased granzyme levels were demonstrated to disrupt the digestive epithelial function in the context of IBD [211]. Additionally, mild crypt necrosis [169] and the induction of apoptosis (Fig. 10) were demonstrated during the peak of infection, which further affect the intestinal barrier integrity and functionality. CMVs have characteristically long replication periods and therefore encounter a large array of innate immune responses such as the induction of apoptosis of infected cells to stop viral spread. In addition, CMVs are able to modulate cellular pathways to block apoptosis [212]. These modulatory functions are in contrast with the increase of apoptotic and necrotic cells observed in the intestine in MCMV-infected mice. Thus, the extensive apoptosis of the epithelial cells may not be induced directly by the virus itself, but could be mediated, e.g., by the TNF-induced apoptosis [213] or by cytotoxic immune cells through the release of cytotoxic molecules such as granzyme B [214]. Both molecules were as aforementioned enhanced in the colonic tissue upon infection.

Interestingly, the breakdown of the epithelial barrier resulting in increased intestinal permeability is also associated with IBD [215]. The CMV-induced pathology observed in this study may have the potential to contribute to and exacerbate intestinal inflammation found in IBD patients. To investigate this effect, MCMV infection was applied in the well-established

DSS-induced colitis mouse model in the present study. Interestingly, infected mice exhibited aggravated and prolonged colitis symptoms compared to uninfected colitis mice (Fig. 11).

The impact of the human CMV on inflammatory processes observed in IBD patients has been widely discussed in the past decades. The first documentation of the association between HCMV and IBD was reported in a case study by Powell *et al*. in 1961 [173] and since then, several human studies were conducted to this topic without achieving a uniform clinical picture. One reason for the controversial results have been the study sizes accompanied by discrepancies in the diagnostic methods used for HCMV detection as well as for the classification of disease severity of IBD [216]. Thus, three different associations between the two diseases arose in the literature. An early HCMV infection was thought to play an important role during the onset of IBD and was discussed as possible cause of the chronic disease [217], but CMV colitis was lately detected most often in pre-existing-IBD patients. Furthermore, HCMV reactivation might occur as consequence of the treatment of IBD patients, who receive immunosuppressive drugs favouring the viral infection. In this context, it is discussed that HCMV occurrence in the intestinal tract might only result as an innocent bystander, in which CMV plays no role in the disease progression. On the other hand the most accepted theory nowadays favours an exacerbating role of the disease progression of IBD patients by promoting intestinal inflammation mediated by HCMV reactivation [133, 177]. The active HCMV replication is thought to enhance the tissue damage during the inflammatory flare-ups and thus worsening the outcome of the IBD patients [218]. The findings of the present study confirm an exacerbating aspect of the CMV infection on colitis associated intestinal inflammation.

One of the key findings of this study was the detrimental effect of the MCMV infection on the intestinal recovery phase following the acute colitis. In addition to the prolonged manifestation of colitis symptoms, infected mice exhibited further evidences for an impaired resolution of the inflammatory processes in the gut. These included an increased colon weight to length ratio (Fig. 11), indicating persistent intestinal inflammation, and histological evidence of persistent severe tissue damage (Fig. 12). To the best of current knowledge, this direct association between HCMV and resolution of inflammation in IBD patients has not yet been studied. However, supporting studies by Zhang *et al*. and Thavamani *et al*. focused on the clinical data on HCMV-related hospitalisations of IBD patients. The in-hospital mortality for IBD patients with HCMV disease was up to 7 times higher, the length of hospital stay 8 to 9 days longer and the hospital care costs significantly increased in comparison to HCMV seronegative IBD patients [219, 220]. The underlying pathophysiological mechanisms, however, were not taken into account in these studies.

In the present work, some elevated inflammatory markers were found in this context. In MCMV-infected mice, enhanced levels of the cytokine TNFα were detected in the colonic tissue during the recovery of DSS-induced colitis (Fig. 14). Consistent with the MCMV-mediated upregulation of TNFα secretion in naive BALB/c mice described above, the pro-inflammatory cytokines may play an important role in the pathological effects in the intestine. TNFα is a key cytokine in the pathogenesis of IBD [221]. Intestinal macrophages, adipocytes, fibroblasts and T cells are known to massively enhance the production of TNFα in IBD, which exerts several pro-inflammatory functions. In addition to the described induction of apoptosis in the target cell by binding to the TNFRs, TNF-signalling further enhances angiogenesis and the damage of IECs, induces the death of Paneth cells via necroptosis and mediates the activation of macrophages and effector T cells [220]. Nowadays, anti-TNFα agents such as infliximab, adalimumab and certolizumab are efficacious treatment options for IBD patients [222]. In the context of HCMV infections, TNFα exhibits a dual role. On one side, HCMV is able to inhibit TNF-signalling by targeting TNFR1 [223] and suppressing the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [224] to diminish antiviral responses. On the other side, it was proven that HCMV induces the production of TNFα by mucosal monocytes and macrophages particularly in AIDS patients with HCMV colitis that leads to the induction of extensive intestinal inflammation [225]. In conclusion, intestinal CMV replication induces enhanced levels of TNFα, which seems to play an important role especially in the pathogenesis of CMV colitis.

Additionally, the MCMV-mediated impairment in the intestinal recovery post colitis was further characterised by severe alterations of the immune cell composition in the lamina propria towards a pro-inflammatory milieu. By analysing the innate and adaptive immune cells, a significant accumulation of macrophages, NK cells, neutrophils, eosinophils, Tregs and cytotoxic $CD8^+$ T cells was detected in the present study in infected mice during the recovery from acute colitis (Fig. 15 and 16). The pathogenesis of IBD is mediated by a complex interplay between genetic, environmental and immunological factors, including the uncontrolled and extensive activation and accumulation of intestinal immune cells. Severe dysregulation of the intestinal immune system is an important driver of mucosal inflammation and can potentiate the tissue destruction in IBD [226].

Macrophages are highly abundant in the gastrointestinal mucosa and can be characterised by a pro-inflammatory or immunomodulatory phenotype [41]. Pro-inflammatory macrophages play an important role in the initial immune response against invading pathogens and are associated with the promotion of intestinal inflammation, which can further stimulate tissue damage, while macrophages with an immunomodulatory phenotype have wound-healing properties and counteract inflammation [227]. The comparison of intestinal macrophages from

IBD patients and healthy individuals indicated extensive changes in the gene expression profiles of the macrophages towards the inflammatory phenotype. Furthermore, also the immune function and metabolic processes were dysregulated resulting in implications for macrophage function and immunometabolism. Alterations of the macrophage phenotype and functions are thought to crucially alter the course of disease progression in IBD patients [228]. Particularly, ae decrease of macrophages with an immunomodulatory phenotype in the intestinal mucosa of IBD patients is detrimental, as the antagonising properties of inflammation and the promotion of wound healing also facilitates the resolution of inflammation [227]. In the present study, further characterisation of the phenotype of the increased macrophage population in the colonic lamina propria was not included. However, the impaired resolution of intestinal inflammation found in the MCMV-infected mice indicates a switch in the phenotype of macrophages towards pro-inflammatory properties, and further characterisation should be included in future studies.

Intestinal NK cells can be found in the lamina propria or attached to the epithelium and play a crucial role in the immunity and the maintenance of intestinal homeostasis. Together with other immune cells such as ILCs and T cells, NK cells respond to inflammation and infections within the intestinal environment [229]. In the context of IBD, accumulation of specific subpopulations of NK cells within the mucosal tissue are reported, contributing to inflammatory processes through the production of pro-inflammatory cytokines such as IFNγ in CD [230] and IL-13 in UC [231]. In line with this, the increased accumulation of NK cells found in the present study of MCMV-infected mice might have contributed to the severe pathology during DSS colitis.

Granulocytes play a crucial role in the immune response associated with IBD. Uncontrolled infiltration of activated granulocytes into the intestinal mucosa was shown to highly contribute to inflammation and tissue damage [232]. Neutrophils are rapidly recruited to the site of inflammation and are considered to be important for the control of invasive microorganisms in the mucosa. Under physiological conditions, neutrophils need to be precisely regulated and properly eliminated after resolution of inflammation [233]. In IBD it is considered that neutrophils contribute to the inflammatory processes by the persistent release of pro-inflammatory cytokines, ROS and NETs, which further induces extensive tissue damage [234, 235]. The accumulation of activated neutrophils is a hallmark of UC and their activity is often used as a marker of disease severity. For instance, the neutrophil to lymphocyte ratio is commonly examined in the peripheral blood of IBD patients [236, 237]. Eosinophils have the capacity to contribute to inflammatory processes by the release of pro-inflammatory cytokines, toxic proteins from the granules and ROS production [238]. In UC, eosinophil numbers are highly increased, but their role in the disease progression is not completely understood [239]. While some studies attribute an important role to eosinophils during wound healing in phases

of remission of UC [238], other studies show an amplification of inflammatory processes in the gut through the complex interaction between eosinophils, neutrophils and mast cells [240]. Nevertheless, the results of the present study are in line with the findings in IBD patients. An accumulation of both neutrophils and eosinophils was observed in DSS-treated mice, which is consistent with the IBD phenotype. Interestingly, the additional MCMV infection further enhanced the accumulation of granulocytes in the colonic tissue, suggesting an amplification of the pathological effects of the immune cells. Together with the increased accumulation of macrophages and NK cells, it was demonstrated that active MCMV replication in the gut during acute colitis leads to an even stronger imbalance of the innate immune response in the mucosa (Fig. 15). This may be a contributing factor to the exacerbated pathology of IBD patients with HCMV association.

In addition to the observed alterations of the innate immune cell composition in the colonic lamina propria, a significant increase in the frequencies of both conventional $CD8^+$ T cells and Tregs was observed in MCMV-infected mice during the recovery phase of acute colitis. The changes in $CD8^+$ T cell frequencies were the most pronounced effect on the intestinal immune cell composition. While MCMV infection alone already induced a strong accumulation in the colonic tissue, this effect became even more prominent during the recovery phase of acute colitis (Fig. 16). Intestinal $CD8^+$ T cells are located in the lamina propria or are attached to the intestinal epithelium as IELs, playing an important role in the adaptive immunity. The majority of these $CD8^+$ T cells exhibit even under homeostatic conditions the phenotype and functional properties of activated cells [241, 242]. Upon cognate antigen encounter, $CD8^+$ T cells proliferate and differentiate into effector T cells and fulfil a crucial role in the immune surveillance and the defence against invading pathogens. Cytotoxic $CD8^+$ T cells, capable of secreting high amounts of effector cytokines such as TNFs, IFNs and cytotoxic granules, are predominant effector T cells in the intestine mediating target cell killing [243]. Interestingly, CMV infections are known to induce a strong $CD8^+$ T cell response. These cells play a critical role in the viral control during the phase of lytic CMV infection, as well as in the prevention and control of viral reactivation [244]. While immune responses in certain organs, such as the spleen [245] or the salivary gland [246, 247], have been the subject of detailed CMV research in the past decades, there is currently limited knowledge regarding the CMV immune response in the gut. However, $CD8^+$ T cells are known to dominate the immune response to a primary CMV infection in most organs where broad T cell specificities against several viral epitopes are induced. Especially in periods of high viral load, the accumulation of CMV-specific $CD8^+$ T cells is required to prevent both the spread within the organ and systemic distribution by their direct cytotoxic activity against infected cells [248]. This is well in line with the findings in the present study. High $CD8^+$ T cell frequencies (Fig. 16) were detected 7 days post MCMV infection, where high viral loads are present within the colonic tissue (Fig. 8). Furthermore, the

occurrence of MCMV-specific $CD8^+$ T cells was analysed using a labelled MHC-tetramer, which directly bound to the respective TCR against the MCMV antigen M45. Despite the strong $CD8^+$ T cell accumulation in the intestine, only a very small proportion of these cells showed a specific response against the M45 epitope (Fig. 17). The broad amount of potential CMV epitopes suggests a $CD8^+$ T cell specific response against a considerable number of other CMV-specific epitopes in the colonic lamina propria. However, no other epitopes could be analysed within this study. In conclusion, the strong accumulation of $CD8^+$ T cells might indicate a crucial role of these cells in the immune response to intestinal CMV replication.

Due to the fact, that the highest frequencies of $CD8^+$ T cells were detected in MCMV-infected mice recovering from the acute colitis, it was hypothesised that the intestinal inflammatory processes during colitis might be favourable for the MCMV replication in the intestinal tissue resulting in enhanced viral loads. Surprisingly, the viral load within the colonic tissue was not altered in DSS-treated mice during the peak of intestinal replication. However, the onset of CMV replication in the colon was accelerated in the inflamed tissue of colitis mice, indicated by the significantly enhanced prevalence of viral DNA within the colonic tissue 3 days post infection (Fig. 13). As before mentioned, CMVs are able to infect a broad variety of cells including myeloid cells. Especially monocytes and DCs are directly targeted by the virus [249]. In murine models it was shown, that infected DCs and monocytes migrated from sites of primary infection such as the lung into the blood stream and enabled MCMV dissemination to secondary organs [250, 251]. Despite the fact that the spread of the infection in the intestinal tract was not investigated in these studies, it can be hypothesised that MCMV reaches the colon through the migration of infected myeloid cells into the mucosal tissue. The increased infiltration of immune cells such as myeloid cells into the colon upon DSS-induced intestinal inflammation [252] could have promoted the viral spread at early time points post infection in this study. However, since the viral load in the colon did not increase further during the course of the infection (Fig. 13) and the frequency of M45-specific $CD8^+$ T cells did not increase in DSS colitis mice (Fig. 17), it can be assumed, that the MCMV infection results in a general accumulation of cytotoxic $CD8^+$ T cells in the colon. This, in turn, might further increase the pro-inflammatory milieu in the intestine during colitis.

To re-establish homeostasis after pathogen clearance, the majority of pathogen-specific cytotoxic $CD8^+$ T cell clones undergo apoptosis and only a small proportion differentiates into memory $CD8^+$ T cells, to provide protection against re-infections [253]. In IBD patients, this sensitive balance of $CD8^+$ T cells is thought to be disrupted, resulting in an ongoing inflammatory state mediated by an accumulation of cytotoxic T cells. While $CD4^+$ T cells have dominated IBD research for decades, there has been a recent shift towards investigating the role of $CD8^+$ T cells in this context. Several studies reported an increased accumulation of

activated cytotoxic $CD8^+$ T cell in the peripheral blood as well as in the inflamed tissue of IBD patients in comparison with healthy controls [254-256]. Furthermore, Lee *et al*. identified elevated levels of a $CD8^+$ T cell subset in severe cases of UC and CD, which indicates an early role of these cells in the development of the disease by promoting the disruption of the intestinal epithelial barrier [257]. Studies with animal models further support the role of cytotoxic $CD8^+$ T cells in the early development of colitis. Westendorf and colleagues demonstrated, that in an adaptive $CD8^+$ T cell transfer model the epithelial-specific antigen expression was sufficient to induce severe $CD8^+$ T cell-mediated intestinal inflammation [258]. Thus, accumulation of cytotoxic $CD8^+$ T cells seems to be of high relevance in the pathogenesis of IBD and have the potential to induce extensive mucosal damage during colitis. This is in accordance with the findings of the present study. The accumulated $CD8^+$ T cells in the MCMV-infected mice recovering from colitis exhibited a highly activated and cytotoxic phenotype, demonstrated by the expression of the early activation marker CD69 and the cytotoxic granule molecule granzyme B (Fig. 17). In conclusion, $CD8^+$ T cells are already part of the dysregulated intestinal immune response of IBD patients inducing detrimental tissue damage in the intestinal mucosa. If HCMV infections in the human intestinal tract exert a similar effect on the immune response to that observed in the mouse model, the accumulation of cytotoxic $CD8^+$ T cells will be dramatically enhanced resulting in further exacerbation of the disease pathology.

In order to provide further evidence that the accumulation of the cytotoxic $CD8^+$ T cells was also responsible for the impaired regenerative phenotype of the DSS-treated mice, experiments with an anti-$CD8^+$ depleting antibody were conducted in the established model. Interestingly, the depletion induced an alleviating phenotype in the mice, as both the exacerbation of colitis symptoms and the impaired resolution of intestinal inflammation were not pronounced in the absence of $CD8^+$ T cells in the MCMV-infected (Fig. 18 and 19). The observed decrease in the severity of colitis is well in line with previous findings in the hapten 2,4 dinitrobenzene sulfonate (DNBS)-induced colitis model from Nancey and colleagues. DNBS sensitised mice developed cytotoxic $CD8^+$ T cell-mediated intestinal inflammation exhibiting pronounced tissue damage after DNBS challenge. $CD8^+$ T cell-depleted mice did not show severe signs of intestinal inflammation [259]. The MCMV-mediated impaired resolution of intestinal inflammation that has been observed in the present study might also be mediated by an impaired regenerative capacity of the colonic tissue. The role of $CD8^+$ T cells in the context of wound healing and tissue repair has so far been inconsistent. In mouse models of skin wound healing and regeneration after myocardial infarction, the cytotoxic T cells were shown to suppress certain aspects of the healing processes. The $CD8^+$ T cell depletion in the skin model resulted in enhanced wound-breaking strength and increased collagen synthesis [260]. $CD8^+$ T cell deficient mice used in an myocardial infarction model exhibited

improved cardiac physiology post infarction [261]. On the other side, $CD8^+$ T cells appear to have a supportive role in the context of bone healing [262]. To the best of our current knowledge, the role of the $CD8^+$ T cells in context of intestinal regeneration remains to be elucidated. However, the phenotype of the $CD8^+$ T cells could play an important role in this context. The results of the present study support a clear detrimental role of the $CD8^+$ T cells especially with a cytotoxic phenotype on the process of intestinal wound healing.

Furthermore, the consequences of the $CD8^+$ T cell depletion for the MCMV load were evaluated. On one side it was expected, that the depletion leads to an uncontrolled viral dissemination in several organs, while on the other side the enhanced intestinal phenotype of the $CD8^+$ T cell depleted mice would rather assume the opposite effect, at least in the intestinal tract. It is interesting to note that a significant increase in the MCMV load was observed in the spleen and liver (Fig. 20). These results are in accordance with the findings of other publications confirming the crucial role of $CD8^+$ T cells for the viral control in these organs. In the liver it was shown that the recruitment of MCMV-specific $CD8^+$ T cells contribute to the protective antiviral response [263]. The study from Lathbury and colleagues further demonstrated that the antiviral $CD8^+$ T cell response to MCMV infection in the spleen and liver is highly dependent on the genetical background of the infected mice. In BALB/c mice, $CD8^+$ T cells were primarily responsible for the clearance of virus from these tissues, while C57BL/10 mice MCMV-control was mediated in by an appropriate NK cell response [264]. This is well in line with the findings of the present study, where the experiments were conducted in BALB/c mice. In contrast, the viral load in the colonic tissue was not altered by the $CD8^+$ T cell depletion (Fig. 20). Despite the strong accumulation of cytotoxic $CD8^+$ T cells in the colon upon colonic MCMV replication, these cells appear not to be crucial, or at least may not be the only cell type capable of mediating the antiviral immune response in the gut. Further research is required to ascertain the extent to which a compensatory effect has occurred in the depletion model or to determine which other immune cells are involved in the control of MCMV replication in the colon.

The last alteration of an intestinal immune cell composition observed in the present study was a significant upregulation of Tregs upon MCMV infection during the recovery phase of acute colitis (Fig. 16). Tregs are crucial mediators in the intestinal tract to maintain homeostasis by preventing excessive inflammation. By the secretion of the immunosuppressive mediators such as IL-10 and TGF-β, they can regulate both innate and adaptive immune responses [67, 79]. In IBD, the balance between anti-inflammatory Tregs and pro-inflammatory T cells is disrupted, with patients exhibiting a decrease in the total numbers of peripheral Tregs and a functional impairment of the remaining Tregs in the mucosal tissue [265, 266]. Despite a slight increase of tissue residual Tregs observed in IBD patients, these cells are insufficient to

counteract the pro-inflammatory milieu induced by the dysregulated innate and adaptive immune response [267]. This phenotype is in accordance with the results of the present study. Although the functionality of the Tregs was not assessed, a slight increase in Treg frequencies was observed. Similar to the findings in IBD patients, this could have occurred as a compensatory effect in order to balance the dominant pro-inflammatory environment. However, this compensatory effect appears to be inadequate in counteracting the substantial accumulation of innate and, particularly, cytotoxic $CD8^+$ T cells, thereby failing to prevent the induction of excessive mucosal tissue damage.

In addition to the MCMV–mediated immunological aspects on the intestinal tract, the consequences of the viral replication were investigated in this study, especially in the context of a potentially impaired regenerative capacity of the gut. The previous findings from Le-Trilling and Ebel *et al*. demonstrate that the murine and human intestinal epithelial cells represent direct targets of the CMV infection. Epithelial cell lines as well as organoids derived from primary intestinal crypt cells were susceptible to the viral infection and it was suggested, that MCMV directly affects the survival of the ISCs in the colonic crypts [169]. The impairment of functionality or the loss of ISCs could have caused the observed restriction of the intestinal regeneration post acute colitis. To further investigate this phenomenon, primary colonic crypts were isolated from MCMV-infected mice during the recovery phase of DSS colitis and the functionality of the ISCs was assessed by analysing the enteroid forming efficacy and the morphology of the generated organoids (Fig. 21). In accordance with the findings from Le-Trilling and Ebel *et al*., the absolute number of intact crypts and the enteroid forming efficacy was significantly reduced already by an MCMV infection alone. A comparable effect was also observed in the organoids of mice recovering from DSS colitis. In this regard, it was demonstrated that organoids generated from tissue biopsies from IBD patients showed morphological dysfunctions in comparison to non-IBD derived organoids, as the formation of crypt-like domains was impaired, the size was reduced and increased cell death was induced [268]. Concomitantly, the most pronounced effect on the enteroid forming efficacy as well as on the organoid morphology was observed with MCMV-infected mice recovering from acute colitis (Fig. 21 and 22), indicating a severe impairment of the functionality of the colonic ISCs. These results are in accordance to the findings of the MCMV-mediated impaired recovery in the *in vivo* experiments.

The impairment of the ISCs was hypothesised to be a consequence of MCMV targeting either the ISCs directly or the adjacent cells in the intestinal crypts, which influence the ISC fate, the stemness and survival by the secretion of Wnt, R-spondin and Notch ligands [18, 19]. To address this, Lgr5-reporter mice were infected with MCMV:*mCherry* and colonic crypts were examined by immunofluorescence microscopy (Fig. 23). Astonishingly, in addition to epithelial

cells, also Lgr5$^+$ ISCs were identified among the target cells of MCMV infection. Further *in vitro* infection experiments confirmed the susceptibility of the ISCs to the viral infection (Fig. 24). CMVs have a broad cell tropism, as a huge range of target cells in different hosts and organ systems have been identified over the past decades. Endothelial and epithelial cells, fibroblasts, smooth muscle cells and immune cells such as the aforementioned monocytes and DCs were found to be permissive to CMV [151]. The ability of the virus to bind to a plethora of target cell receptors such as heparan sulphate, integrins, EGF-R and neuropilin-2 [156-160] as well as its capacity to adapt to different organic niches, suggests that further target cells could be identified in future studies. To the best of current knowledge, the susceptibility of ISCs to viral infections and especially to CMV infections has not been investigated. Interestingly, mouse and human CMVs were shown to infect stem-like cell types in other organ systems. Mutnal and colleagues investigated the pathogenesis of congenital CMV brain infections and identified neural stem cells (NSCs) and neuronal precursor cells as principal target cells of the virus, leading to significant loss of NSCs and subsequent abnormal brain development [269]. Furthermore, the susceptibility of mesenchymal stem cells (MSCs) to CMV infection was examined in detail. MSCs are non-hematopoietic cells that were initially found in the bone marrow but are also located in multiple tissues such as the skin, placenta or in the peripheral blood [270, 271]. Due to their extensive proliferative capacity, the tendency to migrate into damaged tissues orchestrating regenerative processes and the ability of modulating immunologic responses, MSCs have been used in several therapeutic approaches [272]. Nevertheless, the studies from Sundin *et al*. and Avanzi *et al*. demonstrated, that MSCs derived from the bone marrow [273] or the placenta [272] were susceptible to common herpesviruses such as herpes simplex virus 1 (HSV-1) and HCMV. This supports the potential of CMV to directly infect other stem-like cells such as ISCs in accordance with the findings in the present study.

The immediate response of ISCs to MCMV infection was assessed via RNA sequencing. A total of 28,378 DEGs were detected in comparison to uninfected ISCs, indicating massive metabolic changes due to the viral infection, that have to be analysed in detail in future studies. Focusing on the top 15 up- and down-regulated genes one day post infection, the four chemokine-encoding genes *Ccl5, Ccl20, Cxcl10* and *Ccl2* were identified among the up-regulated genes (Fig. 25). These findings are in accordance to the study from Juranic Lisnic *et al*., where the host transcriptome of BALB/c-derived MEFs was analysed during the lytic MCMV infection phase. Remarkably, they also identified an enormous amount of DEGs with the inflammatory chemokine ligands *Ccl5* and *Cxcl10* among the top 5 upregulated genes in infection [274]. Furthermore, all chemokines have already been associated with CMV infections. In detail, CCL2, also known as the monocyte chemotactic protein-1 (MCP-1), is known to be upregulated during HCMV infection and is associated with the facilitation of virus

dissemination and pathogenesis [275]. CCL5 is secreted by various cell types and important for the modulation of the antiviral immune response by attracting immune cells towards the site of infection. For instance, mast cells were shown to recruit $CD8^+$ T cells to CMV-infected lungs to initiate control of viral replication [276]. In renal transplant patients, CMV infections are a significant concern for allograft dysfunction or failure. CMV-mediated secretion of the chemokine CCL20 plays a key role in this process by the recruitment of Th1/Th17 cells to the site of infection, which further enhance allograft damage by the production of TNFα and IFNγ [277]. The secretion of CXCL10 during CMV infection is regulated by the host and the virus. In the host, CXCL10 mediates the recruitment of activated immune cells, such as $CD8^+$ T cells, via the chemokine receptor CXCR3 towards the infected tissue to control viral spread [263]. CMV counteracts the CXCL10 secretion by the expression of a viral homologue to the host cytokine IL-10 (cmvIL-10), which inhibits CXCL10 production through blocking the IL-10 receptor [278]. All together, the chemokines upregulated during ISC infection are all associated with CMV infection and mediate the recruitment of immune cells to the site of infection via chemotaxis. This is also underlined by further analysis in the present study regarding the upregulated biological processes (BP) upon MCMV infection. Gene ontology BP analysis indicated a strong induction of cytokine-mediated signalling pathways as well as the induction of chemotaxis of immune cells such as lymphocytes, monocytes and granulocytes (Fig. 26). Interestingly, these results match with MCMV-mediated increase of innate immune cells including granulocytes and the strong accumulation of the cytotoxic $CD8^+$ T cells during the recovery phase of DSS colitis. Appropriately, $CD8^+$ T cells in the colonic lamina propria showed a significant upregulation of the CXCL10 receptor CXCR3 (Fig. 28), indicating that the accumulation of the cytotoxic T cells during the MCMV infection is CXCL10 – CXCR3 dependent.

In conclusion, the present study has demonstrated that colonic crypt cells including ISCs are susceptible to MCMV infection, resulting in severe pathological consequences for the intestinal tract of the host. Viral replication affects the functionality and transcriptome of ISCs, which may have serious implications for the regenerative capacity of the gut. In addition to CMV-induced accumulation of activated immune cells in the colonic lamina propria, this can result in the development of severe pathologies during colitis. Therefore, it is important to consider CMV infection in the context of chronic inflammatory diseases such as IBD.

A potential approach to protect vulnerable IBD patients, especially those undergoing immunosuppressive treatments, from primary CMV infection is the development of effective vaccines. The present study provides further evidence for the potential of live-attenuated CMVs to serve as vaccines in the context of IBD. CMVs developed a wide range of immune evasion techniques to evade the host's innate immunity [279]. The IFN-pathway is a crucial

component of the antiviral innate immune response. IFNs activate by the binding to their cognate receptors the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway inducing the expression of IFN-stimulated genes (ISGs) for the establishment of a potent antiviral state [189, 279]. However, human and mouse CMVs can induce the proteasomal degradation of the STAT2-protein by the expression of the analogous interferon antagonists pUL145 and pM27 and are therefore capable to evade the IFN-mediated immune response [280, 281]. Consequently, a ΔM27-MCMV mutant was shown to be highly IFN susceptible [281]. Le-Trilling *et al*. recently demonstrated that the live-attenuated mutant was able to replicate at low levels in vaccinated mice, induced a potent humoral immune response, which offered strong protection to challenge MCMV infections [189]. In the present study, vaccination with the live-attenuated ΔM27-MCMV mutant offered full protection against a challenge infection in the acute colitis model. Neither the MCMV-induced aggravated colitis symptoms nor the impaired intestinal recovery were pronounced in the vaccinated mice upon challenge infection (Fig. 29 and 30). Accordingly, the elevated colonic TNFα levels and the accumulation of cytotoxic $CD8^+$ T cells were completely abolished by the vaccination. The significant increase of MCMV-specific $CD8^+$ T cells (Fig. 31) might suggest the induction of a MCMV-specific immunoprotection by the vaccine. Future studies have to examine whether the enhancement of the MCMV-specific $CD8^+$ T cell response was induced by the vaccination, thus exhibiting a memory phenotype, or whether the cells were only enhanced in the colonic lamina propria by the challenge infection. Nevertheless, the live-attenuated ΔM27-MCMV mutant exhibited promising effects in the MCMV-exacerbated DSS colitis model.

The development of HCMV vaccines has been a major goal for many years in order to prevent virus-related complications such as congenital disease, renal allograft dysfunction or end organ disease in immunocompromised individuals such as IBD patients. Notwithstanding the advances that have been made in the research field, a vaccine that has been admitted for use against HCMV is not in sight [282]. The complex biology of the virus and the antigenic variation between a range of HCMV strains is an issue that complicates the development process. Although novel vaccines are designed to induce strong immunity against HCMV, the licensure of a moderately effective vaccine would nevertheless be of great benefit to public health [283]. In immunocompromised IBD patients, the major challenge lies in the generation of sufficient immunity through vaccination. It is vital to note that patients, especially those who are HCMV seronegative, may benefit from a vaccine that stimulates both the cellular and humoral immune responses. This may provide strong protection against primary HCMV infection in IBD patients [284]. So far, different categories of vaccines have advanced to clinical trials. Several subunit vaccines focusing on key viral proteins such as the major viral fusogen gB [285] or IE protein 1 and 2 [286] as well as attempts of attenuated virus vaccines such as the “Towne-Toledo chimera” [287] showed limited efficacy. Consequently, the development of enhanced vaccines

is an objective for the future that could be of particular benefit in the context of IBD. The human analogue of the ΔM27-MCMV mutant utilised in the present study may be a promising candidate.

Nevertheless, in the absence of an effective human vaccine, standardisation of diagnostic methods for the early detection of HCMV infections in IBD patients must be prioritised. The presence of the coinciding clinical or endoscopic symptoms between IBD and HCMV colitis represent a diagnostic challenge. Nowadays, the detection of HCMV markers in multiple fresh tissue biopsies from inflamed intestinal areas via IHC and qPCR are recommended [178, 179]. However, not all global guidelines are consistent and in addition, HCMV colitis is not considered in all patients. The early diagnosis is essential for appropriate management such as the treatment with antivirals. So far, there are few antivirals approved for the treatment of HCMV infections targeting essential mechanisms of the viral replication cycle. Ganciclovir and its prodrug Valganciclovir, Foscarnet and Cidofivir have in common to inhibit viral DNA replication by targeting the viral DNA polymerase. The novel antiviral Letermovir inhibits the terminase complex, important for the encapsulation of viral DNA into new virions, and Maribavir disrupts the assembly of new viral capsids [284]. Due to drug resistances of HCMV, variations in the treatments of patients and the development of novel antivirals are still required. Nonetheless, antivirals induce frequent side effects such as kidney damage (Foscarnet, Cidofivir and Letermovir), liver damage (Ganciclovir and Valganciclovir), and bone marrow suppression (Ganciclovir and Letermovir) [284], which must be taken into account before treatment induction. Nevertheless, upon antiviral administration the HCMV colitis remission rate ranges between 67% to 100% [288], which was also indicated in the present study by endoscopic images of an UC patient with HCMV colitis before and after antiviral treatment (Fig. 32).

In summary, the present study demonstrated that CMV manifestation in the colon has the potential to affect the intestinal milieu both under homeostatic conditions and during intestinal inflammation. In immunocompetent hosts, colonic MCMV replication leads to the induction of a mild pro-inflammatory milieu, mediated by alterations in cytokine secretion, induction of apoptosis and interference with the intestinal barrier integrity. Additionally, MCMV infection during acute colitis exacerbated the inflammatory processes in the gut and impaired the subsequent resolution of inflammation. The accumulation of pro-inflammatory innate and adaptive immune cells during the recovery phase of acute colitis and the susceptibility of the ISCs to MCMV infections both affected the intestinal regenerative capacity and re-establishment of homeostatic conditions in the gut. The ΔM27-MCMV live-attenuated vaccine exhibited strong protection to challenge MCMV infections during acute colitis and emphasises the urgent need for the development of a potent human CMV vaccine in the context of IBD.

5. Literature

1. Helander, H.F. and L. Fändriks, *Surface area of the digestive tract–revisited.* Scandinavian journal of gastroenterology, 2014. 49(6): p. 681-689.
2. Reed, K.K. and R. Wickham. *Review of the gastrointestinal tract: from macro to micro*. in *Seminars in oncology nursing*. 2009. Elsevier.
3. Greenwood-Van Meerveld, B., A.C. Johnson, and D. Grundy, *Gastrointestinal physiology and function.* Gastrointestinal pharmacology, 2017: p. 1-16.
4. Treuting, P.M., M.J. Arends, and S.M. Dintzis, *Upper gastrointestinal tract*, in *Comparative anatomy and histology*. 2018, Elsevier. p. 191-211.
5. Treuting, P.M., M.J. Arends, and S.M. Dintzis, *Lower gastrointestinal tract*, in *Comparative anatomy and histology*. 2018, Elsevier. p. 213-228.
6. Farquhar, M.G. and G.E. Palade, *Junctional complexes in various epithelia.* The Journal of cell biology, 1963. 17(2): p. 375-412.
7. Van Itallie, C.M. and J.M. Anderson, *Claudins and epithelial paracellular transport.* Annu. Rev. Physiol., 2006. 68: p. 403-429.
8. Furuse, M., et al., *Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions.* The Journal of cell biology, 1994. 127(6): p. 1617-1626.
9. Garrod, D. and M. Chidgey, *Desmosome structure, composition and function.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2008. 1778(3): p. 572-587.
10. Pokutta, S. and W.I. Weis, *Structure and mechanism of cadherins and catenins in cell-cell contacts.* Annu. Rev. Cell Dev. Biol., 2007. 23: p. 237-261.
11. Odenwald, M.A. and J.R. Turner, *The intestinal epithelial barrier: a therapeutic target?* Nature reviews Gastroenterology & hepatology, 2017. 14(1): p. 9-21.
12. Shen, L., et al., *Tight junction pore and leak pathways: a dynamic duo.* Annual review of physiology, 2011. 73: p. 283-309.
13. Moran Jr, E., *Anatomy, microbes, and fiber: Small versus large intestine.* Journal of Applied Poultry Research, 2006. 15(1): p. 154-160.
14. Gehart, H. and H. Clevers, *Tales from the crypt: new insights into intestinal stem cells.* Nature reviews Gastroenterology & hepatology, 2019. 16(1): p. 19-34.
15. Carmon, K.S., et al., *R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling.* Proceedings of the National Academy of Sciences, 2011. 108(28): p. 11452-11457.
16. Barker, N., et al., *Identification of stem cells in small intestine and colon by marker gene Lgr5.* Nature, 2007. 449(7165): p. 1003-1007.
17. Marchiando, A.M., et al., *The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding.* Gastroenterology, 2011. 140(4): p. 1208-1218. e2.
18. Sailaja, B.S., X.C. He, and L. Li, *The regulatory niche of intestinal stem cells.* The Journal of physiology, 2016. 594(17): p. 4827-4836.
19. Santos, A.J., et al., *The intestinal stem cell niche: homeostasis and adaptations.* Trends in cell biology, 2018. 28(12): p. 1062-1078.
20. Barker, N., *Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration.* Nature reviews Molecular cell biology, 2014. 15(1): p. 19-33.
21. Mowat, A.M. and W.W. Agace, *Regional specialization within the intestinal immune system.* Nature Reviews Immunology, 2014. 14(10): p. 667-685.
22. Johansson, M.E., et al., *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Proceedings of the national academy of sciences, 2008. 105(39): p. 15064-15069.

23. Tollin, M., et al., *Antimicrobial peptides in the first line defence of human colon mucosa.* Peptides, 2003. 24(4): p. 523-530.
24. Mantis, N.J., N. Rol, and B. Corthésy, *Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut.* Mucosal immunology, 2011. 4(6): p. 603-611.
25. Ayabe, T., et al., *The role of Paneth cells and their antimicrobial peptides in innate host defense.* Trends in microbiology, 2004. 12(8): p. 394-398.
26. Shi, N., et al., *Interaction between the gut microbiome and mucosal immune system.* Military Medical Research, 2017. 4: p. 1-7.
27. Chaplin, D.D., *Overview of the immune response.* Journal of allergy and clinical immunology, 2010. 125(2): p. S3-S23.
28. Ahluwalia, B., M.K. Magnusson, and L. Öhman, *Mucosal immune system of the gastrointestinal tract: maintaining balance between the good and the bad.* Scandinavian journal of gastroenterology, 2017. 52(11): p. 1185-1193.
29. Jung, C., J.-P. Hugot, and F. Barreau, *Peyer' s Patches: The Immune Sensors of the Intestine.* International journal of inflammation, 2010. 2010(1): p. 823710.
30. Willard-Mack, C.L., *Normal structure, function, and histology of lymph nodes.* Toxicologic pathology, 2006. 34(5): p. 409-424.
31. Richards, J.L., et al., *Dietary metabolites and the gut microbiota: an alternative approach to control inflammatory and autoimmune diseases.* Clinical & translational immunology, 2016. 5(5): p. e82.
32. Brandtzaeg, P., et al., *Terminology: nomenclature of mucosa-associated lymphoid tissue.* Mucosal immunology, 2008. 1(1): p. 31-37.
33. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Cellular and Molecular Immunology E-Book: Cellular and Molecular Immunology E-Book*. 2014: Elsevier Health Sciences.
34. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nature immunology, 2015. 16(4): p. 343-353.
35. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. 140(6): p. 805-820.
36. Amarante-Mendes, G.P., et al., *Pattern recognition receptors and the host cell death molecular machinery.* Frontiers in immunology, 2018. 9: p. 2379.
37. Turvey, S.E. and D.H. Broide, *Innate immunity.* Journal of Allergy and Clinical Immunology, 2010. 125(2): p. S24-S32.
38. Li, D. and M. Wu, *Pattern recognition receptors in health and diseases.* Signal transduction and targeted therapy, 2021. 6(1): p. 291.
39. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nature immunology, 2010. 11(5): p. 373-384.
40. Hirayama, D., T. Iida, and H. Nakase, *The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis.* International journal of molecular sciences, 2017. 19(1): p. 92.
41. Gordon, S., *Alternative activation of macrophages.* Nature reviews immunology, 2003. 3(1): p. 23-35.
42. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends in immunology, 2004. 25(12): p. 677-686.
43. Murphy, K. and C. Weaver, *Janeway's immunobiology*. 2016: Garland science.
44. Castanheira, F.V. and P. Kubes, *Neutrophils and NETs in modulating acute and chronic inflammation.* Blood, The Journal of the American Society of Hematology, 2019. 133(20): p. 2178-2185.
45. Giembycz, M.A. and M.A. Lindsay, *Pharmacology of the eosinophil.* Pharmacological reviews, 1999. 51(2): p. 213-340.
46. Duffin, R., et al., *Targeting granulocyte apoptosis: mechanisms, models, and therapies.* Immunological reviews, 2010. 236(1): p. 28-40.

47. Kambayashi, T. and T.M. Laufer, *Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell?* Nature Reviews Immunology, 2014. 14(11): p. 719-730.
48. Rescigno, M., et al., *Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.* Nature immunology, 2001. 2(4): p. 361-367.
49. Mabbott, N.A., et al., *Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium.* Mucosal immunology, 2013. 6(4): p. 666-677.
50. McDole, J.R., et al., *Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine.* Nature, 2012. 483(7389): p. 345-349.
51. Saez, A., et al., *Innate lymphoid cells in intestinal homeostasis and inflammatory bowel disease.* International journal of molecular sciences, 2021. 22(14): p. 7618.
52. Artis, D. and H. Spits, *The biology of innate lymphoid cells.* Nature, 2015. 517(7534): p. 293-301.
53. Spits, H., et al., *Innate lymphoid cells—a proposal for uniform nomenclature.* Nature reviews immunology, 2013. 13(2): p. 145-149.
54. Spits, H. and J.P. Di Santo, *The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling.* Nature immunology, 2011. 12(1): p. 21-27.
55. Biron, C.A., et al., *Natural killer cells in antiviral defense: function and regulation by innate cytokines.* Annual review of immunology, 1999. 17(1): p. 189-220.
56. Burgueño, J.F. and M.T. Abreu, *Epithelial Toll-like receptors and their role in gut homeostasis and disease.* Nature Reviews Gastroenterology & Hepatology, 2020. 17(5): p. 263-278.
57. Gourley, T.S., et al. *Generation and maintenance of immunological memory*. in *Seminars in immunology*. 2004. Elsevier.
58. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* Journal of Allergy and Clinical Immunology, 2010. 125(2): p. S33-S40.
59. Parkin, J. and B. Cohen, *An overview of the immune system.* The Lancet, 2001. 357(9270): p. 1777-1789.
60. Spencer, J. and L.M. Sollid, *The human intestinal B-cell response.* Mucosal immunology, 2016. 9(5): p. 1113-1124.
61. Cambier, J.C., et al., *B-cell anergy: from transgenic models to naturally occurring anergic B cells?* Nature Reviews Immunology, 2007. 7(8): p. 633-643.
62. Stavnezer, J., J.E. Guikema, and C.E. Schrader, *Mechanism and regulation of class switch recombination.* Annu. Rev. Immunol., 2008. 26(1): p. 261-292.
63. Allen, C.D., T. Okada, and J.G. Cyster, *Germinal-center organization and cellular dynamics.* Immunity, 2007. 27(2): p. 190-202.
64. Drayton, D.L., et al., *Lymphoid organ development: from ontogeny to neogenesis.* Nature immunology, 2006. 7(4): p. 344-353.
65. Jenkins, M.K., et al., *In vivo activation of antigen-specific CD4 T cells.* Annual review of immunology, 2001. 19(1): p. 23-45.
66. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu. Rev. Immunol., 2005. 23(1): p. 515-548.
67. Luckheeram, R.V., et al., *CD4$^+$ T cells: differentiation and functions.* Journal of Immunology Research, 2012. 2012(1): p. 925135.
68. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nature immunology, 2004. 5(10): p. 987-995.
69. Afkarian, M., et al., *T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4$^+$ T cells.* Nature immunology, 2002. 3(6): p. 549-557.
70. Murray, H.W., et al., *Human mononuclear phagocyte antiprotozoal mechanisms: oxygen-dependent vs oxygen-independent activity against intracellular Toxoplasma gondii.* Journal of immunology (Baltimore, Md.: 1950), 1985. 134(3): p. 1982-1988.
71. Kaplan, M.H., et al., *Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells.* Immunity, 1996. 4(3): p. 313-319.

72. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. 441(7090): p. 235-238.
73. Manel, N., D. Unutmaz, and D.R. Littman, *The differentiation of human TH-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt.* Nature immunology, 2008. 9(6): p. 641-649.
74. Perez-Lopez, A., et al., *Mucosal immunity to pathogenic intestinal bacteria.* Nature Reviews Immunology, 2016. 16(3): p. 135-148.
75. Aujla, S.J., et al., *IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia.* Nature medicine, 2008. 14(3): p. 275-281.
76. Chen, W., et al., *Conversion of peripheral CD4$^+$ CD25− naive T cells to CD4$^+$ CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3.* The Journal of experimental medicine, 2003. 198(12): p. 1875-1886.
77. Cebula, A., et al., *Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota.* Nature, 2013. 497(7448): p. 258-262.
78. Sakaguchi, S., et al., *Foxp3$^+$ CD25$^+$ CD4$^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease.* Immunological reviews, 2006. 212(1): p. 8-27.
79. Fujio, K., T. Okamura, and K. Yamamoto, *The Family of IL-10-secreting CD4$^+$ T cells.* Advances in immunology, 2010. 105: p. 99-130.
80. Castelli, C., et al., *T-cell recognition of melanoma-associated antigens.* Journal of cellular physiology, 2000. 182(3): p. 323-331.
81. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions.* Nature, 1998. 393(6684): p. 480-483.
82. Mittrücker, H.-W., A. Visekruna, and M. Huber, *Heterogeneity in the differentiation and function of CD8$^+$ T cells.* Archivum immunologiae et therapiae experimentalis, 2014. 62: p. 449-458.
83. Jabri, B. and V. Abadie, *IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction.* Nature Reviews Immunology, 2015. 15(12): p. 771-783.
84. Voskoboinik, I., J.C. Whisstock, and J.A. Trapani, *Perforin and granzymes: function, dysfunction and human pathology.* Nature Reviews Immunology, 2015. 15(6): p. 388-400.
85. Andersen, M.H., et al., *Cytotoxic T cells.* Journal of Investigative Dermatology, 2006. 126(1): p. 32-41.
86. Salmi, M. and S. Jalkanen, *Lymphocyte homing to the gut: attraction, adhesion, and commitment.* Immunological reviews, 2005. 206(1): p. 100-113.
87. Mowat, A.M., *Anatomical basis of tolerance and immunity to intestinal antigens.* Nature Reviews Immunology, 2003. 3(4): p. 331-341.
88. Cepek, K.L., et al., *Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin.* Nature, 1994. 372(6502): p. 190-193.
89. Cheroutre, H., F. Lambolez, and D. Mucida, *The light and dark sides of intestinal intraepithelial lymphocytes.* Nature Reviews Immunology, 2011. 11(7): p. 445-456.
90. Wershil, B.K. and G.T. Furuta, *4. Gastrointestinal mucosal immunity.* Journal of Allergy and Clinical Immunology, 2008. 121(2): p. S380-S383.
91. Maloy, K.J. and F. Powrie, *Intestinal homeostasis and its breakdown in inflammatory bowel disease.* Nature, 2011. 474(7351): p. 298-306.
92. Ramos, G.P. and K.A. Papadakis. *Mechanisms of disease: inflammatory bowel diseases*. in *Mayo Clinic Proceedings*. 2019. Elsevier.
93. Abraham, C. and J.H. Cho, *Inflammatory bowel disease.* N Engl J Med, 2009. 361(21): p. 2066-78.
94. Guan, Q., *A comprehensive review and update on the pathogenesis of inflammatory bowel disease.* Journal of immunology research, 2019. 2019.
95. Li, D., et al., *Lactic Acid Bacteria–Gut-Microbiota-Mediated Intervention towards Inflammatory Bowel Disease.* Microorganisms, 2024. 12(9): p. 1864.

96. Ghione, S., et al., *Dramatic increase in incidence of ulcerative colitis and Crohn's disease (1988–2011): a population-based study of French adolescents.* Official journal of the American College of Gastroenterology| ACG, 2018. 113(2): p. 265-272.
97. Zhao, M., et al., *The burden of inflammatory bowel disease in Europe in 2020.* Journal of Crohn's and Colitis, 2021. 15(9): p. 1573-1587.
98. Alatab, S., et al., *The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017.* The Lancet gastroenterology & hepatology, 2020. 5(1): p. 17-30.
99. Kaplan, G.G., *The global burden of IBD: from 2015 to 2025.* Nature reviews Gastroenterology & hepatology, 2015. 12(12): p. 720-727.
100. Neurath, M.F., *Current and emerging therapeutic targets for IBD.* Nature Reviews Gastroenterology & Hepatology, 2017. 14(5): p. 269-278.
101. Kaser, A. and H. Tilg, *Novel therapeutic targets in the treatment of IBD.* Expert opinion on therapeutic targets, 2008. 12(5): p. 553-563.
102. Duerr, R.H., *Genome-wide association studies herald a new era of rapid discoveries in inflammatory bowel disease research.* Gastroenterology, 2007. 132(5): p. 2045-2049.
103. McGovern, D.P., S. Kugathasan, and J.H. Cho, *Genetics of inflammatory bowel diseases.* Gastroenterology, 2015. 149(5): p. 1163-1176. e2.
104. Ogura, Y., et al., *A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease.* Nature, 2001. 411(6837): p. 603-606.
105. Abraham, C. and R. Medzhitov, *Interactions between the host innate immune system and microbes in inflammatory bowel disease.* Gastroenterology, 2011. 140(6): p. 1729-1737.
106. Franke, A., et al., *Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility.* Nature genetics, 2008. 40(11): p. 1319-1323.
107. Khor, B., A. Gardet, and R.J. Xavier, *Genetics and pathogenesis of inflammatory bowel disease.* Nature, 2011. 474(7351): p. 307-317.
108. Mehta, M., S. Ahmed, and G. Dryden, *Immunopathophysiology of inflammatory bowel disease: how genetics link barrier dysfunction and innate immunity to inflammation.* Innate Immunity, 2017. 23(6): p. 497-505.
109. Zuk, O., et al., *The mystery of missing heritability: Genetic interactions create phantom heritability.* Proceedings of the National Academy of Sciences, 2012. 109(4): p. 1193-1198.
110. Ananthakrishnan, A.N., *Epidemiology and risk factors for IBD.* Nature reviews Gastroenterology & hepatology, 2015. 12(4): p. 205-217.
111. Mahid, S.S., et al. *Smoking and inflammatory bowel disease: a meta-analysis*. in *Mayo Clinic Proceedings*. 2006. Elsevier.
112. Parkes, G.C., K. Whelan, and J.O. Lindsay, *Smoking in inflammatory bowel disease: impact on disease course and insights into the aetiology of its effect.* Journal of Crohn's and Colitis, 2014. 8(8): p. 717-725.
113. Garg, M., et al., *vitamin D and inflammatory bowel disease–established concepts and future directions.* Alimentary pharmacology & therapeutics, 2012. 36(4): p. 324-344.
114. Leslie, W.D., et al., *Vitamin D status and bone density in recently diagnosed inflammatory bowel disease: the Manitoba IBD Cohort Study.* Official journal of the American College of Gastroenterology| ACG, 2008. 103(6): p. 1451-1459.
115. Thia, K.T., et al., *An update on the epidemiology of inflammatory bowel disease in Asia.* Official journal of the American College of Gastroenterology| ACG, 2008. 103(12): p. 3167-3182.
116. Ananthakrishnan, A.N., et al., *Ambient air pollution correlates with hospitalizations for inflammatory bowel disease: an ecologic analysis.* Inflammatory bowel diseases, 2011. 17(5): p. 1138-1145.
117. Dolan, K.T. and E.B. Chang, *Diet, gut microbes, and the pathogenesis of inflammatory bowel diseases.* Molecular nutrition & food research, 2017. 61(1): p. 1600129.

118. Arijs, I., et al., *Mucosal gene expression of antimicrobial peptides in inflammatory bowel disease before and after first infliximab treatment.* PloS one, 2009. 4(11): p. e7984.
119. Marchiando, A.M., W.V. Graham, and J.R. Turner, *Epithelial barriers in homeostasis and disease.* Annual Review of Pathology: Mechanisms of Disease, 2010. 5(1): p. 119-144.
120. Lee, S.H., J. eun Kwon, and M.-L. Cho, *Immunological pathogenesis of inflammatory bowel disease.* Intestinal research, 2018. 16(1): p. 26.
121. Drury, B., et al., *Neutrophil extracellular traps in inflammatory bowel disease: pathogenic mechanisms and clinical translation.* Cellular and molecular gastroenterology and hepatology, 2021. 12(1): p. 321-333.
122. Peng, V., N. Jaeger, and M. Colonna, *Innate lymphoid cells and inflammatory bowel disease.* Innate Lymphoid Cells, 2022: p. 97-112.
123. Monteleone, G., et al., *Bioactive IL-18 expression is up-regulated in Crohn's disease.* The Journal of Immunology, 1999. 163(1): p. 143-147.
124. Monteleone, G., et al., *Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells.* Gastroenterology, 1997. 112(4): p. 1169-1178.
125. Fuss, I.J., et al., *Disparate $CD4^+$ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5.* Journal of immunology (Baltimore, Md.: 1950), 1996. 157(3): p. 1261-1270.
126. Di Sabatino, A., et al., *New pathogenic paradigms in inflammatory bowel disease.* Inflammatory bowel diseases, 2012. 18(2): p. 368-371.
127. Fujino, S., et al., *Increased expression of interleukin 17 in inflammatory bowel disease.* Gut, 2003. 52(1): p. 65-70.
128. Joossens, M., et al., *Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives.* Gut, 2011. 60(5): p. 631-637.
129. Andoh, A., et al., *Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis.* Journal of gastroenterology, 2011. 46: p. 479-486.
130. Shaw, S.Y., J.F. Blanchard, and C.N. Bernstein, *Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease.* Official journal of the American College of Gastroenterology| ACG, 2010. 105(12): p. 2687-2692.
131. Bernstein, C.N., et al., *Population-based case control study of seroprevalence of Mycobacterium paratuberculosis in patients with Crohn's disease and ulcerative colitis.* Journal of Clinical Microbiology, 2004. 42(3): p. 1129-1135.
132. Gradel, K.O., et al., *Increased short-and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis.* Gastroenterology, 2009. 137(2): p. 495-501.
133. Kandiel, A. and B. Lashner, *Cytomegalovirus colitis complicating inflammatory bowel disease.* Official journal of the American College of Gastroenterology| ACG, 2006. 101(12): p. 2857-2865.
134. Yang, C. and D. Merlin, *Unveiling Colitis: A Journey through the Dextran Sodium Sulfate-induced Model.* Inflammatory Bowel Diseases, 2024: p. izad312.
135. Goyal, N., et al., *Animal models of inflammatory bowel disease: a review.* Inflammopharmacology, 2014. 22(4): p. 219-233.
136. Okayasu, I., et al., *A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice.* Gastroenterology, 1990. 98(3): p. 694-702.
137. Perše, M. and A. Cerar, *Dextran sodium sulphate colitis mouse model: traps and tricks.* BioMed Research International, 2012. 2012.
138. Yildiz, H.M., et al., *Size selectivity of intestinal mucus to diffusing particulates is dependent on surface chemistry and exposure to lipids.* Journal of drug targeting, 2015. 23(7-8): p. 768-774.
139. Yu, M., et al., *Molecular structure of dextran sulphate sodium in aqueous environment.* Journal of Molecular Structure, 2018. 1156: p. 320-329.

140. Laroui, H., et al., *Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon.* PloS one, 2012. 7(3): p. e32084.
141. Poritz, L.S., et al., *Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis.* Journal of Surgical Research, 2007. 140(1): p. 12-19.
142. Egger, B., et al., *Characterisation of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency.* Digestion, 2000. 62(4): p. 240-248.
143. Dieleman, et al., *Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines.* Clinical & Experimental Immunology, 1998. 114(3): p. 385-391.
144. Chassaing, B., et al., *Dextran sulfate sodium (DSS)-induced colitis in mice.* Current protocols in immunology, 2014. 104(1): p. 15.25. 1-15.25. 14.
145. Katsandegwaza, B., W. Horsnell, and K. Smith, *Inflammatory bowel disease: A review of pre-clinical murine models of human disease.* International Journal of Molecular Sciences, 2022. 23(16): p. 9344.
146. Sartor, R.B., *Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis.* Nature clinical practice Gastroenterology & hepatology, 2006. 3(7): p. 390-407.
147. Fowler, K., et al., *A systematic literature review of the global seroprevalence of cytomegalovirus: possible implications for treatment, screening, and vaccine development.* BMC Public Health, 2022. 22(1): p. 1659.
148. Landolfo, S., et al., *The human cytomegalovirus.* Pharmacology & therapeutics, 2003. 98(3): p. 269-297.
149. Leruez-Ville, M., et al., *Cytomegalovirus infection during pregnancy: state of the science.* American journal of obstetrics and gynecology, 2020. 223(3): p. 330-349.
150. Ramanan, P. and R.R. Razonable, *Cytomegalovirus infections in solid organ transplantation: a review.* Infection & chemotherapy, 2013. 45(3): p. 260.
151. Söderberg-Nauclér, C., K.N. Fish, and J.A. Nelson, *Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors.* Cell, 1997. 91(1): p. 119-126.
152. Isaacson, M.K. and T. Compton, *Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress.* Journal of virology, 2009. 83(8): p. 3891-3903.
153. Bowman, J.J., et al., *Rhesus and human cytomegalovirus glycoprotein L are required for infection and cell-to-cell spread of virus but cannot complement each other.* Journal of virology, 2011. 85(5): p. 2089-2099.
154. Liu, J., et al., *The human cytomegalovirus trimer and pentamer promote sequential steps in entry into epithelial and endothelial cells at cell surfaces and endosomes.* Journal of virology, 2018. 92(21): p. 10.1128/jvi. 01336-18.
155. Ryckman, B.J., M.C. Chase, and D.C. Johnson, *HCMV gH/gL/UL128–131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors.* Proceedings of the National Academy of Sciences, 2008. 105(37): p. 14118-14123.
156. Vanarsdall, A.L. and D.C. Johnson, *Human cytomegalovirus entry into cells.* Current opinion in virology, 2012. 2(1).
157. Compton, T., D.M. Nowlin, and N.R. Cooper, *Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate.* Virology, 1993. 193(2): p. 834-841.
158. Feire, A.L., H. Koss, and T. Compton, *Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain.* Proceedings of the National Academy of Sciences, 2004. 101(43): p. 15470-15475.
159. Kim, J.H., et al., *Human cytomegalovirus requires epidermal growth factor receptor signaling to enter and initiate the early steps in the establishment of latency in CD34+ human progenitor cells.* Journal of virology, 2017. 91(5): p. 10.1128/jvi. 01206-16.

160. Martinez-Martin, N., et al., *An unbiased screen for human cytomegalovirus identifies neuropilin-2 as a central viral receptor.* Cell, 2018. 174(5): p. 1158-1171. e19.
161. Jentzer, A., et al., *Cytomegalovirus and inflammatory bowel diseases (IBD) with a special focus on the link with ulcerative colitis (UC).* Microorganisms, 2020. 8(7): p. 1078.
162. Fielding, C.A., et al., *Control of immune ligands by members of a cytomegalovirus gene expansion suppresses natural killer cell activation.* Elife, 2017. 6: p. e22206.
163. Berry, R., et al., *Modulation of innate and adaptive immunity by cytomegaloviruses.* Nature reviews immunology, 2020. 20(2): p. 113-127.
164. Griffiths, P. and M. Reeves, *Pathogenesis of human cytomegalovirus in the immunocompromised host.* Nature Reviews Microbiology, 2021. 19(12): p. 759-773.
165. Bravender, T., *Epstein-Barr virus, cytomegalovirus, and infectious mononucleosis.* Adolescent medicine: state of the art reviews, 2010. 21(2): p. 251-64, ix.
166. Britt, W., *Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease.* Human cytomegalovirus, 2008: p. 417-470.
167. Nakase, H. and H. Herfarth, *Cytomegalovirus colitis, cytomegalovirus hepatitis and systemic cytomegalovirus infection: common features and differences.* Inflammatory intestinal diseases, 2016. 1(1): p. 15-23.
168. Sinzger, C., et al., *Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues.* Journal of General Virology, 1995. 76(4): p. 741-750.
169. Le-Trilling, V.T.K., et al., *Acute cytomegalovirus infection modulates the intestinal microbiota and targets intestinal epithelial cells.* European journal of immunology, 2023. 53(2): p. 2249940.
170. Patra, S., et al., *Cytomegalovirus infection of the human gastrointestinal tract.* Journal of gastroenterology and hepatology, 1999. 14(10): p. 973-976.
171. Goodgame, R.W., *Gastrointestinal cytomegalovirus disease.* Annals of internal medicine, 1993. 119(9): p. 924-935.
172. Yerushalmy-Feler, A., J. Padlipsky, and S. Cohen, *Diagnosis and management of CMV colitis.* Current Infectious Disease Reports, 2019. 21: p. 1-6.
173. Powell, R.D., et al., *Cytomegalic inclusion disease and ulcerative colitis: report of a case in a young adult.* The American journal of medicine, 1961. 30(2): p. 334-340.
174. Lv, Y.-l., et al., *Is cytomegalovirus infection related to inflammatory bowel disease, especially steroid-resistant inflammatory bowel disease? A meta-analysis.* Infection and drug resistance, 2017: p. 511-519.
175. Takahashi, Y. and T. Tange, *Prevalence of cytomegalovirus infection in inflammatory bowel disease patients.* Diseases of the colon & rectum, 2004. 47(5): p. 722-726.
176. Papadakis, K.A., et al., *Outcome of cytomegalovirus infections in patients with inflammatory bowel disease.* Official journal of the American College of Gastroenterology| ACG, 2001. 96(7): p. 2137-2142.
177. Kwon, J., et al., *Cytomegalovirus-related colitis in patients with inflammatory bowel disease.* International Journal of Colorectal Disease, 2022. 37(3): p. 685-691.
178. Kucharzik, T., et al., *ECCO guidelines on the prevention, diagnosis, and management of infections in inflammatory bowel disease.* Journal of Crohn's and Colitis, 2021. 15(6): p. 879-913.
179. Roblin, X., et al., *Cytomegalovirus load in inflamed intestinal tissue is predictive of resistance to immunosuppressive therapy in ulcerative colitis.* Official journal of the American College of Gastroenterology| ACG, 2011. 106(11): p. 2001-2008.
180. Eyre-Brook, I. and S. Dundas, *Incidence and clinical significance of colonic cytomegalovirus infection in idiopathic inflammatory bowel disease requiring colectomy.* Gut, 1986. 27(12): p. 1419-1425.
181. Cottone, M., et al., *Prevalence of cytomegalovirus infection in severe refractory ulcerative and Crohn's colitis.* The American journal of gastroenterology, 2001. 96(3): p. 773-775.

182. Kambham, N., et al., *Cytomegalovirus infection in steroid-refractory ulcerative colitis: a case-control study.* The American journal of surgical pathology, 2004. 28(3): p. 365-373.
183. Goto, N., et al., *Lymphatics and fibroblasts support intestinal stem cells in homeostasis and injury.* Cell stem cell, 2022. 29(8): p. 1246-1261. e6.
184. Jordan, S., et al., *Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2.* Journal of virology, 2011. 85(19): p. 10346-10353.
185. Trilling, M., et al., *"Activated" STAT proteins: a paradoxical consequence of inhibited JAK-STAT signaling in cytomegalovirus-infected cells.* The Journal of Immunology, 2014. 192(1): p. 447-458.
186. Trilling, M., et al., *Identification of DNA-damage DNA-binding protein 1 as a conditional essential factor for cytomegalovirus replication in interferon-γ-stimulated cells.* PLoS pathogens, 2011. 7(6): p. e1002069.
187. Andrews, S., *FastQC: a quality control tool for high throughput sequence data.* (No Title), 2010.
188. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. 29(1): p. 15-21.
189. Le-Trilling, V.T.K., et al., *Maternal antibodies induced by a live attenuated vaccine protect neonatal mice from cytomegalovirus.* npj Vaccines, 2023. 8(1): p. 8.
190. Mabrok, H.B., et al., *Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model of breast cancer.* Carcinogenesis, 2012. 33(1): p. 203-208.
191. Brune, W., H. Hengel, and U.H. Koszinowski, *A mouse model for cytomegalovirus infection.* Current protocols in immunology, 2001. 43(1): p. 19.7. 1-19.7. 13.
192. Varani, S. and M.P. Landini, *Cytomegalovirus-induced immunopathology and its clinical consequences.* Herpesviridae, 2011. 2: p. 1-14.
193. Dimitroulia, E., et al., *Frequent detection of cytomegalovirus in the intestine of patients with inflammatory bowel disease.* Inflammatory bowel diseases, 2006. 12(9): p. 879-884.
194. Le-Trilling, V.T.K., et al., *STAT2-dependent immune responses ensure host survival despite the presence of a potent viral antagonist.* Journal of virology, 2018. 92(14): p. e00296-18.
195. Rawlinson, W.D., H.E. Farrell, and B.G. Barrell, *Analysis of the complete DNA sequence of murine cytomegalovirus.* Journal of virology, 1996. 70(12): p. 8833-8849.
196. Zheng, L. and S.-L. Duan, *Molecular regulation mechanism of intestinal stem cells in mucosal injury and repair in ulcerative colitis.* World Journal of Gastroenterology, 2023. 29(16): p. 2380.
197. Sprangers, J., I.C. Zaalberg, and M.M. Maurice, *Organoid-based modeling of intestinal development, regeneration, and repair.* Cell Death & Differentiation, 2021. 28(1): p. 95-107.
198. Ciccocioppo, R., et al., *Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: need for mucosal viral load measurement.* World Journal of Gastroenterology: WJG, 2015. 21(6): p. 1915.
199. Maher, M.M. and M.I. Nassar, *Acute cytomegalovirus infection is a risk factor in refractory and complicated inflammatory bowel disease.* Digestive diseases and sciences, 2009. 54: p. 2456-2462.
200. Criscuoli, V., M.R. Rizzuto, and M. Cottone, *Cytomegalovirus and inflammatory bowel disease: is there a link?* World journal of gastroenterology: WJG, 2006. 12(30): p. 4813.
201. Okumura, R. and K. Takeda, *Maintenance of intestinal homeostasis by mucosal barriers.* Inflammation and regeneration, 2018. 38: p. 1-8.
202. Einbinder, Y., et al., *The clinical spectrum of cytomegalovirus colitis in adults.* Alimentary pharmacology & therapeutics, 2008. 27(7): p. 578-587.
203. Klauber, E., L.E. Briski, and R. Khatib, *Cytomegalovirus colitis in the immunocompetent host: an overview.* Scandinavian journal of infectious diseases, 1998. 30(6): p. 559-564.
204. Ng, F.-H., et al., *Cytomegalovirus colitis in individuals without apparent cause of immunodeficiency.* Digestive diseases and sciences, 1999. 44: p. 945-952.

205. Galiatsatos, P., et al., *Meta-analysis of outcome of cytomegalovirus colitis in immunocompetent hosts.* Digestive diseases and sciences, 2005. 50: p. 609-616.
206. Salehi, M., N. Shafiee, and M. Moradi, *Cytomegalovirus colitis in immunocompetent hosts: A case report and literature review.* Clinical Case Reports, 2024. 12(1): p. e8435.
207. Ko, J.-H., et al., *Clinical presentation and risk factors for cytomegalovirus colitis in immunocompetent adult patients.* Clinical Infectious Diseases, 2015. 60(6): p. e20-e26.
208. Wang, F., et al., *IFN-γ-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction.* Gastroenterology, 2006. 131(4): p. 1153-1163.
209. Meyer, F., et al., *Cytokines and intestinal epithelial permeability: A systematic review.* Autoimmunity reviews, 2023. 22(6): p. 103331.
210. Suzuki, T., N. Yoshinaga, and S. Tanabe, *Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium.* Journal of Biological Chemistry, 2011. 286(36): p. 31263-31271.
211. Hu, M.D., et al., *γδ intraepithelial lymphocytes facilitate pathological epithelial cell shedding via CD103-mediated granzyme release.* Gastroenterology, 2022. 162(3): p. 877-889. e7.
212. Skaletskaya, A., et al., *A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation.* Proceedings of the National Academy of Sciences, 2001. 98(14): p. 7829-7834.
213. Wang, S. and W.S. El-Deiry, *TRAIL and apoptosis induction by TNF-family death receptors.* Oncogene, 2003. 22(53): p. 8628-8633.
214. Lord, S.J., et al., *Granzyme B: a natural born killer.* Immunological reviews, 2003. 193(1): p. 31-38.
215. Leech, B., J. Schloss, and A. Steel, *Association between increased intestinal permeability and disease: A systematic review.* Advances in Integrative Medicine, 2019. 6(1): p. 23-34.
216. Garrido, E., et al., *Clinical significance of cytomegalovirus infection in patients with inflammatory bowel disease.* World Journal of Gastroenterology: WJG, 2013. 19(1): p. 17.
217. Orvar, K., et al., *Cytomegalovirus infection associated with onset of inflammatory bowel disease.* Digestive diseases and sciences, 1993. 38: p. 2307-2310.
218. Christidou, A., E. Zambeli, and G. Mantzaris, *Cytomegalovirus and inflammatory bowel disease: pathogenicity, diagnosis and treatment.* Annals of Gastroenterology, 2007.
219. Zhang, C., et al., *Cytomegalovirus-related hospitalization is associated with adverse outcomes and increased health-care resource utilization in inflammatory bowel disease.* Clinical and translational gastroenterology, 2016. 7(3): p. e150.
220. Thavamani, A., et al., *Cytomegalovirus infection is Associated with adverse outcomes among hospitalized Pediatric patients with inflammatory bowel disease.* Gastroenterology Research, 2023. 16(1): p. 1.
221. Papadakis, K.A. and S.R. Targan, *Role of cytokines in the pathogenesis of inflammatory bowel disease.* Annual review of medicine, 2000. 51(1): p. 289-298.
222. Levin, A.D., M.E. Wildenberg, and G.R. van den Brink, *Mechanism of action of anti-TNF therapy in inflammatory bowel disease.* Journal of Crohn's and Colitis, 2016. 10(8): p. 989-997.
223. Baillie, J., D. Sahlender, and J. Sinclair, *Human cytomegalovirus infection inhibits tumor necrosis factor alpha (TNF-α) signaling by targeting the 55-kilodalton TNF-α receptor.* Journal of virology, 2003. 77(12): p. 7007-7016.
224. Jarvis, M.A., et al., *Human cytomegalovirus attenuates interleukin-1β and tumor necrosis factor alpha proinflammatory signaling by inhibition of NF-κB activation.* Journal of virology, 2006. 80(11): p. 5588-5598.
225. Smith, P.D., et al., *Cytomegalovirus induction of tumor necrosis factor-alpha by human monocytes and mucosal macrophages.* The Journal of clinical investigation, 1992. 90(5): p. 1642-1648.
226. Neurath, M.F., *Targeting immune cell circuits and trafficking in inflammatory bowel disease.* Nature immunology, 2019. 20(8): p. 970-979.

227. Pan, X., et al., *Macrophage immunometabolism in inflammatory bowel diseases: from pathogenesis to therapy.* Pharmacology & therapeutics, 2022. 238: p. 108176.
228. Dharmasiri, S., et al., *Human intestinal macrophages are involved in the pathology of both ulcerative colitis and Crohn disease.* Inflammatory bowel diseases, 2021. 27(10): p. 1641-1652.
229. Poggi, A., et al., *Human gut-associated natural killer cells in health and disease.* Frontiers in immunology, 2019. 10: p. 961.
230. Takayama, T., et al., *Imbalance of NKp44+ NKp46− and NKp44− NKp46+ natural killer cells in the intestinal mucosa of patients with Crohn's disease.* Gastroenterology, 2010. 139(3): p. 882-892. e3.
231. Fuss, I.J. and W. Strober, *The role of IL-13 and NK T cells in experimental and human ulcerative colitis.* Mucosal immunology, 2008. 1: p. S31-S33.
232. Levine, A.P. and A.W. Segal, *What is wrong with granulocytes in inflammatory bowel diseases?* Digestive diseases, 2013. 31(3-4): p. 321-327.
233. Wéra, O., P. Lancellotti, and C. Oury, *The dual role of neutrophils in inflammatory bowel diseases.* Journal of clinical medicine, 2016. 5(12): p. 118.
234. Li, T., et al., *Neutrophil extracellular traps induce intestinal damage and thrombotic tendency in inflammatory bowel disease.* Journal of Crohn's and Colitis, 2020. 14(2): p. 240-253.
235. Chen, H., et al., *Dichotomous roles of neutrophils in modulating pathogenic and repair processes of inflammatory bowel diseases.* Precision Clinical Medicine, 2021. 4(4): p. 246-257.
236. Bressenot, A., et al., *Comparing histological activity indexes in UC.* Gut, 2015. 64(9): p. 1412-1418.
237. Fu, W., et al., *Peripheral blood neutrophil-to-lymphocyte ratio in inflammatory bowel disease and disease activity: a meta-analysis.* International immunopharmacology, 2021. 101: p. 108235.
238. Lampinen, M., et al., *Eosinophil granulocytes are activated during the remission phase of ulcerative colitis.* Gut, 2005. 54(12): p. 1714-1720.
239. Bischoff, S., et al., *Quantitative assessment of intestinal eosinophils and mast cells in inflammatory bowel disease.* Histopathology, 1996. 28(1): p. 1-13.
240. Jeziorska, M., et al., *Distribution and activation of eosinophils in inflammatory bowel disease using an improved immunohistochemical technique.* The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 2001. 194(4): p. 484-492.
241. Zeitz, M., et al., *Lymphocytes isolated from the intestinal lamina propria of normal nonhuman primates have increased expression of genes associated with T-cell activation.* Gastroenterology, 1988. 94(3): p. 647-655.
242. Kim, S.-K., et al., *Activation and migration of CD8 T cells in the intestinal mucosa.* Journal of immunology (Baltimore, Md.: 1950), 1997. 159(9): p. 4295-4306.
243. Koh, C.-H., et al., *CD8 T-cell subsets: heterogeneity, functions, and therapeutic potential.* Experimental & molecular medicine, 2023. 55(11): p. 2287-2299.
244. Klenerman, P. and A. Oxenius, *T cell responses to cytomegalovirus.* Nature Reviews Immunology, 2016. 16(6): p. 367-377.
245. Benedict, C.A., et al., *Specific remodeling of splenic architecture by cytomegalovirus.* PLoS pathogens, 2006. 2(3): p. e16.
246. Campbell, A.E., V.J. Cavanaugh, and J.S. Slater, *The salivary glands as a privileged site of cytomegalovirus immune evasion and persistence.* Medical microbiology and immunology, 2008. 197: p. 205-213.
247. Lucin, P., et al., *Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands.* Journal of virology, 1992. 66(4): p. 1977-1984.
248. Sester, M., et al., *Dominance of virus-specific CD8 T cells in human primary cytomegalovirus infection.* Journal of the American Society of Nephrology, 2002. 13(10): p. 2577-2584.

249. Hahn, G., R. Jores, and E.S. Mocarski, *Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells.* Proceedings of the National Academy of Sciences, 1998. 95(7): p. 3937-3942.
250. Farrell, H.E., et al., *Murine cytomegalovirus spread depends on the infected myeloid cell type.* Journal of virology, 2019. 93(15): p. 10.1128/jvi. 00540-19.
251. Daley-Bauer, L.P., et al., *Cytomegalovirus hijacks CX3CR1hi patrolling monocytes as immune-privileged vehicles for dissemination in mice.* Cell host & microbe, 2014. 15(3): p. 351-362.
252. Kim, Y.-E., et al., *HIF-1α activation in myeloid cells accelerates dextran sodium sulfate-induced colitis progression in mice.* Disease models & mechanisms, 2018. 11(7): p. dmm033241.
253. Konjar, Š., et al., *Intestinal barrier interactions with specialized CD8 T cells.* Frontiers in Immunology, 2017. 8: p. 1281.
254. Funderburg, N.T., et al., *Circulating CD 4$^+$ and CD 8$^+$ T cells are activated in inflammatory bowel disease and are associated with plasma markers of inflammation.* Immunology, 2013. 140(1): p. 87-97.
255. Zundler, S., et al., *Hobit-and Blimp-1-driven CD4$^+$ tissue-resident memory T cells control chronic intestinal inflammation.* Nature immunology, 2019. 20(3): p. 288-300.
256. Boschetti, G., et al., *Enrichment of circulating and mucosal cytotoxic CD8$^+$ T cells is associated with postoperative endoscopic recurrence in patients with Crohn's disease.* Journal of Crohn's and Colitis, 2016. 10(3): p. 338-345.
257. Lee, J.C., et al., *Gene expression profiling of CD8$^+$ T cells predicts prognosis in patients with Crohn disease and ulcerative colitis.* The Journal of clinical investigation, 2011. 121(10): p. 4170-4179.
258. Westendorf, A.M., et al., *Autoimmune-mediated intestinal inflammation–impact and regulation of antigen-specific CD8$^+$ T cells.* Gastroenterology, 2006. 131(2): p. 510-524.
259. Nancey, S., et al., *CD8$^+$ cytotoxic T cells induce relapsing colitis in normal mice.* Gastroenterology, 2006. 131(2): p. 485-496.
260. BARBUL, A., et al., *The effect of in vivo T helper and T suppressor lymphocyte depletion on wound healing.* Annals of surgery, 1989. 209(4): p. 479-483.
261. Ilatovskaya, D.V., et al., *CD8$^+$ T-cells negatively regulate inflammation post-myocardial infarction.* American Journal of Physiology-Heart and Circulatory Physiology, 2019. 317(3): p. H581-H596.
262. Bernhardsson, M., et al., *Depletion of cytotoxic (CD8$^+$) T cells impairs implant fixation in rat cancellous bone.* Journal of Orthopaedic Research®, 2019. 37(4): p. 805-811.
263. Hokeness, K.L., et al., *CXCR3-dependent recruitment of antigen-specific T lymphocytes to the liver during murine cytomegalovirus infection.* Journal of virology, 2007. 81(3): p. 1241-1250.
264. Lathbury, L.J., et al., *Effect of host genotype in determining the relative roles of natural killer cells and T cells in mediating protection against murine cytomegalovirus infection.* Journal of general virology, 1996. 77(10): p. 2605-2613.
265. Duan, S., et al., *Circulating and intestinal regulatory T cells in inflammatory bowel disease: A systemic review and meta-analysis.* International Reviews of Immunology, 2024. 43(2): p. 83-94.
266. Pedros, C., et al., *Disrupted regulatory T cell homeostasis in inflammatory bowel diseases.* World journal of gastroenterology, 2016. 22(3): p. 974.
267. Maul, J., et al., *Peripheral and intestinal regulatory CD4$^+$ CD25high T cells in inflammatory bowel disease.* Gastroenterology, 2005. 128(7): p. 1868-1878.
268. d'Aldebert, E., et al., *Characterization of human colon organoids from inflammatory bowel disease patients.* Frontiers in cell and developmental biology, 2020. 8: p. 363.
269. Mutnal, M.B., et al., *Murine cytomegalovirus infection of neural stem cells alters neurogenesis in the developing brain.* PloS one, 2011. 6(1): p. e16211.
270. Kolf, C.M., E. Cho, and R.S. Tuan, *Mesenchymal stromal cells: biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation.* Arthritis research & therapy, 2007. 9: p. 1-10.

271. He, Q., C. Wan, and G. Li, *Concise review: multipotent mesenchymal stromal cells in blood.* Stem cells, 2007. 25(1): p. 69-77.
272. Avanzi, S., et al., *Susceptibility of human placenta derived mesenchymal stromal/stem cells to human herpesviruses infection.* PloS one, 2013. 8(8): p. e71412.
273. Sundin, M., et al., *Mesenchymal stem cells are susceptible to human herpesviruses, but viral DNA cannot be detected in the healthy seropositive individual.* Bone marrow transplantation, 2006. 37(11): p. 1051-1059.
274. Juranic Lisnic, V., et al., *Dual analysis of the murine cytomegalovirus and host cell transcriptomes reveal new aspects of the virus-host cell interface.* PLoS pathogens, 2013. 9(9): p. e1003611.
275. Hamilton, S.T., et al., *Human cytomegalovirus directly modulates expression of chemokine CCL2 (MCP-1) during viral replication.* Journal of General Virology, 2013. 94(11): p. 2495-2503.
276. Ebert, S., et al., *Mast cells expedite control of pulmonary murine cytomegalovirus infection by enhancing the recruitment of protective CD8 T cells to the lungs.* PLoS Pathogens, 2014. 10(4): p. e1004100.
277. Dhital, R., et al., *Murine cytomegalovirus promotes renal allograft inflammation via Th1/17 cells and IL-17A.* American Journal of Transplantation, 2022. 22(10): p. 2306-2322.
278. Cheeran, M.C.-J., et al., *CXCL10 production from cytomegalovirus-stimulated microglia is regulated by both human and viral interleukin-10.* Journal of virology, 2003. 77(8): p. 4502-4515.
279. Li, S., et al., *The battle between host antiviral innate immunity and immune evasion by cytomegalovirus.* Cellular and Molecular Life Sciences, 2024. 81(1): p. 341.
280. Le-Trilling, V.T.K., et al., *The human cytomegalovirus pUL145 isoforms act as viral DDB1-cullin-associated factors to instruct host protein degradation to impede innate immunity.* Cell reports, 2020. 30(7): p. 2248-2260. e5.
281. Zimmermann, A., et al., *A cytomegaloviral protein reveals a dual role for STAT2 in IFN-γ signaling and antiviral responses.* The Journal of experimental medicine, 2005. 201(10): p. 1543-1553.
282. Schleiss, M.R., S.R. Permar, and S.A. Plotkin, *Progress toward development of a vaccine against congenital cytomegalovirus infection.* Clinical and Vaccine Immunology, 2017. 24(12): p. e00268-17.
283. Permar, S.R., M.R. Schleiss, and S.A. Plotkin, *A vaccine against cytomegalovirus: how close are we?* The Journal of Clinical Investigation, 2025. 135(1).
284. Panda, K., D. Parashar, and R. Viswanathan, *An update on current antiviral strategies to combat human cytomegalovirus infection.* Viruses, 2023. 15(6): p. 1358.
285. Britt, W.J., et al., *Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response.* Journal of virology, 1990. 64(3): p. 1079-1085.
286. Braendstrup, P., et al., *Identification and HLA-tetramer-validation of human $CD4^+$ and $CD8^+$ T cell responses against HCMV proteins IE1 and IE2.* PLoS One, 2014. 9(4): p. e94892.
287. Heineman, T.C., et al., *A phase 1 study of 4 live, recombinant human cytomegalovirus Towne/Toledo chimeric vaccines.* The Journal of infectious diseases, 2006. 193(10): p. 1350-1360.
288. Sager, K., et al., *cytomegalovirus and inflammatory bowel disease.* Alimentary pharmacology & therapeutics, 2015. 41(8): p. 725-733.

6. Appendix

6.1. Abbreviations

AIDS	Acquired immunodeficiency syndrome
AJX	Apical junctional complex
AMPs	Antimicrobial peptides
APCs	Antigen presenting cells
BCR	B cell receptor
BP	Biological process
C	Celsius
Casp3	Caspase 3
cCD4/8	Conventional CD4/8
CD	Crohn’s disease
CD4/8	Cluster of differentiation 4/8
CMV	Cytomegalovirus
Ctrl	Control
DAMPs	Damage-associated molecular patterns
DAI	Disease activity index
DCs	Dendritic cells
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
DNBS	Hapten 2,4 dinitrobenzene sulfonate
dpi	Days post infection
DSS	Dextran sodium sulfate
EDN	Eosinophil-derived neurotoxin
e.g.	Exempli gratia
eGFP	Enhanced green fluorescent protein
EGF-R	Epidermal growth factor receptors
EPO	Eosinophil peroxidase
et al.	Et alii
Fig.	Figure
FOXP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GATA3	GATA-binding protein 3
gB	Glycoprotein B

GI	Gastrointestinal tract
GO	Gene ontology
GrzmB	Granzyme B
h	Hour
HCMV	Human CMV
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency viruses
HSV-1	Herpes simplex virus 1
IBD	Inflammatory bowel disease
IE	Immediate early
IECs	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ILCs	Innate lymphoid cells
ILFs	Isolated lymphoid follicles
i.p.	Intraperitoneal
ISCs	Intestinal stem cells
ISGs	IFN-stimulated genes
iTregs	Inducible Tregs
JAK/STAT	Janus kinase/signal transducer and activator of transcription
kDa	Kilo Dalton
LANUV	Office of nature, environment and consumer protection
L gene	Late gene
Lgr5	Leucine-rich repeat–containing G protein–coupled receptor 5
LPLs	Lamina propria lymphocytes
LPS	Lipopolysaccharides
m	Meter
M cell	Microfold cell
MCFAs	Medium chain length fatty acids
MCMV	Mouse CMV
MCP-1	Monocyte chemotactic protein-1
MEFs	Murine embryonic fibroblasts
mg	Milligram

MHC	Major-histocompatibility-complex
min	Minute
MIT	Massachusetts Institute of Technology
ml	Millilitre
mLN	Mesenteric lymph nodes
mm	Millimetre
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MSCs	Mesenchymal stem cells
MW	Molecular weight
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NSCs	Neural stem cells
nTregs	Natural Tregs
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PFU	Plaque forming unit
PPs	Peyer's patches
pro-EGF	Pro-epidermal growth factor
PRRs	Pattern recognition receptors
RIG-1	Retinoic acid-inducible gene 1
RLRs	RIG-1-like receptors
RORγt	Retinoic acid receptor-related orphan receptor gamma-T
ROS	Reactive oxygen species
SEM	Standard error of the mean
SI	Small intestine
TA	Transit-amplifying
Tab.	Table
T-bet	T-box transcription factor
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th cell	T helper cell

TNF	Tumor necrosis factor
TNFR	TNF receptor
TLRs	Toll-like receptors
Tregs	Regulatory T cells
UC	Ulcerative colitis
WT	Wild type
qPCR	Quantitative polymerase chain reaction
ZO	Zonula Occludens family protein
µg	Microgram
µl	Micro litre
µm	Micrometre

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6.4. Acknowledgments

Die Daten sind in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

6.5. Curriculum vitae

Die Daten sind in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Publications

Le-Trilling VTK#, Ebel JF#, Baier F, Wohlgemuth K, **Pfeifer KR**, Mookhoek A, Krebs P, Determann M, Katschinski B, Adamczyk A, Lange E, Klopfleisch R, Lange CM, Sokolova V, Trilling M, Westendorf AM. (2023). Acute cytomegalovirus infection modulates the intestinal microbiota and targets intestinal epithelial cells. *European journal of immunology*, 53(2), 2249940

Conference contributions

11/2023	**22th Day of Research of the Medical Faculty, University Hospital Essen, Germany** 'Cytomegalovirus infection exacerbates intestinal inflammation in colitis and impairs the subsequent recovery in mice' (Poster)
09/2023	**Joint Meeting of SFI and DGFI, Strasbourg, France** 'Cytomegalovirus infection aggravates intestinal inflammation during colitis and impairs the convalescence of the intestinal niche in mice' (Poster)
08/2023	**Annual Retreat of the Research Training Group 1949, Essen, Germany** 'Another villain in the gut: Cytomegalovirus infection aggravates intestinal inflammation during colitis' (Talk)
11/2022	**21th Day of Research of the Medical Faculty, University Hospital Essen, Germany** 'Cytomegalovirus infection alters intestinal homeostasis and fosters inflammation during colitis in mice' (Poster)
10/2022	**Annual Retreat of the Research Training Group 1949, Essen, Germany** 'Intestinal CMV infection: a risk factor for inflammatory bowel disease?' (Talk)
09/2022	**Joint Meeting of DGfI and ÖGAI, Hannover, Germany** 'Cytomegalovirus infection interferes with intestinal homeostasis and affects the course of colitis in mice' (Poster)

11/2021	**21th Day of Research of the Medical Faculty, University Hospital Essen, Germany** 'Impact of cytomegalovirus infection on the induction and course of colitis in mice' (Virtual Talk)
07/2021	**Annual Retreat of the Research Training Group 1949, Essen, Germany** 'Impact of cytomegalovirus infection on the induction and course of colitis' (Talk)

6.6. Declarations

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „*Cytomegalovirus modulates acute colitis and impairs the resolution of inflammatory processes*“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Kai Robin Pfeifer befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

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