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Interferons in intestinal and airway epithelia: regulation of their synthesis and effects on stem cell growth

Verfasser

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Abstract

Intestinal epithelial cells have evolved to coexist with the rich microflora of the gut lumen. Multiple mechanisms ensure proper reaction to microorganisms – epithelium is usually unresponsive to commensal but can react to pathogenic bacteria. Epithelial cells are the "sensors" of the inside world of the gut lumen. Release of interferons is one of the regulatory mechanisms directing the immune response to pathogens and the inflammatory reaction to epithelial injury. Overreaction to bacteria due to epithelium damage e.g. during the inflammatory bowel disease (IBD), may lead to overstimulation of the immune system and progressing destruction of the intestinal epithelial layer. Earlier studies in Drosophila fruitfly have shown that damage of the intestinal mucosa mobilizes the Jak/Stat signalling pathway which stimulates intestinal epithelial stem cells to regenerate the gut epithelium. Interferon receptors utilize Jak/Stat signalling and mutations in this pathway are linked to IBD in humans. We were interested to investigate the role of interferons in intestinal epithelium regeneration in mouse. We found that mouse colonic epithelial cells CMT-93 produce type I and type III interferon mRNA in response to poly(I:C) - a dsRNA analogue - and to Listeria infection. Interferon beta was found to inhibit CMT-93 proliferation. When comparing type I interferon receptor-deficient (IFNAR1-/-) and wildtype primary mouse intestinal epithelial cells, we found slight differences in their proliferation rates in vitro. Epithelial cells from the small intestine had increased Type I and III interferon mRNA in response to intraoral Listeria but not to Salmonella infections. We have also discovered that *lung* epithelial cells upregulate type I and III interferons in response to coinfection with Influenza virus and *Legionella pneumophila* but not to Legionella infection alone. Finally we have developed a method for short term culture of primary intestinal epithelial cells that we intend to use for future experiments. Although the differences in proliferation between IFNAR1-/- and wildtype primary intestinal epithelial cells are minor, there might be a contributing fact of interferons, as proliferation regulators, to IBD development.

Zusammenfassung

Darmepithelzellen leben im ständigen Kontakt mit der Darmmikroflora. Ihre Reaktion auf pathogene Mikroorganismen unterscheidet sich von der auf kommensale Bakterien. Diese Epithelzellen funktionieren wie Sensoren des Darmlumens. Freisetzung von Interferonen ist dabei einer der Wege auf das antimikrobielle Immunsystem Einfluss zu nehmen. Zu starke Reaktionen auf Bakterien, die durch Schädigung des Epithels während chronisch-entzündlicher Erkrankung (IBD) verursacht werden, führen zur Überreaktion des Immunsystems und zu fortschreitender Schädigung des Epithels. Experimente an Drosophila-Fliegen zeigten, dass im beschädigten Darm der Jak/Stat Signaltransduktionsweg die Stammzellen des Darmepithels stimuliert damit diese die Epithelschicht schnell regenerieren können. Interferon Rezeptoren mobilisieren Jak-Stat Signaltransduktion. Mutationen der im Interferon-Signaltransduktionsweg eingesetzten Jak/Stat Moleküle, wurden mit IBD in Zusammenhang gebracht. Hier untersuchten wir die Rolle der Interferone bei der Darmregeneration in einem Mausmodell. Wir haben festgestellt, dass CMT-93 Maus-Kolonepithelzellen als Antwort auf poly(I:C) - ein dsRNA Analogon - und Listerien-Infektion Typ I und Typ III Interferone produzieren. Interferon-beta Stimulation senkte die Proliferationsrate von CMT-93 Zellen. Zwischen Mäusen ohne Typ I Interferon Rezeptor (IFNAR1-/-) und C57BL/6 Wildtyp-Mäusen haben wir *kleine* Unterschiede in Proliferationsraten von Primärdarmepithelzellen ermittelt. Dünndarm-Epithelzellen exprimierten auch mehr Typ I und III Interferon-mRNA nach Infektion mit Listerien, nicht aber mit Salmonellen. Wir haben auch entdeckt, dass Typ I und III Interferone nach Koinfektion mit Influenza Virus und Legionellen in Lungenepithelzellen produziert wurden, nicht aber nach Infektion mit Legionella alleine. Für weiterführende Studien wurde eine Methode zur kurzfristigen Kultur von primären Darmepithelzellen entwickelt. Obwohl die Unterschiede in der Proliferation zwischen Epithelzellen aus IFNAR1-/- und Wildtyp-Mäusen gering waren, es ist möglicht dass Interferone zu IBD-Entwicklung beitragen indem sie die Proliferationsraten von Darm-Stammzellen beeinflussen.

Contents

Introduction

First to see and first to react – the innate immune system

The innate immune system is the host's primary defense mechanism against a plethora of invading organisms. It can recognize pathogens and use cell mediated or humoral immunity to eliminate them. Furthermore it alerts the cells of the adaptive immune system, T and B lymphocytes, that carry specific antigen receptors and perform various functions. Lymphocytes can kill infected cells directly or indirectly by providing help to the innate immune system - activating phagocytic cells and natural killer (NK) cells. Lymphocytes also produce antibodies that increase the visibility of the pathogens and neutralize them.

To combat specific infections and to keep the immune system in shape cells need a way to convey their messages to a specific audience, i.e. target cells. Cell-cell contacts are often used for local signalling e.g. during lymphocyte maturation and on-site activities of adaptive immune response, but to mediate long-range signals, cells secrete signalling molecules called cytokines that can stimulate proliferation of specific cells, shift the flavour of immune responses or bring the response to a halt once the pathogen has been eradicated. Infected cells produce cytokines that signal the immune system about infection and can also induce a protective state in the neighbouring cells. One class of such molecules are interferons (IFNs), named for their ability to interfere with virus replication in cells that have already been exposed to inactivated virus (Isaacs & Lindenmann 1957). They induce an antiviral state in the infected and neighbouring cells (Fig. 1) by inducing protein kinase R (PKR), 2'5'-oligoadenylate synthetase (2'5'-OAS), RNases, Mx proteins and other proteins that lead to inhibition of virus transcription, translation and virus particle assembly, lead to viral RNA degradation etc.. Interferons act also on non-infected cells - they activate players of the innate immune system e.g. NK cells and macrophages, to destroy infected and tumor cells. Interferons affect adaptive immune cells either *directly*, by stimulating Th1 cell development that promotes destruction of infected cells by macrophages, or *indirectly*, by increasing presentation of viral antigens via upregulation of class I and class II MHC molecules on the infected cells.

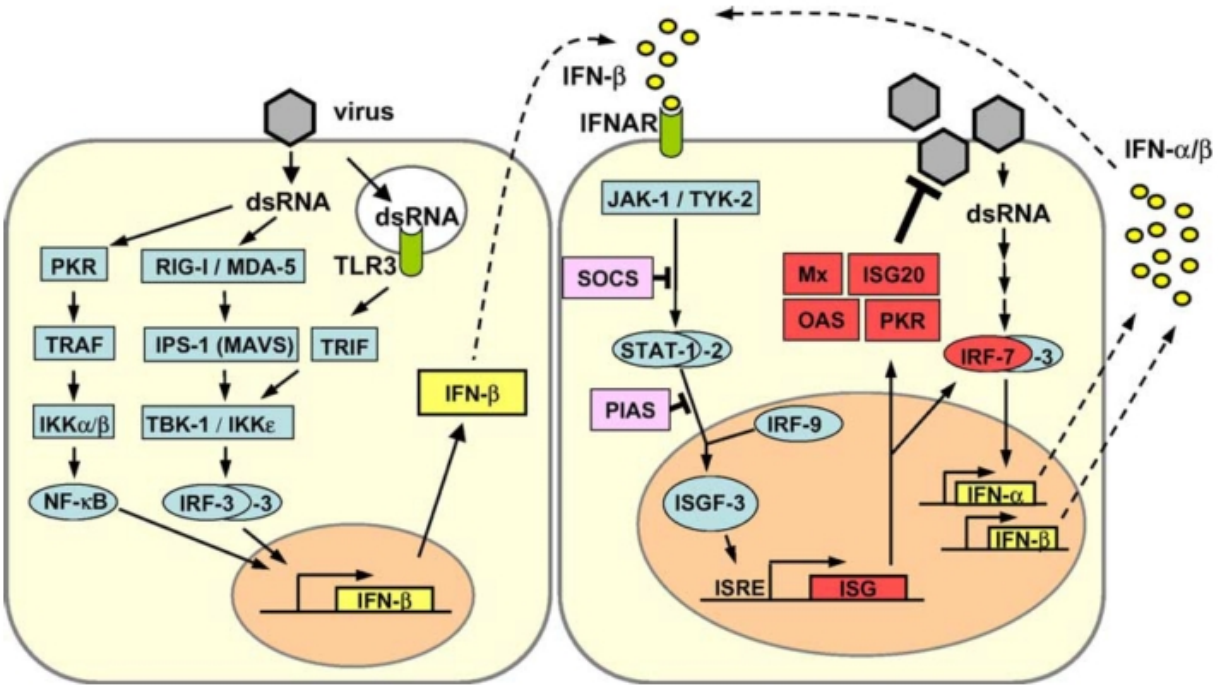


Fig. 1. A virus-infected cell produces Type I Interferons and alerts neighbouring cells. Some of the cytoplasmic PRRs (Rig-I, Mda-5, PKR) are depicted here and are referred to in the text (O. Haller et al. 2006).

Sensing pathogens

Recognition of the pathogen is a first step required for the immune response. Antigen sensing is performed either by soluble factors, like antibodies or the complement system present in blood and mucus, or by receptors on the cell surface, in the cell cytoplasm or in phagocytic vesicles, formed after the pathogen has been engulfed. Cells not belonging to the immune system and cells of the innate immune system recognize specific molecules by pathogen associated molecular pattern (PAMP) recognition receptors (PRRs), that evolved to recognize a spectrum of pathogen molecules that are both essential for the pathogen i.e. cannot be lost to prevent immune system recognition, and shared by multiple species of pathogens. Such antigens can be displayed either on the surface of the pathogen, like peptidoglycan, lipopolysaccharide (LPS) and flagellin on the bacteria, mannans on the cell walls of fungi, envelope proteins of viruses, or the antigens can be sensed by the PRRs once the pathogen has been either lysed in a lysosome or, as in case of viruses, has been exposed in the host cytoplasm. Nucleic acids are a good example for such antigens – they are both essential to the pathogen and are

present in all pathogenic organisms. They are recognized by cytoplasmic sensors and by *endosomal* toll like receptors (TLRs) via their Leucine-Rich Repeat domains (LRR) (Fig. 2). Both single-stranded (ss) and double-stranded (ds) RNA and also DNA molecules are detected in order to cover a broad spectrum of pathogens. In endosomes, TLR-3 recognizes dsRNA, TLR-7 and -8 – ssRNA (TLR-8 is non-functional in mice), and TLR-9 recognizes CpG DNA, i.e. unmethylated CpG-repeats that are present in higher amount in bacteria than in animals. Once activated, Toll Like Receptors dimerize and associate with adapter molecules (MyD88/MAL, TRIF/TRAM) via the cytoplasmic TIR homology domain. Adapters then interact via their Death domain with downstream signalling kinases IRAK1/4, TAK1, IKK and TBK1 that lead to activation of AP1 and NFkB transcription factors and production of pro-inflammatory cytokines and to IRF3, IRF5 and IRF7 transcription factor activation that results in production of Type-I interferons (Fig. 3, reviewed in (West et al. 2006)).

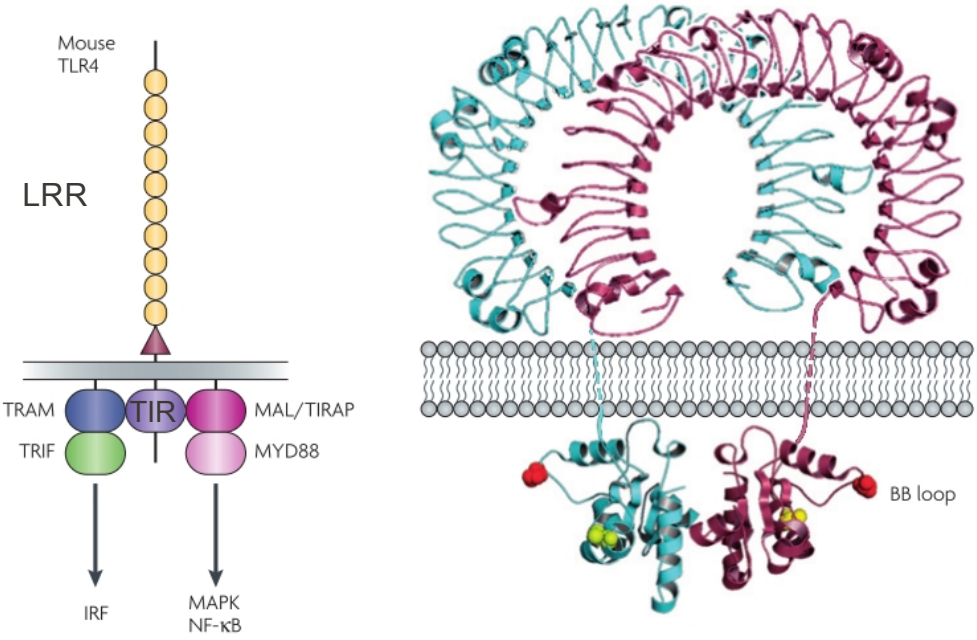


Fig. 2. TLR4 as an example of Toll Like Receptor PRRs, contains the extracellular pathogen molecule sensing Leucine-Rich Repeat domain LRR and intracellular Toll/Interleukin-1 receptor domain (TIR) that interacts with adapter proteins once TLR is activated and dimerized. LRR forms a ring like structure (shown right, structure based on human TLR3 and TLR2); modified from (Leulier & Lemaitre 2008).

Cytoplasmic PRRs are useful to detect pathogens living inside of the cell and include RNA and DNA sensing proteins and also NLR receptors. They enable every cell to induce pro-inflammatory cytokines

as a response to infections and alert both the immune system and neighbouring cells about the infection. Nucleotide-binding oligomerization domain (NOD)-like receptors, NLRs, contain a leucine-rich repeat domain (LRR), just like TLRs, and a caspase activation and recruitment domain, CARD, that is necessary for the recruitment of downstream effectors that activate pro-inflammatory NF-kB signalling. Whereas NLRs recognize peptidoglycan components of Gram-positive and Gram-negative bacteria, bacterial DNA and some stress-related molecules, other cell proteins like RNA helicases Rig-I and Mda5, and DNA sensing proteins, like DAI, are important for anti-viral defense once viral nucleic acids have entered the cytoplasm and are not available for endosomal TLR recognition. Rig-I and Mda5 are involved in recognition of double-stranded *RNA* molecules and lead to the activation of TBK1/IKK-i pathway and subsequent pro-inflammatory cytokine production. Cytoplasmic *DNA* receptors like DAI, IFI16, RNA polymerase III or AIM2 lead to production of interferons via the TBK1-IRF3 pathway, activation of pro-inflammatory cytokines via NFkB and to production of IL1 and IL18 that leads to Th and B cell activation and to activation of macrophages via IFN-γ ((Akira et al. 2006) (Proell et al. 2008) (Uematsu & Fujimoto 2010) (Unterholzner et al. 2010)).

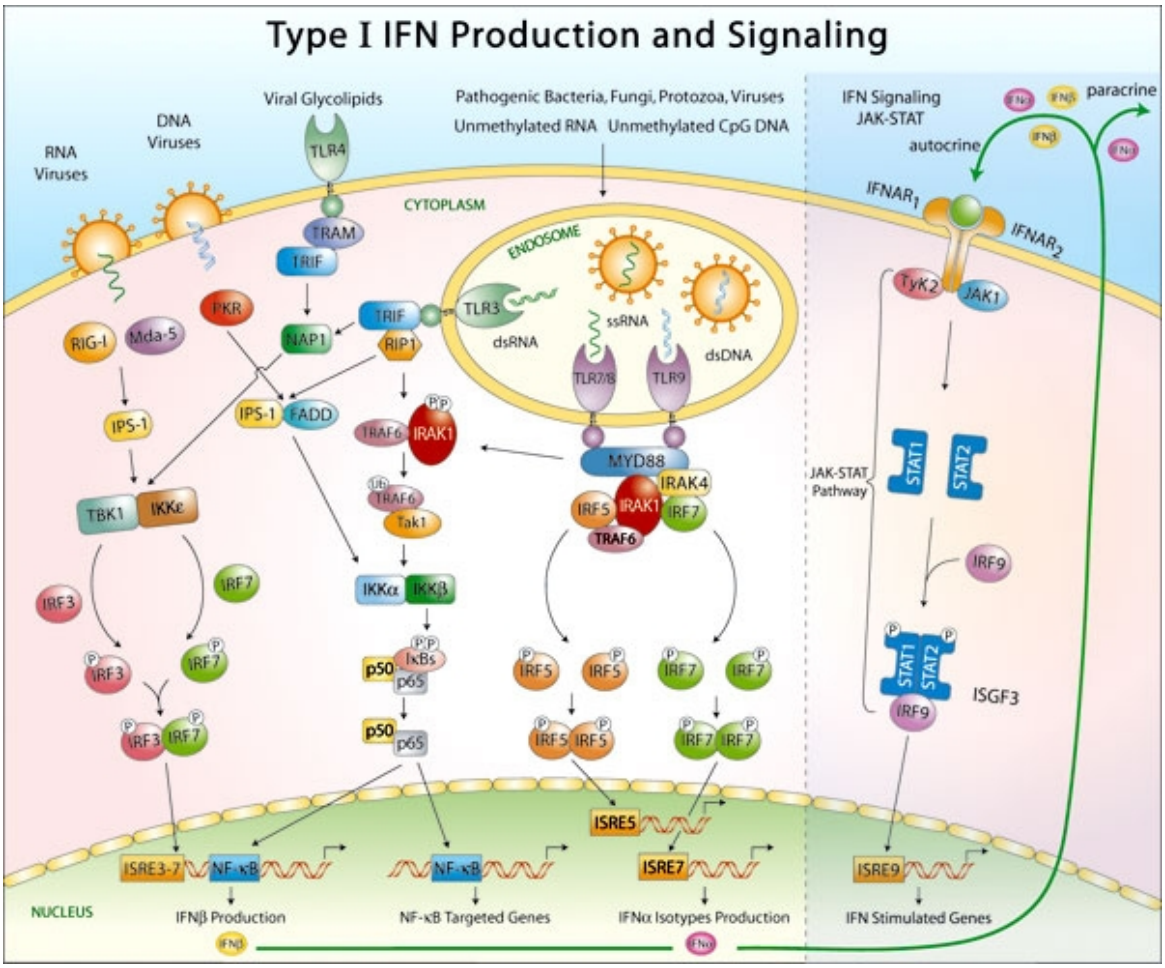


Fig. 3. PAMP-receptors lead to production of pro-inflammatory cytokines, including interferons. Source: http://www.invivogen.com/ressource.php?ID=20

Interferons interfere with infections and with cell cycle

As previously described, interferons (IFNs) are a major class of cytokines that are induced upon infection. They serve as a protective armour that is worn against pathogens by different cells of the body, and as a bright banner that raises innate and adaptive immunity to arms. Most cells of the body induce IFNs via *intracellular* PAMP recognition receptors, whereas specialized IFN-producing plasmacytoid dendritic cells (pDCs) stimulate induction of IFNs via TLRs (Fig. 1, Fig. 3). Once secreted, IFNs bind to neighbouring cells and signal via the Jak/Stat pathway. Upon IFN binding, its receptors dimerize and activate receptor-bound tyrosine kinases of the Jak family (Jak1, -2, -3 and Tyk2), that phosphorylate IFN receptors. This stimulates binding of signal transducer and activator of transcription molecules (STATs) via their SH2 domain to receptors and exposes STATs to Jak-mediated phosphorylation. Once phosphorylated, STATs dimerize and translocate to the nucleus in order to stimulate interferon responsive genes marked by specific regulatory sequences ISRE (type I IFN targets) and GAS (type II IFN targets) in their enhancer regions (Fig. 4).

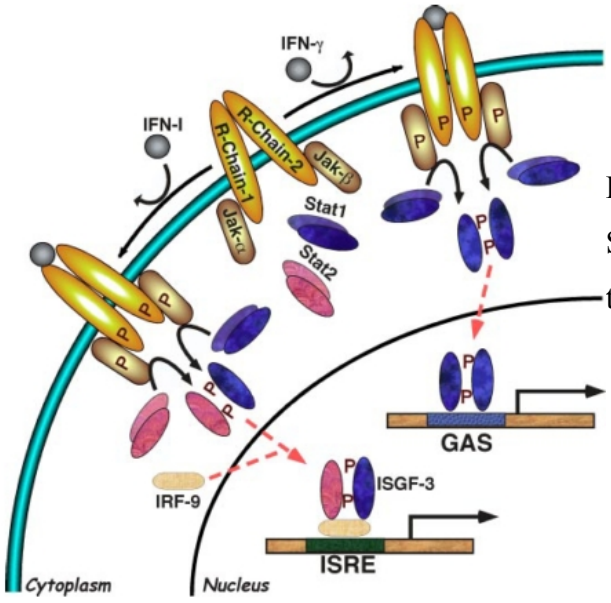


Fig. 4. Type I and Type II Interferon signalling with STATs serving both as sensors of information and as transcription factors; from (Schindler et al. 2007)

Interferons not only induce an anti-viral state in the cell but also activate its *pro-apoptotic* program, possibly via altering p53 expression and via MAVS, common adapter of Rig-I and Mda5 localized on

the mitochondrial membrane. IFNs have also *non cell-autonomous functions* that lead to death of infected cells - they stimulate cells of the innate and adaptive immune system, NK and cytotoxic T cells (CTLs), to kill infected cells. Furthermore CTLs have a more favourable environment for clonal expansion in type I IFN presence and IFN-recieving DCs are more active in cross presentation (Stetson & Ruslan Medzhitov 2006).

Interferons were shown to alter the *cell cycle*. Interferon treatment of transformed cells induces G0 cycle arrest via IFN-induced genes like 2´,5´-OAS, OAS-dependent RNaseL, PKR and IRF1. OAS produces short 2'-5' phosphodiester-linked adenosine chains from ATP upon sensing dsRNA. This activates endonuclease RNase L that cleaves viral RNA but also host mRNA and rRNA, slowing translation and, as a result, also the cell cycle. Produced RNA fragments are sensed again by cytoplasmic sensors stimulating IFN production and also may trigger RNA damage stress response, leading to cell apoptosis (Silverman 2007). Interferons can inhibit cell cycle dependent kinases (Cdk-s) by induction of Cdk-inhibitors, e.g. Cdk inhibitor p21 is under transcriptional control of STAT1 (Chin et al. 1996). Furthermore, IFNs regulate tumor suppressors and oncogenes, e.g retinoblastoma tumor suppressor (Rb) protein gets activated by hypophosphorylation and c-myc oncogene synthesis is inhibited. IFNs can interfere with the IL2 signalling pathway and can inhibit IL2-dependent clonal expansion of T cells (reviewed in (Sangfelt et al. 2000)). It has also been shown, that the newly discovered type III IFNs (IFN-λ, IFN-λ) also have an anti-proliferative effect on intestinal epithelial cell lines (Brand et al. 2005).

Type III Interferons and epithelia

Type I interferons can be produced by different cell types in response to PAMPs, but are synthesized in high amounts by plasmacytoid dendritic cells (Y.-J. Liu 2005). Production of type II interferon, IFN-γ, is restricted to NK, Th1 activated DCs and macrophages and has a pronounced macrophage-activating function (Decker et al. 2002). Type III IFNs, also called IFN-λ or IL28/29, are genetically and structurally close to IL10. There are three isoforms of type III IFNs in humans, IFN-λ 1, 2 and 3, whereas in mice IFN-λ 1 is a pseudogene. Type III interferons bind to the receptor consisting of IL10R-beta and IL28R alpha subunits but its downstream signalling pathway is shared with Type I interferons (Fig. 5) – activation of STAT1 and 2, interaction with IRF9, expression of antiviral proteins and

activation of MAPK kinase pathway. While having the same target genes, type I and type III IFNs have still been preserved throughout evolution. IFN-λ apparently plays a role in epithelial cells of mucosal surfaces, that respond stronger to it than other tissues due to higher IFN-λ receptor levels, and may provide an additional boost to these epithelial cells that are both the barrier against and the sensor of pathogens. Type III IFNs are produced by epithelial cells, monocyte-derived DCs, and pDCs in response to the same stimuli as type I IFNs, e.g. virus infections. A study shows that a dsRNA analogue, poly(I:C) can induce IFN-λ in blood mononuclear cells (Sheppard et al. 2003), possibly through TLR3. Receptors to IFN-λ are highly expressed in epithelia of lungs and intestine and in keratinocytes and make the host resistant to some RNA viruses (Sommereyns et al. 2008)(Mordstein et al. 2010)(Pulverer et al. 2010). For that reason, during our studies we also investigated whether intestinal and lung epithelial cells produce IFN-λ.

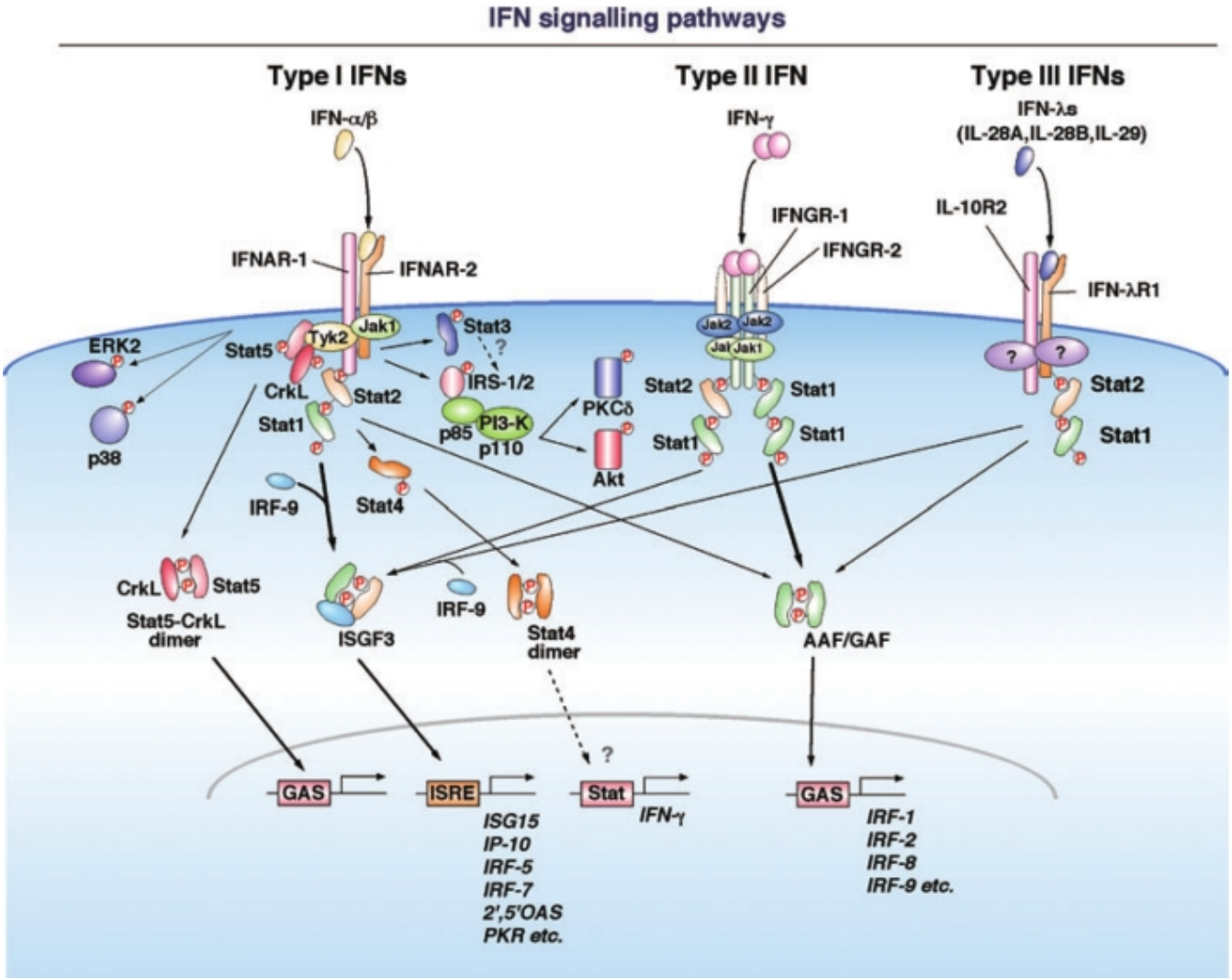


Fig. 5. Three types of interferons use different receptors but converge on shared downstream signalling molecules. From (Takaoka & Yanai 2006).

Why investigate gut epithelial regeneration – Inflammatory Bowel Disease

Our primary goal was to find out *whether IFNs can alter intestinal epithelial cell proliferation*. The main incentive for such research was that mutations in the IFN signalling pathway were linked in genetic association studies to the development of Inflammatory Bowel Disease (IBD) in humans (Anderson et al. 2009).

IBD encompasses two different diseases, ulcerative colitis (UC) and Crohn's Disease (CD), that although affecting different parts of the intestine, share some common features in their pathogenesis. UC primarily affects the mucosa in rectum, colon and caecum in a continuous fashion while CD is characterized by patchy, transmural tissue destruction in the whole gastrointestinal tract. Growing incidence (Loftus 2004) of IBD led to increased scientific interest and as a result to important insights into what may trigger the development of the disease. Although there is low concordance of IBD in identical twins, arguing that diet/environment, microbiota and state of the host's immune system are important factors, genetic predisposition is still regarded as one of the contributing factors of IBD. Prominent genes linked to IBD include: NOD2 (pathogen recognition), ATG16L1 (autophagy), Tyk2 , Stat3 and IRF5 (IFN-Jak/Stat signalling), MUC2 (mucus production), IL23 receptor and others (Franke et al. 2010). Some pathways are now being linked, e.g. in dendritic cells NOD and ATG16L may act together in order to display intracellular pathogens via class II MHC and to stimulate NFkB-mediated cytokine release (Ramjeet et al. 2010).

Trigger events for IBD are the destruction of the protective epithelial layer accompanied by decrease in antimicrobial proteins, tight junction proteins, Goblet cells and over-stimulation of dendritic cells (DCs) with bacterial antigens derived either from commensals or pathogens. The resulting uncontrolled inflammation of the submucosa leads to recruitment and activation of different T helper populations, NK and NKT cells, monocytes and macrophages - this leads to further epithelial damage and, if untreated, the patient requires surgical resection of the intestine in order to remove affected tissue (reviewed in (Matricon et al. 2010). On early stages, IBD can be treated with immunosuppressants and anti-inflammatory drugs like sulfasalazine and corticosteroids, but 20% of patients are not treatable by

these drugs. Antibiotics and probiotics are given to patients in order to change host microbiota and potentially outcompete pathogens. Other drugs include anti-TNFa antibodies infliximab, adalimumab, that not only block TNF that recruits neutrophils, macrophages and lymphocytes, but also induce apoptosis in lymphocytes and monocytes, block IFN-γ and GMCSF synthesis by T lymphocytes and restore the gut barrier (Lawlor & Moss 2009). However, 30% of patients do not respond to such treatment and 50% of sensitive patients loose responsiveness after one year (Triantafillidis et al. 2011). Further studies are necessary to understand IBD and to develop new therapies.

The aberrant activation of the innate immune system, that is in close contact with the epithelial layer (Fig. 6), is believed to occur at early stages of IBD. Pathogen molecule receptors PRRs, are under close study in models of intestinal inflammation, because their activation leads to upregulation of various cytokines. There are different views on the role of TLRs in IBD development. TLR signalling has been implicated in the release of a proliferation-inducing ligand (APRIL) and thyminee stromal lymphopoietin (TSLP) signalling molecules from IECs, which stimulate synthesis of protective IgA by B cells and prime DCs to stimulate regulatory T cells (reviewed in (Abreu 2010; Wells et al. 2011)). TLR-MyD88 signalling, possibly induced by commensal bacteria, stimulates the production of cytoprotective IL6, TNF and KC-1 cytokines and inhibits the development of spontaneous colitis in IL10 deficient mice (Rakoff-Nahoum et al. 2004). MyD88 signalling induced via TLR4 can lead to upregulation of EREG and AREG that activate epidermal growth factor receptor, EGFR, on the epithelial cells and protect against colitis (Brandl et al. 2010). Apical stimulation of TLR9 in polarized cultured cells leads to production of protective IFN-β and TLR9 deficient mice are more susceptible to DSS-induced colitis (reviewed in (Clavel & D. Haller 2007)). Further studies showed that TLR9 stimulation leads to production of "anti-inflammatory" IFN-β via DNA-PK, IRF1, IRF7 and IRF8 and that IFN alpha receptor deficient mice (IFNAR KO) develop a more severe colitis upon DSS treatment. In order to find out what cell population provides this protective effect adoptive transfer (injection) of immune cells deficient in specific genes was performed. Wild type mice injected with IFNa/b receptor-deficient macrophages and stimulated with TLR9 agonist were not protected against DSS-colitis like mice that received wt macrophages, pointing towards the involvement of macrophages in IBD development (Katakura et al. 2005). Stimulation of TLR3 via subcutaneous injection of double-stranded RNA homologue, poly(I:C), into mice also led to protection against DSS-induced colitis and here IFNs were again regarded as *negative* regulators of colitis (Vijay-Kumar et al. 2007). Transfecting

poly(I:C) into intestinal epithelial cell lines leads to activation of cytoplasmic Rig-I and IPS1 nucleic acid sensors and to production of IFN-β (Hirata et al. 2010). Fukata et al. (2010) show, however, that activation of PRRs may lead to pathologies - TLR4 mediated signalling leads to a strong inflammatory response and to colitis-induced tumorigenesis. Local delivery of IFN-β, one of the target genes of TLR signalling, by IFN-β-producing Lactobacilli increases sensitivity to DSS-induced colitis by upregulating TNF-alpha, IFN-γ, IL17A (McFarland et al. 2011). However, mice deficient in IRF1, a transcription factor responsible for IFN-β and IFN-β-inducible genes upregulation, develop worse DSS-induced colitis and affected cells show less caspase activity and antigen presentation genes (Mannick et al. 2005). This was unexpected, because IRF1 *deficiency* usually plays a protective role in other inflammatory disease models like diabetes, encephalomyelitis and arthritis. It seems that the degree of TLR activation, sites of stimulation (systemic vs. local) and, possibly, the location of the TLR stimulus on the polarized epithelial cells may influence whether TLR signalling is protective or not. The exact role of IFNs in immune and non-immune system cells involved in IBD development still remains to be defined.

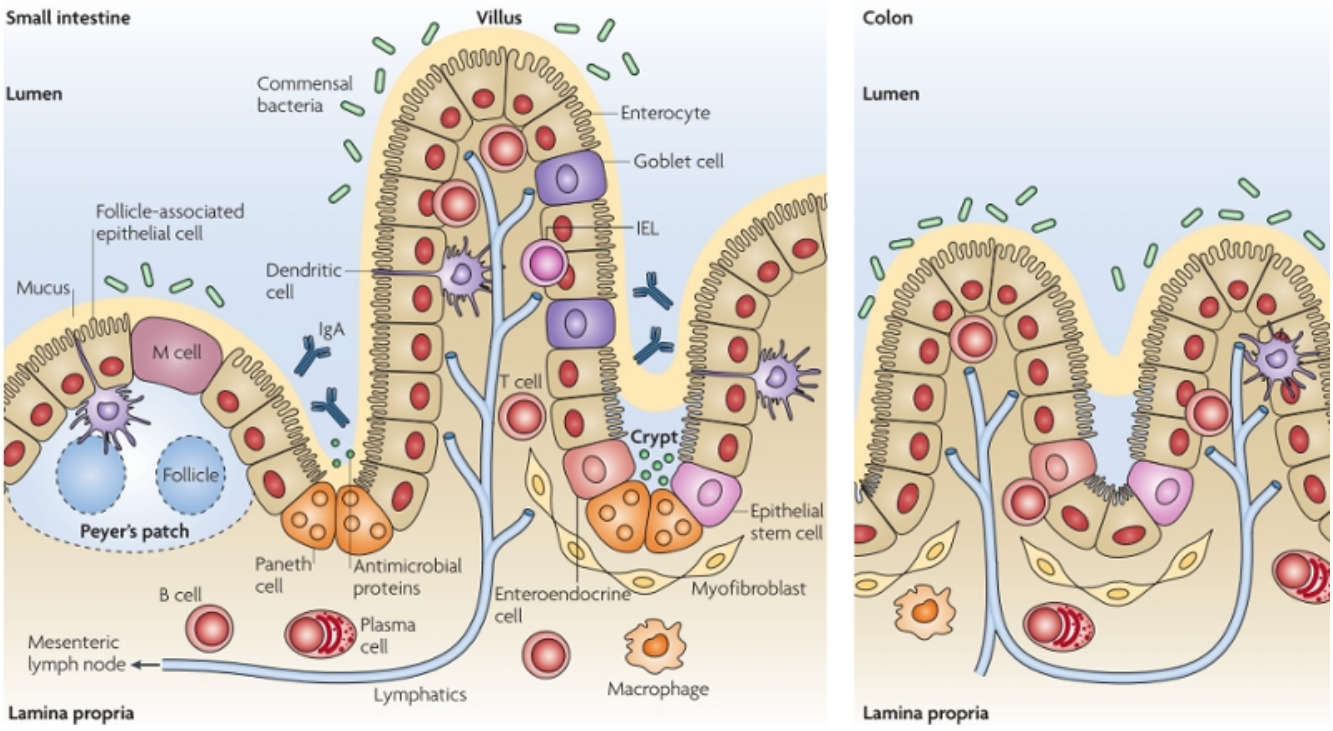


Fig. 6. Intestinal epithelial layer and immune system cells provide a barrier against commensal and pathogenic bacteria. IEL: intraepithelial lymphocyte. From (Abreu 2010)

Mouse models are indispensable for studying disease development, associated risk factors and potential treatments. There are several models to induce IBD in mice. Disrupting epithelial layer by various chemicals may give us the answer whether the defects in the epithelium are causative in case of IBD or arise due to the already progressing disease. One of the commonly used experimental model utilizes dextran sulfate sodium (DSS), which is a polysaccharide that, if given to drinking water of mice, is destroying the mucus layer thus enabling bacteria to have direct contact with intestinal epithelial cells (IECs) and dendritic cells. DSS is used to start an acute inflammation reminiscent of UC and helps to investigate the role of the epithelium as a barrier as well as the involvement of the innate immune system in colitis development. Prolonged exposure to DSS can also lead to chronic colitis and, if combined with carcinogen azoxymethane, will lead to colon cancer development. In mice, prominent, but not unique, features of DSS-induced colitis include loss of body weight due to bloody diarrhea, shortening of the colon, high levels of myeloperoxidase (MPO) i.e. high amounts of neutrophils recruited to affected tissues, increased mucosal permeability and increased inflammatory mediators like IL1b, IL6, IL12 and IFN-γ in the distal colon (Yan et al. 2009). There are other mouse models of colitis, including haptenizing chemicals, total and tissue-specific gene knockout mice, adoptive transfer of T cell populations etc. (Jurjus et al. 2004; A. Mizoguchi & E. Mizoguchi 2008; Wirtz & Neurath 2007).

Intestinal epithelium and its stem cells

The epithelial barrier of the intestine has high turnover rate and consists of various types of differentiated cells. Intestinal epithelial stem cells, ISCs, are located at the bottom of the crypts of the intestinal epithelium and divide asymmetrically to give rise to transient amplifying cells that proliferate and differentiate into absorptive enterocytes, secretory enteroendocrine cells and mucus-producing Goblet cells. ISCs also give rise to Paneth cells, that produce antimirobial peptides and thus keep the crypt free of microbes. In search of localizing ISCs, several markers have been suggested, including musashi1, DCAMKL1, p-PTEN and others (Samuel et al. 2009). BrdU or H3-thymidine label retaining cells, LRCs, positioned at +4 position in the crypts, above Paneth cells, are considered to be quiescent stem cells and columnar based cells, CBCs, localized at the bottom of the crypt, are thought to be actively dividing stem cells (Fig. 7) (Neal et al. 2010). This “zoned” system of two stem cell populations is thought to protect the quiescent stem cells from accumulating mutations during division

and allowing them to reconstitute only the active stem cell population whereas the active stem cells can rapidly regenerate the epithelial layer during injury (L. Li & Clevers 2010). A G-protein coupled receptor, Lgr5, that lies downstream of Wnt signalling pathway, is now regarded as bona fide active ISC marker and the Lgr5+ cells reside at the crypt base (Barker et al. 2007). Attempts have been made to define the niche that provides Wnt/beta-catenin, EGFR and Delta/Notch proliferation signals for these stem cells, even myofibroblasts were suggested to play a role in the niche (Samuel et al. 2009). In 2009 Sato et al. have defined the conditions for growth of ISCs in an artificial extracellular matrix, Matrigel, that contains laminin, collagen and several growth factors, with addition of Wnt, BMP and EGF stimuli. These cells formed organoids, i.e. clusters of crypts reminiscent of intestinal epithelium in mice, and ISC markers were retained in cells at the base of the crypt. Later, Paneth cells were found to provide a niche for the ISCs to keep their stemness via Wnt, EGF, TGFb, Dll4 (Notch ligand) signalling pathways (T. Sato et al. 2010).

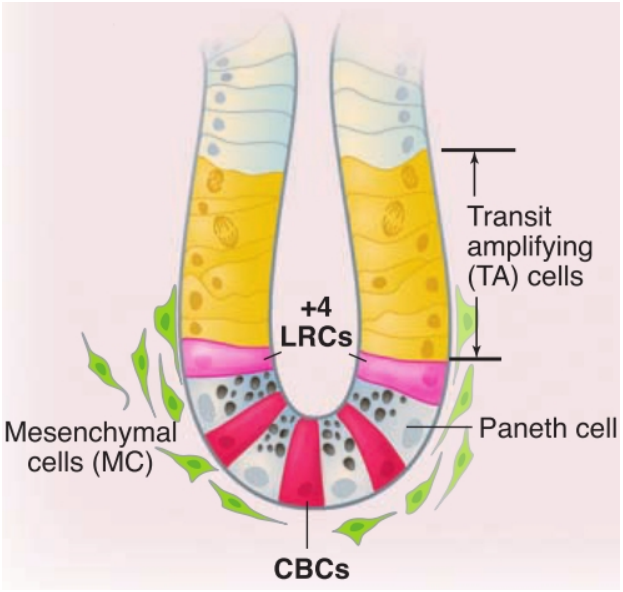


Fig. 7. Intestinal epithelial stem cells reside in crypts and give rise to transit amplifying cells that differentiate into enterocytes, enteroendocrine cells and Goblet cells. +4 LRCs, label retaining cells at position 4 from the bottom of the crypt, are active stem cells, whereas columnar based cells (CBCs) are quiescent stem cells. Paneth cells provide a niche for CBCs supplying the latter with Wnt, Notch and EGFR ligands. From (L. Li & Clevers 2010).

Gut regeneration after infection or after stress can be studied also in a genetically simpler model. Fruit fly Drosophila melanogaster provides a good model for the mammalian gastrointestinal tract – it has somewhat similar intestine compartmentalization and it possesses intestinal epithelial stem cells, that give rise to undifferentiated enteroblasts that differentiate further into absorptive enterocytes and secretory enteroendocrine cells (Pitsouli et al. 2009). From studies done in D. melanogaster, it is known that upon infection or dietary stress, signalling from dying differentiated intestinal epithelial cells via

Jak/Stat pathway (in Drosophila: *domeless* receptor, *hopscotch* kinase and *Stat92E* as signal transducer) via stress cytokines Upd2 and Upd3 acts as a proliferation signal on ISCs and, together with Notch signalling, as a differentiation signal on enteroblasts (Jiang et al. 2009). EGF signalling has also been implicated in gut regeneration (Jiang et al. 2011) and TLR signalling may stimulate ISCs by inducing EGF receptor ligands ampiregulin and prostaglandin E2 (Abreu 2010). Such combined signalling leads to rapid regeneration of the gut epithelium within a few days, if bacteria or stress have been removed. It also correlates with mouse studies, where it has been shown that epithelial damage in DSS-inflicted colitis stimulates the proliferation of ISCs. We wanted to investigate, whether we can address the potential effect of IFNs on the proliferation of ISCs i.e. whether in mice they act similar to the Upd cytokines in the fruit fly.

Flu-Legionella Coinfections and Interferons

Because of our interest in the role of interferons during the infection of epithelia, we also conducted experiments on co-infecting mouse lungs with virus and bacterium (Influenza and Legionella pneumoniae) to find out how flu infection can affect the course of the following Legionella-caused pneumonia. We also performed pilot experiments with intra-oral infections of mice with Gram-negative Salmonella and Gram-positive Listeria bacteria in order to find out whether these bacteria can induce Type III IFNs in gut epithelium.

Common flu-bacteria coinfections include such pathogens as S. pneumoniae, H. influenzae, various Staphylococcus and Streptococcus species (X.-Y. Wang et al. 2011). It has been noted, that, compared to influenza infection alone, combined flu and pneumonia leads to higher mortality, probably due to higher dissemination of bacteria if the lung epithelium is already damaged by virus, and by neutrophil mediated friendly fire, that leads to tissue destruction (DeLeo & Musser 2010). Possible mechanisms of mice being prone to following *bacterial* infections after the viral infection can also be desensitization of macrophages' Toll like receptors (Didierlaurent et al. 2008), downregulation of neutrophil chemoattractants (Shahangian et al. 2009), decrease of phagocytosis of alveolar macrophages via IFN-γ signalling (K. Sun & Metzger 2008), decrease of IL12, a CTL and NK cell activator, released from DCs by viral haemagglutinin (Noone et al. 2005) etc. - the research in this field has to be continued to provide the whole picture of lung sensitization to bacterial infections by preceding exposure to viruses.

Infections with Legionella bacterium can lead to pneumonia, that ranges from mild cough to respiratory failure (Stout & V. L. Yu 1997). Legionella is a genus of Gram-negative bacteria that made its first appearance in 1976 when a large group of people contacted a previously unknown airborne bacterial pathogen that caused pneumonia. Legionella usually lives in amoeba and has evolved mechanisms to live in these phagocytic cells. Thus it is not surprising, that in animals it primarily infects phagocytic alveolar macrophages. It contains Type 2 and Type 4 secretion systems and uses the latter, called Dot/Icm, to inject its effector proteins from the phagosome into the host cells' cytoplasm. This leads to altered vesicle transport of the cell and prevents the phagosome from fusing with lysosomes, creating a protected niche for Legionella called Legionella-Containing Vacuole, LCV. Infected cells respond to infection by recognizing Legionellas' flagellin via cytoplasmic Naip5 inflammasome, by sensing peptidoglycans via Nod1/2 and by detecting RNA via Rig-I and Mda5. This leads to proinflammatory NFkB signalling and to production of IFN-β ((Hubber & Roy 2010) (Newton et al. 2010) (Schuelein et al. 2011) (Vance 2010)).

It has recently been shown, that flu infection leads to a general stress response and to increase of glucocorticoid serum levels that act as immunosuppressants. This leads to reduced IL6, IFN-γ and other chemokines in lung tissue and to lower amount of infiltrating cells of the immune system to the infected tissue. Complications with secondary pulmonary bacterial, e.g. Legionella, infection follow but also *prevention* of lethal immunopathology during such coinfections occurs (Jamieson et al. 2010). With this in mind, we wanted to investigate the effect of flu infection on subsequent Legionella-caused disease development, in particular concentrating on the IFN production by lung epithelial cells.

Aims summary

In this project we were interested in the role of IFNs in epithelial layers. Our main goal was to find out, whether IFNs can affect the regeneration of the epithelial layer by directly influencing the proliferation of the intestinal epithelial stem cells (ISCs). The aim of my thesis was to establish an *in vitro* method for studying the effect of IFN-β on primary intestinal epithelial cell proliferation and also to analyze a mouse colon epithelial cell line for responses to PRR stimulation. We conducted experiments on epithelial cell line to study its potential as a model of gut epithelium responses to pathogen molecules and infections. These studies are a part of a lager project that involves *in vivo* studies of changes in

intestinal epithelium, associated immune system and intestinal microbiota in mice with chemically induced colitis.

In addition, we wanted to investigate the role of IFNs during lung coinfections. We wanted to find out whether there are indicative levels of inflammatory mediators that can explain worse outcome of pulmonary coinfections as compared to infections with either virus or bacterium alone.

Methods overview - *in vitro veritas*

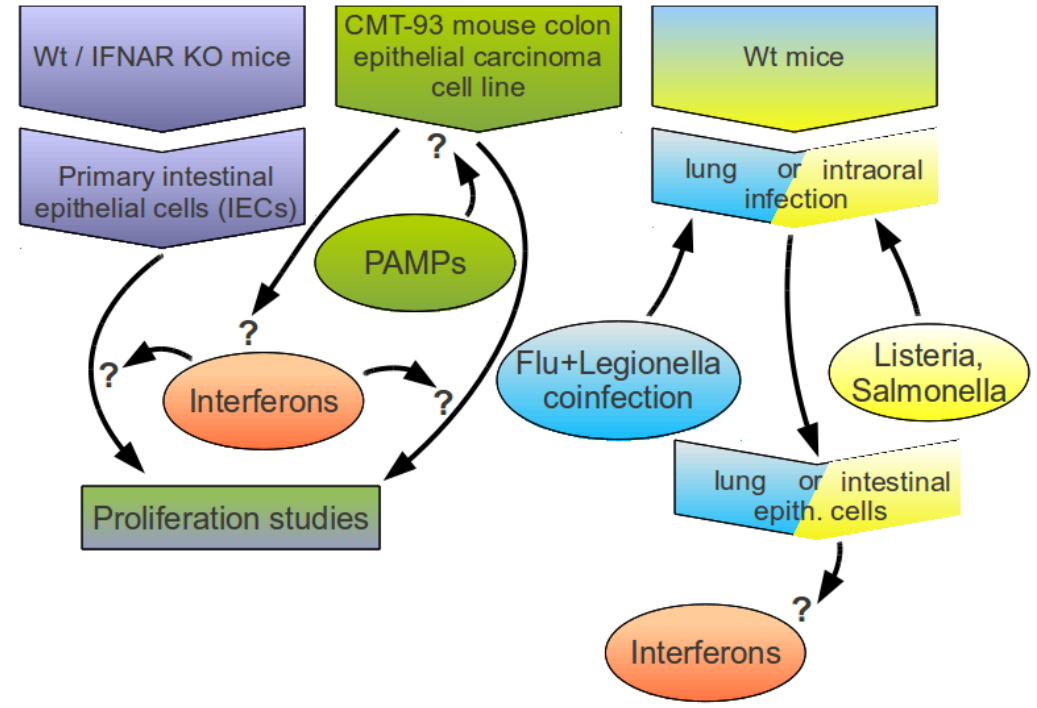
In order to *model* responses of gut epithelium to PRRs we first concentrated on stimulating the mouse colon epithelial carcinoma cell line, CMT-93, with different TLR ligands. To stimulate TLR4 we used lipopolysaccharide, LPS, a molecule containing a set of fatty acid chains, an oligosaccharide and glycan polymers, present on the surface of Gram-negative bacteria. TLR3 was stimulated by polyinosilic-polycytidylic acid, poly(I:C), a double-stranded RNA analogue containing a strand of cytidines paired to the strand of inosines, derivatives of adenines. To stimulate TLR9 we used type C CpG DNA that contains CpG repeats on the phosphorothioate backbone and is a potent inducer of IFN-β in pDC and B cells. Unmethylated CpG sequences are present in bacteria and serve as PAMP, whereas CpG in mammals are mostly methylated and are also present in relatively lower quantity because of CpG suppression, the fact that upon spontaneous deamination, methylated cytidines are converted to thymines that can escape DNA-repair mechanisms and lead to point mutations (CpGs reviewed in (R Medzhitov 2001)). We used poly(deoxyadenylic-deoxythymidylic) acid poly(dA:dT) as a stimulant of intracellular DNA receptors DAI, AIM2, LRRFIP1, and also of dsRNA sensor Rig-I, once poly(dA:dT) is transcribed (Ablasser et al. 2009). We wanted to find out whether CMT-93 cells produce Type I and epithelium-specific Type III interferons in response to TLR stimuli and also whether these IFNs have an effect on CMT-93 cells. To answer the first question, we monitored IFN-β and IFN-λ mRNA induction with qPCR upon TLR stimulation. To address responsiveness to IFNs, we observed the activating phosphorylation of tyrosine on STAT1, a molecule downstream of IFN receptors, upon incubating cells with IFNs. We also tested the effect of IFN-β on the proliferation rate of CMT-93.

To further investigate the question of IFN-β influence on epithelial cells, we isolated gut epithelial

cells, possibly containing intestinal epithelial stem cells, from intestine of either wild type or IFN-alpha receptor knockout mice and were monitoring their proliferation in vitro. We tested several isolation protocols, with and without enzymatic digestion, and considered different growth conditions. Finally we decided to grow intestinal epithelial cells after EDTA isolation without enzymatic digestion in soft agarose/agar culture (modified from Booth & O'Shea (R. I. Freshney & M. G. Freshney 2002)). We seeded mostly single cell suspensions into agarose mixed with growth medium and cultured for more than a month in incubator – we expected that transiently amplifying cells and stem cells would form colonies (for a review about primary IEC culture: (Kaeffer 2002)).

Because primary IECs cultured without additional growth factors (unlike in (T. Sato et al. 2009)) were growing very slowly, we also devised a method for short-term culture of colonic epithelial cells. After trying different coatings of plates, we decided to grow EDTA-isolated crypts (modified from (Bartsch et al. 2004)) on extracellular matrix layer left on the dish after osmotic lysis of endothelial cells. This basement membrane contains collagens (mostly Type III and Type I), proteoglycans (heparin, chondroitin sulphate), laminin, fibronectin and elastin (Gospodarowicz et al. 1984). Isolated IECs attached and even spread out if seeded at lower densities to the endothelial basement membrane and experiments are planned to treat these cultures with IFNs to test for their effect on proliferation.

A)



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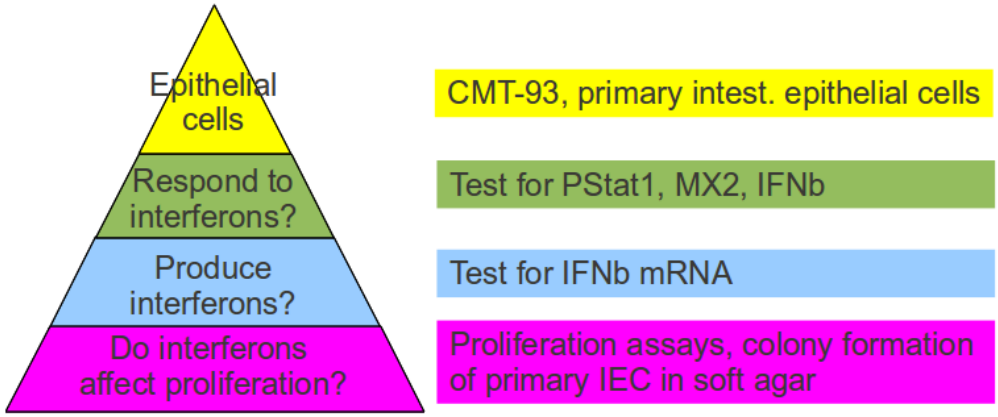


Fig. 8 Overview of three projects (A) of the thesis and methods (B) used to tackle intestinal epithelial responses to interferons.

Results

CMT-93 cells respond to limited TLR stimuli

To investigate the potential of the mouse epithelial colon carcinoma cell line CMT-93 as a model for gut responses, these cells were cultured in presence of LPS to stimulate surface TLR4, poly(I:C) to stimulate endosomal TLR3 or Rig-I/Mda5, CpG to stimulate endosomal TLR9, and poly(dA:dT) to stimulate cytoplasmic DNA receptors and indirectly RNA receptors. Upregulation of IFN-β, antiviral MX2 and IFN-λ mRNA was used as the readout of this experiment. Poly(I:C) and poly(dA:dT) were applied on the cells with and without the transfection reagent that helps these chemicals to cross the cell membrane. CMT-93 cells responded strongly to transfected poly(I:C) – the upregulation of IFN-β and MX2 mRNA was seen 8h and 24h after transfection and upregulation of IFN-λ after 24h (Fig. 9). CMT-93 did not react to variable LPS concentrations and to CpG (Fig. 9). On protein level, CMT-93 cells had increased levels of PStat1 after poly(I:C) transfection indicating the production of IFNs by these cells (Fig. 10, 11). When tested for responsiveness to IFNs, CMT-93 cells responded to IFN-γ and weaker to IFN-β both at 1h and 24h after stimulation (Fig. 11). Different transfection reagents were tested for poly(dA:dT) in order to be sure that it is transfected successfully into CMT-93 cells but still no response was observed (Fig. 11).

Because of high background and low signal of P-Stat1 after poly(I:C) stimulation, we sought to monitor also phosphorylation of Stat2, that is involved in Type I IFN signalling. We observed an unspecific background band in all CMT-93 samples and a weak specific P-Stat2 band only after IFN-β and poly(I:C) stimulation (Fig. 11). Other P-Stat2 detection antibodies from several suppliers did not show better detection (Fig. 12).

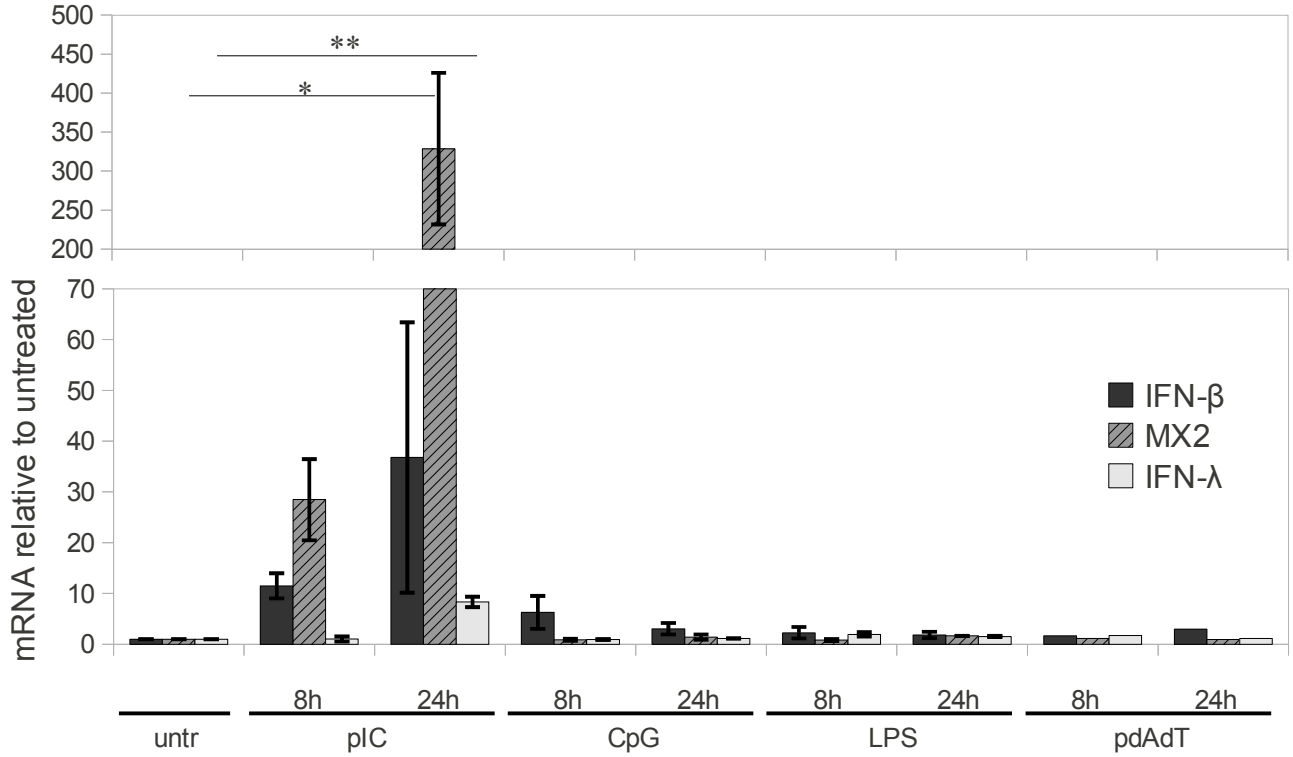


Fig. 9. CMT-93 cells were treated with different PAMPs to stimulate TLRs and cytoplasmic nucleic acid receptors. CMT-93 produce IFN-β, IFN-λ and MX2 mRNA only in response to poly(I:C), that stimulates TLR3 and Mda5. Increase in IFN-β and MX2 may be explained by the autocrine IFN-β loop. Conc.: poly(I:C): 5µg/mL, poly(dA:dT): 5µg/mL, LPS: 100ng/mL, CpG: 10µg/mL (n=3 except poly dAdT: n=2).*: p=0.028, **: p=0.0035

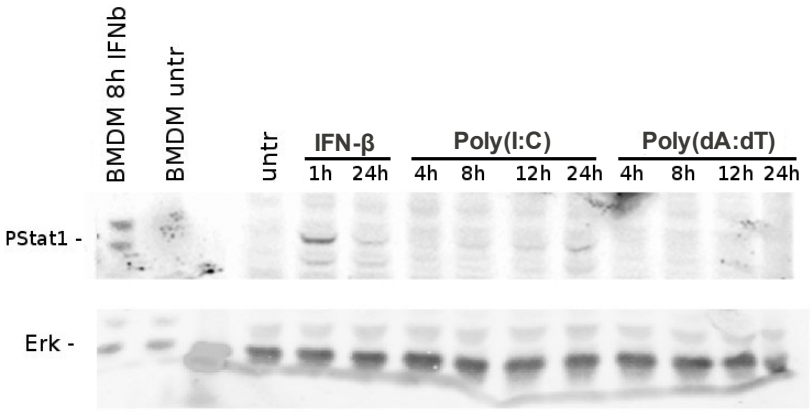


Fig. 10. CMT-93 cells respond to IFN-β and to poly(I:C), as tested by WB against P-(Y701)Stat-1. IFN-β-treated bone marrow-derived macrophages (BMDMs) were used as positive control, Erk as loading control. Conc.: IFN-β: 50U/mL, poly(I:C): 5µg/mL, poly(dA:dT): 5µg/mL

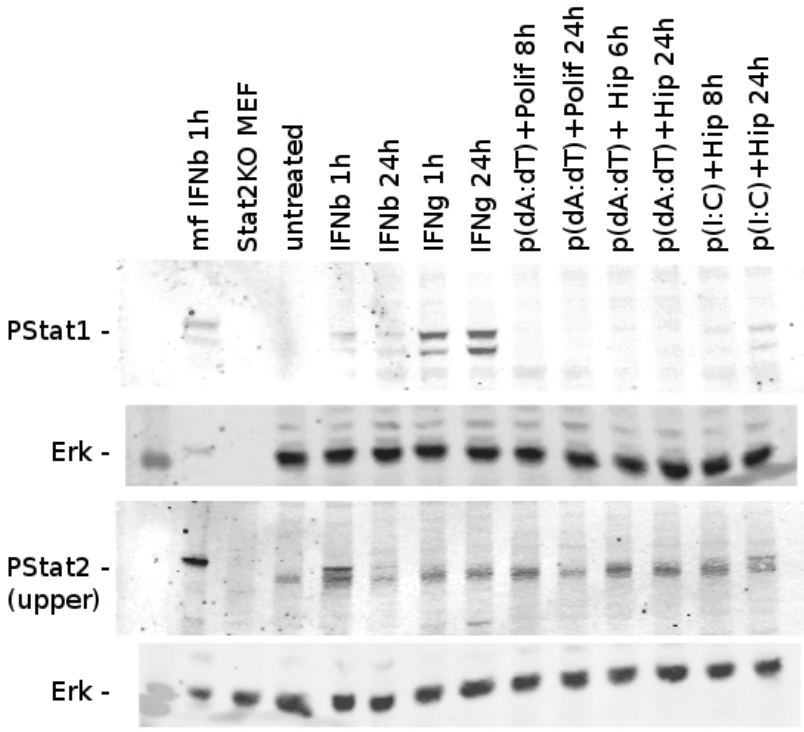


Fig. 11. CMT-93 react to IFN-γ and weakly to IFN-β and poly(I:C), but not to poly(dA:dT) transfected with Polyfect or Hiperfect transfection reagents, as seen by phosphorylation of Stat1. Stat2 is strongly phosphorylated in response to IFN-β 1h after stimulation and also in response to poly(I:C) 24h after transfection. An unspecific band is seen in all CMT-93 samples. IFN-γ: 0.5µg/mL, IFN-β, poly(I:C) and poly(dA:dT) as in Fig. 10.

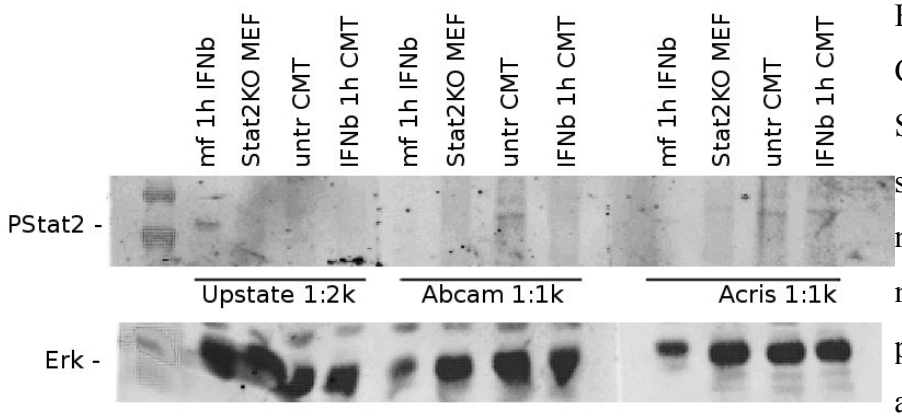


Fig. 12. Detecting P-Stat2 in CMT-93 cells: none of the P-Stat2 antibodies from several suppliers improved signal-to-noise ratio of WB. IFN-β-treated macrophages were used as positive control, Stat2-KO MEFs as negative control.

CMT-93 do not respond to human IFN-λ

After observing the increase in IFN-λ mRNA levels after poly(I:C) stimulation, we wanted to find out whether CMT-93 cells are responsive to IFN-λ the same way as they respond to IFN-β. We decided to screen for P-Stat1, that is shared by both type I and type III Interferon signalling pathway. We have obtained IFN-λ (IL28B) of human origin and were interested whether CMT-93 cells would react to it because Type I interferons are regarded to be species-specific (Sakaguchi et al. 1982) and type II

Interferon protein, IFN-γ, is only 40% homologous between mice and humans (Gray & Goeddel 1983). In our case, human IFN-λ stimulated only the *human* colon cell line LS174T but not *mouse* CMT-93, whereas the situation was reversed for mouse IFN-β (Fig 13A). In order to definitely determine whether these cells are responsive to IFN-λ, it would be necessary to stimulate CMT-93 and LS174T also with mouse IFN-λ, make IFN-λ titrations and validate IFN-λ receptors in CMT-93. On mRNA level, CMT-93 cells also did not respond to IFN-λ (Fig. 13B).

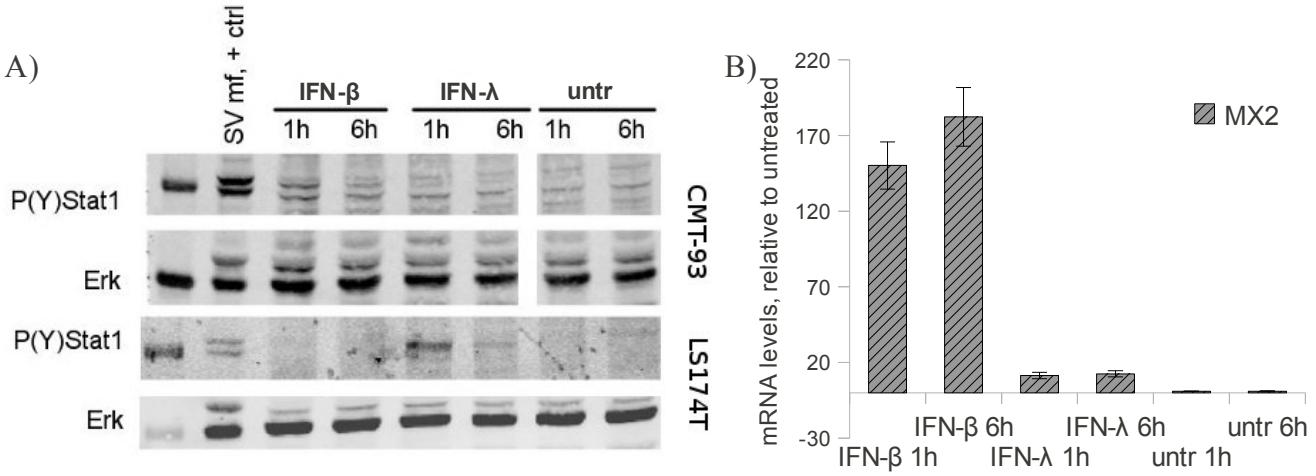


Fig. 13. Interferons act in a species-specific fashion on intestinal epithelial cell lines. When analyzed for Stat1 phosphorylation (A), mouse IFN-β stimulates only the mouse-derived CMT-93 cell line, human IFN-λ stimulates only the human-derived LS174T cell line. On mRNA level, no IFN-responsive MX2 gene upregulation is seen when CMT-93 cells are treated with human IFN-λ (B).

Testing for mucus production by CMT-93 cells

While further studying CMT-93 cells as model of colon epithelial cells, we tested whether CMT-93 cells can produce mucus, that later can be harvested and used to grow intestinal bacteria (Dr. David Berrys' project). We tried washing the potential mucus layer with PBS from CMT-93 cells grown for several weeks without splitting. Then we analyzed proteins on SDS-PAGE gel and tried to see bands appearing at >200 kDa. To stain glycosylated proteins, including mucins, we tried oxidation with periodic acid followed by Schiffs reagent staining accompanied by silver nitrate staining for better sensitivity. This protocol failed to detect any specific bands (Fig. 14). However, at the time of performing these experiments, we also did not have a positive control, so a problem with the staining procedure is also possible. As the next step, we stained the gel with Coomassie, cut out bands of

interest and sent to mass spectrometry analysis (15). We failed to detect mucin proteins and could not use CMT-93 for mucus production.

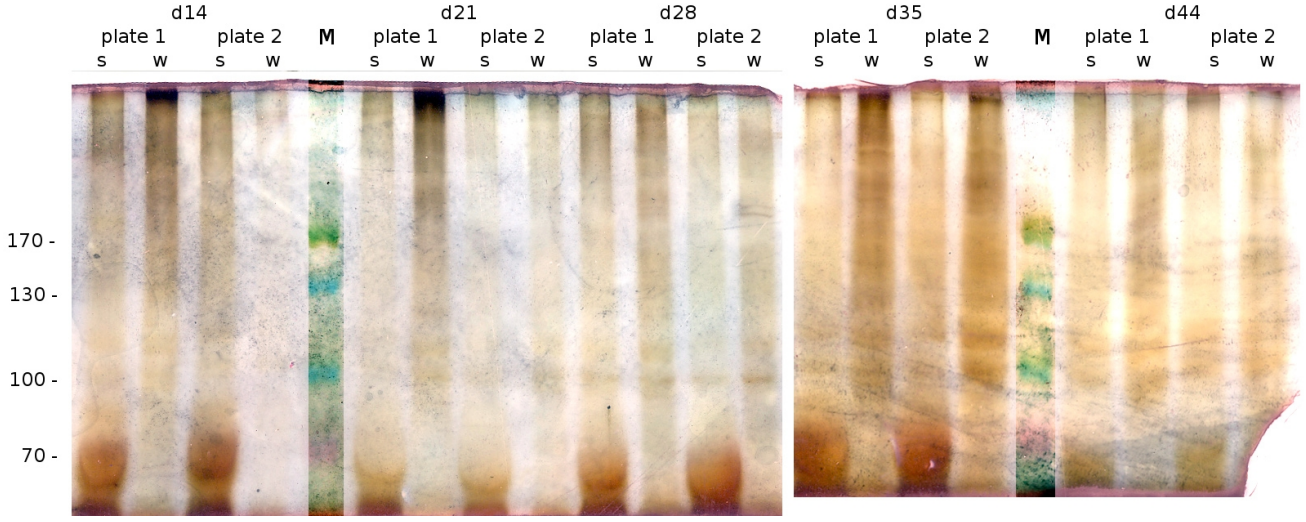


Fig. 14. PAS-silver nitrate staining does not reveal mucin proteins. dXX: amount of days CMT-93 were in culture without splitting; s: cell culture medium supernatant; w: PBS wash of cell layer, M: weight marker

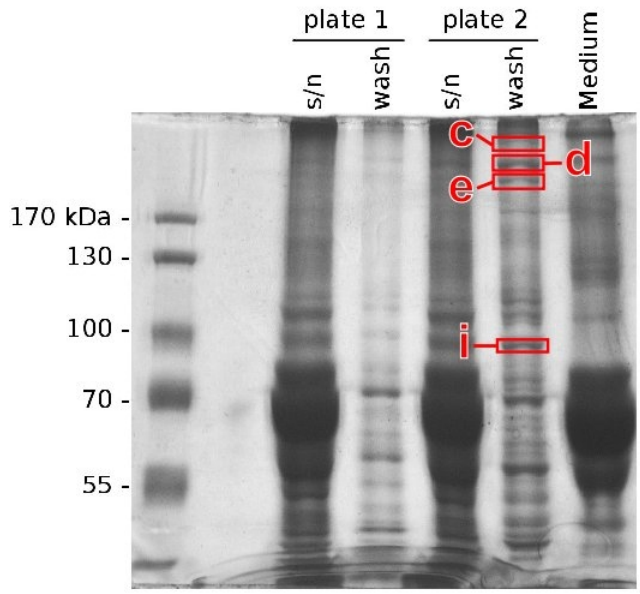


Fig. 15. Coomassie-stained SDS-PAGE gel depicting washes from confluent CMT93 cells grown on plate without splitting for 3 wk., bands marked with letters were sent to mass spectrometry analysis to search for mucin proteins. Major components of the bands were determined to be:

Band	Major components
C	plectin
D	filamin, spectrin
E	myosin, afadin
I	heat shock protein (HSP 90)

Epithelium of small intestine produces IFN-λ upon Listeria infection

After in vitro experiments with intestinal epithelial cell lines we wanted to find out whether Type III Interferons are upregulated also in response to bacterial infections *in vivo*. First we infected CMT-93 cells with Gram-negative Salmonella and Gram-positive Listeria bacteria and screened for IFN-β, MX2 and IFN-λ upregulation (Fig. 16) and then we infected mice and isolated intestinal epithelial cells to screen for the same set of genes.

Salmonella are known to induce inflammatory NFkB activation in human epithelial cells in vitro by injecting Sop proteins into cytoplasm bypassing MyD88-dependent TLR signalling and Caspase-1 dependent inflammasome signalling (Bruno et al. 2009) although the recognition works differently in macrophages (Franchi et al. 2009). Whether Salmonella can induce type III interferons is unknown. Listeria recognition is also not completely understood. Once the bacteria are in the cytoplasm, they activate TBK1 and lead to IFN-β production via IRF3 together with RNA helicase DDX3X stimulation (Soulat et al. 2008), but there is little data on induction of IFN-λ by Listeria. Recently, Lebreton et al., (2011) showed that in LoVo intestinal epithelial cell line IFN-λ is strongly upregulated as a response to infection with Listeria.

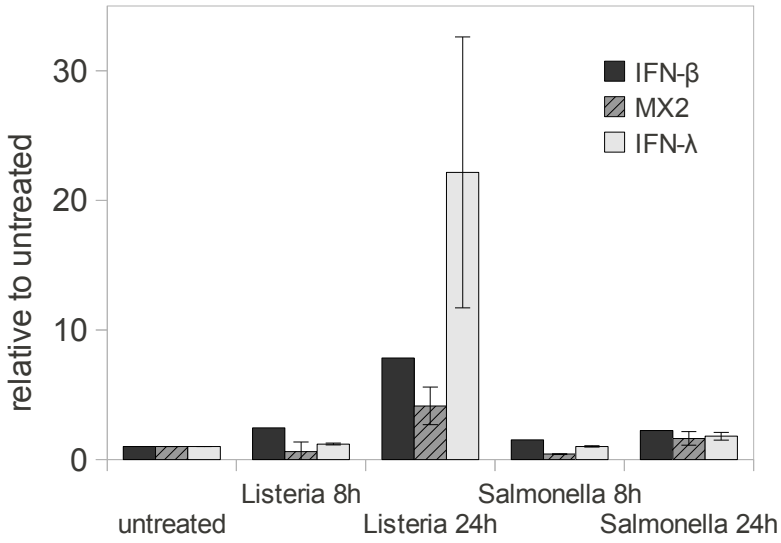


Fig. 16. CMT-93 cells upregulate IFN-β, MX2 and IFN-λ in response to Listeria (MOI 100) but not to Salmonella (MOI 10) infection.

We have found out, that epithelial cells from small intestine respond to Listeria infections when tested for IFN-β, IFN-λ and MX2 mRNA (Fig. 17). Infection with Salmonella did not lead to strong upregulation of IFN-β and MX2 (not shown) and to IFN-λ (Fig. 18).

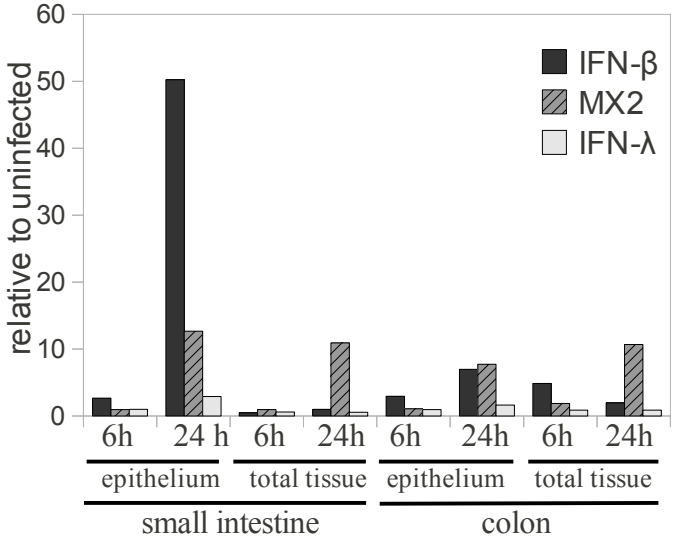


Fig. 17. Mouse epithelial layer responds to intraoral Listeria infection by upregulating IFN-β and MX2 and IFN-λ mostly after 24h.

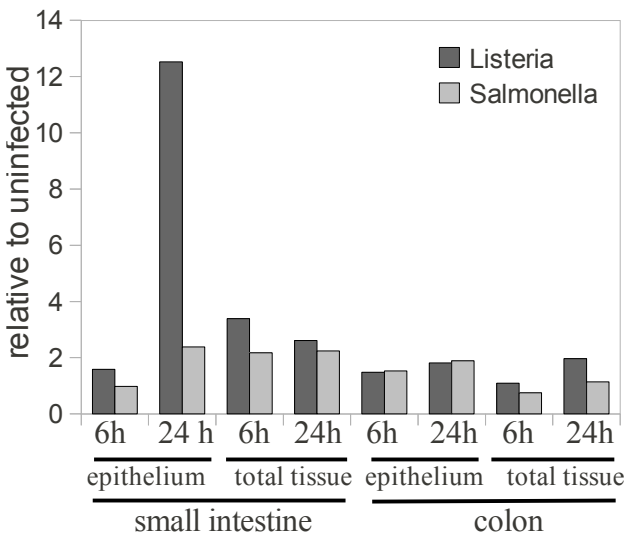
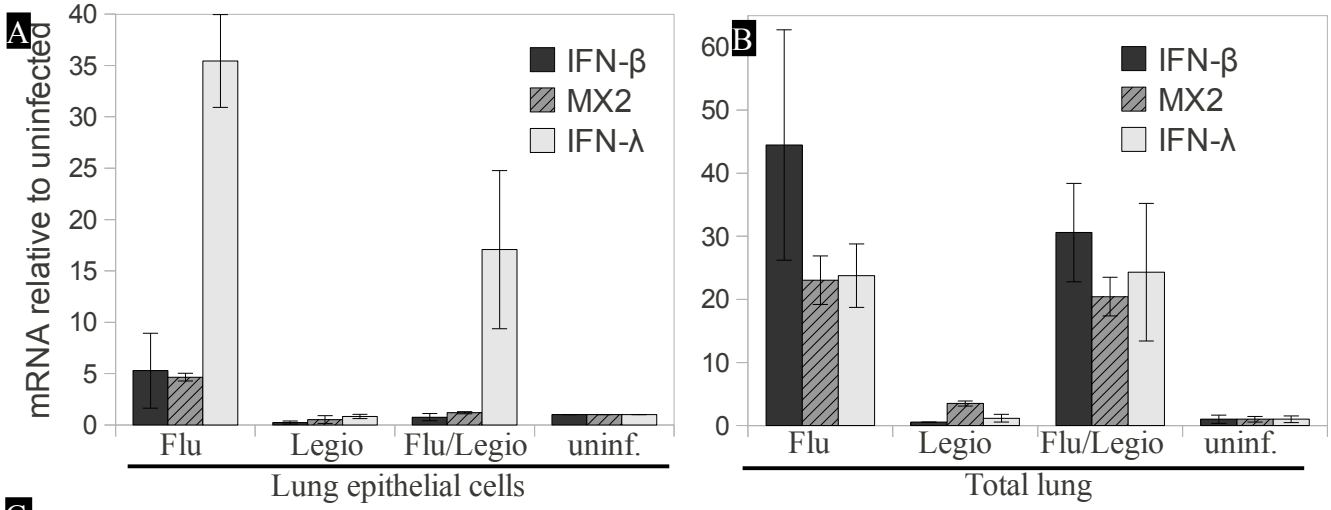


Fig. 18. IFN-λ is strongly upregulated only in the epithelial layer of the small intestine 24h after Listeria infection but not after Salmonella infection.

Viral-bacterial coinfections in lungs

As mentioned in the introduction, viral pulmonary infections may lead to increased sensitivity of patients towards bacterial infections. In order to test whether interferon production is induced during coinfection, we infected mice intra-nasally with influenza virus and 3 days later with Legionella bacterium. We did not know whether the levels of IFNs will be lower in coinfected mice because of e.g. glucocorticoid release, damaged epithelium, impaired immune cell migration etc. or the levels will be higher because Legionella can activate further cytoplasmic PRRs e.g. Nod1/2 with its' PAMPs.

To our surprise we observed less IFN-β and IFN-λ mRNA in lung epithelial cells of coinfected mice as compared to cells from influenza-only infected mice (Fig. 19A). We did not see, however, such difference in total lung tissue or in lung lavage, that contains cells from alveolar fluid (Fig. 19B, C). Genes associated with tissue repair - Gcnt2 and Timp4 - are also not significantly higher in coinfected lungs (Fig. 19D, E). Future experiments, like determining the viral/bacterial load in the lungs in short intervals after infection, staining for viruses/bacteria in the lungs etc. might provide an explanation to the fact why there is less IFNs in coinfected lungs.



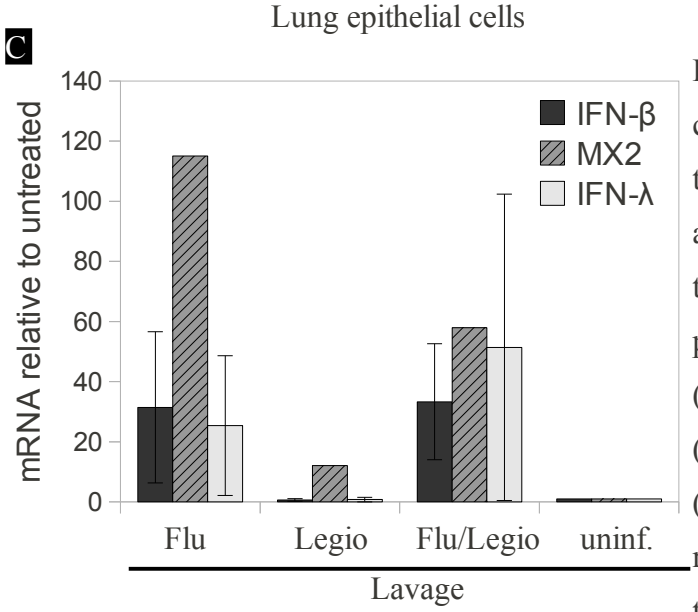
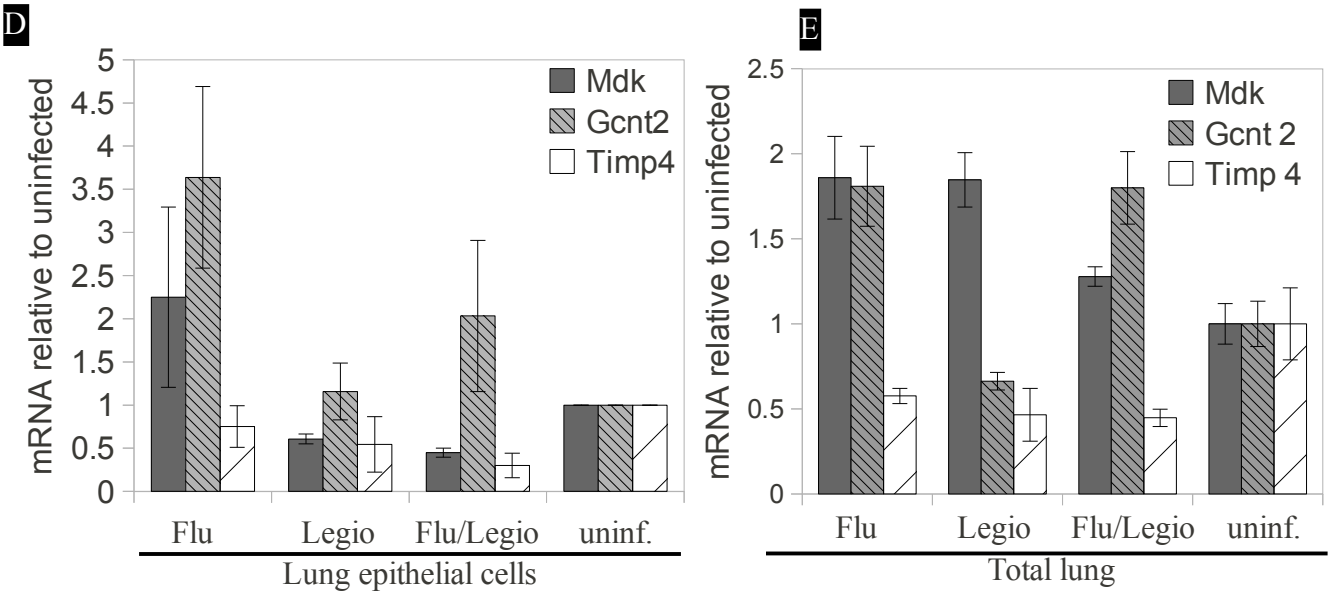


Fig. 19. Relative to influenza infection alone, coinfection with influenza and Legionella leads to decreased production of inflammatory IFN-β and IFN-λ, anti-viral MX2, and, to lesser extent, tissue repair genes Mdk, Gcnt2 and Timp4. A: primary lung epithelial culture from infected mice (n=2); B: total lung tissue (n=1); C: lung lavage (cells washed out together with alveolar fluid) (IFN-β, IFN-λ data: n=2; MX2 data: n=1); tissue repair genes in lung epithelial cells (D, n=2) and total lung (E, n=1)



Interferons influence cell proliferation

As mentioned earlier, destruction of gut epithelium leads to unrestricted contacts between intestinal microflora and intestinal epithelial and immune system cells. “Friendly fire” from recruited immune system cells inflicts further damage to surrounding tissue. Intestinal epithelial stem cells need to be activated in order to regenerate the protective epithelial barrier. We were interested in the effect of IFNs on the proliferation rate of intestinal epithelial cells because of in vivo studies of colitis in mice. Our hypothesis for different recovery rate of IFNAR KO mice from DSS-induced colitis when compared to wt mice is that IFNs may affect the proliferation rate of intestinal epithelial stem cells. *In vivo* studies of epithelial crypt cell proliferation in wt and IFNAR mice, using BrDU label incorporation into cycling cells and Ki67 proliferation marker staining, did not show significant differences in proliferation of colon epithelial cells during DSS-induced colitis (studies done by Dr. Isabella Rauch). There could be, however, subtle differences in proliferation that are not seen by these methods or that DSS effects just override the effects of IFNs on proliferation. However in other IFN-inducing situations (e.g. during Listeria infections etc.) the potential effect of IFNs on proliferation could be important. Thus we decided to test the effect of IFNs on CMT-93 cell line proliferation and also to isolate and culture mouse intestinal epithelial cells and observe IFN effect on their proliferation rate *in vitro*.

We found out, that IFN-β inhibits proliferation of CMT-93 cell line (Fig. 20) which is in concordance with similar studies, showing that IFNs have an anti-proliferative effect. We also tested whether IFN-β can penetrate the cells that are growing in soft agar system (Fig. 21) i.e. culture conditions used for primary intestinal epithelial cell growth. For this, IFN-β solution was applied to the wells with CMT-93 and primary epithelial cells, both grown in soft agar, and after a period of time mRNA was harvested using Trizol chemical, that uses the phenol/chloroform RNA extraction principle. RNA quantity was too low from wells with primary intestinal epithelial cells, that is why only CMT-93 data are presented. We found out that CMT-93 cells were responsive to IFN-β and poly(I:C) treatment when grown in soft agar (Fig. 22).

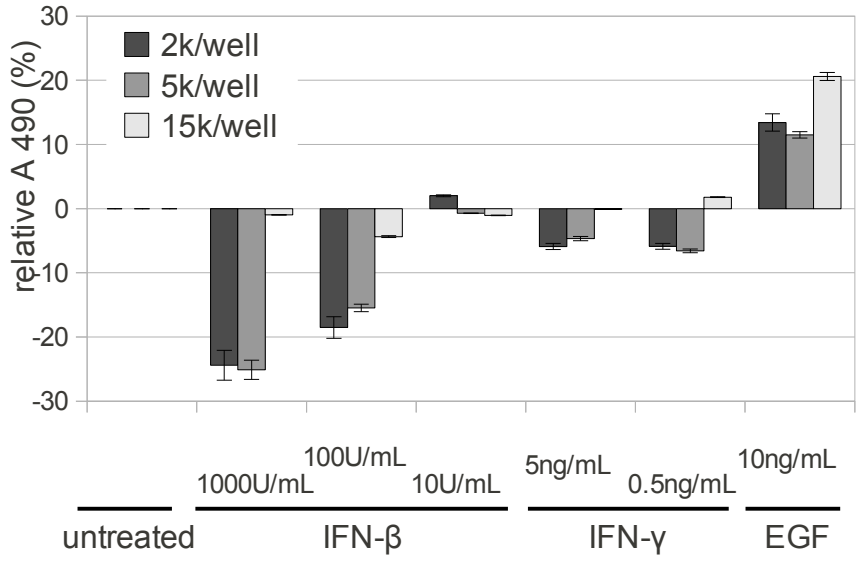


Fig. 20. Type I IFNs inhibit the growth of CMT-93 cells as tested by MTS proliferation assay. Cells were seeded in different concentrations in order to avoid false negative results caused by seeding the cells too densely and inhibiting their proliferation due to contact inhibition. EGF was used as positive control.

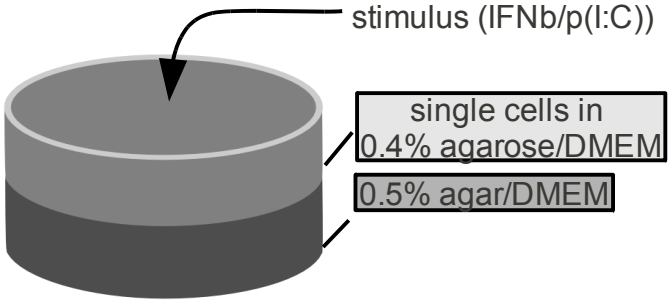


Fig. 21. Schematics of the soft agar system used for growing intestinal epithelial cells. Every well of a 24-well plate is covered with agar/DMEM mixture, left to solidify, then covered with a single cell suspension mixed with warm agarose and DMEM. Stimulation at d0: stimulus added to top layer of agarose, later stimulations: stimulus added in DMEM on top of agarose at the specified time. The top layer was always kept moist by applying 250µL DMEM/2.5% FCS, adding more when necessary.

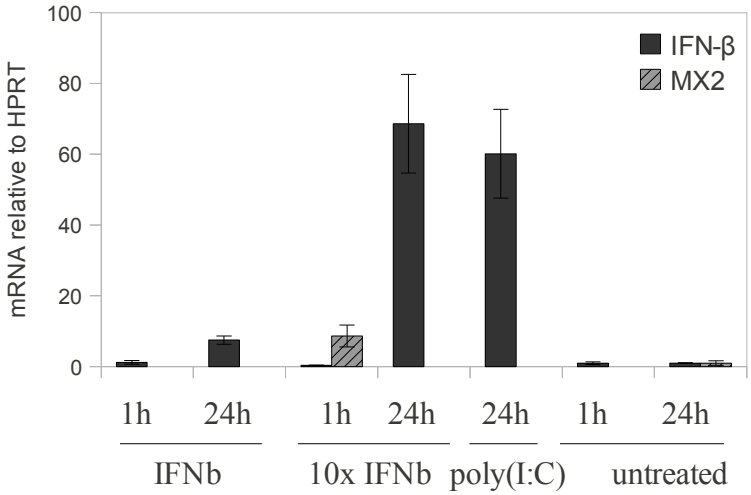


Fig. 22. CMT-93 cells remain responsive to IFN-β and poly(I:C) even when cultured in agarose – the expression of IFN-β mRNA increases after stimulation with IFN-β for 24h. Conc.: IFN-β: 3.3U/well, 10x IFN-β: 33U/well, poly(I:C): 2µg/well

When seeding primary intestinal epithelial cells, we took care to seed single cell suspensions so that the colonies grown after the incubation period were definitely *in vitro* grown colonies and not groups of cells left undissociated after isolation procedure. We have monitored the size of colonies from primary epithelial cells over time in order to ensure that the colonies grow. We observed a decrease of smaller and an increase of larger colonies with ongoing cultivation (Fig. 23).

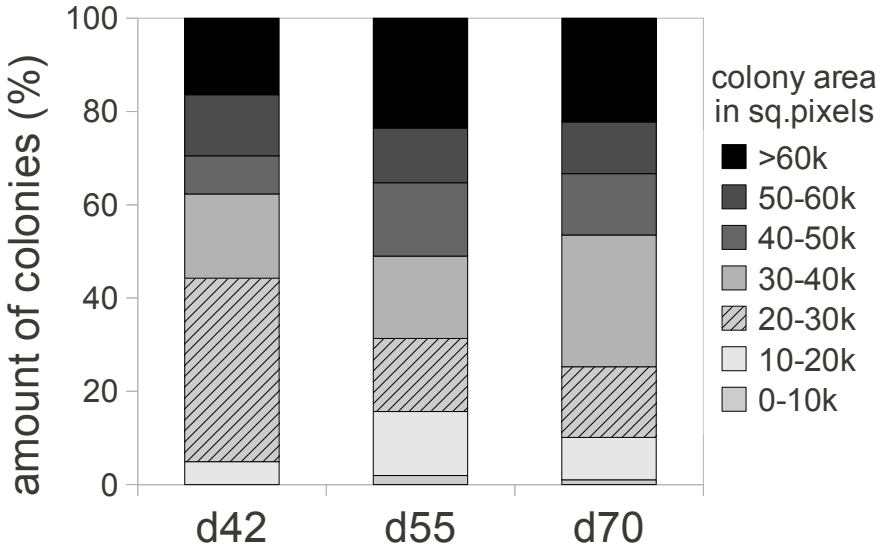


Fig. 23. Primary epithelial cells grow in vitro in soft agar. Colony growth assessed by measuring colony size at day 42-70 after isolation.

Next we isolated mouse intestinal epithelial cells from the small intestine and colon of wt C57BL/6 mice and IFNAR1 KO mice as single cells and seeded them into soft agar. After 4 months we determined the colony amount and colony size in order to find out whether the stem cell amount and the stem cell activity are different between wt and IFNAR KO mice. There was no pronounced difference in the colony count between the wild type and the knockout mice when the single measurements were pooled (Fig. 24).

When the colony sizes were analyzed, no significant differences were found between colonies from wt and IFNAR KO intestinal epithelial cells. Nevertheless, there is a trend of IFNAR KO colonies from small intestine to be smaller (Fig. 25A, mice pairs 1-3) and from colons to be bigger (Fig. 25B). When tested for sensitivity to IFN-β at different stages, we expected IFNAR KO primary cells not to respond – we have seen non-significant differences between cells treated with IFN-β immediately after seeding, 2 weeks after seeding or in absence of IFN-β treatment both in wt and IFNAR KO cells. The only significant difference was observed between non-treated IFNAR KO cells and wt cells treated 2 weeks later with IFN-β (Fig. 26).

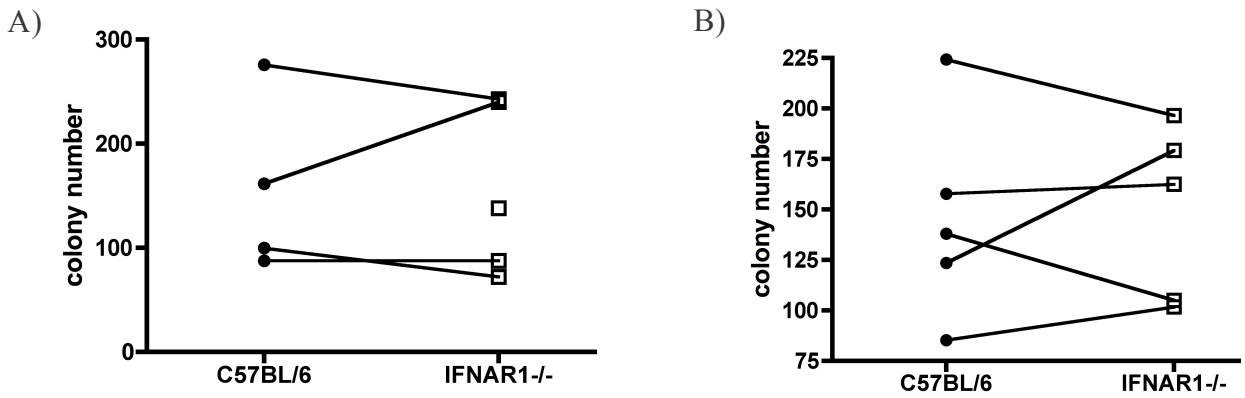


Fig. 24. Numbers of colonies of intestinal epithelial cells from small intestine (A) and colon (B) do not differ between wt C57BL/6 mice and IFNAR KO mice. Groups of cells isolated and seeded on the same day are joined by lines.

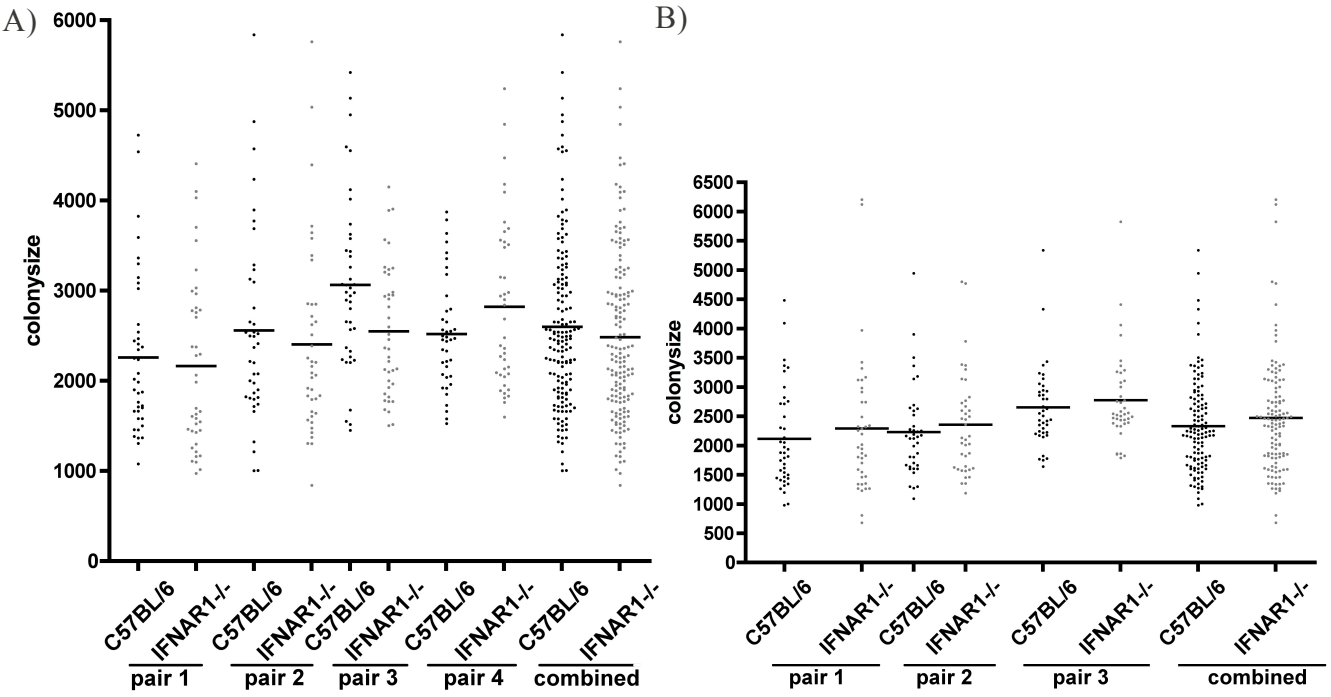


Fig. 25. Intestinal epithelial cells isolated from the small intestine of wt mice form, in general, bigger colonies (except of the 4th pair of mice) than colonies from IFNAR KO mice (A), although the situation is reversed in cells isolated from colon (B).

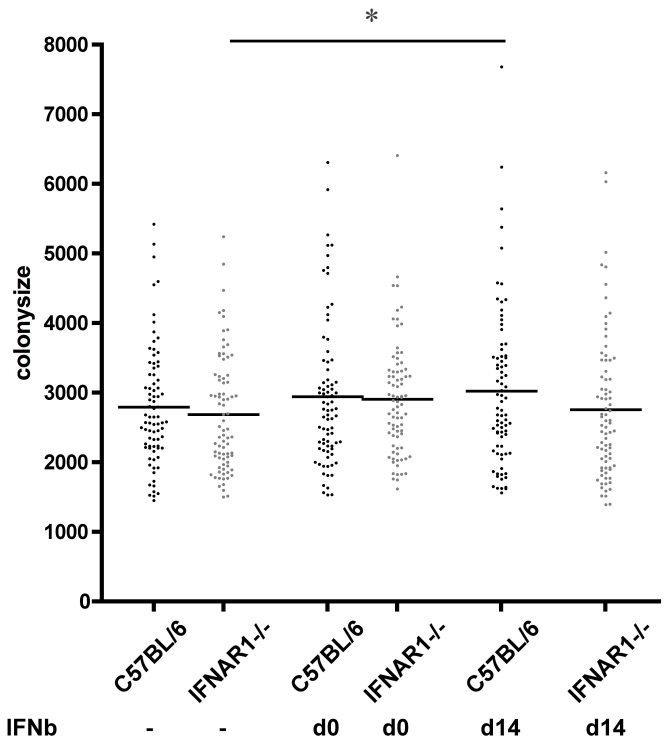


Fig. 26. Primary intestinal epithelial cell colonies grown under IFN-β stimulus. IFN-β was added either during seeding (d0) or 2 weeks after seeding (d14). There is a significant difference ($p>0.05$) in colony size between untreated IFNAR1 -/- and wt C57BL/6 cells treated with IFN-β 2 weeks after seeding. (n=2)

Because of slow cell growth we tried to determine whether colonies that we see after some period in soft agar culture still contain living cells. Trypan blue (TB) staining did not show clearly positive (dead) and negative (alive) cells even in CMT-93 cell colonies. This may be explained by several technical problems: concentrated TB stain was hard to remove from the agarose block, whereas too diluted TB did not give strong staining. Furthermore, because the agarose block is optically denser than fluid medium and is also uneven, differential contrast microscopy did not work on our samples. That is why we decided to try fluorescent staining. We picked ethidium bromide as the stain that would penetrate dead cells and stain DNA, whereas acridin orange (AO) would counterstain live cells. Enzymatic conversion of fluorescin diacetate was a second method of choice for staining live cells. In CMT-93 colonies clear distinction between live and dead cells was possible but colonies from primary epithelial cells remained weakly but uniformly stained with both EtBr and AO/FDA stains (Fig. 27).

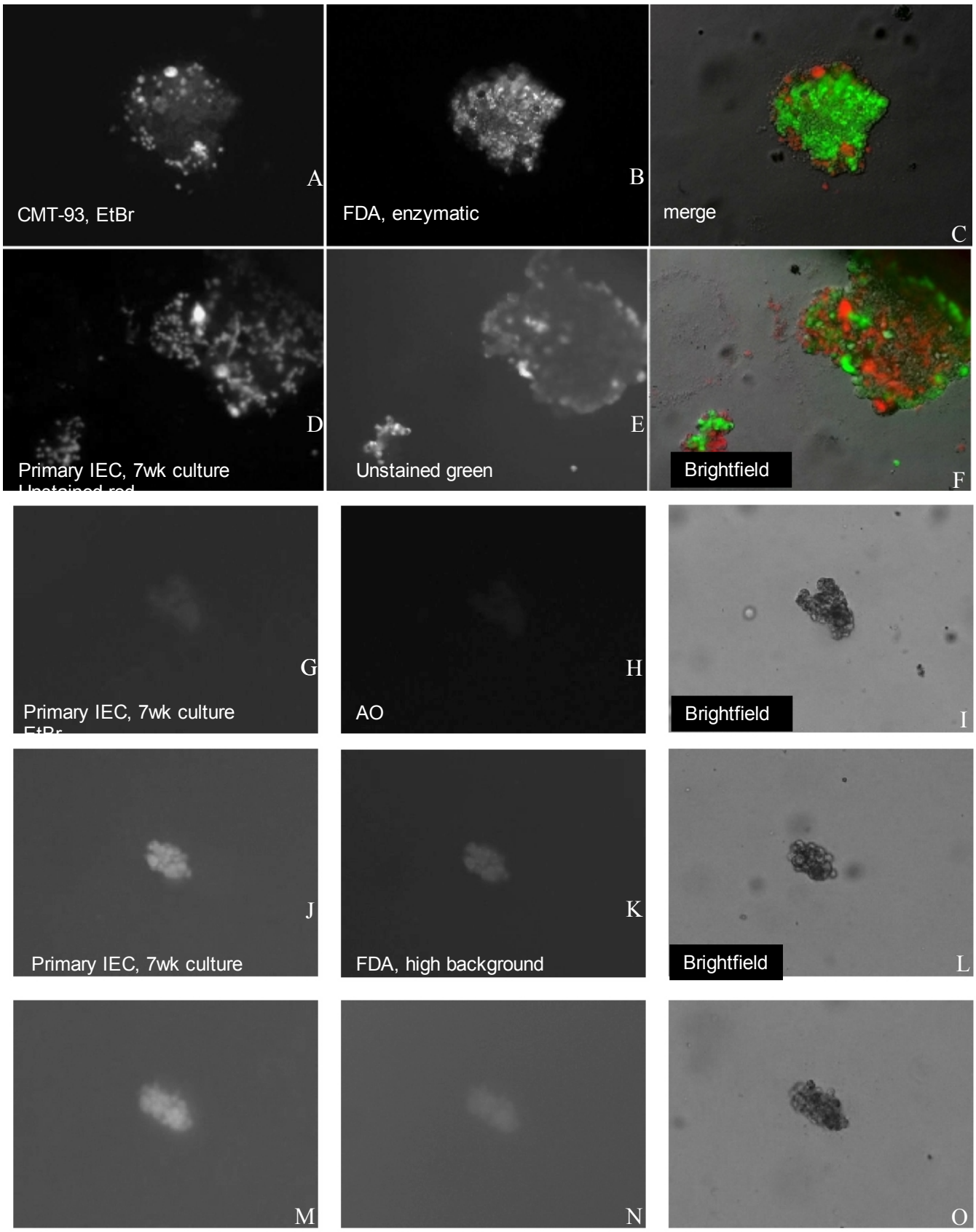

A
CMT-93, EtBr
B
FDA, enzymatic
merge
C
D
Primary IEC, 7wk culture
E
Unstained green
Brightfield
F
G
Primary IEC, 7wk culture
H
AO
Brightfield
I
J
Primary IEC, 7wk culture
K
FDA, high background
Brightfield
L
M
N
O

Fig. 27. Viability staining tested to establish whether colonies from primary intestinal epithelial cells are viable. CMT-93 cells were used as control cells (A-F). Both acridine orange (AO) and enzymatic fluorescein diacetate (FDA) stain live cells, whereas ethidium bromide (EtBr) that stains dead cells. Staining of primary intestinal epithelial cells (G-O) gave inconclusive results.

We have also devised a method for culturing epithelial cells from colonic crypts for short period of time. After we managed to isolate relatively intact crypts using chelating EDTA, we tested several conditions in order to keep cells adherent. We tried non-coated tissue treated, gelatin coated and collagen coated plates and in all cases the isolated primary intestinal epithelial cells failed to attach. Then we tried to seed cells on a feeder cell layer consisting of mitomycin treated i.e. non-proliferating mouse embryonic fibroblasts (MEFs) (E Michalska 2007) but encountered the problem of MEFs detaching from the plate. Finally, we plated CMT-93, MEF and endothelial cells and, after reaching a confluent layer, osmotically lysed the cells with ammonium hydroxide in order to obtain plates covered with extracellular matrix (ECM) components left by these cells. Only endothelial cells lysed nicely and left ECM to which primary intestinal epithelial cells were able to attach (Fig 28C). In the future, experiments may be performed on these short-term cultures including stimulation with PRR agonists, proliferation studies in presence of IFNs etc.

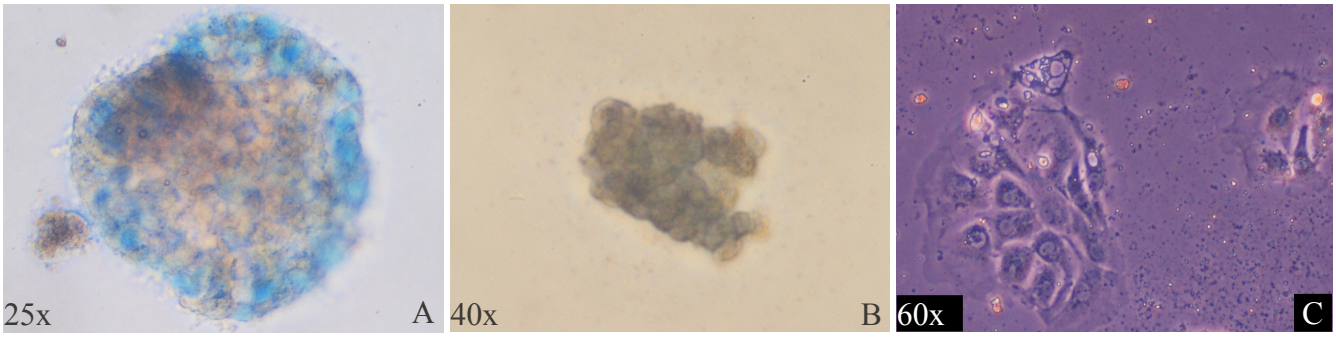


Fig. 28. Gut epithelial cells of different shape. In soft agar, CMT-93 carcinoma cells form round colonies (A), whereas primary intestinal epithelial cells often form crypt-like colonies (B). Staining with trypan blue was performed in an attempt to check the viability of cells in colonies. Colon crypt cells grown on ECM from endothelial cells adhere and spread if seeded not too densely (C).

Discussion

One of our starting questions was whether we can simulate the responses of intestinal epithelial cells to pathogens and their molecules in vitro in order to better understand the gut defence mechanisms against infection. We were interested to find out how the cells react to the microbiota, what pathogen associated molecular patterns are recognized and what the responses are that help the epithelial layer in fighting off pathogenic microbes.

First we dissected the responses of immortalized CMT-93 intestinal epithelial cells to several PAMPs. We found out that these cells respond by upregulation of IFN-β, IFN-λ and MX2 to poly(I:C), a dsRNA analogue and a strong TLR3 stimulant. The cells did not respond to other nucleic acid-derived stimuli, like poly(dA:dT) and CpG, no matter whether the transfection reagent was used or not. Applying poly(I:C) without the transfection reagent also did *not* lead to upregulation of IFN-β, IFN-λ and MX2 (not shown). The transfection procedure leads to poly(I:C) being located inside of the cytoplasm, that may lead to Mda5-mediated IFN production (Kato et al. 2006). Additionally, it has been shown for human intestinal epithelial cells, that transfected poly(I:C) leads to upregulation of IFN-β not via the TLR3 but via the RigI pathway (Hirata et al. 2011). Thus CMT-93 cells' TLR3 signalling may not play the role in responses to poly(I:C) – to test this hypothesis we would need to either knock down TLR3 or to inhibit its downstream signalling pathway, e.g. to block TRIF adapter protein.

Unresponsiveness to CpG was unexpected because intestinal epithelial cells should express TLR9 and upon CpG stimulation should activate NFkB pathway, although the studies showing such findings were performed on *polarized* IECs (Takeshita & Ishii 2008). It was also shown that TLR9 is expressed on the *surface* of mouse intestinal epithelial cells and on human colon epithelial HT-29 cells (Ewaschuk et al. 2007), that is why we first decided to stimulate cells with CpG without transfection. Maybe, the spatial localization of TLR9 is important for responses to CpG, but we have not grown CMT-93 cells in polarizing conditions (growing polarized CMT-93 described in: Iliev et al. 2009).

Testing several different concentrations of CpG, poly(dA:dT) and LPS did not lead to upregulation of IFN-β, MX2 and IFN-λ mRNA. We wondered whether CMT-93 express needed TLRs – we have performed qPCR check for TLR3, and 9 and we obtained a weak signal (not shown) for these PRRs.

We were surprised to see absence of IFN and MX2 upregulation after treatment with LPS even at very high concentrations. Intestinal epithelial cells should be sensitive towards this major bacterial component. Studies in human intestinal epithelial cell lines show that stimulation of TLR4 leads to upregulation of epiregulin, EGFR stimulant, that leads to increased proliferation (Hsu et al. 2010). Simulating CMT-93 cells with LPS leads also to MAPK phosphorylation within 5 minutes after LPS is applied (Cario et al. 2000). We were mostly interested in the response of cells to IFNs, but in the future proliferation studies on CMT-93 cells treated with various PRR stimuli can be performed. If there is a response in proliferation rate, then in CMT-93 cells there might be a defect on the path between TLR and IRFs, the transcription factors for interferons, but the other signalling pathways might be intact.

During our screens for IFN-λ upregulation we have noticed the presence of two peaks in the melting curves of qPCR products probably meaning that we were detecting two IFN-λ isoforms: IL28A and IL28B i.e. IFN-λ 2/3. An interesting detail of responsiveness of CMT-93 cells to PAMPs was that one of the isoforms prevailed if cells were stimulated with poly(I:C). However, we were not able to verify it with isoform-specific primers due to low signal from qPCR analysis, apparently due to low efficiency of these primers.

The finding that interferon beta inhibited cell proliferation of the CMT-93 cell line was an expected result as it was shown before that antiviral activities of interferons impair translation and slow down the cell cycle (Brand et al. 2005), (Hoffmann et al. 2011), (Sangfelt et al. 2000). However, the effect of IFNs on the proliferation of the primary intestinal epithelial cells and especially on the intestinal epithelial stem cells was harder to determine. At first, we had to establish a protocol that would yield single cell suspensions. Digestion of intestinal tissue with collagenase, dispase or pancreatin did not produce desired single cell suspensions. Addition of DNase I, that should aid in dissolving cell clumps held by DNA aggregates also did not improve the isolation. Treating intestinal tissue with chelating EDTA solution yielded the suspension of single cells and small clumps of cells, the latter were filtered out through cotton wool. This rather rough treatment of the primary intestinal cells, and also wash of tissue with anti-fungal amphothericin in order to combat contamination, may have lead to low colony growth efficiency of the primary cells. The other factors leading to such low efficiency might have been: the absence of defined growth factors, except 2.5% serum in the medium; fluctuating (20-70%) viability of freshly isolated cells; absence of extracellular matrix components, unlike in Matrigel. We

have also tried to culture the cells in agarose gels of different strength, in order to have gel tensile forces similar to Matrigel, used for culturing such cells in vitro. T. Sato et al. (2010) found out that Paneth cells provide the niche for intestinal epithelial stem cells. After seeding cell doublets, that contained Lgr5+ ISCs and Paneth cells, into Matrigel, the authors have observed drastic increase in colony formation efficiency as compared to seeding Lgr5+ single cells only. Also, when sorted Paneth cells and ISCs were mixed together, both cell types were forming contacts with each other and were effectively forming the colonies. We incubated the single cell suspension in liquid medium prior to seeding them into agarose in attempt to obtain cell pairs, that potentially might contain a Paneth cell and an ISC. These experiments however failed to increase the number of colonies. The low efficiency and long incubation time needed for the colonies to grow make the soft agar method not well suited for studying intestinal epithelial cell growth.

The colonies that we have grown in soft agar did often resemble the cylindrical shape of intestinal crypts, but we did not develop a method for staining cells in soft agar with e.g. epithelial cell marker antibody in order to confirm their identity. Future experiments may include confirming the intestinal epithelial stem cell character of the cells after long-term growth in soft agar by running a PCR for Lgr5 gene, that is a recognized marker for such cells. After the colonies were formed, we expected to see clearly two groups of colonies: smaller colonies formed by transiently amplifying cells, that have a limited proliferation potential and larger colonies formed by bona fide intestinal epithelial stem cells, but this was not observed. Probably, due to lack of specific growth factors in soft agar system, only the ISCs or TA cells are able to grow.

A defining experiment of testing whether IFNs influence the proliferation rate was when we treated both wt and IFNAR1 KO primary epithelial cells with IFN-β and monitored colony growth. There was only a small difference between non-treated IFNAR1-/- and IFN-treated wt cells in colony size even though we knew that IFN-β can penetrate the soft agar. Wt cells formed bigger colonies that can suggest proliferatory function of Type I IFNs in intestinal epithelial layer. An important test lacks here: after finding out that IFNs have anti-proliferative effect on CMT-93 cells in standard growth conditions, we should have tested the effect of IFNs on proliferation of these cells in soft agar.

The epithelium of the small intestine and colon differs in several ways: there are no protruding villi in

the colon, the colonic mucus consists of two layers, bacterial composition is different suggesting that there might be differences in epithelium-bacteria interactions (Wells et al. 2011). Based on our results there is a clear trend of untreated IFNAR KO cells forming larger colonies when isolated from colon, and forming smaller colonies when isolated from small intestine when compared to wt cells. This may further highlight the differences between colonic and small intestinal epithelium.

Based on our results of IECs growth in soft agar, the different recovery of IFNAR KO mice from DSS-induced colitis when compared to wt C57Bl/6 mice is, possibly, not entirely explained by small differences in proliferation rates of ISCs. An alternative explanation of different recovery rates could be altered recruitment and/or activity of the cells of the immune system at the damaged site. To rule out the involvement of immune cells in the recovery process, a tissue-specific IFNAR knock-out would be needed, e.g. using a floxed IFNAR gene and a Cre recombinase under the control of Lgr5 promoter. Also it would be beneficial to use mouse infection models where the epithelium of either small intestine or colon is affected. Maybe Listeria can play a role of such pathogens as we show that they induce IFNs mostly in the small intestine.

Less tissue destruction during colitis might lead to better recovery, although the histology data from Dr. Isabella Rauch show that the destruction of the tissue is similar at the point where the mice are most affected by DSS administration. And the data from Drosophila (Jiang et al. 2009) clearly argue for the involvement of Jak/Stat pathway in the stimulation of epithelial layer regeneration. The question of influence of IFNs on the ISC proliferation in mice remains to be answered.

In the search of an alternative approach, we have developed a short term culturing method of intestinal epithelial cells on ECM left by endothelial cells. One of the possible future experiments could be to apply IFNs onto such cultures and watch for an effect in proliferation, although we still have not tested whether the cells in such culture conditions actually proliferate. Furthermore, we could test the sensitivity of such primary cells towards PAMPs that we have tested on CMT-93 cells and correlate the results with already known cell line data and in vivo data. In addition, testing for epithelial antigens with fluorescent antibody staining is more simple that in soft agar system. The developed method might provide some valuable insights into relatively freshly isolated cells and allows time for cell manipulation.

The data from intestinal cells of mice infected with bacterial pathogens suggest higher expression of Type I IFNs 24h after infection with highest expression in small intestine epithelial cells from Listeria infected mice. Type III IFN was strongly upregulated only in Listeria infection and not in Salmonella infection. Low inducibility by Salmonella infection corroborates findings of (Salzman et al. 1998) that observed IFN-β upregulation in Salmonella infected mice only with pre-treatment of cells with IL1 or LPS. It would be interesting to discover why Listeria have stronger effect on the upregulation of IFN-λ when compared to Salmonella and also to make a comparison with bona fide inducers of IFNs, i.e. viruses.

Because we used different amounts of bacteria for infections, we might have also monitored a gene that is more characteristic for anti-bacterial responses then IFN, e.g. iNOS – this would give us a relative measure for the activation of IECs by these two different bacteria. Other genes could have also been monitored - (Eckmann et al. 2000) shows for human IEC lines that upon Salmonella infection G-CSF, MIP2-alpha, IRF1 and other genes are strongly upregulated as early as 3h after infection. We have noticed from the CMT-93 studies (not shown) that the cells respond with different strength to same MOI of Salmonella and Listeria.

Upregulation of IFN-λ upon bacterial infection in intestinal epithelial cells points towards the importance of this IFN class in the epithelial cells during the bacterial infection. It would be also interesting to screen for IFN-λ upregulation in associated immune cells. Performing infections in ligated intestinal loop model may be considered if more controlled infection conditions are required for future experiments because of high variation between individual mouse infections.

Further finding supporting the importance of Type III interferons in epithelial cells comes from viral-bacterial coinfection studies in lungs. We see upregulation of both type I and III IFN upon (co-)infection with Influenza virus, however we see no upregulation of these genes in case of bacterial (Legionella) infection only. The picture is less clear when lung epithelial cells, total lung tissue and alveolar fluid (lavage) are tested for these genes. However, highest IFN-λ levels were observed in flu-infected lung epithelial cells and lowest in Legionella-infected lung epithelial cells. This data combined with the results from intestinal infections indicate that some bacteria (Listeria) might, as viruses (Mordstein et al. 2010), induce IFN lambda in epithelial cells, whereas the other bacteria (Legionella)

do not induce IFN-λ.

From histology data (Dr. Amanda Jamieson) it is known that Influenza/Legionella coinfections lead to higher epithelial layer damage, but we have seen less upregulation of IFNs in coinfected mice. Our working hypothesis is that epithelial layer suffers larger destruction during coinfection (confirmed by histology done by my supervisor Dr. Amanda Jamieson) and cannot keep up with the developing infection. However, the levels of genes linked to tissue remodelling, Gcnt2 and Timp4, were not significantly higher in coinfected lungs. Why the interferon level is lower in coinfected lungs remains an open question. One of the possible explanations is that the mice clear up the infections faster because of a very strong immune response when Legionella is introduced into Influenza-infected mice but this has to be verified by histology and CFU/PFU data.

To sum up, we found out that IFNs can be induced by poly(I:C) in CMT-93 cells and that these cells are responsive to type I IFN and, in particular, IFN-β has an anti-proliferative effect on them. We have also found that type I and III IFNs are upregulated in mouse intestinal epithelial cells upon Listeria infection and in lungs upon Influenza infection and Influenza/Legionella coinfection. The data on IFNAR KO primary intestinal epithelial cell proliferation is not conclusive and at the moment we cannot link how exactly mutations in the Jak/Stat signalling pathway might contribute to IBD development. Future experiments are needed not only to study the epithelial homeostasis of the gut and the factors that influence it but also to provide clues whether IFNs can be used to treat IBD as there are conflicting reports at present (Axtell et al. 2011), (Musch et al. 2007), (Nikolaus 2003), (Pena-Rossi et al. 2008).

Materials and methods

qPCR

H2O	9.39µL
25mM $MgCl_2$	1.5µL
10x buffer (Fermentas, +KCl, -$MgCl_2$)	1.5µL
10mM dNTPs	0.3µL
primer fw (100µM)*	0.045µL
primer rev (100µM)*	0.045µL
SYBR green (in DMSO)** (freshly diluted 1:100 in H2O)	0.6µL
Taq Pol	0.12µL
DNA (1:3 dilution in H2O)	1.5µL

Temperature cycle:

- 1 cycle 95 degC 10 min
- 45 cycles 95 degC 15 sec → 60 degC 20 sec → 72 degC 20 sec
- 1 cycle 95 degC 1 min → 55 degC → melting curve 20 min → 95 degC 15 sec

*for 4µM primers (mixed) add 1µL of primer mix and only 8.39 µL H2O
**SYBR green is diluted 1:15 in DMSO and stored in dark at -20degC, then diluted 1:100 in water just before usage

Alternatively, mix 1µg RNA, primers (as above), bring to 7.5µL with H2O and add 2x SYBR Mix solution (Fermentas).

qPCR analysis

Melting curves are checked for consistency and compared to negative controls (H2O). Threshold for Ct values is set to appx. 3000. If values are deviating between duplicates for more than 1 cycle then they are not considered true. Following formula is used to calculate RNA levels:

RNA level: $2^{(Ct_{HPRT}-Ct_{sample})}$
RNA level relative to untreated sample: $[2^{(Ct_{HPRT}-Ct_{sample})}] / [2^{(Ct_{HPRT,\ untreated}-Ct_{sample,\ untreated})}]$

qPCR primers:

HPRT fw:	5'-GTTGGATACAGGCCAGACTTTGTTG-3'
HRRT rev:	5'-GAGGGTAGGCTGGCCTATTGGCT-3'
IFN-β fw:	5'-TCAGAATGAGTGGTGGTTGC-3'
IFN-β rev:	5'-GACCTTTCAAATGCAGTAGATTCA-3'
MX2 fw:	5'-CCAGTTCTTCTCAGTCCCAAGATT-3'
MX2 rev:	5'-TACTGGATGATCAAGGGAACGTGG-3'
IFN-λ 2/3 fw:	5'-AGCTGCAGGCCTTCAAAAAG-3'

IFN-λ 2/3 rev: 5'-TGGGAGTGAATGTGGCTCAG-3'
IL-28A fw: 5'-AAGGACCTGAGGTGCAGTTCC-3' (low efficiency)
IL-28A rev: 5'-GGCCAGGGCTGAGTCAGT-3' (low efficiency)
IL-28B fw: 5'-AGGACATGAGGTGCAGTTCCC-3' (low efficiency)
IL28B rev: 5'-GGTGGTCAGGGCTGAGTC-3'(low efficiency)

cDNA synthesis

- in PCR tube: put 1µg RNA and bring to 10µL with H2O
- add 1µL oligo-dT
- 5 min 70 degC (PCR machine)
- 5 min on ice
- add 4µL 5x RT buffer (Fermentas), 2µL 10mM dNTPs, 2µL H2O (make master mix for multiple rxns)
- 5 min 37 degC (PCR machine)
- add 1µL reverse transcriptase (RevertAid Fermentas)
- 1h 42 degC (PCR machine)
- 10 min 70 degC (PCR machine)
- store at -20 degC

RNA isolation (MN NucleoSpin RNA II kit, cells grown in 6-well plates)

- wash cells from medium with 2mL PBS
- apply 350µL RA1 buffer (complemented with 1% beta-Mercapto-Ethanol)
- if necessary, freeze in liquid N2 and store at -80 degC
- filter through violet collar insert tube by centrifuging 1 min 11,000 g
- mix f/t with same amount of 70% EtOH (not denatured), triturate x5
- apply liquid to blue collar insert tube, centrifuge 11,000 g 30 sec
- discard f/t, exchange collection tube, apply 350µL MDB buffer, centrifuge 11,000 g 1min
- discard f/t, apply 95µL DNase solution (diluted 1:10 in DNase buffer), incubate 15 min RT
- wash with 200µL RA2 (11,000 g 30 sec), change collection tube
- wash with 600µL RA3 (11,000 g 30 sec)
- wash with 250µL RA3 (11,000 g 2 min)
- elute RNA with 40µL RNase-free water (11,000g 1 min)
- if possible, use immediately for cDNA synthesis, store at -80 degC

RNA isolation (PureLink RNA Micro Kit Invitrogen)

- before isolation:
  - add 1% of beta-Mercapto-Ethanol to Lysis buffer
  - add EtOH to Wash buffer II as indicated
  - dissolve DNase as indicated
  - Carrier RNA: dissolve 1:100 in Lysis Buffer (final conc.: 5ng/µL), use 5µL of this solution

per isolation
- [colonies were picked with a thin Pasteur pipette and collected in a drop of medium (isolation was not successful)]
- centrifuge cells 2 000 g 5 min
- discard s/n, resuspend pellet in 350 µL Lysis buffer (beta-Merc-EtOH added)
- add 5 µL of 5ng/µL Carrier RNA solution
- triturate
- homogenize by passing 5-10 times through G21 needle
- add 350 µL of 70% ethanol, mix thoroughly
- transfer 700 µL of cell lysate to column
- centrifuge 12 000 g 1 min RT, discard f/t
- add 350 µL Wash buffer I, centrifuge 12 000 g 1 min RT, discard f/t, replace collection tube
- mix 10 µL reconstituted DNase and 10 µL 2x DNase buffer, apply onto center of the column membrane, incubate 15 min RT
- wash with 350 µL Wash buffer I, centrifuge 12 000 g 15 sec RT
- wash with 500 µL Wash buffer II (ethanol added), centrifuge 12 000 g 15 sec RT
- repeat 2 previous steps
- centrifuge 12 000 g 1 min, discard collection tube and insert column into recovery tube
- apply 12 µL Rnase-free H2O, incubate 1 min RT, centrifuge 12 000 g 2 min RT
- store at -80 degC
- use 1:3 dilution for qPCR

<u>Intestinal Epithelial Cells isoation (after Hornef, for RNA)</u>

- isolate intestine
- rinse in ice-cold PBS
- use half of small intestine/colon for **total tissue** lysate:
  - put tissue to ice-cold PBS
  - vortex shortly, remove PBS
  - add 700 µL RA1 buffer (MN kit)
  - homogenize with Polytron homogenizer, put on ice for ~ 1 min for the foam to settle down
  - transfer liquid homogenate to 1.5 mL tube
  - store at -80 degC
- for **epithelial layer**, put other half of colon and representative parts of small intestine (beginning, middle part and ending, ~ 3 cm each, mixed together) into ice cold PBS
  - remove PBS
  - incubate in 30mM EDTA/PBS 10 min 37 degC
  - shake vigorously
  - remove s/n, add PBS
  - centrifuge 1200 g 5 min, resuspend pellet in RA1 buffer (MN kit)
  - store at -80 degC

<u>Single cell isolation for soft agar</u>

- prepare:
  - 2x DMEM medium: 5.352 g DMEM powder (Gibco), 400 µL 100x Pen/Strep, 4 mL 100mM sodium pyruvate, 1.48 g $NaHCO_3$, 20 mL FCS, 200 µL 50mg/mL gentamycin; filter under sterile conditions
  - 3mM EDTA/PBS, pH 7.4 (set with NaOH), add DTT to 0.5mM final concentration (50mL EDTA, 20µL of 1M DTT), filter
  - Pen/Strep/Amphotericin sterilization solution: 40 mL PBS, 40 µL 1000x PenStrep, 800 µL of 250µg/mL amphotericin (Sigma)
  - 1% agar in H2O, keep at 40 degC before mixing 1:1 with 2x medium
  - 0.8% agarose in H2O, keep at 40 degC before mixing 1:1 with 2x medium
  - pre-warm 1x- and 2x DMEM
  - coat wells with 0.5% agar/DMEM: use 500µL per well of 24-well plate, coat 2 plates per mouse, use 2x6 wells for colon and 2x18 wells for small intestine (or You can coat during EDTA incubation step)
  - put cotton wool into Pasteur pipettes (ca. 1 cm), do not press in very hard, autoclave in aluminum foil
- cut out small intestine and colon
- flush with ~ 10 mL PBS
- open intestines with scissors, cut into small pieces with scalpel
- wash 3x in 50 mL tubes with PBS (use 10 mL pipette)
- incubate for 5 min in sterilization solution (amphotericin)
- wash 2-3 times with PBS, decant all liquid, transfer to 15 mL tubes
- incubate in 8-10 mL 3mM EDTA 90 min RT under slow tilting
- [here You can coat wells with agar/DMEM]
- discard EDTA s/n from cells
- add 10-13 mL PBS and pass tissue several times through 10 mL pipette, transfer s/n to 50 mL tube, repeat in total 4 times pooling together s/n from every step (avoid air bubbles)
- centrifuge 400 rpm 1-2 min RT, carefully remove s/n
- resuspend in 5 mL PBS
- centrifuge 1 000 rpm 5 min
- remove s/n, resuspend in several mL EDTA (what is left from initial 50mL solution, distribute equally between intestines)
- pass through 40 µm strainer
- centrifuge 1 000 rpm 5 min
- resuspend in 5 mL pre-warmed DMEM
- pass **gently** through 18G and 21G needles (3 times each)
- pass through cotton wool-filled Pasteur pipette
- centrifuge 1 500 rpm 5 min and resuspend in small volume of 1xDMEM (small intestine – 500 µL, colon – 100 µL)
- count cells using trypan blue (~ 1:20 dilution for colon, 1:80 (or higher) dilution for small intestine)
- bring small intestine cells from genotypes to same concentration, same with colon cells
- plate cells out (mix with agarose one by one, do not allow agarose to cool down, plate rapidly, avoid air bubbles)

- **small intestine**
  - IFN treatment (x14 master mix, 12 wells):
    - 875 000 cells
    - 1750 µL minus cells volume (µL) of 2x DMEM (pre-warmed) (total volume of 2xDMEM and cells has to be 1750 µL)
    - 140 µL IFN-β (final concentration is 50 U/mL)
    - 1750 µL 0.8% agarose (40 degC)
  - no IFN treatment (x28 master mix, x24 wells)
    - 1 750 000 cells
    - 3500 µL minus cells volume (µL) of 2x DMEM (pre-warmed)
    - 280 µL 1x DMEM
    - 3500 µL 0.8% agarose (40 degC)
- **colon** (use half of amount if not enough cells)
  - IFN treatment (x5 master mix, 4 wells):
    - 312 500 cells
    - 625 µL minus cells volume (µL) of 2x DMEM (pre-warmed)
    - 50 µL IFN-β (final concentration is 50 U/mL)
    - 625 µL 0.8% agarose (40 degC)
  - no IFN treatment (x10 master mix, x8 wells)
    - 625 000 cells
    - 1250 µL minus cells volume (µL) of 2x DMEM (pre-warmed)
    - 100 µL 1x DMEM
    - 1250 µL 0.8% agarose (40 degC)
- 2 weeks after plating, add 200 µL of IFN-β solution where applicable and 1x DMEM to the rest

<u>Lung epithelial cell (LEC) isolation</u>

- coat tissue culture-treated 10cm dishes with anti- mouse CD45 and anti- mouse CD16/32
  - add 50µg of each antibody per 5mL PBS per plate, observe sterile conditions
  - seal with Parafilm and incubate 4 degC o/n
- coat 12 well plates with collagen type IV
  - 1 mL of 1µg/mL collagen in PBS per well, store at 4 degC o/n
  - before use, wash 2x with PBS
- prepare:
  - sterile 0.9% NaCl, 20mL per mouse
  - 1% (aq.) low melting agarose, autoclave and keep at 40 degC before use, 0.45 mL per mouse
  - Dispase I, 1U/mL in PBS, 4mL per mouse
  - 0.01% DNase I (0.01g/100mL) in DMEM, 7mL per mouse
  - DMEM 10% FCS, pre-warm
  - Ham's F12 complemented with:
    - 15 mM Hepes
    - 0.8 mM $CaCl_2$

    - 0.25 % BSA
    - 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite (1884-1VL Sigma)
    - 2% FCS
  - ice
- euthanize mice by CO_2
- open chest to have access to heart, lungs and trachea
- make small incision at top left of heart (right atrium)
- clear lungs from blood by injecting 20 mL NaCl solution to top right (left atrium) portion of the heart, lungs become white if perfusion performed correctly
- take a piece (~ 7 cm) of suture thread, make a knot around trachea but do not pull tight yet
- cut a small hole in upper part of trachea
- fill syringe with 1mL PBS, attach to needle fitted with silicon tube (~ 5-7 cm), insert silicon tube into trachea, pull the suture thread to prevent leakage (not too tight so that the tube is not closed), flush lungs with PBS in syringe (this will collect lung fluid and some cells/pathogens from the lung), keep the silicon tube in trachea
- transfer lavage fluid into 1.5 mL tube, centrifuge 1500 rpm 5 min, discard s/n, resuspend pellet in 350 µL RA1 buffer (MN kit), store at -80 degC
- fill lungs with 3 mL Dispase solution
- remove syringe, keep tube in trachea, fill lings with 0.45 mL agarose
- put ice on lungs for ~ 1-2 min until agarose solidifies
- cut the lungs out and put into 15 mL tube filled with rest of dispase, incubate 45 min RT
- pour 7mL DNase/DMEM into Petri dish
- take the lung out of tube, rip with forceps into small pieces, filter through 70 µm cell strainer into DNase/DMEM
- centrifuge cells 130 g 12 min 4 degC
- discard s/n, resuspend in 10 mL DMEM 10% FCS
- pour cells onto antibody-coated plates, incubate 2h 37 degC (panning)
- collect s/n and centrifuge it 130 g 12 min 4 degC
- remove s/n, resuspend in 10 mL Ham's F12 with additives, incubate 2h or o/n
- remove non-adherent cells, wash gently with PBS, apply RA1 (MN kit) lysis buffer, store RNA at -80 degC

<u>Oral infection with Salmonella/Listeria</u>

- take away food from mice for o/n
- grow bacteria o/n (~ 3 mL, Salmonella in LB, Listeria in BHI)
- measure OD_{600} (OD_{600}1=5*10^8 cfu/mL Salmonella and 1*10^9 cfu/mL Listeria (LO28))
- infect with 100 µL bacteria suspension (gavage)
  - 1-2 *10^9 cfu/mouse of Listeria
  - 1 *10^6 cfu/mouse of Salmonella
- sacrifice mice when necessary, isolate RNA from intestines (total tissue or epithelial layer only)

<u>MTT proliferation assay</u>

- cells seeded in 96-well plates and treated where necessary
- remove medium
- add 50 µL 10% MTT/medium solution
- incubate 3-4 h
- carefully aspirate medium
- add 50 or 100 µL of solvent solution
- stir gently
- measure specific absorption at 570 nm and background at 690 nm

MTS proliferation assay (Promega)

- thaw the reagent
- add 20 µL reagent per well containing cells in 100 µL medium
- incubate in incubator at 37 degC 5% CO_2 for 1-4 h
- measure absorption at 490 nm

Protein detection via Western Blot

- separating gel:
  - 7.5% : 4 mL H2O, 2 mL 1.5M Tris HCl pH 8.8, 2 mL acrylamide, 160 µL 10% SDS, 24 µL 20% APS, 24 µL TEMED
  - 10% : 3.3 mL H2O, 2 mL 1.5M Tris HCl pH 8.8, 2.7 mL acrylamide, 160 µL 10% SDS, 24 µL 20% APS, 24 µL TEMED
  - 12% : 2.8 mL H2O, 2 mL 1.5M Tris HCl pH 8.8, 3.2 mL acrylamide, 160 µL 10% SDS, 24 µL 20% APS, 24 µL TEMED
- stacking gel, 4%: 2.5 mL H2O, 1 mL 0.5M Tris HCl pH 6.8, 0.5 mL acrylamide, 80 µL 10% SDS, 12 µL 20% APS, 12 µL TEMED
- 5-20 µL of sample loaded (mix sample with 4x sample buffer and cook 5 min 95 degC, centrigufe 1 min max speed)
- 3 µL size marker loaded
- gel runs at 80V (running into separating gel), at 120V until necessary
- semi-dry blotting: anode (positive, down) | 6x 3MM Whatman in anode buffer 1 | 3x 3MM Whatman in Anode buffer 2 | membrane incubated in H2O | separating gel incubated in cathode buffer | 9x 3MM Whatman incubated in cathode buffer
- blotting running at 32 mA (fixed) 25 V 1.5h
- if necessary, colour with Ponceau red stain, decolorate with H2O, destain with TBST
- cut membrane if necessary
- block in 5% milk/PBS (3% BSA/PBS for phospho-specific antibodies)
- wash 3x 10 min TBST
- incubate with 1-ary antibody o/n 4 degC (antibody diluted in 2% BSA 0.05 % sodium azide)
- wash 3x 10 min TBST
- in the dark:
  - incubate with 2-ary antibody (1:20 000 in TBST) for 30 min on shaker
  - wash 3x 10 min TBST

- scan on IR imager
- if necessary, strip membrane in order to incubate with an other 1-ary antibody:
  - wash 3x 10 min TBST
  - strip 5 min in stripping buffer
  - wash with H2O
  - scan on IR imager to check for background, if necessary, strip further
  - wash 3x 10 min TBST
  - block membrane again, proceed with 1-ary and 2-ary antibody staining as before
- 4x sample buffer: 0.25 M Tris pH 6.8, 20% glycerol, 1.6% SDS, 20% beta-mercaptoethanol
- SDS-PAGE running buffer: 0.25 M Tris, 1.92 M glycin, 1% SDS, pH 8.3
- Anode buffer 1: 0.3 M Tris, 20% methanol, pH 10.4
- Anode buffer 2: 2.5 mM Tris, 20% methanol, pH 10.4
- Cathode buffer: 0.04 M 6-aminocapronic acid, 20% methanol, 0.01% SDS
- stripping buffer: 200 mM Glycin pH 2.8, 150 mM NaCl, 0.5 % Tween 20, H2O

Colonic crypt cell isolation for short term culture

- seed bovine aorta derived endothelial cells CW1 (~ 100 000 cells per well) on collagen coated wells (1µg/mL), let them grow until confluency (1d)
- remove medium, wash several times with PBS, lyse cells with 5mM ammonia solution (observe swelling and destruction of cells under microscope) for several minutes, wash several times with H2O, store in cold PBS if necessary – this is ECM-coated plate
- sacrifice mouse, remove colon, rinse in ice-cold 0.91% NaCl
- close smaller end of Pasteur pipette with fire, insert Pasteur pipette into cap of 50mL tube
- fix one end of colon to small end of Pasteur pipette with thread, cut excess of thread away, invert and extend colon, fix other end with thread
- incubate in EDTA solution for 50 min 37 degC 100rpm
- scrape epithelial layer with scalpel into Petri dish, add 1 mL DMEM, count crypts under microscope, seed in up to 1000 crypts/1mL/well in ECM-coated plates, use DMEM 5 % FCS/PenStrep as medium
- next day, remove unattached cells, add 1 mL DMEM 5% FCS
- exchange half of medium every 2nd day
- EDTA solution: 1.5mM ETDA, 109 mM NaCl, 2.4 mM KCl, 1.5 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 10 mM glucose, 5 mM glutamine
- medium for endothelial cells: 377.5mL DMEM high glucose, 100mL FCS, 5mL 100x non-essential amino acids, 5mL 100mM (100x) sodium pyruvate, 12.5mL 1M HEPES

Salmonella/Listeria infection of CMT-93 cells

- inoculate single colony of Salmonella or Listeria into 3 mL LB or BHI respectively, grow o/n 37 degC shaking
- Salmonella only: add 78 µL 5M NaCl to fresh 3 mL LB (final concentration 300 mM), add 75 µL of Salmonella o/n culture, mix, incubate 3h 37 degC (no shaking) → this induces invasiveness

- measure OD_{600} (OD_{600}1=5*10^8 cfu/mL Salmonella and 1*10^9 cfu/mL Listeria (LO28))
- Salmonella:
  - wash bacteria 3x in pre-warmed DMEM, centrifuge at max speed 2 min in table top centrifuge
  - add bacteria in total volume of 100 µL to cells
  - spin bacteria onto CMT-93 cells by centrifuging at 1000 rpm 5 min
  - incubate 1h 37 degC in incubator
  - while cells are incubating, dilute bacterial inoculum and plate on LB plates to determine actual MOI (grow at 37 degC o/n)
  - wash cells 1x with PBS, replace with medium containing 100 µg/mL gentamicin for 1.5-2 h to kill extracellular bacteria
  - replace medium with antibiotics-free medium
- Listeria:
  - apply necessary amount of Listeria
  - incubate 2h with CMT 93 cells, remove s/n
  - while cells are incubating, dilute bacterial inoculum and plate on BHI plates to determine actual MOI (grow at 37 degC o/n)
  - add medium containing 50 µg/mL gentamicin, culture for 1h in incubator
  - exchange medium to 10 10µg/mL containing medium
- LB: 10g Bacto-tryptone, 5g yeast extract, 10g NaCl, set pH to 7.5, add 15g agar, fill with H2O to 1L, autoclave
- BHI: 37 g/L BHI (brain heart infusion), 1% agar, aqueous solution

<u>Protein lysis</u>

- remove s/n from cells
- wash with PBS, remove s/n
- add 80 µL complemented Frackelton lysis buffer per well (6 well plate)
- scrape, transfer lysate to 1.5 mL tube
- store at -20 degC
- complementing Frackelton buffer:
  - 50 µL protease inhibitor mix (“pill”, diluted in 5mL H2O according to manufacturer's protocol)
  - 10 µL PMSF (100 mM in isopropanol)
  - 10 µL sodium orthovanadate (10 mM in H2O)
  - 1 µL DTT (1M in H2O)
  - 1mL Frackelton buffer (10 mM Tris-HCl, 30 mM Na_4P2O_7, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, pH 7.1, store at 4 degC), complement freshly

<u>Coomassie staining</u>

- fix SDS-PAGE gel in 40% ethanol 10% acetic acid aqueous fixing solution for 30 min
- wash 2x with H2O
- stain with Colloidal Coomassie (prepare freshly, 50 mL methanol, 200 mL Colloidal Coomassie

stock) for up to 3 h
- no need to destain
- fixing solution: 40% ethanol, 10% concentrated acetic acid, dissolved in H2O
- Colloidal Coomassie stock: 50g ammonium sulphate, 6 mL 85% phosphoric acid, 490 mL H2O, 10 mL 5% Coomassie Brilliant Blue G-250 (aq.)

Mucus isolation

- plate CMT 93, wait until confluent
- collect s/n, wash cells with additional 3mL PBS, pool to s/n → **s/n fraction**
- add 5 mL PBS onto cells, pipette roughly (to potentially isolate the mucus layer), collect into 15 mL tube, repeat 2 times pooling washes together → **wash fraction**
- add medium to CMT 93 cells and continue growing them (no splitting), collect s/n and wash fractions after 1-2-3 weeks again
- centrifuge fractions 1500 rpm 5 min RT, remove s/n
- add 50 µL of rehydration buffer
- transfer to 1.5 mL tube, store at -20 degC
- rehydration buffer: 8M urea, 2M thiourea, 4% CHAPS, 0.5% Triton X100, 0.005% bromphenol blue, H2O to 100 mL

Alcian blue staining

- grow cells on cover slips until they supposedly produce mucus layer
- remove medium
- fix cells with Carnoy's solution 10 min RT (60 % ethanol, 30% chloroform, 10% glacial acetic acid)
- wash 2x PBS
- stain mucus polysaccharides and glycosaminoglycans with cationic 1% alcian blue 1h RT
- wash with H2O
- stain cell nuclei with kernechtrot 5-10 min RT
- wash with H2O
- embed and observe
- to prepare kernechtrot staining solution dissolve 0.1g kernechtrot in 100mL 5% boiling aluminium sulfate

Alcian-PAS staining

- stain with 1% alcian blue 30 min
- wash with 5% sodium tetraborate
- wash with H2O
- incubate with 0.5% periodic acid 10 min
- wash with H2O
- incubate with Schiff's reagent 30 min
- wah extensively with H2O

- stain with hemalaun 30 sec
- wash with H2O
- dry and embed in Dako

Immunohistochemistry, FL antibodies

- grow cells on cover slips
- fix cells in 4% PFA 10-20 min RT
- wash 2x PBS
- permeabilize in 0.1% Triton X 100, 2-10 min RT
- wash 2x PBS
- block with 5% goat serum 30 min RT
- stain with 1:40 phalloidin (for actin) 40 min RT
- stain with primary antibodies of choice:
  - anti-E-cadherin, mouse, 1:50 in 2% BSA
  - pan-Cytokeratin-FITC, 1:50 in 2% BSA
- wash 1x PBS
- stain with secondary antibody:
  - anti-mouse 488, 1:1000 in 5% normal goat serum, 2h RT
- wash 1x PBS
- stain with 25 µL 1:1000 Dapi for few seconds
- wash 2x PBS
- wash 1x H2O
- mount on DAKO mounting medium on slide
- let dry o/n
- store at 4 degC

Probe preparation for Mass Spectrometry

- wear gloves & change them frequently to avoid keratin contamination
- cut out gel pieces with band of interest
- add 100 µL ABC buffer, 80 µL acetonitril (ACN) solution and shake 15 min 900 rpm 20 degC
- remove fluid, repeat if gel piece still has some colour
- remove s/n, add 80 µL ACN, shake 5 min 900 rpm 20 degC
- remove ACN
- dry in vacuum centrifuge 10 min RT
- add 200 µL 10 mM DTT (15mg/10mL in ABC buffer), shake 30 min 900 rpm 56 degC
- remove s/n, add 80 µL ACN, shake 5 min 900 rpm 20 degC
- remove ACN
- add 100 µL iodo acetamide (10mg/mL in ABC buffer), incubate in dark 20 min RT, no shaking
- remove s/n
- wash 3x with 200 µL ABC buffer (10 min 900 rpm), remove s/n
- add 80 µL ACN, shake 5-10 min 900 rpm 20 degC

- remove s/n, remove rest in vacuum centrifuge 10 min RT
- make Trypsin Gold working dilution (12.5 ng/µL) in 70 µL ABC buffer
- add 5-10 µL of Trypsin Gold soluiton onto every gel piece
- 5 min on ice, gel absorbs trypsin
- remove s/n if any
- cover gel piece with ABC buffer to avoid drying-out
- o/n digestion 37 degC (12-16 h)
- stop digestion with 10% trifluoracetic acid (TFA) (final concentration should be 1% TFA)
- sonicate 10 min (peptides go to solution, 1st extraction), collect fluid into small PCR tube
- add 10 µL 0.1% TFA to gel pieces, sonicate 10 min (2nd extraction), pool with 1st extraction
- freeze at -80 degC
- ABC buffer: 50 mM ammonium bicarbonate (198 mg in 50 mL MQ H2O)

<u>CMT-93 stimulation (poly-IC, poly-dAdT, IFN-γ, IFN-β, LPS, CpG), calculations per well of 6-well plate</u>

- poly-IC:
  - 10 µg pI:C + 1 mL DMEM + 3 µL Hiperfect (mix in this order) → 10 min RT
  - remove s/n from cells, apply this solution
  - 6h later add 1mL DMEM 20% FCS
- poly dAdT:
  - 10 µg pdA:dT + 3 µL Polyfect → 10 min RT → + 1 mL DMEM 10% FCS
  - remove s/n from cells, apply this solution
  - 6h later add 1 mL DMEM 10% FCS
- IFN-γ: add directly 1µg/well
- IFN-β: remove 400 µL s/n, add 5x IFN-β solution (5x stock is 250U/mL)
- IFN-λ (human IL28B): add directly to medium (100ng/mL)
- LPS: add directly to medium (1ng/mL-10µg/mL)
- CpG: add directly to medium (10µg/mL)

<u>Silverstain of SDS-PAGE gels</u>

- wash gel 2x 5min H2O
- fix gel 2x 15 min in 30% ethanol 10% acetic acid
- wash gel 2x 5 min 10% ethanol
- wash gel 2x 5 min H2O
- incubate 1 min with Sensitizer solution (0.1mL/50mL H2O)
- wash 2x 1 min H2O
- stain 30 min with Stain Working Solution (1mL Enhancer + 50 mL Stain)
- wash 2x 20 sec H2O
- develop until bands appear
- stop reaction with 5% acetic acid for 10 min
- put gel onto Whatman or onto Saran foil, can be dried with vacuum drier

PAS staining of SDS-PAGE gels

- fix gel with 40% ethanol 10% acetic acid 0.5-1h
- incubate in 1% periodic acid 7% acetic acid for 30 min
- incubate in 7% acetic acid for 30 min
- apply Schiff's reagent for 30 min
- wash with water 30 min

Also unsuccessful staining of mucins attempted according to "Staining of Glycoproteins/Proteoglycals in SDS-Gels" (paragraph 3.2), Moller and Poulsen, Protein Protocols Handbook, 2nd edition

RNA isolation with Trizol (colonies from agar)

- pass gel pieces with 1 mL Trizol through needle until homogenous
- add 200 µL chloroform, shake 15 sec and incubate 5 min RT
- centrifuge 15 min 12 000 rpm 4 degC
- take out water (upper) phase and mix it with 500 µL of isopropanol
- incubate 10 min RT
- centrifuge 10 min 12 000 rpm 4 degC
- remove s/n, put 1 mL 75% ethanol
- centrifuge 5 min 7 500 rpm 4 degC
- remove ethanol by pipette and air-dry pellet (do not dry too much)
- resuspend pellet in 200 µL water (DEPC, o not use DEPC water if RNA to be used for microarray), incubate 10 min at 55 degC 300 rpm
- add 20 µL 3M sodium acetate (pH 5.2) and 500 µL 96% ethanol
- precipitate o/n at 20 degC
- centrifuge 30 min full speed 4 degC
- remove s/n, wash pellet with 1mL 75% ethanol
- centrifuge 10 min full speed 4 degC
- rmeove ethanol, air-dry pellet
- resuspend in 30 µL H2O (DEPC) 10 min 55 degC 300 rpm
- store at -80 degC
- chloroform: mix 49+1 with isoamyl alcohol, add 2 mL autoclaved H2O, shake, resolve phases, remove H2O, repeat 3x, leave some H2O over chloroform, store in dark

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I'm sitting now on softest cloud.

For future that is still not set,
For smiles that we might someday get,
For finding way on path that's mined,
For lives I dedicate my life.

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- 2006-2011 Universitaet Wien, Diplomstudium Molekularbiologie

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Stipendien:

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Laboratory rotations:

Dr Javier Martinez, IMBA, Vienna, Austria, 4 months:

RNA biochemistry. A library of short hairpin RNA constructs was created and transfected into HeLa cells and radioactive RNA ligation assays were performed to screen for RNA ligation inhibition. In parallel, immunofluorescence studies were performed with polyclonal antibodies to identify possible localization of putative tRNA ligase. Acknowledged in: Popow et al., HSPC117 Is the Essential Subunit of a Human tRNA Splicing Ligase Complex, Science 2011, 331 (6018): 760-764.

Dr Balbino Alarcon, CBMSO, Madrid, Spain, 2 months:

T cell receptor signalling. FLIM-FRET and other live fluorescent microscopy experiments were performed to test for possible interaction between signalling molecules, Western blot analysis was done to determine the dynamics of protein levels and phosphorylation after T cells were activated by target cells.

Dr Maria Luisa Toribio Garcia, CBMSO, Madrid, Spain, 2 months:

T cell development. Viral transduction of SNP-bearing receptor into T cell line and observation of proliferative capacity (FACS). Immunohistochemical studies of Notch pathway molecules localization in human thymus. FTOC: fetal thymic organ culture: hematopoietic progenitors, deficient in specific signalling molecules, were cultured in isolated mouse embryonal thymus stroma to define signalling pathways involved in T cell development. Human cord blood hematopoietic progenitors were isolated for in vitro differentiation studies.

Dr Juan-Jose Garrido, CBMSO, Madrid, Spain, 1 month:

Neuronal cell development. Isolation and culture of mouse hippocampal neurons transfected with genes involved in signalling pathways, treatment of primary neurons with stimulatory/inhibitory molecules to determine their effect on axonal growth and morphology , studies of radial glia cell differentiation in vitro (fluorescence microscopy).

Scholarships:

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