Interleukin-10-Loaded Nano- and Microparticles for the Local Treatment of the Intestinal Mucosa and the Deep Lung

Dissertation

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Zusammenfassung

Das antiinflammatorische Zytokin Interleukin-10 (IL-10) gilt als vielversprechender Wirkstoff zur Behandlung entzündlicher Erkrankungen wie chronisch-entzündlicher Darmerkrankungen und dem akuten Atemnotsyndrom (ARDS). Die lokale Anwendung von IL-10 ist der systemischen vorzuziehen, da eine höhere Effektivität und geringere Nebenwirkungen zu erwarten sind. Die vorliegende Arbeit befasst sich daher mit der Entwicklung von *Drug Delivery*-Systemen zur lokalen Freisetzung von IL-10 an der Darmschleimhaut und in der tiefen Lunge.

Mittels einer Doppelemulsionsmethode wurden IL-10-beladene Nanopartikel (NP) aus Polylactid-co-Glycolid (PLGA) entwickelt. Die NP wiesen jedoch nur eine geringe *in vitro*-Freisetzung von IL-10 auf, das außerdem nicht mehr bioaktiv war.

Mittels Sprühtrocknung wurden IL-10-beladene Mikropartikel (MP) hergestellt, die eine hohe IL-10-Bioaktivität und gute Langzeitstabilität aufwiesen. Um die Magensaftresistenz zu erhöhen, wurden die MP in Mikrosphären aus Eudragit L 100-55 oder S 100 verkapselt. Jedoch verursachte die Inkubation in sauren Medien trotzdem eine fast vollständige Denaturierung des IL-10.

Die MP wurden mit Leucin modifiziert, so dass ein Trockenpulver mit sehr guten aerodynamischen Eigenschaften erzielt wurde. Die Applikation in einem entzündeten alveolaren Kokulturmodell reduzierte die inflammatorischen Marker IL-6 und TNF signifikant und zeigt das Potential für die klinische Entwicklung von IL-10-beladenen Trockenpulvern zur Inhalation.

Abstract

The anti-inflammatory cytokine Interleukin-10 (IL-10) has been proposed as drug for the treatment of acute and chronic inflammatory diseases, such as inflammatory bowel disease (IBD) or the acute respiratory distress syndrome (ARDS). The local delivery of IL-10 is preferable to systemic application, as fewer side effects are expected. Therefore, the present study deals with the development of drug delivery systems for the local delivery of IL-10 to the intestinal mucosa and the deep lung.

IL-10-loaded poly(lactic-co-glycolic) acid (PLGA) nanoparticles were developed by a double emulsion method, with bovine serum albumin (BSA) as stabiliser for the IL-10. These particles showed slow IL-10 release and poor bioactivity.

IL-10-loaded microparticles (MP) were prepared by spray drying. These particles showed high remaining IL-10 bioactivity and promising long-term stability. For local targeting of the intestinal mucosa, the particles were encapsulated in Eudragit L 100-55 or S 100 microspheres. However, exposure to gastric conditions resulted in almost complete denaturation of the IL-10.

Using leucine for the preparation of the spray-dried MP, dry powder particles with excellent aerodynamic properties were produced. Applied to an inflamed human alveolar co-culture model, the dry powder significantly reduced the inflammatory markers IL-6 and TNF as compared to blank particles, indicating the potential for a clinical development of IL-10-based dry-powder formulations for inhalation.

Contents

Acronyms

ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BSSB	Balanced salt solution B
CBA	Cytometric bead array
CCD	Central composite design
CD	Crohn's disease
CMWT	Culture medium without T-STIM
DCM	Dichloromethane
DLS	Dynamic light scattering
DMF	Dimethylformamide
DoE	Design of experiment
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
EMA	European Medicines Agency
EPR	Enhanced permeability and retention
EtOAc	Ethyl acetate
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCCD	Face-centred central composite design
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FPF	Fine particle fraction
GM-CSF	Granulocyte-macrophage colony-stimulating-factor
GSD	Geometric standard deviation (GSD)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hIL-10	human interleukin-10
HP-β-CD	(2-Hydroxypropyl)-β-cyclodextrin
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-1ß	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
iNOS	inducible nitric oxide synthase

IP	Intraperitoneal
IV	Intravenous
JAK1	Janus kinase 1
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MMAD	Mass median aerodynamic diameter
MMP-9	Matrix metallopeptidase-9
MOC	Micro-orifice collector
MOF	Multi organ failure
MP	Microparticle(s)
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor κB
NGI	Next Generation Impactor
NiMOS	Nanoparticle-in-microsphere oral system
NP	Nanoparticle(s)
O/W	Oil-water
PADDOCC	Pharmaceutical Aerosol Deposition Device on Cell Cultures
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PDI	Polydispersity index
PEG	Polyethylene glycol
PLGA	Poly(lactic-co-glycolic) acid
PVA	Polyvinyl alcohol
RH	Relative humidity
rhIL-10	recombinant human interleukin-10
rmIL-4	recombinant murine interleukin-4
RT	Room temperature
SAGM	Saline-adenine-glucose-mannitol
SC	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
siRNA	small interfering ribonucleic acid
SOCS3	Suppressor of cytokine signalling 3
S/O/O	Solid-in-oil-in-oil
S/O/W	Solid-in-oil-in-water
STAT	Signal transducer and activator of transcription
TARC	Thymus- and activation-regulated chemokine
Th	T helper
TLR	Toll-like receptor
TNBS	Trinitrobenzenesulfonic acid

TGF	Transforming growth factor
TNF	Tumour necrosis factor
UC	Ulcerative colitis
W/O/W	Water-in-oil-in-water

1 General Introduction

1.1 Interleukin-10

Interleukin-10 (IL-10) is a potent immunoregulatory pleiotropic cytokine. It was originally discovered by Fiorentino et al. in 1989 as a factor that inhibits the Interferon-γ (IFN-γ) synthesis by T helper (Th) 1 cell clones (Fiorentino et al., 1989). The novel cytokine had originally been named cytokine synthesis inhibitor factor and was later renamed to IL-10 (Steidler et al., 2009). While its key features relate mainly to its strong immunosuppressive effects, IL-10 has immunostimulatory properties as well. It plays a crucial role in preventing overshooting inflammatory responses and autoimmune pathologies. Together with the interferons, the IL-10 family forms the class II cytokine family (Ouyang et al., 2011).

1.1.1 Structure und receptor binding

Human interleukin-10 (hIL-10) is composed of two identical polypeptide chains with 160 amino acids and a molecular mass of 18.6 kDa each (Vieira et al., 1991). The two non-covalently-bound subunits form an intercalated homodimer (Zdanov et al., 1995). Each subunit contains two intra-chain disulfide bonds, one between the residues 12 and 108 and the other between the residues 62 and 114 (Windsor et al., 1993). The crystal structure of hIL-10 revealed that each subunit of the protein consists of six α-helical segments (A-F) and their connecting loops (depicted in Fig. 1.). The non-covalent dimeric structure is stabilised by intertwining of the helices E and F. The dimer topology and architecture of IL-10 is very similar to that of IFN-γ (Zdanov et al., 1995), (Zdanov et al., 1996). Native IL-10 has an apparent isoelectric point of pH 8.2 (Bondoc et al., 1997).

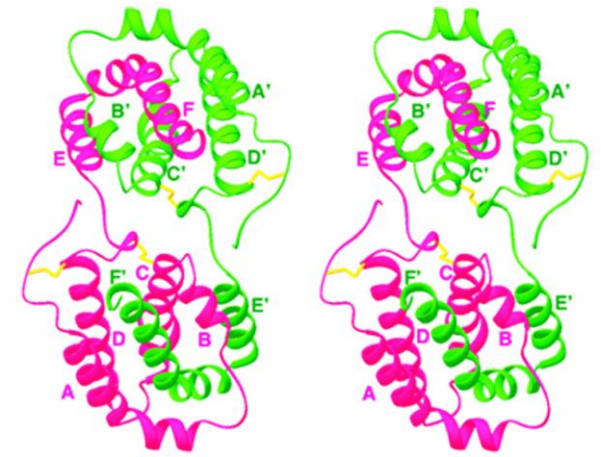

Fig. 1: Stereo diagram of the 3D structure of hIL-10 (Zdanov, 2010). The two monomers that form the dimer are shown in violet and green. Disulfide bridges are depicted in yellow. Reprinted from (Zdanov, 2010) with permission from Elsevier.

Full cellular responses to IL-10 require the sequential assembly of two different class II cytokine transmembrane receptor subunits, namely IL-10R1 (also known as IL-10RA) and IL-10R2 (also known as IL-10RB) on the cell surface (Liu et al., 1994), (Kotenko et al., 1997). Initially, IL-10 binds with high affinity to IL-10R1. This binding induces a conformational change of IL-10 that regulates binding, with low affinity, to IL-10R2, resulting in the formation of a ternary receptor complex. The structure of the whole receptor complex has not yet been determined (Zdanov, 2010), (Acuner-Ozbabacan et al., 2014). So far, only a crystal structure of the 1:2 complex of hIL-10 with the extra-cellular, water-soluble domain of IL-10R has been elucidated (Josephson et al., 2001).

1.1.1 Biology of IL-10

The biological activities of IL-10 are mediated by receptor engagement, followed by formation of the ternary receptor complex, which activates the JAK/STAT signalling pathway (Fig. 2). First, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) are activated. They both autophosphorylate themselves and phosphorylate specific tyrosine residues of the intracellular part of IL-10R1. Subsequently, signal transducer and activator of transcription 3 (STAT3) binds to these tyrosine residues. In turn, STAT3 is activated by phosphorylation (Finbloom and Winestock, 1995), (Kotenko et al., 1997), (Riley et al., 1999), (Wehinger et al., 1996). In addition, STAT1, and in some cells additionally STAT5, molecules are activated (Wehinger et al., 1996). Homo- and heterodimers of these transcription factors are formed.

These dimers translocate to the nucleus where they bind to several promoters and induce the transcription of various genes (Weber-Nordt et al., 1996).

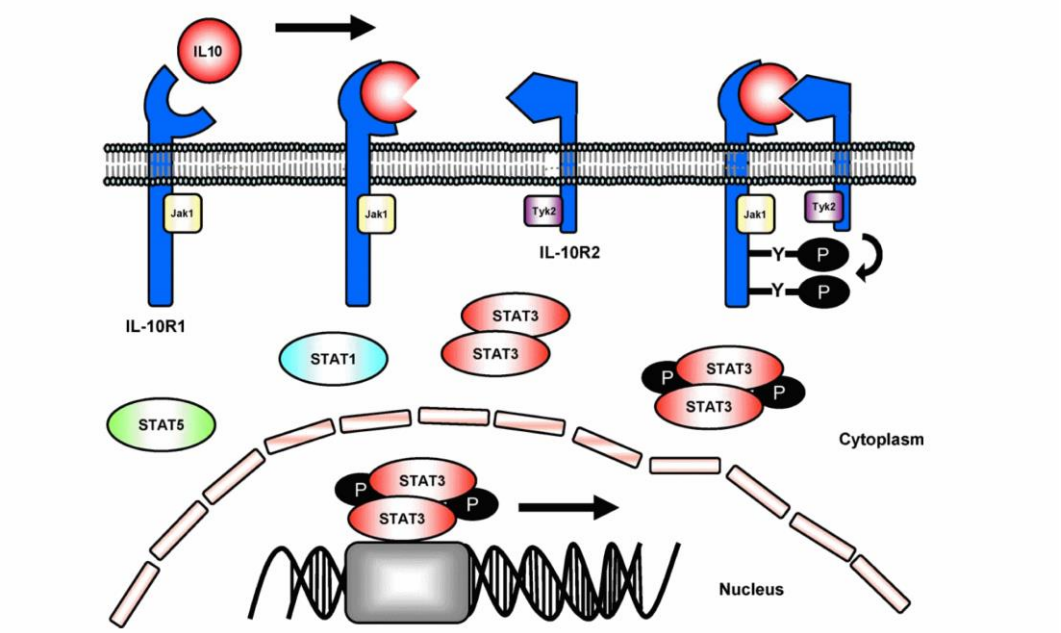

Fig. 2: The interaction of IL-10 with its receptors and the IL-10 signalling pathway (Sabat et al., 2010). IL-10 first binds to IL10R1 followed by a conformational change of IL-10 which regulates additional binding to IL10R2 resulting in the formation of a ternary receptor complex. The receptor associated kinases JAK1 and TYK2 are activated followed by tyrosine phosphorylation of the intracellular part of IL10R1. This initiates the binding of STAT3, STAT1 and, in some cell types, additionally STAT5. STAT homo- and heterodimers translocate into the nucleus where they bind to STAT-binding elements of several promoters and induce transcription of the respective genes. Reprinted from (Sabat et al., 2010) with permission from Elsevier.

A main mechanism of the IL-10-mediated immunosuppression is the inhibition of the nuclear translocation and the DNA binding of the nuclear factor κB (NF-κB), as well as the blocking of the inhibitor of κB kinase activity (Schottelius et al., 1999). Furthermore, IL-10 inhibits the Toll-like receptor (TLR)-induced synthesis of pro-inflammatory mediators by inhibition of MyD88 translation (Dagvadorj et al., 2008).

IL-10 is synthesised by almost all leukocytes in the human body. Originally, it had been discovered as a factor secreted by Th2 cells (Fiorentino et al., 1989). Monocytes and macrophages are additional important cell sources but IL-10 is also produced by dendritic cells, B cells, natural killer cells and eosinophilic and neutrophilic granulocytes. The local concentration of IL-10 depends on the type of tissue and the specific immune stimulation. IL-10 secretion by monocytes and

macrophages is induced by several endogenous and external stimulators, such as catecholamines and lipopolysaccharide (LPS) (Sabat et al., 2010).

The biological effects of IL-10 are very complex and versatile. Monocytes and macrophages are the main target cells of inhibitory IL-10 effects (Sabat et al., 2010). In peripheral blood mononuclear cells (PBMCs) of psoriasis patients, IL-10 treatment upregulates 1600 genes and downregulates another 1300 genes (Jung et al., 2004). IL-10 supresses mainly the active, proinflammatory role of monocytes/macrophages in innate and specific immunity, whereas inhibitory, tolerance-inducing functions are enhanced. The release of immune mediators, antigen presentation and phagocytosis are each affected (Sabat et al., 2010). Specifically, IL-10 inhibits the IFN-γ- and LPS-induced production of the mostly pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor (TNF), granulocyte-macrophage colony-stimulating-factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) from monocytes and macrophages on the transcriptional level (de Waal Malefyt, Abrams, et al., 1991), (Fiorentino et al., 1991). In contrast, the production of IL-1 receptor antagonist and soluble TNF receptors, which act as anti-inflammatory mediators, are enhanced (Jenkins et al., 1994), (Hart et al., 1996). Additionally, IL-10 inhibits the antigen presentation by macrophages and monocytes by reduction of both the constitutive and the IFN-γ-induced expression of major histocompatibility complex (MHC) class II proteins on the cell surface (de Waal Malefyt, Haanen, et al., 1991). The differentiation of monocytes to dendritic cells is inhibited by IL-10, whereas the maturation to macrophages is enhanced (Allavena et al., 1998). IL-10 also acts on other cell types, for example on T cells. It inhibits both the proliferation and the cytokine synthesis of type 1 and type 2 CD4+ cells (Del Prete et al., 1993). In neutrophilic and eosinophilic granulocytes, IL-10 inhibits the LPS-induced synthesis of several pro-inflammatory cytokines (Cassatella et al., 1993), (Takanaski et al., 1994). Additionally, IL-10 inhibits the production of cyclooxygenase-2 in neutrophils and thus the synthesis of pro-inflammatory prostaglandin E2 (Niiro et al., 1997). IL-10 also inhibits both the spontaneous and antigen-induced production of nitric oxide and pro-inflammatory cytokines like TNF by mast cells (Arock et al., 1996), (Lin and Befus, 1997). The

expression of immunoglobulin (Ig) E receptors and the IgE-mediated activation of mast cells is inhibited by IL-10 as well (Kennedy Norton et al., 2008).

IL-10 has not only inhibitory functions but also acts on different immune cells in a stimulatory way. For example, it enhances IL-2-dependent proliferation and cytokine production as well as the cytotoxic activity of natural killer cells (Carson et al., 1995). Furthermore, it enhances proliferation and MHC II expression of B cells and their differentiation into plasma cells (Go et al., 1990), (Rousset et al., 1995).

It has been shown that several types of pathogens exploit the immunosuppressive effect of IL-10 for their own benefit. Co-evolution of viruses with their hosts has led to the encoding of orthologs of IL-10, which are called viral IL-10s. Several viruses seem to have independently acquired these orthologs from their hosts. To date, viral IL-10 orthologs in at least 21 viruses have been reported, among them the human cytomegalovirus and the Epstein-Barr virus. In comparison with their cellular orthologs, viral IL-10s show a bioactivity profile that is more restricted to immunosuppressive activities (Ouyang et al., 2014). Other pathogens are known for exploiting the effects of IL-10 as well. Infection with the tick-borne bacterium B*orrelia burgdorferi* causes lipoprotein-mediated elicitation of IL-10 by human immune cells, which seems to suppress immune activities that are critical for the early control of the infection, enhancing the chances of the bacterium to evade early clearance and allowing them to persist in the host (Chung et al., 2013). Parasitic worms induce IL-10 expression in the host as well, to evade clearance by the immune system (Specht et al., 2012). On the other hand, there is also clear evidence that enhanced IL-10 expression might be beneficial to ameliorate severe systemic effects caused by infections such as malaria (Freitas do Rosario and Langhorne, 2012).

IL-10 was also found to play an important role in the pathogenesis of various non-infectious diseases. These diseases can be subdivided into the ones associated with IL-10 deficiency and those associated with IL-10 overproduction (Sabat et al., 2010). The development of *Lupus erythematosus* and melanoma seems to be favoured by enhanced IL-10 concentrations. Enhanced concentrations of IL-10-producing cells were found in *Lupus erythematosus* patients and their IL-10 serum

level corelates with disease activity (Gröndal et al., 1999), (Houssiau et al., 1995). Furthermore, patients treated with anti-IL-10 antibodies showed a reduction of disease activity. These clinical findings were mainly attributed to the decreased expression of several activation markers on endothelial cells and their reduced spontaneous release by PBMCs (Llorente et al., 2000). IL-10 is also a growth factor for human melanoma cells (Yue et al., 1997), which indicates a tumour-promoting activity. Overall, however, the role of IL-10 in the development and progression of cancer is diverse, complex and not fully understood. As IL-10 induces immunosuppression, it can assist in the escape of malignant cells from tumour immune surveillance. On the other hand, IL-10 exerts both proliferative and inhibitory effects on tumours, e.g. in breast cancer (Hamidullah et al., 2012). It promotes tumour-specific immune surveillance and inhibits pathogenic inflammation in cancer and has thus even emerged as a new potential drug for immune intervention in cancer (Dennis et al., 2013). Evidence of antitumor activity of PEGylated IL-10 was shown in a phase I study in patients with advanced solid tumours (Naing et al., 2016). A phase III trials that investigates PEGylated IL-10 in combination with chemotherapy in patients with metastatic pancreatic cancer is currently in the recruiting phase (ClinicalTrials.gov identifier: NCT02923921, 2017).

In diseases associated with an absolute or relative IL-10 deficiency, the persistent overshooting immune activation causes chronic inflammation. This is e.g. the case in inflammatory bowel disease (IBD) which is described in detail in chapter 1.2 and in autoimmune diseases like multiple sclerosis and psoriasis. Low IL-10 mRNA expression was found in psoriatic skin lesions as compared to other inflammatory dermatoses. Furthermore, patients on conventional anti-psoriatic therapy showed increased IL-10 expression by PBMCs, which suggests anti-psoriatic activity of IL-10 (Asadullah et al., 1998). There is also evidence for the role of IL-10 deficiency in multiple sclerosis: B-cells from patients were found to have a reduced capacity to produce IL-10 (Hirotani et al., 2010).

In conclusion, IL-10 is a potent immune suppressor that inhibits both the innate and the adaptive immune system. As a potent anti-inflammatory mediator, it plays an important role in limiting immune responses and can prevent tissue damage caused

by overshooting and prolonged inflammatory immune responses. On the other hand, IL-10 also exhibits immunostimulatory functions. The effects of IL-10 are complex and depend on the specific tissue and target cells.

1.1.2 Potential as an anti-inflammatory drug

Due to its potent anti-inflammatory properties, IL-10 has been proposed as a potential drug for the treatment of several autoimmune and immune-related diseases like rheumatoid arthritis, psoriasis, multiple sclerosis, type I diabetes and IBD. Consequently, substantial efforts were directed at the (mostly systemic) investigation of the therapeutic efficacy of IL-10 against several of these diseases. However, most studies that investigated the systemic administration of IL-10 in patients with auto-immune diseases resulted in conflicting or insignificant results (Saxena et al., 2014).

So far, the most promising results of systemic IL-10 application have been obtained in the treatment of psoriasis, a chronic inflammatory disease of the skin at which leukocytes migrate from the blood vessels to the epidermis where they form papulosquamous plaques (Griffiths and Barker, 2007). High levels of pro-inflammatory cytokines like IFN-γ and TNF are found in the skin lesions, while IL-10 concentrations are lower than in other dermatoses (Griffiths and Barker, 2007), (Asadullah et al., 1998). In a phase II trial, patients were treated with subcutaneous (SC) injections of IL-10 for 7 weeks. Anti-psoriatic effects were found in 9 of 10 patients and a significant decrease in both the affected area and the disease index was observed (Asadullah et al., 1999). In a second placebo-controlled phase II trial, patients with chronic psoriasis in submission received SC injections of IL-10 for 4 months. The incidence of relapses was significantly lower in the IL-10-treated group than in the placebo group (Friedrich et al., 2002). The mechanisms of IL-10 activity on psoriasis are still not well understood but are thought to be caused by effects on T cells and antigen-presenting cells (Asadullah et al., 2001). In contrast, a placebo-controlled double-blind phase II trial with psoriasis patients receiving IL-10 SC for 12 weeks showed only a marginal clinical improvement compared to the placebo group, although a clear shift of the T cell response from the Th1 to Th2 type was observed (Asadullah et al., 1998).

Multiple sclerosis is a chronic inflammatory, demyelinating disease of the central nervous system characterized by focal inflammatory lesions, which are associated with irreversible damage to axons and myelin. Autoreactive T cells and B cells play an important role in the demyelination (Lassmann et al., 2012), while IL-10-producing B cells contribute to inhibiting inflammatory responses that enhance the destruction of myelin (Saxena et al., 2014). However, B cells from patients with multiple sclerosis have a diminished capacity to excrete IL-10 (Hirotani et al., 2010). It was also shown that the treatment with Interferon-β enhances the IL-10 serum levels in multiple sclerosis patients. Therefore, it was suggested that an upregulation of IL-10 production is possibly the positive mechanism of action of Interferon-β in multiple sclerosis (Rudick et al., 1996). However, systemic IL-10 treatment failed to attenuate demyelination in animal models of multiple sclerosis and is thus currently not thought to be effective in ameliorating the disease (Saxena et al., 2014).

Rheumatoid arthritis, an autoimmune disorder primarily affecting the joints, is characterised by synovial inflammation that causes destruction of cartilage and bone, autoantibody production and, in some patients, systemic inflammation (McInnes and Schett, 2011). The disease is driven by the expression of TNF and other pro-inflammatory cytokines, which enhance the inflammatory immune responses and play an important role in the destruction of the joints. IL-10 seems to play an ambivalent role in the disease by suppressing inflammatory cytokines while simultaneously enhancing the humoral immune response (Saxena et al., 2014). Its stimulatory effect on the expression of IgG receptors on monocytes and macrophages stimulates their pro-inflammatory and tissue-destructive activity (Van Roon et al., 2003). A clinical study investigating a treatment by IL-10 combined with methotrexate in patients with rheumatoid arthritis showed a slight clinical improvement compared to treatment with only methotrexate (Weinblatt et al., 1999). However, as IL-10 was not as potent in inhibiting the symptoms of rheumatoid arthritis as TNF antibodies, the development was stopped. The short half-life of IL-10 may have played a role in its limited efficacy (Schwager et al., 2009) (Weinblatt et al., 1999). Dekavil, a fusion protein of an antibody directed against the extra-domain A of fibronectin with IL-10, is currently in clinical development. Dekavil inhibits progression of arthritis in the collagen-induced

mouse model (Schwager et al., 2009). In a phase Ib clinical trial of a combination therapy with methotrexate, 15 of 23 patients experienced a therapeutic benefit (Galeazzi, Bazzichi, Sebastiani, Neri, Giovannoni, et al., 2014), (Galeazzi, Bazzichi, Sebastiani, Neri, Garcia, et al., 2014).

1.1.3 Stability of IL-10

The first publication on IL-10 already described its high susceptibility to acidic conditions (Fiorentino et al., 1989). After incubation at pH 2 for 1 h, almost no bioactivity remained. This allowed an early distinction from other cytokines that are much less prone to acidic denaturation. This initial observation was confirmed and further investigated by Syto et al.: if preincubated at a pH below pH 6, the bioactivity of IL-10 in solution decreases in a pH-dependent manner with an apparent pK_a value of about 4.8. The loss of activity is linearly correlated with the irreversible dissociation of the non-covalent dimer. As the monomer is not bioactive, the bioactivity of IL-10 bioactivity depends on preservation of the dimer. Syto et al. also investigated the influence of temperature stress on IL-10 solutions. Heating of 0.3 mg/ml IL-10 in aqueous solution to 55°C for one hour resulted in 22% dissociation of the dimer as compared to only 2% dissociation after heating to 37°C. When the IL-10 concentration was reduced to 0.05 mg/ml, the formation of the monomer was enhanced to 55% and 10% after incubating the solution for 1h at 55°C and 37°C, respectively, indicating that the rate of the dissociation of the dimer dependent on the initial concentration of IL-10. Again, a linear dependence of the IL-10 bioactivity on the remaining amount of dimer was observed (Syto et al., 1998). Additionally, the two intact disulfide bridges formed by the four cysteines of each IL-10 molecule are essential for the preservation of IL-10 bioactivity. Reduction of these bonds destabilises the protein and reduces its α-helical content from 60% to 53%. The reduced IL-10 possesses no *in vitro* biological activity (Windsor et al., 1993).

Carvalho et al. investigated the structural stability of 0.25 mg/ml IL-10 in phosphate-buffered saline (PBS) at 37°C by circular dichroism. After 6 days, the measured spectrum completely lost its characteristic pattern, indicating quantitative denaturation of the protein (Carvalho et al., 2010). The stability of IL-10 in serum samples was assessed at temperatures of 4°C, 20°C, 30°C and 40°C over 21 days.

The concentration of IL-10, as determined by an enzyme-linked immunosorbent assay (ELISA), remained constant at 4°C but significantly decreased at the higher temperatures. At 20°C, the concentration declined to 63% after 14 days. At 40°C, it decreased to 70% after only one day (Kenis et al., 2002).

1.2 Inflammatory bowel disease (IBD)

IBD is an umbrella term for a group of chronic relapsing diseases of the gastrointestinal tract. The most common disorders of this group are Crohn's disease (CD) and ulcerative colitis (UC). They are both characterised by epithelial damage and intestinal inflammation (Abraham and Cho, 2009). A minority of IBD patients additionally suffers from extraintestinal manifestations, most commonly of the musculoskeletal and dermatologic systems. (Bernstein et al., 2001). CD can discontinuously affect any region of the intestine (Fig. 3). The inflammation is transmural, that is, it spans into the entire depth of the intestinal wall and forms ulcers. Sometimes, these ulcers even completely extend through the intestinal wall, creating channel connections to other organs called fistulas. In contrast, in UC the inflammation is solely confined to the mucosa and does not affect deeper tissue layers. The rectum, and often additionally the colon, are affected in a continuous pattern. (Abraham and Cho, 2009).

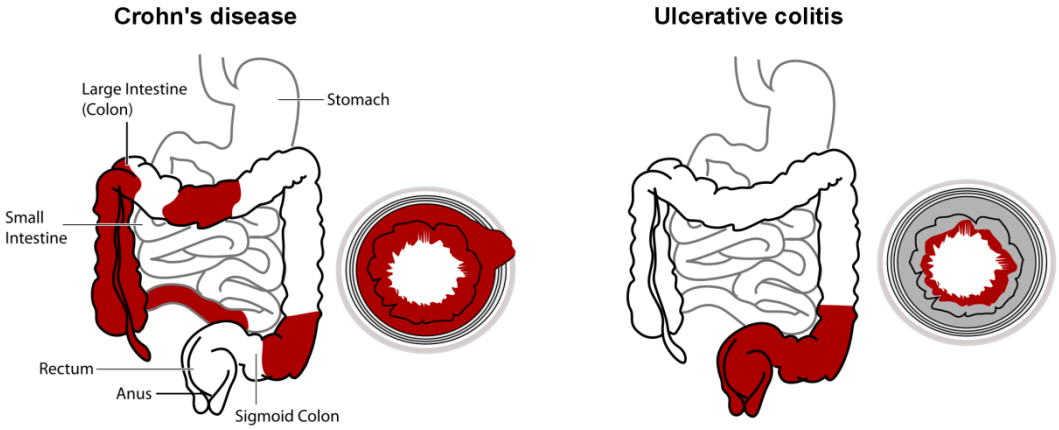

Fig. 3: The localisation of inflammation in CD and UC in the gastrointestinal system. The affected intestinal tissue layers are shown in schematic cross sections. Figure adapted from (Lock, 2004).

Common symptoms of both diseases comprise (often bloody) diarrhoea and abdominal pain. For IBD patients, the risk of development of intestinal cancers is drastically higher than for healthy persons (Lukas, 2010), (Laukoetter et al., 2010).

It has been estimated that more than 3.5 million people in the world are affected by IBD, thereof more than 2 million in Europe (Burisch et al., 2013), (Loftus Jr, 2004).The direct healthcare costs of IBD in Europe are estimated at about 5 billion Euros per year (Burisch et al., 2013). The incidence of IBD in developing countries is generally rising, which seems to be associated with the spread of the Western life style (Loftus Jr, 2004).

A combination of genetic and environmental factors such as hygiene and diet is thought to be responsible for the development of IBD (Van Limbergen et al., 2014), (Cosnes, 2010). Interactions between the intestinal immune system and the intestinal microbiota are thought to play a major role in the onset of IBD. In the healthy intestine, the immune system adapts to the commensal bacteria, so that pro-inflammatory and immunoregulatory responses are well balanced (homeostasis). In IBD, a dysregulation of the normal response of the immune system to intestinal bacteria is believed to disrupt this homeostasis and cause chronic active inflammation (Barbosa and Rescigno, 2010).

Currently, no cure is available for IBD. Therefore, patients often depend on lifelong pharmaceutical therapy to control the disease. The acute disease is treated with the goal to reduce inflammation and induce remission of the symptoms. Once remission is achieved, medical treatment is continued to avoid recurrence of the symptoms. The current guidelines of the European Crohn's and Colitis Association recommend the treatment with aminosalycilates (e.g. mesalazine), systemic and local corticosteroids (mainly budesonide), immunomodulators (azathioprine, 6-mercaptopurine, methotrexate) and TNF antibodies. The choice of medication depends on the disease, its localisation and the severity of the symptoms (Dignass et al., 2010), (Dignass et al., 2012). The introduction of TNF antibodies, like infliximab, in the therapy of IBD was a major improvement for many patients, especially those suffering from severe therapy-resistant IBD. Unfortunately, even infliximab fails to maintain remission in more than 50% of the treated patients (Hanauer et al., 2002). The therapy with TNF antibodies also has major drawbacks. E.g., it increases the risk for serious bacterial infections in comparison to therapy with immunomodulators (Schneeweiss et al., 2009). Furthermore, a dose-dependent increase in the risk of malignancies was observed in rheumatoid arthritis patients

treated with TNF antibodies (Bongartz et al., 2006). In 2008, natalizumab, a humanised monoclonal antibody against the cell adhesion molecule α4-integrin, was approved by the FDA for the treatment of CD. This antibody inhibits the infiltration of lymphocytes into the intestinal tract (Ghosh, 2003), (FDA, 2008). Despite these recent valuable additions to the existing therapeutic spectrum, the available pharmacological options are still not sufficient to ensure a satisfactory long-term treatment of many patients. Consequently, the rate of intestinal surgery in Europe has recently still been at least 30% for patients with CD and 10% for patients with UC within the first 10 years after diagnosis (Burisch et al., 2013). This reveals the urgent need for an improved pharmacological therapy – including novel drugs – in the management of IBD.

1.2.1 Nano- and microparticles as novel drug delivery systems for the treatment of IBD

Drug delivery systems based on nanoparticulate carrier systems have been widely investigated over the last years. The large functional surface offers many possibilities for drug loading and interactions with biological barriers and single cells, allowing e.g. a specific active targeting by coupling with antibodies. Nanoparticles (NP) can be optimised for a sustained and controlled release of drugs. Furthermore, NP can be used for the passive targeting of specific tissues. A widely known and often exploited example is the enhanced permeability and retention (EPR) effect in tumours (Matsumura and Maeda, 1986). The EPR enables the selective passive targeting of tumour tissue by intravenous (IV) administration of NP (Brannon-Peppas and Blanchette, 2012).

It is tempting to assume that small particles may also selectively accumulate in inflamed intestinal tissue, as the intestinal barrier is severely disrupted in the inflamed areas. In IBD, microerosions of the epithelial barrier and discontinuous tight junction strands are commonly found, even in patients with only moderate disease activity (Zeissig et al., 2007), (Schmidt et al., 2013), (Salim and Söderholm, 2011). Indeed, Lamprecht et al. observed an adherence of NP (100 nm in size) in the inflamed tissue of a trinitrobenzenesulfonic acid (TNBS) rat model that was six times higher than in healthy tissue. For microparticles (MP, 1 µm in size), the deposition was 5-fold higher in inflamed tissue than in the healthy control. The

accumulation was mainly attributed to the enhanced mucus production in inflamed regions. It was also shown that a substantial amount of NP still resided in the inflamed colon 72 h after treatment (Lamprecht, Schäfer, et al., 2001). This selective and prolonged accumulation results in both enhanced drug concentration and sustained drug release in inflamed tissue as compared to adjacent normal tissue (Lamprecht, Ubrich, et al., 2001), (Lamprecht et al., 2005b). Recently, the results obtained in animal models were partially confirmed in humans as well. Poly(lactic-co-glycolic) acid (PLGA) particles, 250 nm (NP) or 3 µm (MP) in size, were rectally applied to IBD patients. The accumulation of the MP in the ulcerous lesions was significantly enhanced. In contrast, only traces of NP were observed on the mucosal surface. It was suggested that they persorbed into deeper tissue layers through cellular voids (Schmidt et al., 2013). These results indicate that a passive, specific targeting of inflamed intestinal tissue in humans is feasible and that nanoparticular drug delivery systems are a very promising approach for an improved treatment of IBD.

Over the last years, a huge number of different nanosized carrier systems has been developed for the treatment of IBD. They have mainly been based on one of the following principal carrier systems:

- NP/MP made of natural or synthetic polymers or solid lipids
- Liposomes
- Emulsions
- Polymeric micelles

Most frequently, polymeric particles have been utilized as carrier systems. A wide variety of established and potential drugs were formulated with this approach. Many studies have focused on the local delivery of established drugs such as 5-aminsolicylates, corticosteroids, and immunosuppressants (tacrolimus and cyclosporine). However, also novel therapeutic principles, such as small interfering RNA (siRNA) and antisense DNA, have been formulated. A very comprehensive overview on the use of nanocarriers for the treatment of IBD was published by Viscido et al. (Viscido et al., 2014). Most of the recent studies on the treatment of IBD with NP utilised either one of the polymers PLGA or chitosan or copolymers

based on methacrylic acid and methyl methacrylate (Eudragit® S 100 and L 100) (Coco et al., 2013). Due to its advantageous properties, PLGA is widely considered as material of choice. It is biocompatible and biodegradable. *In vivo*, hydrolysis of the ester bonds results in the release of its nontoxic monomers lactic acid and glycolic acid, which are subsequently metabolised (Wang and Wu, 1997). PLGA is approved by both the U.S Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use in humans (Gentile et al., 2014). It is widely used for the encapsulation of both hydrophobic and hydrophilic small molecules and peptides as well as of larger biomolecules like RNA, DNA or proteins (Danhier et al., 2012).

1.2.2 IL-10 as a potential drug for the treatment of IBD

IL-10 is an important immunoregulatory and anti-inflammatory cytokine that has been proposed as a potential drug for the treatment of IBD (Herfarth et al., 1996). Its great importance for the maintenance of immunotolerance against commensal bacteria was shown in multiple animal models. IL-10-deficient (IL-$10^{-/-}$) mice spontaneously develop severe chronic enterocolitis when grown up under normal conditions. In contrast, if the mice are kept under germ-free conditions, the inflammation is highly annihilated, suggesting an exuberant immune response to enteric antigens caused by the absence of IL-10 (Kühn et al., 1993). It was further shown that the onset of colitis in this model is dependent on intact TLR signalling pathways (Rakoff-Nahoum et al., 2006). TLR are essential for the initiation of immune responses to bacteria by detecting conserved microbial macromolecules, such as LPS (Kawai and Akira, 2006). It was thus suggested that IL-10, which is produced in response to TLR-dependent bacterial stimulation, is indispensable for the maintenance of immune tolerance towards commensal bacteria (Rakoff-Nahoum et al., 2006). It has also been proposed that especially IL-10-producing CD4+ cells play an important role in the preservation of the intestinal homeostasis in the healthy intestine (Asseman et al., 1999). When IL-10-deficient mice were daily treated with intraperitoneal (IP) injections of IL-10 beginning at an age of 3 weeks, the onset of colitis was completely prohibited. In contrast, IL-10 treatment of adult mice with already developed colitis only reduced the progression of symptoms but was not curative (Berg et al., 1996). As expected, mice with deficient

IL-10 receptors developed chronic colitis as well when raised under conventional conditions, which provides further evidence for the pivotal role of IL-10 in the healthy intestine (Spencer et al., 1998).

In several animal models of IBD, IL-10 treatment was able to attenuate the intestinal inflammation. IP application of IL-10 showed good anti-inflammatory efficacy in mice that were simultaneously treated with intrarectal TNBS to induce colitis. It was shown that administration of TNBS breaks the tolerance of mice against their autologous intestinal flora, while the treatment with IL-10 restores this tolerance (Duchmann et al., 1996). Herfarth et al. intramurally injected streptococcal peptidoglycan-polysaccharides in rats. Prior and subsequent SC injection of IL-10 attenuated the induced acute enterocolitis in a dose-dependent manner (Herfarth et al., 1996). Grool et al. induced acute colitis in rabbits by rectal instillation of formalin followed by IV injection of heat-aggregated rabbit immunoglobulin. Prophylactic treatment with IL-10 reduced the intestinal inflammation and decreased the tissue myeloperoxidase levels and leukotriene B levels in the rectal dialysate (Grool et al., 1998).

Further evidence for the protective role of IL-10 at the onset of IBD was found in the genome analysis of IBD patients. Mutations of the IL-10 receptor were found in patients with early-onset colitis, that is, patients who developed chronic colitis already during childhood. These mutations abrogate IL-10-induced signalling. In accordance with this finding, PBMCs from these patients secrete TNF, IL-6 and other pro-inflammatory cytokines in enhanced amounts, which indicates a disruption of the normal immunoregulation (Glocker et al., 2009). Correa et al. showed reduced IL-10 secretion of dendritic cells in a subpopulation of patients with severe forms of CD in response to LPS stimulation in comparison to healthy controls. It was proposed that treatment with IL-10 could be of high benefit to these patients (Correa et al., 2009). It was also shown that recurrence in CD patients occurred with double the frequency in a subgroup with low ileal IL-10 mRNA concentrations (Meresse et al., 2002).

The treatment of IBD patients with IL-10 was tested in several clinical studies. At first, the one-time IV injection of IL-10 doses from 1 to 25 µg/kg was tested in healthy volunteers (Chernoff et al., 1995). The treatment was well tolerated. *Ex*

vivo, the endotoxin-stimulated production of TNF and IL-1β was inhibited up to 95%. A first study in three UC patients tested the topical treatment with IL-10 enemas. A local and systemic downregulation of the pro-inflammatory cytokines IL-1β and TNF was observed (Schreiber et al., 1995). Later, several larger studies were conducted to assess the systemic treatment of CD patients with IL-10. In a trial comprising 46 patients with active steroid-resistant CD, 0.5–25 µg/kg bodyweight IL-10 was administered IV for seven consecutive days. The CD scores in IL-10-treated patients were lower than in the placebo group at the end of the trial. The IL-10 was well tolerated. 50% of the patients treated with IL-10-treated were in remission in the three-week follow-up period as compared to only 30% of the placebo group. IL-10 was rapidly cleared from the body with a half-life of 2.6 hours on day one and of only 1.1 hours on day seven (van Deventer et al., 1997). However, the overall promising results could not be confirmed in later trials. In a subsequent study, 329 therapy-refractory CD patients received 1–20 µg/kg IL-10, administered SC, daily over 28 days. The treatment was well tolerated up to a daily dose of 8 µg/kg. This dose group also showed a slight tendency to clinical improvement. However, no remission was observed and overall, no clinical efficacy of the IL-10 treatment could be shown. Also, patients in the 20 µg/kg group experienced side effects such as headache, fever, dizziness and thrombocytopenia significantly more frequently than the placebo group (Schreiber et al., 2000). In another study, 95 patients with mild to moderately active CD were daily treated with SC IL-10 in the dose range of 1–20 µg/kg for 28 days. 24% of the patients that received 5 µg/kg IL-10 experienced clinical remission, compared to 0% in the placebo group. Higher doses of IL-10 were less effective and cases of reversible anaemia and thrombocytopenia were observed (Fedorak et al., 2000). In another clinical trial, patients with CD who had undergone intestinal resection were treated with SC IL-10 injections, either 4 µg/kg IL-10 once daily or of 8 µg/kg twice weekly, over 12 weeks. The treatment was well tolerated but no prevention of the recurrence of CD was observed (Colombel et al., 2001). Thus, overall, the results of the clinical studies were disappointing, showing no or only slight benefits of systemic IL-10 treatment for CD patients. Several possible reasons for the overall lack of efficacy were discussed in the literature. Among them are:

1) The short serum half-life of IL-10 of only 1.1–2.6 hours after systemic administration may result in systemic clearance before the drug can reach an efficacious concentration in the intestinal tissue (Li and He, 2004), (Marlow et al., 2013).
2) The tested dose levels, upwards limited by detrimental systemic effects, were too low.

Therefore, a promising strategy could be the local administration of IL-10 to the intestinal mucosa. This approach may help to overcome the above mentioned limitations of a systemic administration (Herfarth, 2002), (Marlow et al., 2013).

1.2.3 Attempts to locally deliver IL-10 to the intestinal mucosa

Several studies explored the potential therapeutic effects of local intestinal IL-10 application. As already mentioned above, a topical treatment with IL-10 formulated as enema (IL-10 in a solution of 0.9% NaCl + 1% human serum albumin) was tested by Schreiber et al. in three UC patients. During treatment, local and systemic downregulation of the pro-inflammatory cytokines IL-1β and TNF was observed (Schreiber et al., 1995).

The daily oral administration of transgenic tobacco that expresses IL-10 to IL-10-deficient mice over 4 weeks was shown to significantly improve the histological appearance of the gut as compared to untreated controls and to reduce TNF mRNA levels in the small intestine (Menassa et al., 2007). The oral application of IL-10-containing milk fermented by a strain of IL-10-producing *Lactococcus lactis* was reported to reduce damage scores and IFN-γ levels in a TNBS colitis model (del Carmen et al., 2011). However, it is highly doubtful whether a simple oral administration of IL-10 without any protection against the gastric environment can be successful in humans, considering the complete irreversible loss of IL-10 bioactivity in acidic conditions as present in the human stomach (Syto et al., 1998).

Nakase et al. prepared microspheres of gelatine that were crosslinked and freeze dried before an IL-10 solution was dropped on the dried spheres to be absorbed by the gelatine. IL-10-deficient mice were either treated with this formulation or an IL-10 solution by rectal administration three times per week for four weeks. The

presence of IL-10 was prolonged after administration of the microspheres in comparison to the IL-10 solution, indicating both prolonged colonic retention and prolonged local release from the microspheres. Less than 0.1% of the administered IL-10 was found in the blood, indicating that systemic side effects after local administration are unlikely. The histologic scores of the colon, based on the degree of visible inflammation, were significantly lower in mice treated with IL-10 microspheres than in both untreated mice and mice treated with IL-10 solution (Nakase et al., 2002). It has to be pointed out that gelatine microspheres would not be a suitable delivery system for an oral drug application, since they get quickly digested during the intestinal passage and do not offer any protection from the degrading environment in the stomach.

Recently, (Capurso and Fahmy, 2011) developed a drug delivery system based on an aqueous gelatine core loaded with IL-10 that was subsequently coated with methacrylic acid copolymer (Eudragit® S 100). The microspheres were prepared by a double emulsion method. The encapsulation efficiency, as determined by an ELISA, was 14%. However, the remaining bioactivity of the IL-10 after encapsulation and release was not determined.

Another formulation of IL-10-loaded MP, this time polylactic acid microspheres prepared by a modified double emulsion-method, is described in a patent application by Egilmez et Sikora. First, an emulsion of a solution of IL-10 in PBS containing bovine serum albumin (BSA) and polysorbate 20 in a solution of polylactic acid in dichloromethane (DCM) was prepared by orbital shaking and the emulsion was instantly flash frozen. After lyophilisation, the residue was dispersed in DCM and discharged into petroleum ether to form polymeric microspheres. The final formulation contained 1% BSA and 0.5% IL-10. IL-10 knockout mice were fed with these particles, corresponding to 1 µg IL-10 per mouse, twice a week over three weeks. The administration was started when the mice reached an age of 5 weeks. The treatment ameliorated the onset of colitis as determined based on histological scores, body weight and serum amyloid A levels. Surprisingly, mice fed with higher amounts of the particles, corresponding to either 5 µg or 20 µg IL-10 per animal, showed no improvement as compared to the negative control (Egilmez

and Sikora, 2005). To the author's knowledge, neither these results nor any follow-up studies were published in the scientific literature.

A very innovative approach for the local treatment with IL-10 was presented by Steidler et al. They genetically transformed a *Lactococcus lactis* strain to express biologically active murine IL-10. 3-week old IL-10-deficient mice that had not yet developed colitis were treated daily with intragastric inocula of these bacteria for 4 weeks. In the treated group, the colonic inflammation as measured by the histological score, was significantly lower than in the untreated control group. Efficacy was also observed in a dextran sulfate sodium-induced colitis mouse model (Steidler et al., 2000). Subsequently, *L. lactis* was genetically modified to express human IL-10 by replacing the thymidylate synthase gene with a synthetic human IL10 gene (Steidler et al., 2003). In IL-10-deficient mice, uptake of these bacteria by the paracellular route, from the defective mucosal barrier to inflamed ileum and colon tissue, could be shown (Waeytens et al., 2008). In another study, enteric-coated capsules containing freeze-dried, viable *L. lactis* were developed (Huyghebaert et al., 2005). The formulation was administered to ten CD patients twice daily over seven days in a phase I safety and tolerability study. Proton pump inhibitors and cholate acid binders were given as co-medication to improve the viability of the bacteria. A decrease in disease activity was observed (Braat et al., 2006). A subsequent phase II trial was announced but, to the author's knowledge, has not been published so far (Steidler et al., 2009).

Another approach to reach enhanced local concentrations of IL-10 in the inflamed intestinal tissue is the local overexpression of the IL-10 gene by local transfection. A single rectal infusion of IL-10-encoding adenoviral vectors was able to significantly reduce colitis in IL-10-deficient mice (Lindsay et al., 2003). IL-10-expressing plasmid DNA was encapsulated in gelatine NP that were further encapsulated in poly(ε-caprolactone) microspheres. Oral administration of these particles in a TNBS-induced colitis mouse model resulted in reduced inflammation and suppression of the levels of pro-inflammatory cytokines (Bhavsar and Amiji, 2008).

1.3 The potential of IL-10 for the treatment of lung diseases

The acute respiratory distress syndrome (ARDS) is characterised by the abrupt onset of significant hypoxaemia accompanied by pulmonary oedema resulting from increased vascular permeability in the lung. The onset can be triggered by direct injuries to the lung, caused by e.g. by pneumonia or inhalation of smoke or by indirect injuries caused by e.g. a severe bacterial sepsis or a haemorrhagic shock (Wheeler and Bernard, 2007). During severe systemic inflammation, pro-inflammatory cytokines such as IL-6, IL-8, IL-1β and TNF are released (Kobbe et al., 2008). Enhanced plasma levels of these cytokines are associated with an enhanced risk for the development of ARDS (Roumen et al., 1993). Thus, the attenuation of the excessive pro-inflammatory cytokine release has been proposed as a promising therapeutic strategy for the treatment of ARDS (Finnerty et al., 2006).

Indeed, an early systemic treatment with IL-10 after haemorrhagic shock can significantly reduce the severity of the triggered pulmonary inflammation in animal models (Kobbe, Schmidt, et al., 2009), (Kobbe, Stoffels, et al., 2009). On the other hand, it was shown that the attenuation of the systemic inflammation can increase the chance for the development of severe infections or even sepsis (Ayala et al., 1994), (Song et al., 1999), (Kobayashi et al., 2001). It was thus proposed to selectively ameliorate the inflammation in the lung by local application of IL-10.

To test this hypothesis, mice previously subjected to haemorrhagic shock were treated by inhalation of nebulised recombinant murine IL-10. The local application resulted in an attenuation of pulmonary inflammation, while the systemic concentrations of pro-inflammatory cytokines were not modified as compared to the untreated control (Kobbe et al., 2012). In another study, inhalation treatment with IL-10 of mice that had bilateral femoral fractures attenuated the pulmonary infiltration by neutrophils and decreased the expression of the intercellular adhesion molecule 1 (ICAM-1). The IL-10 application did not show any significant effect on the systemic inflammatory response (Lichte et al., 2015). These results indicate that a local treatment of the inflamed lung with IL-10 might also be a promising treatment option for humans with ARDS.

Asthma is another lung disorder that may be alleviated by treatment with IL-10. Bronchoalveolar lavage samples of both paediatric and adult asthma patients are characterised by significantly reduced IL-10 concentrations as compared to those of healthy controls (Borish et al., 1996), (Gupta et al., 2014). Furthermore, alveolar macrophages of asthma patients show a significantly decreased spontaneous and stimulated IL-10 expression (John et al., 1998). Haplotypes associated with a low IL-10 production are associated with severe forms of asthma (Lim et al., 1998). These findings suggest that the local pulmonary application of IL-10 might be a promising option for the treatment of asthma as well.

1.2 Aims of this work

Drug delivery systems for the local application of IL-10 to the intestine as well as to the lung would allow to further investigate the local anti-inflammatory effects of IL-10 and enable the development new treatment options for IBD and ARDS. In the case of IBD, passive targeting via NP or MP appears to be a promising option because of the ability of these particles to locally accumulate in the inflamed mucosal areas of the intestine after oral application. This would enable a controlled and sustained release of effective doses without systemic side effects.

For the treatment of ARDS and other inflammatory lung diseases, a dry powder formulation based on NP or MP would be a promising new therapeutic option. It would enable the deposition of efficacious amounts of IL-10 in the deep lung while avoiding systemic side effects. To the author's knowledge, no solid formulation for the inhalation of IL-10 has been developed so far.

Therefore, one aim of this work was the development of a nanoparticulate drug delivery system, based on the biocompatible and biodegradable polymer PLGA, for the oral delivery of IL-10. As the remaining bioactivity of IL-10 after formulation was poor and attempts to optimise the formulation process failed, an alternative system was developed. It was based on spray-dried IL-10-loaded MP that were encapsulated into microspheres comprised of the gastro-resistant polymers Eudragit L 100-55 or S 100.

The second aim of this thesis was to develop a MP formulation for the local delivery of IL-10 to the deep lung. This part included the modification of the spray-dried MP, to obtain an inhalable dry powder. The anti-inflammatory potential efficacy of these particles was assessed in an inflamed *in vitro* model of the deep lung. To reach the alveolar space in the human lung, the aerodynamic properties of the particles, which determine their behaviour in the inhalation process, are of utmost importance. Thus, another important subtask was the characterisation of these properties.

As it became evident during the present study that IL-10 is very sensitive to degradation, which is in accordance with results from the literature, an additional objective was pursued: to search for potentially more stable and less expensive substitutes for IL-10. To this end, four nonapeptides, which are homologous to IL-10, were investigated on their IL-10-mimetic bioactivity.

2. Practical work

2.1 IL-10-loaded PLGA nanoparticles for the treatment of IBD

2.1.1 Preliminary remarks

The practical work presented in this chapter was exclusively planned, conducted and evaluated by the present author.

2.1.2 Introduction

2.1.2.1 Methods for the preparation of polymeric nanoparticles loaded with protein drugs

Typical polymers used for preparing micro- and nanoparticles are PLGA, chitosan or copolymers based on methacrylic acid and methyl methacrylate (Coco et al., 2013) Several techniques have been developed for the production of protein-loaded polymeric micro- and nanoparticles on the laboratory scale. The most commonly used approach is the water-in-oil-in-water (W/O/W) double emulsion solvent evaporation method. First, an aqueous solution of the protein to be encapsulated is emulsified in an organic solvent (W/O emulsion). Subsequently, this emulsion is dispersed in an outer aqueous continuous phase to form the W/O/W double emulsion. Finally, the organic solvent is evaporated to form solid particles. Alternative processes for the final solvent evaporation were also developed, namely solvent extraction, spray drying and spray-freeze-drying. A modification of the W/O/W method is the solid-in-oil-in-water (S/O/W) method, where solid protein particles instead of a protein solution are dispersed in the organic solvent (van der Walle et al., 2009), (Wang et al., 2013). Ethyl acetate (EtOAc) and DCM are the most frequently used organic solvents. EtOAc is usually used for the formulation of nanoparticles as the use of DMC results in the formation of larger particles, usually in the micrometre range (Bilati et al., 2005b), (Bilati et al., 2005c).

Another option for the formulation of protein-loaded PLGA NP is the nanoprecipitation method. It requires the use of two miscible solvents. PLGA and the protein drug have to be soluble in the first solvent but must not dissolve in the

second one. The polymer-protein solution is poured or injected into the second organic solvent, the so-called non-solvent, and nanoprecipitation occurs by rapid desolvation of the polymer (Bilati et al., 2005a).

PLGA varieties with uncapped carboxylic groups are commonly used for the encapsulation of proteins, as the ionic interactions between polymer and protein seem to enhance entrapment of the protein. On the other hand, carboxylic end groups often reduce the release rate of the encapsulated protein (Blanco and Alonso, 1997).

Recently, co-axial electrospray processes were developed for the formulation of protein-loaded PLGA MP. In short, an inner aqueous phase is localised in the centre of the spray needle, which is encompassed by the outer continuous organic phase in which the polymer is dissolved. Thus, this method avoids an emulsification step. However, it is based on complicated and expensive equipment that is not widely available (Xie et al., 2008).

It is generally difficult to encapsulate bioactive proteins without loss of bioactivity caused by the formulation process. In many cases, denaturation of the encapsulated protein was observed after preparation with the W/O/W method. The O/W interface during the first emulsification step is regarded to be the most significant factor contributing to protein denaturation. Other factors causing protein instability include the W/O interface, shear stress and heat or cavitation stress during homogenisation (Wang et al., 2013).

Van de Weert et al. investigated the effect of emulsification in water/DCM using a rotor/stator homogeniser on the recovery and structure of lysozyme. They observed noncovalent precipitation of the protein at the interface as well as reduced recovery. The recovery of the lysozyme could be improved by the addition of BSA and PVA but not by the addition of polysorbate 20 and 80 or sucrose (van de Weert, Hoechstetter, et al., 2000). In another study, the stability of lysozyme in water/DCM emulsions in the presence of PLGA was investigated. The emulsions were prepared by ultra-sonication. Both (2-Hydroxypropyl)-β-cyclodextrin (HP-β-CD) and polyethylene glycol (PEG) 400 increased the lysozyme recovery, in contrast to mannitol and trehalose (Kang et al., 2002). Pérez et al. showed that different polyols

ameliorated lysozyme denaturation during the emulsification of an aqueous lysozyme solution in DCM. Maltose, lactose and especially sorbitol were highly effective stabilisers while the addition of sucrose and trehalose even reduced the remaining enzymatic activity (Pérez and Griebenow, 2001).

Denaturation during emulsification was observed for various other proteins as well. For example, the bioactivity of the enzyme α-chymotrypsin was reduced to 70% after rotor/stator emulsification of the aqueous enzyme solution with DCM. The addition of maltose enhanced the remaining activity to 82% and the addition of PEG 4000 even to 96%. In contrast, the addition of various other sugars, like sucrose, trehalose or fructose, did not stabilise the enzyme (Perez-Rodriguez et al., 2003). Morlock et al. prepared erythropoietin-loaded PLGA microspheres by the W/O/W technique. The first homogenisation step with a rotor/stator homogeniser led to the formation of 5% aggregates. When ultra-sonication or vortexing were applied for homogenisation instead, aggregation slightly increased to more than 10%. The addition of BSA, arginine or HP-β-CD to the internal aqueous phase significantly reduced the aggregation, in contrast to the addition of mannitol, trehalose and PEG grades of various molecular weights (Morlock et al., 1997). Son et al. prepared water/DCM emulsions with the DNA-binding monoclonal antibody 3D8 scFv. Even without the addition of any stabiliser, the DNA-binding activity was not reduced after emulsification. The addition of HP-β-CD, PEG 600, trehalose or mannitol did not affect the remaining activity, whereas heparin significantly reduced it. However, when PLGA was added to the primary emulsion, only mannitol protected the bioactivity of the antibody, whereas all other excipients had a negative impact on bioactivity (Son et al., 2009). Cleland et al. investigated the stability of IFN-γ, a protein that has a structure very similar to that of IL-10 (see chapter 1.1.1), after homogenisation with DCM. The addition of trehalose led to full recovery of the dimeric protein as compared to only 52% recovery without the addition of any excipient. Mannitol had a slight protective effect, whereas polysorbate 20 even decreased the recovery (Cleland and Jones, 1996). Interestingly, Maa et al. showed that, the homogenisation of an aqueous solution of human growth hormone by a rotor-stator caused severe irreversible aggregation of the protein at the air-liquid interface, even without any addition of organic solvent. Aggregation was attenuated by addition of polysorbate 20 and, to a higher extent,

by poloxamer 238 (Maa and Hsu, 1997). These results indicated that protein denaturation at the air-liquid interface may play a more significant role than generally assumed.

It is currently not possible to adopt a common strategy for the stabilisation of proteins, as the specific factors affecting the stability of a specific protein are still poorly understood. The addition of an excipient to the solution of a given protein may enhance its stability to denaturation, whereas it may be detrimental to the stability of another protein. Therefore, an empirical trial and error approach is still needed for the formulation of proteins (Bilati et al., 2005d). Unfortunately, the significance of many published studies on the development of nanoscale drug delivery systems for the encapsulation and delivery of protein drugs is limited, as these studies only focused on the formulation of model proteins, like BSA, ovalbumin or antibodies. These model proteins are very robust against destabilising chemical and physical conditions as compared to many bioactive proteins such as cytokines. Also, in many studies neither the remaining bioactivity nor changes in the conformation of the protein after formulation were investigated.

2.1.2.2 Aim of the study

The targeted local delivery of IL-10 to the inflamed intestinal mucosa is a promising strategy for the treatment of IBD (Herfarth, 2002), (Marlow et al., 2013). Polymeric NP are considered to be a suitable drug delivery system for the specific targeting of the inflamed regions of the intestine and for a prolonged drug release (Lamprecht, Schäfer, et al., 2001), (Lamprecht, Ubrich, et al., 2001). The administration of IL-10-loaded polylactide microspheres showed promising results in a murine IBD model (Capurso and Fahmy, 2011). In comparison to MP, NP showed higher accumulation in the inflamed intestinal regions in a rat model of ulcerative colitis (Lamprecht, Schäfer, et al., 2001). Therefore, in the present study it was aimed to prepare IL-10-loaded NP for the local delivery of IL-10 to the inflamed intestinal mucosa. As the method of choice, the double emulsion solvent evaporation technique was selected, and PLGA was chosen as polymer for the preparation of the NP. Important subgoals were the determination of the encapsulation efficacy, of

the IL-10 release from the particles and, most notably, of the bioactivity remaining after the formulation process.

2.1.3 Materials and methods

2.1.3.1 Materials

All chemicals used were of analytical or higher grade. PLGA with a D,L-lactide:glycolide ratio of 50:50, free carboxylic end groups and an inherent viscosity, measured at a concentration of 0,1% in $CHCl_3$ at 25°C, of 0.44 dl/g (Resomer® Sample CR). The polymer was a gift from Boehringer Ingelheim (Ingelheim, Germany). NaCl was purchased from VWR International (Radnor, USA). HP-β-CD, 2-mercaptoethanol, BSA of 96% purity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin/streptomycin, poloxamer 188 and trehalose dihydrate were bought from Sigma-Aldrich (St. Louis, USA). Polyvinyl alcohol (PVA) 4-88 (Mowiol® 4-88) was purchased from Merck (Darmstadt, Germany). Recombinant human interleukin-10 (rhIL-10) and recombinant murine interleukin-4 (rmIL-4) were obtained from peprotech (Rocky Hill, USA). The murine mast cell line MC/9 (CRL-8306™) was purchased from ATTC (Manassas, USA). RPMI 1640 with L-glutamine and without phenol red was purchased from PAA Laboratories (Pasching, Austria). Heat inactivated fetal bovine serum (FBS) was bought from Lonza (Cologne, Germany). Rat T-cell culture supplement with ConA (IL-2 culture supplement) was purchased from Becton, Dickinson and Company (Franklin Lakes, USA). Cytometric bead array (CBA) flex sets comprising IL-10 antibody-coated beads and IL-10 detection reagent were purchased from Becton, Dickinson and Company.

2.1.3.2 Pretrial to determine the IL-10 stability in the release medium

100 µl aliquots of a 1 µg/ml (all concentrations w/V, unless indicated otherwise) IL-10 solution in PBS pH 7.4 + 0.1% BSA were carefully pipetted into 50-ml polypropylene tubes. The tubes were then either slowly filled up with pure purified water, 10 mM PBS pH 6.8, 10 mM PBS pH 6.8 + 0.1% BSA or PBS pH 6.8 + 1% BSA, up to a final concentration of 2.5 ng/ml IL-10. The final pH of the solutions

was checked with a pH meter. The tubes were incubated at 37°C and 90 rpm on a shaker incubator. 1-ml samples were taken at defined time points and instantly frozen at -80°C until further analysis. The thawed samples were incubated with fluorescently labelled beads coated with IL-10 antibodies, followed by a second incubation with fluorescently labelled reporter antibodies (CBA Flex Set). All samples and standards were prepared according to the manufacturer's instruction. Briefly, 50 µl of each sample was pipetted into a 4-ml polypropylene tube, mixed with 50 µl of diluted antibody-coupled beads and incubated at RT for 1 h. 50 µl of the solution of IL-10 reporter antibodies were added and, after mixing, the samples were incubated at RT for another 2 h. The IL-10 standards were prepared accordingly. The fluorescence intensities of the beads were analysed with a FACSCalibur™ flow cytometer (Becton, Dickinson and Company). For each sample and standard, approximately 300 single beads were measured, and the mean fluorescence intensity of the bead population was determined by nonlinear regression from the data of the standards. The 5-parameter logistics model of the software FCAP Array (version 3, Soft Flow, Pecs, Hungary) was used for the calculations.

2.1.3.3 Preparation of IL-10-loaded nanoparticles

The protein-loaded NP were prepared by a double emulsion solvent evaporation method (Fig. 4). 80 ± 2 mg PLGA and 2.0 ml EtOAc were added in a round bottom glass ultracentrifugation tube (100x16 mm, Duran Group, Germany) and the polymer was dissolved by magnetic stirring. After removing the magnetic stirrer, 200 µl of 1% BSA in 10 mM PBS pH 7.4 containing 1 µg IL-10 were added as internal water phase. To limit the increase in temperature during the homogenisation steps, the tube was immerged in a beaker filled with water. The mixture was then homogenised with a dispersing aggregate PT-DA 12/2 that was attached to a Polytron® PT 2500E homogenizer (both from Kinematica, Luzern, Switzerland). The homogenisation was performed at 18000 rpm for 60 s, unless indicated otherwise. Subsequently, 2 ml of 2% PVA dissolved in deionised water were added to the previously prepared oil-in-water (O/W) emulsion and the mixture was homogenised (18000 rpm for 60 s, unless indicated otherwise) to form the W/O/W emulsion. This emulsion was filled into a scintillation vial and magnetically stirred

at 250 rpm. After 5 min, 2 ml of 0.1% BSA dissolved in PBS pH 7.4 were added. The samples were stirred in darkness overnight to let the EtOAc completely evaporate (Fig. 4), so that solid NP were formed. NP loaded with the model protein fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) were prepared according to the process described above but using 200 µl of a solution of 0.2% FITC-BSA and 0.8% BSA in PBS as the internal water phase. Blank NP (not containing IL-10) were also prepared in accordance with the process described above, except that no IL-10 was added during the preparation.

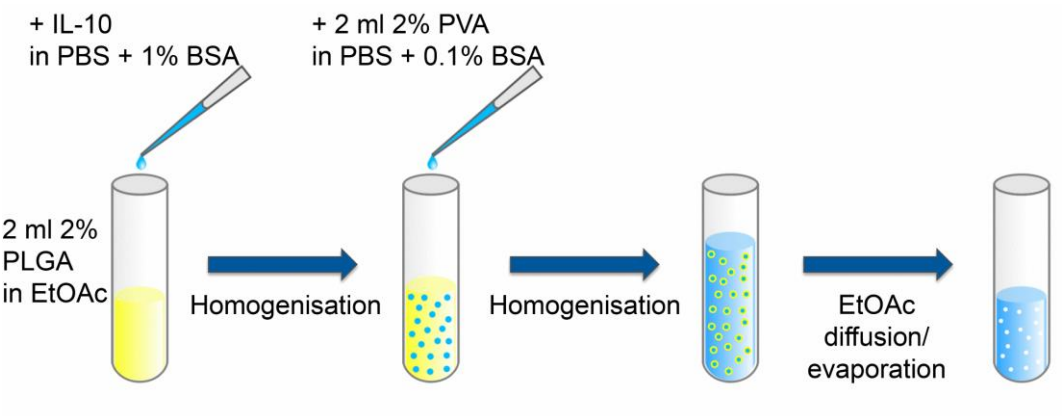

Fig. 4: Preparation of the IL-10-loaded NP by a W/O/W double emulsion method. 200 µl of PBS containing IL-10 and 1% BSA were added to 2 ml of 2% PLGA dissolved in EtOAc. Homogenisation by a high shear homogeniser formed an O/W emulsion. 2 ml of a solution of 2% PVA and 0.1% BSA in PBS were added and the mixture was homogenised to form a W/O/W double emulsion. By evaporation of the EtOAc solid IL-10-loaded nanoparticles were formed.

2.1.3.4 Determination of NP size and charge

To determine the particle-size distribution, the NP suspensions were diluted 1:20 with purified water. The mean hydrodynamic diameter of the particles and the polydispersity index (PDI), a measure for the size distribution of the particles, were both determined by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Each sample was measured in triplicate at 25°C and a scattering angle of 173°. The Z-average diameter, which can be described as the intensity-based harmonic mean, and the PDI were calculated by the device software with the autocorrelation function set to the general-purpose mode. Subsequently, the means of the respective three measurements were calculated. Additionally, the particle size and size distribution of some samples were also determined by NP tracking analysis with a NanoSight LM 10 HS, (NanoSight,

Amesbury, UK). Before the measurements, the particle suspensions were diluted with purified water to a particle concentration in the range of 5 to $10*10^8$ particles per ml as determined by the software of the NanoSight. The particles were tracked at RT for 45 s and the mean size and standard deviation (SD) of the size distribution were calculated by the device software. The zeta potential of the diluted particle suspensions was determined by laser Doppler electrophoresis in the Zetasizer Nano ZS. Each sample was measured in triplicate and the mean was calculated.

2.1.3.5 Scanning electron microscopy

Scanning electron microscopy (SEM) was employed to take images of the NP. Samples were prepared by centrifugation (10000 xg, 5 min) of the NP suspension followed by discarding the supernatant, by addition of 4 ml purified water and by the redispersion of the particles with a pipette. Each washing step was repeated three times. The washed particle suspension was diluted 1:20 with purified water. One drop of the diluted suspension was pipetted onto a small fragment of a silicon wafer so that the suspension completely wetted the surface and excess liquid flowed off from the wafer. After drying at RT, the wafer fragment was sputter-coated with a gold film of about 20 nm thickness. Scanning electron micrographs were obtained with a 7000F (JEOL, Akishima, Japan) by a secondary electron detector at an accelerating voltage of 20 kV.

2.1.3.6 Determination of encapsulation efficacy and *in vitro* release profiles of IL-10 and FITC-BSA

For these experiments, 0.1% BSA in 10 mM PBS pH 7.4 was added to the particle suspension up to a final volume of 25 ml. 2 ml aliquots of the homogeneous suspension were transferred into 2-ml polypropylene micro tubes. Subsequently, the samples were centrifuged (10000 xg at 10°C, 5 min) and 1.8 ml supernatant was removed from each tube. The encapsulation efficacy of the particles was indirectly determined by the analysis of the concentration of IL-10 or FITC-BSA in the supernatant. The encapsulation efficiency was calculated by the following formula:

$$EE_{indirect}[\%] = \frac{(\mathrm{m}(\mathrm{API}_{initial}) - \mathrm{m}(\mathrm{API}_{supernatant}))}{m(\mathrm{API}_{initial})} * 100 \qquad \text{(eq. 1)}$$

where $m(API_{initial})$ is the initial amount of the drug used for the preparation of the particles and $m(API_{supernatant})$ the amount of drug recovered in the supernatant.

The washing of the particles was continued by adding 1.8 ml of 0.1% BSA in PBS pH 6.8 to the precipitated particles. The precipitate was resuspended with a pipette and the complete washing procedure was repeated twice. Afterwards, the washed samples were incubated at 37°C, 90 rpm on a shaker. At the dedicated time points, samples (complete tubes) were withdrawn and centrifuged (10000 xg at 10°C for 5 min). 1.8 ml supernatant was sampled and frozen at -80°C until further analysis. For the determination of the IL-10 concentration in the supernatant, thawed samples were analysed as described in chapter 2.1.3.2.

FITC-BSA concentrations were determined by measurement of the fluorescence intensity of the thawed supernatants and comparison with that of standards. The standards were prepared by dissolution and further dilution of FITC-BSA in the supernatants of BSA-loaded particles. 200 µl of the sample or standard was pipetted into the well of a polystyrene 96-well microtiter plate. The fluorescence intensity was determined at an excitation wavelength of 490 nm and an emission wavelength of 525 nm with a microplate reader (Infinite M200, Tecan Group AG, Maennedorf, Switzerland). Each standard was measured in triplicate and each sample in duplicate. The means of the replicates were calculated.

2.1.3.7 Design of experiment

In the course of the present experiments, it became evident that IL-10 considerable denatured during the preparation of the nanoparticles and/or the release. To get a better understanding of the impact of critical factors on the denaturation and to potentially improve the stability of IL-10 in the NP, a design of experiment (DoE) based on the response surface methodology was conducted. A face-centred central composite design (FCCD) was applied to evaluate the influences of four independent formulation variables on the remaining IL-10 concentration and the

remaining IL-10 bioactivity. These variables were the process parameters homogenisation speed (factor A) and homogenisation time (factor B), the concentration of the IL-10 carrier protein and stabiliser BSA in the inner aqueous phase (W1) of the emulsion (factor C), as well as the concentration of an additional potential protein stabiliser that might enhance IL-10 stability, namely poloxamer 188 in W1 (factor D). For each factor, three levels were specified: a high level (+1), a low level (-1) and a medium level (0). The factors and their levels are shown in Tab. 1. As responses, the remaining IL-10 bioactivity after homogenisation and solvent evaporation (Y1) and the remaining IL-10 concentration (Y2) were determined. The former was determined by the MC/9 cell assay, described in detail in chapter 2.1.3.7.2, and the latter by using antibody-coated beads.

		Coded level		
Factors		**-1**	**0**	**+1**
A	**Homogenisation speed [rpm]**	**0**	**10000**	**20000**
B	**Homogenisation time [s]**	**30**	**75**	**120**
C	**c(BSA) [%]**	**0.01**	**2.5**	**5**
D	**c(poloxamer 188) [%]**	**0**	**2.5**	**5**

Tab. 1: Independent factors A–D and their coded levels investigated in the FCCD.

Based on these factors and their levels, the FCCD was designed with the statistical software Design-Expert®, version 9 (Stat-Ease, Minneapolis, US). The design consisted of 30 single experiments (runs), including six centre points to evaluate the experimental error. The experimental series was performed in two blocks and the experimental runs were conducted in random order to minimise the effect of any drift that might occur during the series of experiments. The runs and the assigned values of the input factors are shown in table 2 in chapter 2.1.4.3. The input factors and the obtained response values (Y1 and Y2) of the DoE were statistically evaluated with the software Design Expert. In the model created for the remaining IL-10 concentration (Y2), the response data were square root-transformed based on the Box-Cox plot for power transforms, which showed that transformation with a power of 0.49 minimises the residual sum of squares. The model calculations considered all single factors (A, B, C and D), as well as all potential two factor interactions (AB, AC, AD, BC, BD and CD) and quadratic interactions (A^2, B^2, C^2

and D^2). Subsequently, the model was reduced by backward elimination. Terms that were not significant for the model, that is, had a p-value < 0.1 based on the analysis of variance (ANOVA, partial sum of squares type III), were successively eliminated. Terms that were not significant but contributed to significant terms of higher order were not eliminated.

2.1.3.7.1 Preparation and execution of the experimental runs of the DoE

Aqueous solutions of BSA and poloxamer 188 in 10 mM PBS pH 7.4 were prepared for each run. The pH value of the solutions was set to 7.4 by the addition of 1 N NaOH. For each run, 190 µl of the respective solution were added to a thawed aliquot of 1 µg IL-10 in 10 µl PBS pH 7.4 + 0.1% BSA. Subsequently, the combined solution was carefully pipetted in a round bottomed glass ultracentrifuge tube (16x100 mm). 2.0 ml of water-saturated EtOAc were slowly added on top of the aqueous solution by pipetting. Extensive care was taken to avoid early mixing of the two phases. The phase mixture was then homogenised with a Polytron® PT 2500E and the attached dispersing aggregate PT-DA 12/2 (Kinematica, Luzern, Switzerland). In the runs without homogenisation step (0 rpm homogenisation speed), the dispersing aggregate was inserted into the centrifuge tube with the sample for the corresponding time in the same way as for the homogenised samples, but without activation. During the homogenisation, the solution was cooled by immersion of the tube in an ice-water mixture. Subsequently, the sample was pipetted into a weighed 20 ml glass snap vial to 4.0 ml of 0.25% BSA in PBS pH 7.4. The vials were put under a laboratory fume hood for 18 h to completely evaporate the EtOAc. Afterwards, the remaining solution was filled up with purified water to 4.00 g. For the further calculations, it was assumed that each vial contained now a volume of exactly 4.00 ml. The samples were further analysed on their remaining cytokine concentration and IL-10 bioactivity.

2.1.3.7.2 Determination of the IL-10 bioactivity

The remaining IL-10 bioactivity was determined by the quantification of the rmIL-4-dependent growth stimulation of MC/9 cells (Thompson-Snipes et al., 1991). The cells were grown in a medium consisting of RPMI 1640 with L-glutamine and without phenol red, supplemented with 10% heat-inactivated FBS,

5% rat T-STIM with ConA, 0.05 mM 2-mercaptoethanol, 100 I.U./ml penicillin and 100 µg/ml streptomycin. The cells were twice centrifuged and washed with cell culture medium without T-STIM (CMWT). They were then diluted in CMWT to a concentration of $2*10^5$ cells/ml. 24 µl of each sample were pipetted to 976 µl CMWT and allowed to homogenise for 15 minutes at RT. Samples were prepared from thawed IL-10 aliquots by dilution in CMWT. The MC/9 cell assay was conducted in a polystyrene 96-well microtiter plate (Greiner Bio One, Kremsmuenster, Austria). 50 µl of the diluted IL-10 solution were added to 100 µl CMWT containing $2*10^5$ cells/ml and 7.5 pg/ml IL-4. For each sample, six wells were prepared and for each standard 3 wells. Subsequently, the 96-well plates were incubated for 48 h at 37°C, 5% CO_2. The IL-10-dependent proliferation of the cells was measured by a colorimetric MTT assay (Hansen et al., 1989). Briefly, 20 µl of 5mg/ml MTT in 10 mM PBS pH 7.4 were added to each well and the plates were incubated for 4 h at 37°C, 5% CO_2. The formed formazan crystals were then solubilised with a lysis buffer consisting of 20% (w/v) SDS dissolved in 47.8% dimethylformamide (DMF), 47.8% demineralised water, 2.0% glacial acetic acid and 2.5% 1 N HCl (all V/V). 100 µl of this lysis buffer were pipetted to each well. The plates were then incubated overnight at 37°C. Subsequently, the absorbance at 570nm and 690 nm was measured by a plate reader (Infinite M200, Tecan Group AG, Maennedorf, Switzerland). The absorbance measured at 690 nm was subtracted from the one at 570 nm to correct for cell debris.

The IL-10 bioactivity of the samples was calculated by applying a nonlinear regression of the data from the standards, whose bioactivity was assumed to be 100%, using the software SigmaPlot (Systat Software, San Jose, USA). The following 4-parameter logistic equation was used:

$$y = min + \frac{max - min}{\left(1 + \frac{x}{EC_{50}}\right)^{-Hillslope}} \qquad \text{(eq. 2)}$$

where y represents the corrected absorbance, x the concentration of bioactive IL-10 and EC_{50} the half-maximal effective concentration of IL-10. Max and min are the minimum and maximum asymptote of the curve and the hillslope is a slope factor. For each sample, the mean concentration of six replicates was calculated. To reduce

the influence of factors like the used cell passage or potential variabilities between the used microtiter plates, standards were run on every plate.

2.1.3.7.3 Determination of IL-10 recovery by a bead-based immunoassay

The remaining IL-10 concentration in the FCCD samples after the experimental runs was determined with antibody-coated beads. From each sample, 8 µl were diluted in 992 µl 10 mM PBS pH 7.4 + 0.25% BSA. Standards were prepared by diluting thawed IL-10 aliquots with the same BSA-containing PBS buffer. The samples were further prepared and analysed as described in chapter 2.1.3.2.

2.1.4 Results and discussion

2.1.4.1 IL-10 stability in the release medium

To simulate the drug release of nanoparticles in the small intestine, PBS pH 6.8 is commonly used as release medium. BSA is widely used as protein carrier/stabiliser for the reconstitution, dilution and freezing of cytokine solutions. The addition of 0.1–1% BSA or an equivalent protein, e.g. human serum albumin, is recommended by most cytokine suppliers for the stabilisation of their products. The use of BSA as stabiliser for cytokines is also recommended in published protocols for bioassays (Choi et al., 2011) and analytical assays (Sack et al., 2006). In the present study, the stability of IL-10 was tested at low concentrations in the range expected for *in vitro* release profiles of IL-10-loaded NP. When IL-10 was diluted to 2.5 ng/ml, the concentration measured in water and PBS was already clearly reduced even before incubation at 37°C, 90 rpm (Fig. 5, squares). During incubation, the measured concentration drastically decreased further. In comparison, the addition of 0.1% or 1% BSA to the PBS buffer resulted in the expected concentration after dilution and additionally enhanced the IL-10 stability during the incubation under release conditions (Fig. 5, circles and triangles). Nevertheless, the detectable concentration of IL-10 after incubation for 72 h i decreased to 58 ± 7% and 65 ± 7%, respectively. No significant difference in IL-10 stability between the solution with 1% and 0.1% BSA was observed. Therefore, PBS with a concentration of 0.1% BSA was selected as release buffer for the IL-10 loaded particles.

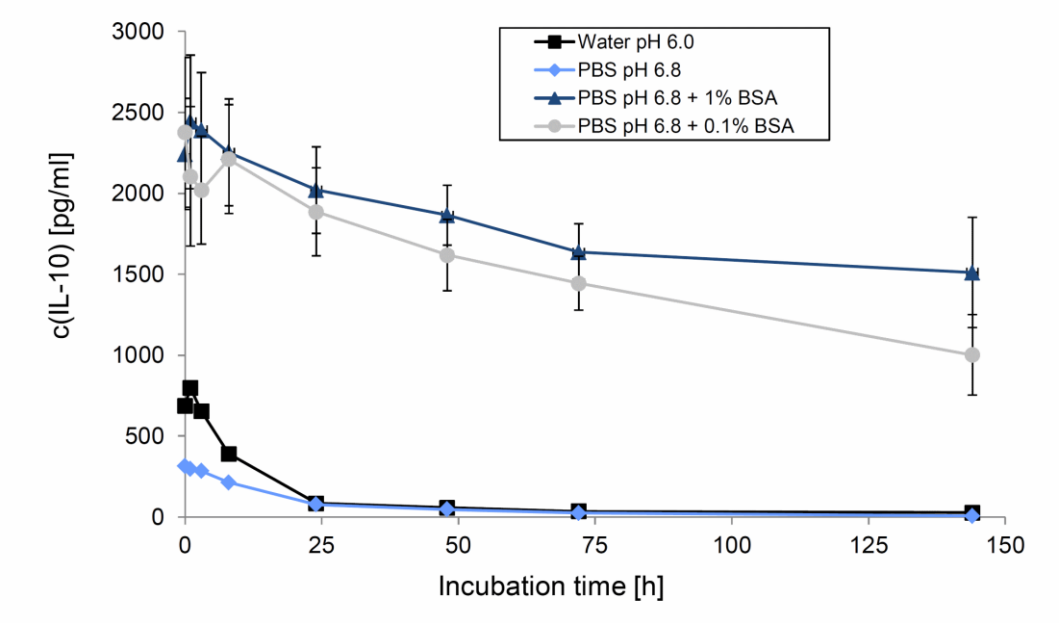

Fig. 5: Concentration of IL-10 in various release media as function of the incubation time. Samples were incubated at 37°C, 90 rpm. Data are shown as mean ± SD. n = 3–4 for PBS pH 6.8 + 0.1% BSA and PBS pH 6.8 + 1% BSA; n = 1 for water and PBS pH 6.8.

2.1.4.2 Properties of the IL-10-loaded NP

Process parameters that are suitable for the preparation of nanoparticles with a mean size of about 300 nm and a narrow particle size distribution were determined in pretrials. Homogenisation speeds in the range of 9000–18000 rpm for each homogenisation step and homogenisation times in the range of 30–120 s were applied. As expected, both the mean size and the size distribution (represented by the PDI) increased with decreasing homogenisation time or speed (Fig. 6, upper diagrams).

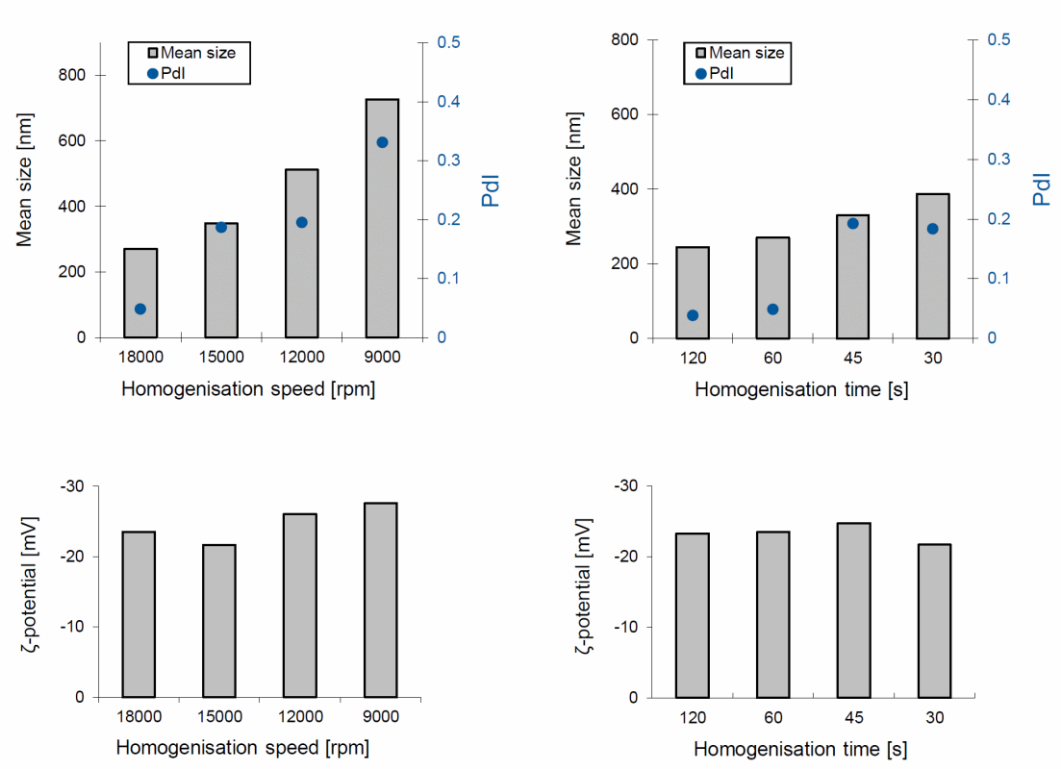

Fig. 6: Effect of the homogenisation speed (left graphs) and the homogenisation time (right graphs) on the mean size and PDI (upper graphs) and on the zeta potential (lower graphs) of BSA-loaded PLGA NP. The homogenisation speed was varied while keeping the homogenisation time constant at 60s. Conversely, the homogenisation time was varied while keeping the homogenisation speed constant at 18000 rpm.

Based on these results, a homogenisation speed of 18000 rpm and a homogenisation time of 60 s were chosen for the further preparation of the IL-10-loaded nanoparticles to ensure both a small size and a narrow size distribution. 1 µg of IL-10 was used for the preparation of each particle batch. For the above parameters, the mean size and PDI of the IL-10-loaded particles, as determined by DLS, were 300 ± 35 nm and 0.09 ± 0.02 (n = 3, mean ± SD). Similar results were also obtained by nanoparticle tracking analysis with a mean size of 242 ± 20 nm and a SD of the size distribution of 77 ± 27 nm (n = 3, mean ± SD). The particle size distribution curves showed unimodal size distribution with both measurement techniques. The mean size measured by nanoparticle tracking analysis was slightly smaller in comparison to that determined by DLS, an effect that is commonly observed (Filipe et al., 2010). The zeta potential of the particles was -22.4 ± 6.4 mV (n = 3, mean ± SD), indicating an acceptable stability of the suspension in water.

SEM micrographs of the particles (Fig. 7) show round to slightly angular-shaped spherical particles with a smooth surface and a size range of about 100–400 nm.

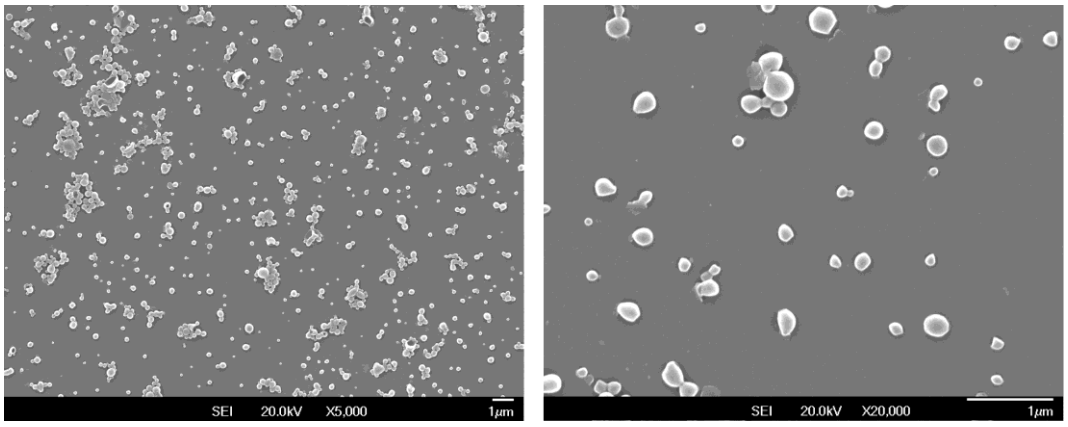

Fig. 7: SEM micrographs of the IL-10-loaded PLGA NP at a magnification of 2000x (left) and 20000x (right).

To reduce early denaturation as well as adsorption of IL-10 to the surface of the vessel containing the release medium, 0.1% BSA was added to both the washing solution and the release buffer. The encapsulation efficacy of the IL-10-loaded NP was indirectly determined by measurements of the IL-10 concentration in the supernatant after centrifugation. Fluorescently labelled antibody-coupled beads were used for quantification. Only 5.0 ± 3.2% of the initially added IL-10 (n = 3, mean ± SD) were found in the supernatant. Thus, the theoretical encapsulation efficiency was 95 ± 3.2%. In comparison, for FITC-BSA-loaded particles prepared with the same process, 30.0 ± 6.0% (n = 3, mean ± SD) of the initial FITC-BSA were found in the supernatant, which corresponds to an encapsulation efficiency of 70.0 ± 6.0%.

The release of IL-10 from the washed particles was determined in PBS pH 6.8 at 37°C, 90 rpm. The initial burst release accounted to only 0.8 ± 0.3% of the initial IL-10 amount (Fig. 8). After 24 h, no further increase in the released IL-10 concentration was observed. Considering that IL-10 is not stable in the release medium, it seems likely that the release of IL-10 from the particles continues even after 24 h on a very low level. Furthermore, it must be taken into account that IL-10 can only bind to the antibody-coupled beads if the epitope is still intact and on the surface of the protein. During the particle preparation, a major part of the IL-10 might have been degraded, which could result in a reduced or even completely lost binding affinity to the antibody and thus to an underestimation of the released amount of IL-10. In comparison, particles prepared in the same way but loaded with the model protein FITC-BSA showed a steadier release with a much higher relative

amount released: 8 ± 1% (n = 3, mean ± SD) of the initial amount of FITC-BSA and 12% of the encapsulated amount were released within 24 h and 12 ± 3% and 16% were released after 48 h, respectively.

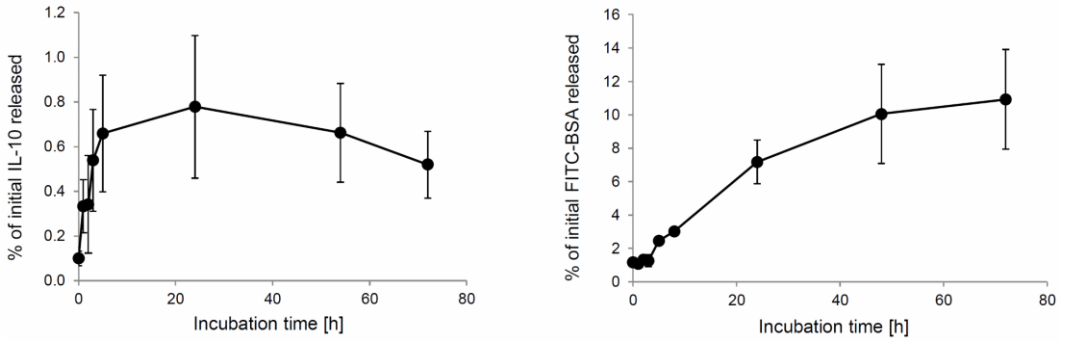

Fig. 8: Release profiles of IL-10-loaded PLGA NP (left) and FITC-BSA-loaded NP (right). The particles were incubated at 37°C, 90 rpm in PBS pH 7.4 + 0.1% BSA. The ordinate shows the drug amount released relative to the initial amount used for the particle preparation. n = 3, mean ± SD.

In another experiment, 1 mg/ml of the washed IL-10-loaded NP was added to MC/9 cells costimulated with 5 pg/ml IL-4 and incubated in the culture medium for 48 h at 37°C. No induction of cell proliferation and hence no IL-10 bioactivity was observed (data not shown), which strongly indicates that the IL-10-loaded NP did not release significant amounts of bioactive IL-10 within the first few hours.

In conclusion, the developed PLGA NP are in principle suitable for the encapsulation of proteins. This is suggested by the high encapsulation efficiency of 70% for the model protein FITC-BSA and by the relatively low but controlled *in vitro* release of about 16% of encapsulated model drug within 48 h. However, in comparison, a much lower drug release was detected for the IL-10-loaded NP. Furthermore, no IL-10 bioactivity could be observed on MC/9 cells. These results indicate that PLGA NP prepared by a double emulsion solvent evaporation method are not a suitable drug delivery system for IL-10. It appears likely that the major part of the encapsulated IL-10 was already denatured during the particle preparation.

2.1.4.3 DoE of the formulation parameters

To obtain a better understanding of the factors responsible for the negative results in the preceding chapter, a design of experiment (DoE) was conducted. The DoE

allowed investigating the influence of process parameters of the NP preparation on the denaturation of the IL-10 in more detail. To this end, a central composite design (CCD) was applied. It consists of a factorial design comprising a group of axial points for assessing curvature, as well as centre points for the determination of experimental reproducibility. In general, both factorial designs and CCD provide good statistical optimisation for a set of experiments. They enable the analysis of many variables with relatively few experimental runs in comparison to the classic experimental approach, where only the value of one factor is varied at a time. Furthermore, CCD can show functional relationships between variables that cannot be discovered by one factor at a time approaches. In comparison to factorial designs, CCD can also describe curvature. For the determination of curvature, the axial points should ideally lie outside of the factorial box. However, this results in only a small space within the tested factorial range where the fitted model is reliable. As it was the aim to obtain maximum information over the whole tested factorial range, a FCCD was chosen, where the axial points lie within the factorial range.

The input factor levels and the measured responses of all 30 experimental runs are summarised in table 2. The remaining bioactivity of the IL-10 solutions after the emulsification process (Y1) was in the range of 0.1 to 2.7% of the initial bioactivity. The remaining amount of IL-10, detected by antibody-coupled beads, ranged from 0.4 to 42.9%.

Tab. 2: FCCD matrix with experimental runs in standard order with the assigned independent input factor values and the measured responses. Input factor A represents the homogenisation speed [rpm], B the homogenisation time[s], C the BSA concentration in the water phase (w/v) [%], D the poloxamer 188 concentration in W1 (w/v) [%]. Response Y1 represents the remaining IL-10 bioactivity and Y2 the remaining IL-10 amount [%]. The Y1 response value for sample 15 (bracketed) was identified as an outlier and was thus not included in the data set used for the modelling.

			Input factors				Responses	
Sample	**Run**	**Block**	**A**	**B**	**C**	**D**	**Y1**	**Y2**
1	18	1	0	30	0.01	0	2.3	12.7
2	19	1	20000	30	0.01	0	2.4	3.6
3	12	1	0	120	0.01	0	2.0	13.7
4	9	1	20000	120	0.01	0	0.1	0.4
5	2	1	0	30	5.00	0	1.3	19.5
6	15	1	20000	30	5.00	0	1.5	12.6
7	3	1	0	120	5.00	0	0.7	15.7
8	11	1	20000	120	5.00	0	2.6	24.9
9	5	1	0	30	0.01	5.00	2.7	20.5
10	8	1	20000	30	0.01	5.00	0.1	3.0
11	10	1	0	120	0.01	5.00	4	42.9
12	4	1	20000	120	0.01	5.00	0.1	2.9
13	13	1	0	30	5.00	5.00	2.7	28.8
14	16	1	20000	30	5.00	5.00	2.2	14.8
15	6	1	0	120	5.00	5.00	(22)	34.1
16	17	1	20000	120	5.00	5.00	1.1	11.4
17	7	1	10000	75	2.50	2.50	1.0	15.0
18	1	1	10000	75	2.50	2.50	0.3	15.0
19	20	1	10000	75	2.50	2.50	0.6	4.1
20	14	1	10000	75	2.50	2.50	2.2	17.7
21	22	2	0	75	2.50	2.50	1.8	23.2
22	26	2	20000	75	2.50	2.50	1.3	12.8
23	28	2	10000	30	2.50	2.50	2.4	21.7
24	25	2	10000	120	2.50	2.50	0.2	9.0
25	30	2	10000	75	0.01	2.50	1.2	10.1
26	24	2	10000	75	5.00	2.50	0.1	9.5
27	21	2	10000	75	2.50	0	0.7	4.8
28	27	2	10000	75	2.50	5.00	1.6	17.8
29	23	2	10000	75	2.50	2.50	0.3	9.2
30	29	2	10000	75	2.50	2.50	1.5	9.9

Based on these values and on the input factors statistical models were created with the software Design Expert. For the remaining detected amount of IL-10 (Y2), the model reduction resulted in a reduced two factor interaction model. The equation in terms of coded factors is the following:

$$Y2 = 3.58 - 0.97 * A + 0.61 * C + 0.46 * D + 0.53 * AC - 0.44 * AD \quad \text{(eq. 3)}$$

The model has a p-value (ANOVA, partial sum of squares type III) of < 0.0001, indicating that the model is highly significant. The factors A, C and D as well as the interactions AC and AD are significant model terms ($p < 0.05$, see Tab. 3), while B is not significant and was therefore removed from the equation. The adjusted R^2 for the model is 0.664 and the predicted R^2 0.541.

Tab. 3: Overview of the model terms that significantly affect the remaining detected amount of IL-10 (Y2).

	Actual model term	**Coding**	**p-value**
Factors	Homogenisation speed	A	<0.0001
	BSA concentration	C	0.0026
	Poloxamer 188 concentration	D	0.0178
Interactions	Homogenisation speed * BSA concentration	AC	0.0100
	Homogenisation speed * poloxamer 188 concentration	AD	0.0300

The contour plot of the model for Y2 with homogenisation speed (A) and BSA concentration (C) as selected input factors input factors is shown in Fig. 9. The poloxamer 188 concentration was set to 2.5 %. At a BSA concentration of 0.01%, the measured remaining amount of IL-10 (Y2) is reduced from about 20% to less than 5% if the homogenisation speed is increased from 0 to 20000 rpm (Fig. 9). With increasing BSA concentration, this effect is reduced. For c(BSA) = 5%, more than 10% of the initial IL-10 are still detected by the antibody-coupled beads.

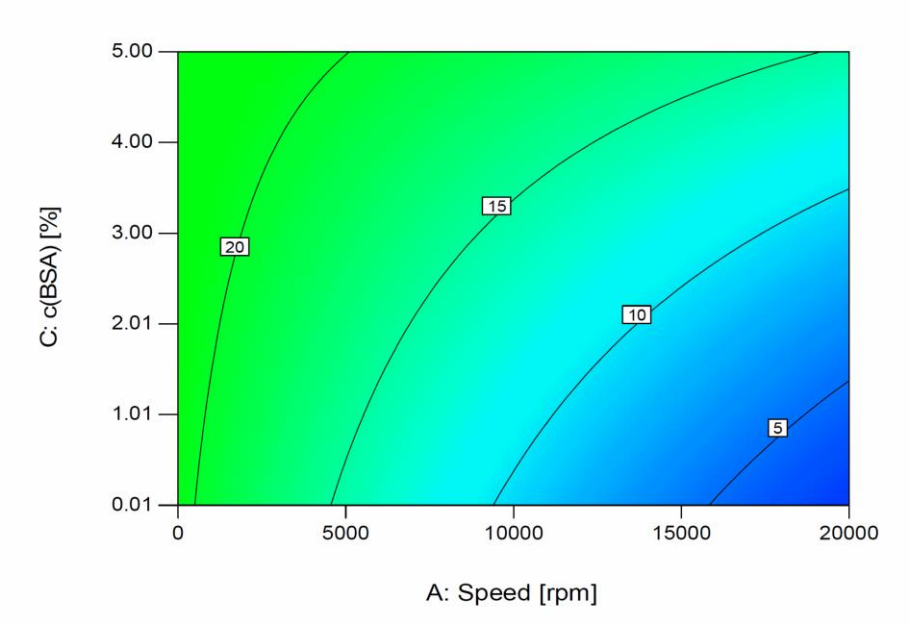

Fig. 9: Modelled influence of the factors A (homogenisation speed) and C (BSA concentration) on the remaining IL-10 concentration after emulsification (Y2), as indicated by colour and contour lines. The numbers on the contours show the relative amount of IL-10 (%) remaining after emulsification. Factor D (poloxamer 188 concentration) was set to its mid value of 2.5%. Y2 decreases with increasing homogenisation speed and increases with increasing BSA concentration.

The contour plot for A vs. D (poloxamer 188 concentration) reveals a similar interaction. With increasing poloxamer concentration, the negative effect of the increasing homogenisation speed on the remaining detected IL-10 amount is reduced, especially at low speeds (Fig. 10). Overall, the model shows that an increase of the homogenisation speed clearly reduces the remaining detectable amounts of IL-10 in the homogenisation/emulsification process, while both BSA and poloxamer 188 have a concentration-dependent protective effect.

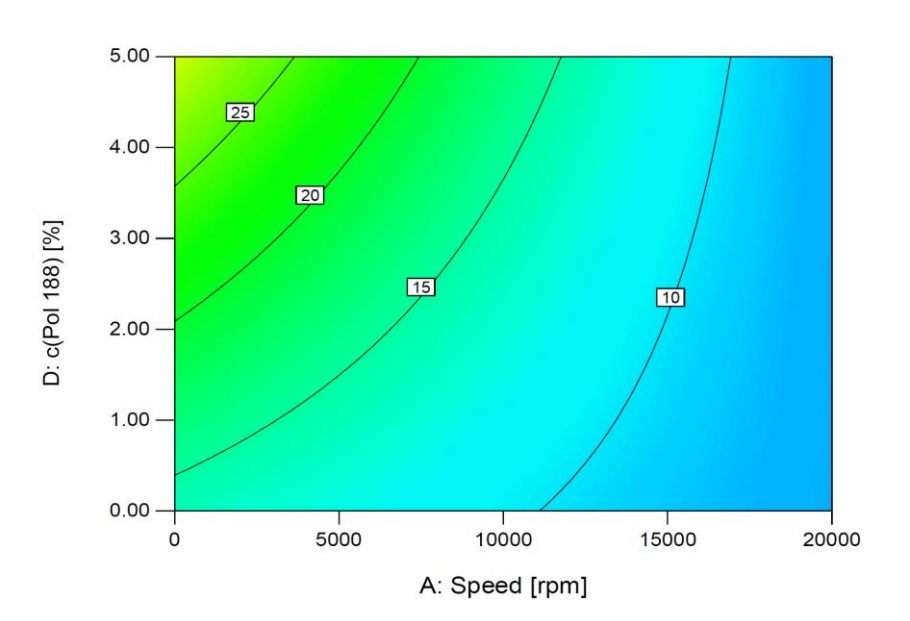

Fig. 10: Modelled influence of the factors A (homogenisation speed) and D (poloxamer 188 concentration) on the remaining IL-10 concentration after emulsification (Y2), as indicated by colour and contour lines. The numbers on the contours show the relative amount of IL-10 (%) remaining after emulsification. Factor C (BSA concentration) was set to its mid value of 2.5%. Y2 decreases with increasing homogenisation speed and increases with increasing poloxamer 188 concentration.

The model for the response Y1 (remaining IL-10 bioactivity) has the following equation:

$$Y1 = 1.02 - 0.47 * A - 0.02 * C + 0.18 * D + 0.54 * AC - 0.54 * AD + 0.67A^2 \quad \text{(eq. 4)}$$

This model has a p-value (ANOVA, partial sum of squares type III) of 0.004, indicating that the model is significant. The factor A and the interactions AC and AD are significant model terms ($p < 0.05$, see tableTab. 4). The adjusted R^2 for the model is 0.44. The predicted R^2 is only 0.19, indicating that the model has poor precision and predictive power.

Tab. 4: Overview on the model terms significantly affecting the remaining IL-10 bioactivity (Y1).

	Actual model term	Coding	p-value
Factors	**Homogenisation speed**	**A**	**<0.0001**
	BSA concentration	**C**	**0.9252**
	Poloxamer 188 concentration	**C**	**0.3366**
Interactions	**Homogenisation speed * BSA concentration**	**AC**	**0.0100**
	Homogenisation speed * poloxamer 188 concentration	**AD**	**0.0300**
	Homogenisation speed * Homogenisation speed	**A^2**	**0.6909**

The contour plot of the model generated for homogenisation speed (A) vs. BSA concentration (C) shows, for lower BSA concentrations in the range of 0.01 to about 2%, a decreasing IL-10 bioactivity with increasing homogenisation speed from more than 2.5 to less than 1% (Fig. 11). For higher concentrations, no clear trend can be observed.

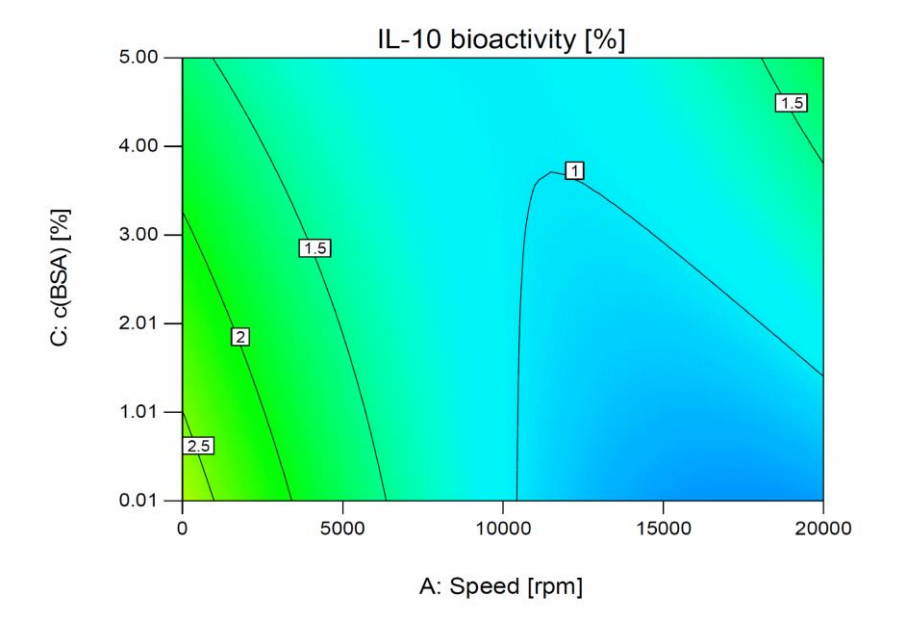

Fig. 11: Modelled influence of the factors A (homogenisation speed) and C (BSA concentration) on the remaining IL-10 bioactivity after emulsification (Y1), as indicated by colour and contour lines. The numbers on the contours show the relative amount of IL-10 bioactivity (%) remaining after emulsification. Factor D (poloxamer 188 concentration) was set to its mid value of 2.5%. At low BSA concentrations (0.01 to about 2%), Y1 decreases with increasing homogenisation speed. For higher BSA concentrations, no clear trend is observable.

The contour plot for A vs. D (poloxamer 188 concentration) indicates, that, at low homogenisation speeds, an increase in the poloxamer concentration slightly stabilises IL-10 during the emulsification, and thus increases the remaining bioactivity (Fig. 12). At higher speeds (> 5000 rpm), the bioactivity is reduced regardless of the poloxamer concentration.

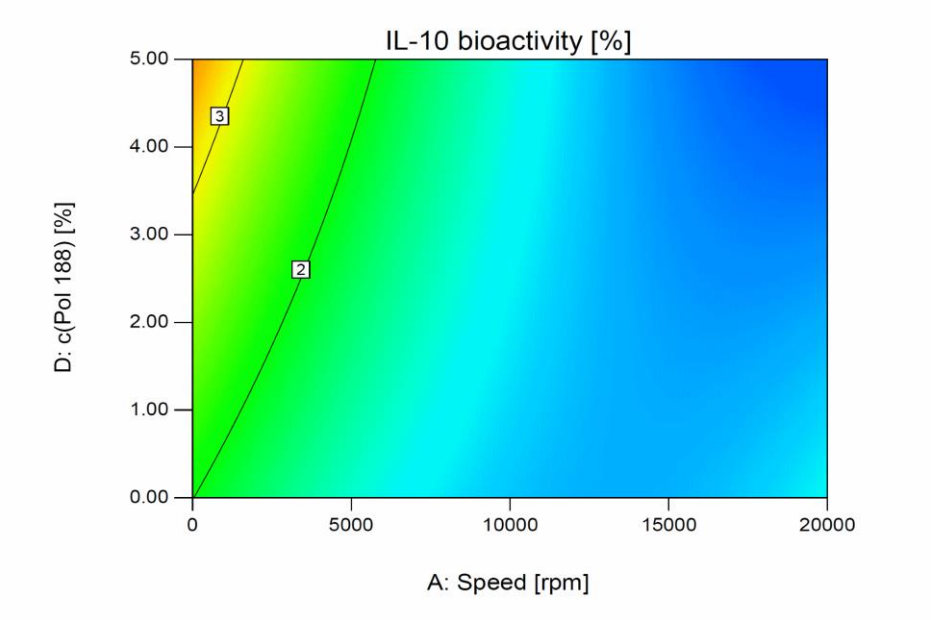

Fig. 12: Modelled influence of the factors A (homogenisation speed) and D (poloxamer 188 concentration) on the remaining IL-10 bioactivity after emulsification (Y1), as indicated by colour and contour lines. The numbers on the contours show the relative amount of IL-10 bioactivity (%) remaining after emulsification. Factor C (BSA concentration) was set to its mid value of 2.5 %. The IL-10 bioactivity (Y1) decreases with increasing homogenisation speed and increases with increasing poloxamer concentration.

The results of the model calculations for Y1 should not be overinterpreted in detail because its statistical figures, such as the adjusted R^2 and the predicted R^2, indicate that the model is of rather poor quality. This is probably caused by the poor accuracy of the experimental values of Y1. These measurements were performed with an *in vitro* MC/9 cell assay and the accuracy and reproducibility of bioassays are generally very limited in comparison to conventional quantitative assays. However, overall, the model still shows that an increase of the homogenisation speed considerably reduces the remaining IL-10 concentration. On the other hand, both BSA and poloxamer 188 have only a limited stabilising effect at low homogenisation speeds. Thus, even if the homogenisation speed is set to 0, that is, the aqueous phase and the EtOAc phase are not homogenised and therefore not emulsified at all, the dissolved IL-10 is significantly degraded and its remaining

bioactivity is reduced to less than 5%. No combination of the tested factors improves the remaining IL-10 bioactivity to a suitable level. Obviously, IL-10 is highly prone to degradation in EtOAc/water mixtures. Interestingly, the remaining IL-10 amount determined by the antibody-based immunoassay was significantly higher than the remaining IL-10 bioactivity determined by the cell assay. On average, the remaining IL-10 amount (relative to the initial amount) was 17-fold higher (mean for n = 29; sample 15 was excluded) than the remaining IL-10 bioactivity. The correlation between the two responses is low as can be seen in Fig. 13.

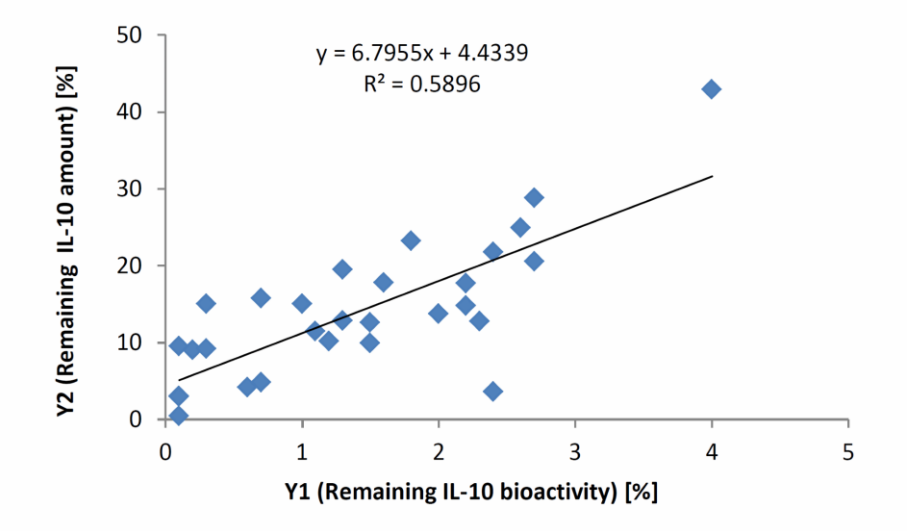

Fig. 13: Response Y1 (remaining IL-10 bioactivity) vs. response Y2 (remaining IL-10 amount). The line shows the linear regression function that was calculated for the data points. The equation is shown in the graph. Sample 15 was excluded from the graph and the regression analysis.

That the amount of IL-10 determined with the immunoassay is so much higher than the remaining bioactivity clearly suggests that the applied immunoassay is not very sensitive to the denaturation of the native IL-10 structure that occurs during emulsification. The used immunoassay, the IL-10 CBA Flex Set from Becton Dickinson, consists of antibodies which are covalently bound to the surface of polystyrene-based microspheres. These beads were incubated with the sample solutions to allow binding of the IL-10 to the antibodies. Quantitative detection of the IL-10 is then mediated by incubation with a second IL-10 antibody conjugated with phycoerythrin, a red fluorescent pigment (Morgan et al., 2004). For the native protein, the fluorescence intensity is proportional to the concentration of IL-10 in the sample (Morgan et al., 2004).

As in the case of the widely used enzyme-linked immunosorbent assay (ELISA), two separate antibodies that bind to two different epitopes of the detected protein are used for the CBA assay. Thom et al. observed that ELISA for IL-10 from different manufactures indicated, depending on the confirmation of the analysed IL-10, different concentrations. The antibodies employed in the Biosource ELISA were conformation-sensitive, while the ones of the R&D system were insensitive to several conformational changes. They detected both native IL-10 and IL-10 previously inactivated by disulfide bond reduction with dithiothreitol. Furthermore, the antibodies even detected IL-10 with similar sensitivity that was previously heat-stressed and thus dissociated to monomers (Thom et al., 2005). Several monoclonal antibodies against IL-10 were investigated by de Groote et al. They found three major antigenic regions on the protein. However, only one of these regions comprises epitopes involved in receptor binding (De Groote et al., 1994). The Biosource ELISA utilised a capture antibody that binds to an epitope involved in receptor binding. Thus, this antibody seemed to be able to differentiate between native and chemically reduced or heat-denatured IL-10 (Thom et al., 2005). Diverging results between ELISAs and bioactivity tests were also reported for the heat-inactivated cytotoxic protein Shiga toxin 1. The tested ELISA was sensitive to inactivation of epitopes on the B subunit of the toxin, whereas a bioassay was sensitive to the inactivation of the bioactive site on the A subunit (Lumor et al., 2012).

In the present study, the bioactivity of IL-10 was determined with a MC/9 cell assay that depends on direct cytokine-receptor interaction and subsequent receptor activation. Thus, it is plausible that a huge discrepancy between the IL-10 amount determined with the CBA IL-10 immunoassay and the remaining bioactivity was observed. These results strongly indicate that the immunoassay has only limited power to discriminate between the native conformation of IL-10 and the denatured state caused by the interaction of the aqueous IL-10 solution with EtOAc.

Additional experiments were performed to evaluate whether the supplementary addition of the excipients trehalose or HP-β-CD in addition to 2.5 % BSA has a stabilising effect on IL-10 in solution when EtOAc is added and evaporated at RT. The results for the remaining IL-10 concentration and the remaining IL-10

bioactivity are displayed in Fig. 14. In the aqueous solution without organic solvent (w/o OS), both remaining IL-10 concentration and remaining IL-10 bioactivity were close to 100%. Addition of EtOAc caused a drastic reduction of the remaining concentration and bioactivity of IL-10 about 10% and 3%. A slight increase of the remaining IL-10 concentration and remaining IL-10 bioactivity was obtained by addition of 2.5% HP-ß-CD and a somewhat larger increase was observed when 2.5% trehalose was added (third and fourth category in Fig. 14). Although fraught with high uncertainties, the remaining IL-10 amount and, in particular the remaining bioactivity were still disappointingly low. The substitution of EtOAc by DCM, an alternative solvent for the preparation of PLGA particles by the double emulsion method, did not result in an improved remaining IL-10 concentration and remaining IL-10 bioactivity (fifth category in Fig. 14) either. The denaturation caused by addition of DCM was comparable to that caused by EtOAc.

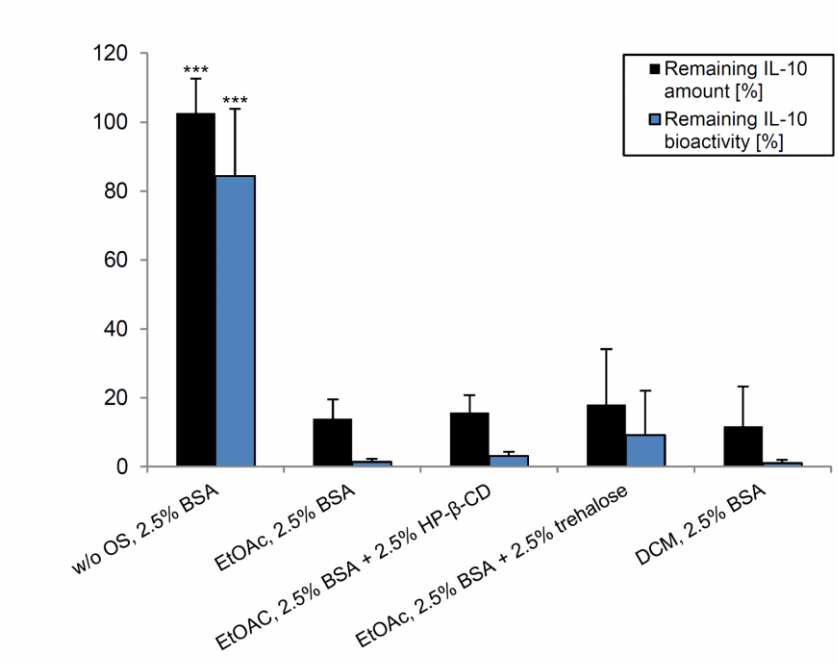

Fig. 14: Remaining IL-10 amount/concentration and remaining IL-10 bioactivity in aqueous solutions containing 5µg/ml IL-10 and 2.5% BSA without added organic solvent (w/o OS, positive control), with added EtOAc and with added DCM. In two groups, also 2.5% HP-β-CD or 2.5% trehalose were added to the aqueous phase. Results are presented as mean ± SD for n = 3. *** $p < 0.001$ (ANOVA, Holm-Sidak). The values for the solution of 2.5% BSA with added EtOAc were predicted with the respective DoE models (mean ± SD).

It has to be pointed out that the concentration of IL-10 used in these experiments was very low, only 5 µg/ml in the aqueous phase. For most published experiments on the stability of proteins during emulsification, much higher concentrations of the

investigated protein were used, e.g. 20–100 mg/ml (Son et al., 2009). Several studies reported that the extent of interfacial denaturation is highly dependent on protein concentration. At low concentrations, protein is often considerably adsorbed and denatured at the W/O interface, whereas at higher concentrations (in the mg/ml range) protein denaturation seems to be limited by a self-protecting effect (Bilati et al., 2005d), (Wolf, 2003). Unfortunately, in the present study it was not possible to test whether this effect also occurs in the case of IL-10, as the high costs for commercially available rhIL-10 (more than 4000 € for 1 mg) prevented the use of IL-10 in high concentrations.

2.1.5 Conclusion

IL-10-loaded PLGA NP were prepared by a double emulsion solvent evaporation method with EtOAc as the solvent for PLGA and BSA as the carrier/stabiliser of IL-10. Only marginal amounts of IL-10, still detectable by a bead-based immunoassay, were released from the NP in PBS pH 6.8. Therefore, a DoE was conducted to evaluate the influence of different process factors and the concentrations of stabilisers on the stability of IL-10 during the first O/W emulsification step. The DoE revealed that IL-10 was largely denatured during emulsification. Even if the organic and the aqueous phase were not homogenised, the remaining IL-10 bioactivity was not significantly enhanced. Furthermore, enhanced concentrations of stabilisers such as BSA, poloxamer 188, HP-β-CD or trehalose did not sufficiently stabilise the IL-10. When the organic solvent EtOAc was substituted by DCM, a similar amount of denaturation was observed. Overall, the double emulsion method appears to be unsuitable for the formulation of IL-10-loaded polymeric nanoparticles. Further combinations of protein-stabilising excipients as well as of different buffer systems and pH values should be investigated in the future to find optimised formulations which provide an enhanced remaining bioactivity of IL-10. Very likely, it will be necessary to employ emulsions with a significantly higher concentration of IL-10 than the one used in the present study (5 µg/ml) to benefit from the self-protecting effect of proteins.

2.2 Spray-dried IL-10-loaded microparticles encapsulated in Eudragit microspheres

2.2.1 Preliminary remarks

The practical work presented in this chapter was exclusively planned, conducted and evaluated by the present author.

The investigations reported in the preceding chapter showed that PLGA NP prepared by a double emulsion solvent evaporation method are not a suitable approach for the formulation of IL-10, at least at the low concentrations of IL-10 available for the present studies. Therefore, an alternative system was tested, namely IL-10-loaded MP prepared by spray drying of aqueous IL-10 solutions with BSA and trehalose added as stabilising excipients and poloxamer 188 as surfactant. To enable the oral delivery of these MP to the intestinal mucosa, they were encapsulated in microspheres made of the enteric methacrylic acid copolymers.

2.2.2 Introduction

2.2.2.1 Spray drying of proteins

Proteins in the solid state generally show higher physical stability than in solution (Maltesen and van de Weert, 2008). Traditionally, freeze drying is the method of choice for the dehydration of protein formulations but spray drying has emerged as a viable alternative over the last two decades (Maltesen and van de Weert, 2008), (Sollohub and Cal, 2010). Spray drying is well established in the pharmaceutical industry for the production of dry powders for inhalation (Vehring, 2008), (Sollohub and Cal, 2010). The continuous process comprises three major phases. The atomisation of the liquid stream by an appropriate device is followed by the drying of the fine droplets with a drying gas and the collection of the dry powder (Cal and Sollohub, 2010). Spray drying is a particularly suitable method for the production of protein-loaded dry powders for pulmonary delivery. It is a scalable and cost-effective process that enables the engineering of particles with defined size, shape and surface properties (Depreter et al., 2013).

However, spray drying of protein solutions without addition of stabilising excipients usually results in substantial aggregation and denaturation of the protein (Lee, 2002). Heat stress, shear stress, adsorption at the air/liquid interface and dehydration are the main sources of protein denaturation during the spray drying process (Maltesen and van de Weert, 2008). Thermal denaturation is usually avoidable, as the temperature of the drying gas can be optimised. Furthermore, the heat loss upon evaporation keeps the temperature of the atomised droplets significantly lower than that of the drying gas. The denaturation temperature of proteins in the solid state is dependent on their water content and usually increases once more and more water is removed by evaporation (Depreter et al., 2013). Mechanical shear stress occurs mainly during the atomisation of the liquid feed into fine droplets. Adsorption to the air/water interface is generally considered to be the major factor promoting protein degradation during spray drying (Maa and Hsu, 1997) (Lee, 2002). Most proteins are amphiphilic and thus adsorb to the large air/water interface area that is created by the droplet formation. This adsorption may cause unfolding, as the exposition of hydrophobic residues of proteins to interfaces is usually thermodynamically favoured. These conformational changes can result in protein aggregation and irreversible denaturation (Maltesen and van de Weert, 2008). Additionally, the drying of the droplets may cause structural perturbation of proteins by dehydration stress (Maltesen and van de Weert, 2008). Consequently, stabilising excipients are commonly added to protein solutions to be spray dried to improve their process stability.

Surfactants, like polysorbate 20, polysorbate 80 or sodium dodecyl sulfate (SDS), are often used to reduce the extent of protein aggregation and denaturation of the protein at the air/water interface (Adler and Lee, 1999), (Adler et al., 2000), (Maa and Hsu, 1997), (Maa et al., 1998). It was shown that surfactants reduce the protein concentration on the surface of the spray dried particles, which indicates an exclusion of the protein from the air/liquid interface of the droplets during the drying step (Adler and Lee, 1999), (Adler et al., 2000). Frequently, about 0.05 to 0.1% (w/v) surfactant is added to the spray solution, a concentration that was found to maximise the protective effect against aggregation and denaturation of the protein (Lee, 2002). Additionally, surfactants also influence the morphology of the

spray-dried particles, enhancing sphericity and surface smoothness (Adler and Lee, 1999), (Maa et al., 1998).

Various sugars and polyols are known to protect proteins in solution, during drying as well as in the solid state from aggregation and denaturation (Depreter et al., 2013). Their protective effect is explained by various theories. The preferential exclusion theory proposes that carbohydrates sequester water molecules from proteins, thereby increasing their compactness and stability. The water replacement theory assumes the substitution of water molecules by hydrogen bonds formed between protein and carbohydrate, thereby stabilising the proteins. The vitrification theory assumes that the carbohydrates form an amorphous glassy matrix around the protein during dehydration, thereby protecting its native structure (Jain and Roy, 2009). Trehalose and sucrose are thought to be the most effective sugars for stabilising sensitive proteins during spray drying and subsequent storage. They are both non-reducing and thus not prone to Maillard reactions with amino acids of proteins (Lee, 2002). In various studies, a sugar-protein ratio in the range of about 1:4 to 1:1 was found to be optimal for maintaining the stability of the protein during spray drying, e.g. in the case of trypsinogen, lysozyme and a monoclonal antibody (Andya et al., 1999), (Liao et al., 2002), (Tzannis and Prestrelski, 1999).

Various other excipients were successfully employed to enhance the stability of proteins during spray drying, too. For example, the remaining bioactivity of alcohol dehydrogenase after spray drying was enhanced by the addition of the proteins BSA and β-lactoglobulin. As well as surfactants, these two proteins are thought to exclude other protein from the surface of the formed droplets (Yoshii et al., 2008).

The Büchi B-90 nano spray dryer is a novel laboratory scale spray dryer that can generate and collect particles in the low micron and submicron size range. In contrast to conventional spray dryers that utilise nozzles or rotary atomizers to form spray droplets, the B-90 uses a vibrating mesh technology. The spray mesh is a thin steel membrane with precise micron-sized holes (e.g. with a diameter of 4 µm). A piezoelectric element incorporated into the spray head and driven at an ultrasonic frequency of 60 kHz causes the mesh to vibrate. In this way, an aerosol of very fine droplets is generated from the liquid feed (Heng et al., 2011). The mean size of droplets of distilled water generated with the 4 µm mesh is 5 µm (Schmid et al.,

2011). The generation of very fine droplets enables the preparation of submicron-sized primary particles whose actual size is also dependent on the concentration of the dissolved solids (Lee et al., 2011). The collection of particles smaller than 2 µm is challenging with most of the conventional spray dryers that use cyclone collectors or bag filters. The B-90 therefore incorporates an electrostatic particle collector (Fig. 15) that consists of a grounded star-shaped cathode and a cylindrical particle-collecting anode. This particle collector enables the collection of small particles with a higher yield than conventional collectors for spray-dryers (Heng et al., 2011).

(Lee et al., 2011) explored the feasibility of the B-90 to produce BSA NP, using polysorbate 80 as excipient. Under optimised process conditions, they obtained smooth spherical particles with a median size of 460 nm. (Bürki et al., 2011) used the B-90 to spray dry β-galactosidase together with trehalose as protein stabiliser. The residual enzyme activity was found to be dependent on the inlet air temperature. Using a 4 µm mesh, full enzyme activity was retained after spray drying at an inlet temperature of 80°C, whereas an inlet temperature of 120°C caused a reduction in bioactivity of about 20%. (Schmid, 2011) found that the temperature of the spray mesh correlates with both the temperature of the inlet air and the spray intensity. At an inlet temperature of 30°C and a spray intensity of 100%, the mesh reached a temperature of 31°C, while an inlet temperature of 60°C and a spray intensity of 100% resulted in a mesh temperature of 52°C. It can be assumed that the spray solution reaches a similar temperature during droplet formation and thus is potentially exposed to significant temperature stress. To examine the effect of the inlet air temperature on the bioactivity of proteins, a solution of 0.1% lactic dehydrogenase, 5% trehalose and 0.1% polysorbate 80 was spray-dried at various temperatures. An inlet air temperature of 60°C reduced the enzyme activity in the dried particles to about 30%. In contrast, 80% of the original bioactivity remained when an inlet temperature of 30°C was applied (Schmid, 2011). These results indicate that proteins that are spray dried with the B-90 at high inlet air temperatures are indeed exposed to significant temperature stress.

2.2.2.2 Enteric-coated drug delivery systems for the treatment of IBD

Enteric-coated drug delivery systems are commonly used for the treatment of IBD: The enteric coating protects the drug against the gastric environment and allows a targeted release in the affected sections of the intestine. Besides derivatives of cellulose, polymers used for enteric coatings are mostly based on methacrylic acid and methyl methacrylate (Lautenschläger et al., 2014). Depending on the specific composition of the copolymer, the pH threshold at which the polymer starts to dissolve and with it the targeted part of the intestine varies. Eudragit L 100-55 dissolves at pH values above 5.5, which are reached in the duodenum following the gastric passage. Eudragit L 100 dissolves at a pH above 6.0, which is usually reached before during passage of the distal small intestine. Both polymers are suitable for the treatment of CD, where inflamed areas in these regions of the intestine are common. The highest pH threshold of the acrylate methacrylate copolymers is exhibited by Eudragit S 100 with pH 7.0, which is suitable for colon targeting (Lautenschläger et al., 2014), (Evonik Industries, n.d.). Numerous Eudragit-coated oral dosage forms for the treatment of IBD are approved and marketed, proving the safety and therapeutic efficacy of such delivery systems. Examples are beclomethasone tablets coated with Eudragit L 100-55 (Clipper®), budesonide capsules coated with Eudragit L 100 (Entocort™ EC) and mesalamine tablets coated with Eudragit S (Asacol®) (Rizzello et al., 2002), (Edsbäcker and Andersson, 2004), (Faber and Korelitz, 1993).

Besides the coating of conventional oral dosage forms, enteric polymers have also widely been used to develop novel oral dosage forms. One promising approach is the design of multiparticulate systems consisting of drug-loaded NP or MP encapsulated in Eudragit microspheres. These systems can protect the drug from the gastric environment and enable improved site-specific and controlled release compared to conventional systems. (Lorenzo-Lamosa et al., 1998) encapsulated diclofenac-loaded chitosan MP in Eudragit L and Eudragit S, preventing drug release at acidic pH *in vitro*, while achieving continuous release above the pH threshold of the respective Eudragit. (Rodríguez et al., 1998) microencapsulated budesonide-loaded cellulose acetate butyrate MP in Eudragit S. The encapsulation prevented drug release *in vitro* below pH 7 and the cellulose acetate butyrate MP

controlled the drug release after dissolution of the Eudragit S at pH 7.5. The administration of the formulation in a rat colitis model significantly reduced inflammation in comparison to a budesonide suspension (Rodriguez et al., 2001). (Lamprecht et al., 2005a) developed tacrolimus-loaded PLGA NP entrapped in microspheres of the pH-sensitive polymer Eudragit P-4135F. The microspheres showed pH-dependent release kinetics *in vitro*. *In vivo*, the system was superior in terms of the anti-inflammatory response in a rat colitis model as compared to both a drug solution and the drug-loaded NP without enteric coating, suggesting a colon-specific release of the drug from the microspheres.

2.2.2.3 Aims of this study

During the previously conducted study, it became apparent that IL-10 in aqueous solution at low concentrations is largely degraded once exposed to interfaces with organic solvents (see chapter 2.1). Therefore, a new strategy for the preparation of IL-10-loaded particles for the oral treatment of IBD was pursued. A focus was to avoid water/organic solvent interfaces during the formulation preparation to reduce denaturation of the IL-10. To this end, an aqueous solution containing IL-10 and stabilising excipients was spray dried to obtain solid IL-10-loaded particles. In these particles, IL-10 is expected to be less prone to denaturation by organic solvents than IL-10 in aqueous solution. The B-90 nano spray dryer was used to produce particles in the low micron and submicron range. The IL-10-loaded particles were then encapsulated in Eudragit microspheres to protect them against the gastric environment after oral intake and to enable a pH-modified release of the IL-10. The final subgoal was to determine the remaining bioactivity of the IL-10 after incubation of the microspheres in acidic release media that mimic the gastric environment.

2.2.3 Materials and methods

2.2.3.1 Materials

NaCl was purchased from VWR International (Radnor, USA). 2-mercaptoethanol, BSA (≥ 96% purity), MTT, penicillin/streptomycin, poloxamer 188 and trehalose

dihydrate were purchased from Sigma-Aldrich (St. Louis, USA). Dimethylsiloxane (Baysilone® M50) was produced by Bayer (Leverkusen, Germany). Poly(methacrylic acid-co-ethyl acrylate) 1:1 (Eudragit® L 100-55) and poly(methacrylic acid-co-methyl methacrylate) 1:2 (Eudragit® S 100) were purchased from Evonik Industries (Essen, Germany). Soy lecithin (phospholipid content >96%) was obtained from Caesar & Loretz (Hilden, Germany). Paraffin viscous (Ph. Eur./USP) with a dynamic viscosity of 110–230 mPa*s at 20°C was purchased from Merck (Darmstadt, Germany). RhIL-10 and rmIL-4 were purchased from peprotech (Rocky Hill, USA). The murine mast cell line MC/9 (CRL-8306™) was purchased from ATTC (Manassas, USA). RPMI 1640 with L-glutamine and without phenol red was purchased from PAA Laboratories (Pasching, Austria). Heat-inactivated FBS was purchased from Lonza (Cologne, Germany). Rat T-cell culture supplement with ConA (IL-2 culture supplement) was obtained from Becton, Dickinson and Company (Franklin Lakes, USA). IL-10 antibody-coated beads and IL-10 detection reagent (CBA Flex Set) were purchased from Becton, Dickinson and Company.

2.2.3.2 Determination of IL-10 denaturation caused by heat stress and pH-reduction

The following experiments were carried out to obtain information on appropriate conditions for the spray-drying process with the B-90 nano spray dryer. 25 ng/ml IL-10 was added to an aqueous solution of 0.25% BSA, 0.25% trehalose dihydrate and 0.05% poloxamer 188. The final solution had either a pH of 5.3 (pH not adjusted) or 7.4 (adjusted by addition of 0.1 N NaOH). 2 ml aliquots of the solutions were filled into 2-ml polypropylene micro reaction vessels. The vessels were submerged into a beaker filled with water of defined temperature for 5 min. During incubation, the temperature in the beaker was kept constant by a hot plate coupled with a thermosensor that was submerged into the vessel. The water in the vessel was stirred to accelerate the temperature equilibration between the beaker and the samples. After incubation, the samples were cooled down to RT by submerging them RT water that was stirred. Subsequently, 120 µl of each sample were diluted with 880 µl CMWT and the remaining IL-10 bioactivity was determined by the MC/9 cell assay (see chapter 2.2.3.9). Each sample was measured 5-fold. For the

analysis of the IL-10 recovery by the bead-based immunoassay (CBA), samples diluted in the culture medium were frozen at -80°C until further analysis (see chapter 2.2.3.10).

2.2.3.3 Preparation of spray-dried MP

IL-10-loaded MP were prepared by spray drying aqueous solutions containing IL-10 and the protein stabilisers BSA, trehalose and poloxamer 188 with a B-90 nano spray dryer (Büchi Laboratoriums Technik AG, Flawil, Switzerland). Unless otherwise indicated, the pH of the solutions was adjusted to 7.4 by addition of 0.1 N NaOH before the IL-10 was added. Lee and his co-workers thoroughly described the underlying physical and technical details of the nano spray drying process (Lee et al., 2011). Briefly, the prepared solution was fed to the spray head by a peristaltic pump (Fig. 15). In the spray head, a mesh with a size of 4 µm dispersed the solution to fine droplets. Heated air moved the droplets downwards and dried them to solid particles. The particles were collected on a charged, cylinder-shaped electrode. The reservoir for the solution was ice-cooled during the spay-drying process to minimise heat-induced degradation of the IL-10. The inlet air temperature of the spray dryer was set to 50°C. An air flow rate of 140 l/min was used to dry the sprayed droplets. The resulting outlet air temperature was in the range of 32–36°C. After the process, the dried particles were scraped off the collecting electrode with a plastic scraper.

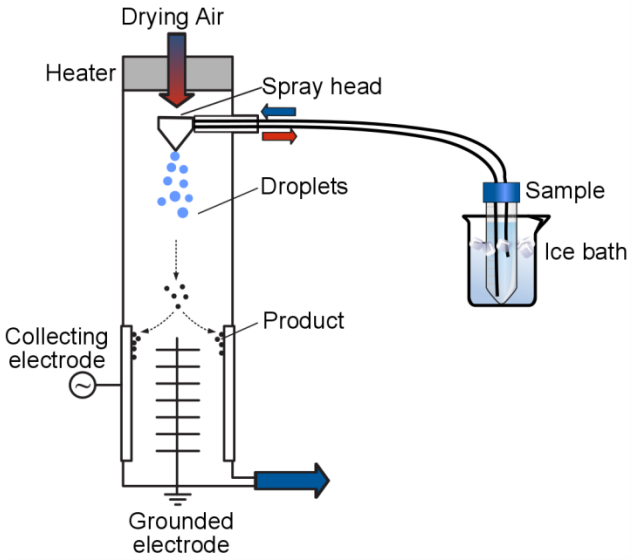

Fig. 15: Preparation of IL-10-loaded spray-dried MP with the B-90 nano spray dryer. Details see text. Parts of this figure were adopted from the operation manual of the B-90 (Büchi Labortechnik, n.d.).

The volume of sprayed solution was determined by volumetric measurement of the solution in the reservoir before and after the process. Based on this volume and the known concentration of dissolved excipients, the yield of each batch was calculated.

2.2.3.4 Preparation of the microspheres

The spray-dried MP were microencapsulated in Eudragit with a modified solid-in-oil-in-oil (S/O/O) emulsion evaporation process based on the method published by (Chan et al., 2005). 75 ml paraffin viscous, 750 mg soy lecithin and 75 µl dimethylsiloxane were added in a 150-ml low form glass beaker (height: 80 mm, external Ø: 60 mm, model VWR 213-1123, VWR International, Radnor, USA). A 3-bladed propeller stirrer (R 1381, IKA, Staufen im Breisgau, Germany) propelled by an overhead stirrer (IKA Eurostar) was immersed into the mixture at a fixed height so that the lower end of the blade would just have touched the surface of a fill volume of 25 ml. The soy lecithin was dissolved by slight stirring and warming on a heat plate. Upon completion, the heating was stopped to let the solution cool down to RT. 40 mg of spray-dried particles were added to 1 ml of ethanol (99.8%) in a 2-ml polypropylene micro reaction vessel. The particles were dispersed with a 3-mm microtip attached to a sonifier (Type 250, Branson Ultrasonics, Danbury, USA) at 10% amplitude for 20 s, while the vessel was cooled in an ice bath. 1 ml

of a 10% (w/v) solution of methacrylic acid-ethyl acrylate copolymer (Eudragit® L 100-55 or S 100) in ethanol was added to the dispersion and the vessel was briefly vortexed. Subsequently, the dispersion was added to the soy lecithin solution and the mixture was stirred at a fixed rotational speed (600 rpm if not indicated otherwise) to form an emulsion. Stirring of the emulsion was continued overnight at the same speed until the ethanol completely evaporated. The particles were separated from the paraffin with a glass suction filter (VitraPor®, pore size 1–1.6 µm, ROBU® Glasfilter-Geraete, Hattert, Germany). and washed with hexane three times. The loading of the microspheres with spray-dried particles was calculated by dividing the amount of dry powder used for the formulation of the microspheres by the weight of the washed microspheres.

2.2.3.5 Investigation of particle size and morphology

The spray-dried MP were characterised by SEM and the microspheres by optical microscopy. The samples for SEM imaging were prepared by spreading a small amount of the dry powder on adhesive tape (Tesa Transparent, Tesa, Norderstedt, Germany). To reduce the thickness of the particle layer, excess powder was subsequently removed by stripping off the coated tape with a second piece of tape. The remaining powder was then deposited onto a double sided adhesive carbon disc and coated with a gold film of about 20 nm thickness by a W150R QS sputter coater (Quorum Technologies, East Grinstead, UK). SEM micrographs were obtained with an EVO HD15 (Carl Zeiss, Oberkochen Germany) at an accelerating voltage of 5 kV or 15 kV. The mean size and the size distribution of the primary spray-dried particles were obtained by image analysis of the SEM micrographs. In the image files, the single particles were manually overlaid with ellipsoids of matching size and axial ratio. The ferret diameter of these ellipsoids was then calculated using the software Image J (National Institutes of Health, USA). For each sample, at least 100 particles were measured and the number-based mean size and the SD of the size distribution were calculated.

Images of the microspheres were taken with a BH-2 light microscope (Olympus, Tokyo, Japan). Focus-stacked images of the microspheres were reconstructed from confocal laser scanning microscopy (CLSM) images obtained at different focal

lengths (z-stack) with a Zeiss LSM 510 (Carl Zeiss Microscopy GmbH, Jena, Germany).

The size distribution of the Eudragit microspheres was determined by laser diffraction in a Mastersizer 2000 equipped with the Hydro µP wet dispersion unit (both from Malvern Instruments, Malvern, UK). The microspheres were dispersed in 2.5 mM acetic acid and a drop of polysorbate 80 was added to prevent floating of the particles. Each batch was measured in triplicate and the mean value was calculated. The volume based mean size (diameter) and the width of the distribution were calculated. The latter was expressed in terms of the span which is an indicator for the range of the volume distribution of the particles:

$$span = \frac{d_{90} - d_{10}}{\mathrm{d}_{50}} \qquad \text{(eq. 5)}$$

where d10, d50 and d90 are the volume-based diameters at 10, 50 and 90% of the cumulative volume, respectively.

2.2.3.6 *In vitro* release of MP from the microspheres

The MP consisted predominantly of BSA and trehalose. Therefore, the IL-10 release from the microspheres could be indirectly determined by measuring the amount of the released BSA. 1 mg/ml microspheres loaded with spray-dried particles were suspended in 2.5 mM HCl (pH 2.4) in a 50-ml polypropylene falcon tube and incubated at 37°C, 100 rpm. For sampling, the shaking of the tubes was briefly stopped to allow sedimentation of the microspheres. 1 ml of supernatant was sampled. 1 ml of 2.5 mM HCL was added to the release vessel to substitute the removed volume and the incubation was continued. The pulled sample was centrifuged, and the supernatant was further analysed as described below. After 60 min, a salt mix equivalent to the addition of 10 mM PBS pH 7.4 was added to the release vessel. After dissolution of the buffer salts, a volume of 1 N NaOH equivalent to the addition of 10 mM NaOH was added with a pipette to set the pH to 7.4. Subsequently, sampling of the supernatant was continued as described above. The concentration of the released BSA in the supernatant was determined by a bicinchoninic acid (BCA) assay (Quanti Pro™. Sigma-Aldrich). 100 µl

sampled supernatant were pipetted in the well of a polystyrene 96-well plate and 100 µl BCA working reagent was added. For calibration of the assay, standards were prepared from pure BSA. The covered plate was incubated at 37°C for two hours. After cooling down the plate to RT, the absorbance at 562 nm was measured by a plate reader (Infinite M200, Tecan Group AG, Maennedorf, Switzerland).

2.2.3.7 Determination of the acid resistance of the microspheres

An important question of the present study is whether the Eudragit microspheres provide sufficient protection for the encapsulated IL-10-loaded spray-dried MP under acidic gastric conditions that correspond to the gastric environment. To clarify this point, the microspheres were incubated at a concentration of 10 mg/ml, either in 2.5 mM HCl (pH 2.4) or in 2.5 mM acetic acid (pH 4.2), at 37°C, 100 rpm for 60 min. After this treatment, the microspheres were filtered and dissolved in cell culture medium. The remaining IL-10 bioactivity was determined as described in the next section.

2.2.3.8 Determination of the IL-10 bioactivity

The bioactivity of IL-10 was determined by utilising its ability to induce a rmIL-4-dependent growth stimulation of MC/9 cells (Thompson-Snipes et al., 1991). Cells were grown and prepared as described in chapter 2.1.3.7.2. For the determination of the IL-10 bioactivity in the spray-dried particles, the collected dry powder was dissolved in the cell culture medium and suitably diluted. This solution was then added to the MC/9 cells in the 96-well plate. To normalize for any influence of the formulations on bioactivity of the cells, the standards were prepared with matching concentrations of spray-dried particles containing no IL-10 (placebo formulation). For the determination of the remaining IL-10 bioactivity in the Eudragit microspheres, the microspheres were dissolved and prepared accordingly. Placebo microspheres were used to prepare the standards. The IL-10-dependent proliferation of the MC/9 cells was determined by a colorimetric MTT assay (Hansen et al., 1989) as described in chapter 2.1.3.7.2.

2.2.3.9 Long term stability testing under accelerated conditions

The spray-dried particles were aliquoted in high-performance liquid chromatography (HPLC) glass vials and closed with a perforated cap to simulate unclosed ensure a quick equilibration of humidity. The particles were then incubated at 25°C/60% RH in a controlled climate chamber for up to six months, in accordance with the ICH Q1A accelerated conditions for drug products intended for long-term storage in the refrigerator (ICH, 2003).

2.2.3.10 Determination of IL-10 recovery

The amount of IL-10 recovered from the spray solution and from the MP after various treatments was determined with a bead-based immunoassay (CBA Flex Set). The sample preparation was performed according to the manufacturer's manual. Briefly, 50 µl of the respective standards and samples were pipetted into a 4-ml polypropylene tube, mixed with 50 µl of diluted antibody-coupled beads and incubated at RT for 1 h. 50 µl of fluorescent IL-10 antibodies were added, the samples mixed and then incubated at RT for 2 h. The concentration of IL-10 in the samples was calculated from the fluorescence intensity of the beads, which was determined by a FACSCalibur™ flow cytometer (BD Biosciences) as described in chapter 2.1.3.7.3.

2.2.4 Results and discussion

2.2.4.1 IL-10 denaturation caused by heat stress and acidic environments

IL-10 is very sensitive to both elevated temperatures and to acidic environments (Syto et al., 1998), (Fiorentino et al., 1989). Its biological activity is dependent on the intactness of the noncovalent dimer. This is indicated by the observation that the amount of dissociation is proportional to the loss of bioactivity on MC/9 cells (Syto et al., 1998). The temperature-induced dissociation is furthermore dependent on the concentration of IL-10. Syto et al. reported 22% dissociation of the dimer for a solution of 0.3 mg/ml IL-10 and 55% for 0.05 mg/ml, in each case after incubation at 55°C for 1h. The dimer is also acid-labile. When IL-10 was incubated in a concentration of 0.1 mg/ml at pH values below 6, the dimer lost its bioactivity on

MC/9 cells in a pH-dependent manner with an apparent pK_a value of approximately 4.8 (Syto et al., 1998).

Due to the limited amount of IL-10 available for the present study, it was planned to spray dry IL-10 solution of a concentration of 0.25 ng/ml, which is significantly lower than the ones mentioned above. In these low-concentrated solutions, IL-10 might exhibit an enhanced lability to dissociation. Therefore, at first, the stability of IL-10 in the spray-drying solution against heat stress and reduced pH values was investigated. For this, samples were incubated for 5 min at different temperatures, rapidly cooled down to RT and then analysed. Pretrials showed that the temperature of the samples equilibrated to the incubation temperature within 1 min. The results for the recovered IL-10 concentration, expressed as percentage of the initial IL-10 concentration determined by a bead-based immunoassay, are displayed in Fig. 16. Also shown is the remaining bioactivity on MC/9 cells, expressed as percentage of the initial IL-10 bioactivity. In the solution containing 0.25% BSA, 0.25% trehalose dihydrate and 0.05% poloxamer 188, the IL-10 bioactivity was significantly decreased after incubation at 50°C or higher temperatures (graph A). Incubation at 50°C for 5 minutes caused a reduction of the bioactivity to 60% and incubation at 60°C even to only 2%. When the pH of the solution was not adjusted to 7.4 before the addition of the IL-10, the final pH of the solution was 5.3. At this pH, the IL-10 bioactivity was reduced to 9% when the sample was incubated at 25°C and further reduced to 2% when incubated at 50°C (graph B). Overall, IL-10 in the tested low-concentrated solutions was much more sensitive to the applied stress factors than reported by Syto et al (Syto et al., 1998). The results confirm that the lower IL-10 concentration used in this study is associated with a higher susceptibility to denaturation. A likely reason is the lack of the “self-protecting effect” of the protein (Bilati et al., 2005d), (Wolf, 2003) at this low concentration.

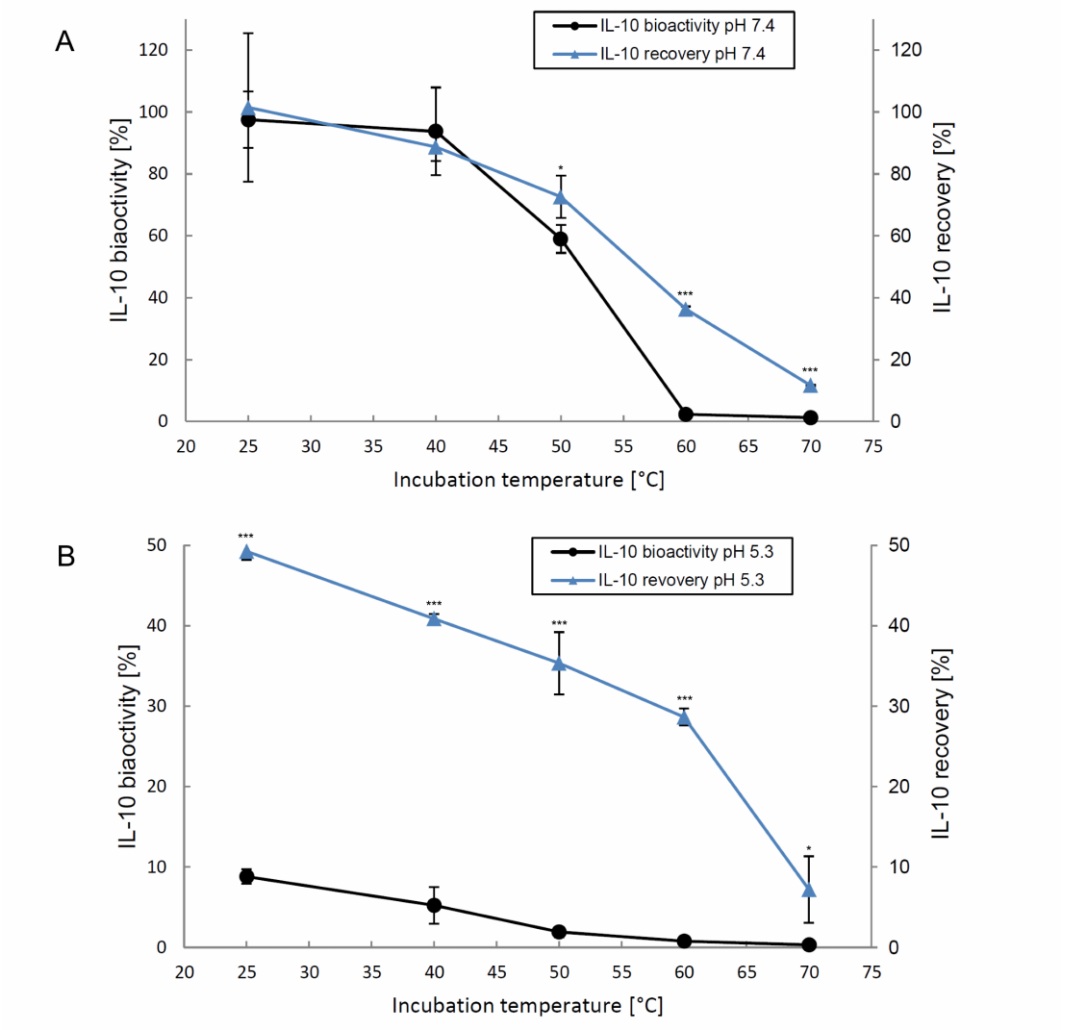

Fig. 16: Dependence of the IL-10 recovery and of the remaining bioactivity on the incubation temperature. A: IL-10 solution with pH 7.4. B: IL-10 solution with pH 5.3. Data are shown as mean ± SD for n = 3. The measured recoveries and bioactivities for the respective temperature conditions were compared by t-tests. Significant differences as indicated by the two-tailed p-values are marked in the graphs: * ($p \leq 0.05$), *** $p \leq 0.001$.

Interestingly, the results for the IL-10 recovery determined with the bead-based immunoassay did not correlate well with those for the bioactivity from the bioassay. The reduction of the former by both heat-stress and acidic pH was appreciably less pronounced than that of the latter (Fig. 16). For the solution of pH 7.4 incubated at 60°C, an IL-10 recovery of 36% was determined, whereas a remaining bioactivity of only 2% was measured. The corresponding values for the samples of pH 5.3 incubated at 25°C were 49% and 9.1%. As already observed in case of the denaturation caused by O/W interfaces (see chapter 2.1.4.3), the immunoassay is little sensitive to heat- and pH-induced denaturation of IL-10. As both stress factors cause a dissociation of the dimer, it can be assumed that the antibodies used in the immunoassay possess high binding affinity to the IL-10 monomer, too. The CBA

Flex Set from Becton, Dickinson and Company (BD) uses proprietary monoclonal antibodies. The characteristics of these antibodies and the specific epitopes of IL-10 to which they bind have not been published. Unfortunately, the manufacturer did not disclose them on request of the author either.

2.2.4.2 Characteristics of the spray drying process

In view of the results shown in Fig. 16, an inlet temperature of 50°C was chosen for the spray-drying of IL-10 solution with the B-90 nano spray dryer. The solutions contained different ratios of the excipients BSA and trehalose as well as poloxamer 188 as surfactant. It was shown that the addition of surfactants, such as polysorbate 80, induces the formation of smooth, spherical BSA particles when spray dried with the B90, whereas particles without surfactant were commonly irregular, wrinkled and donut-shaped (Lee et al., 2011). The use of polysorbate 80 was omitted in this work as it was found to be cytotoxic on MC/9 cells at concentrations as low as 10 µg/ml (data not shown). Instead, poloxamer 188, which proved to be non-cytotoxic on MC/9 cells, was used as a non-ionic surfactant. The solutions contained a low total concentration of 0.55% (w/v) solid excipients to reduce the particle size (Lee et al., 2011). The yield of the collected particles was determined after spray drying for 2h. The highest yield was obtained for the formulation containing a 1:1 ratio of BSA and trehalose (Fig. 17). The lowest yield was obtained for the formulation without BSA. It should be noted that the spray-dried particles of the latter formulation were hardly removable from the surface of the collection electrode as compared to the other powders and thus were not collectable in high yield.

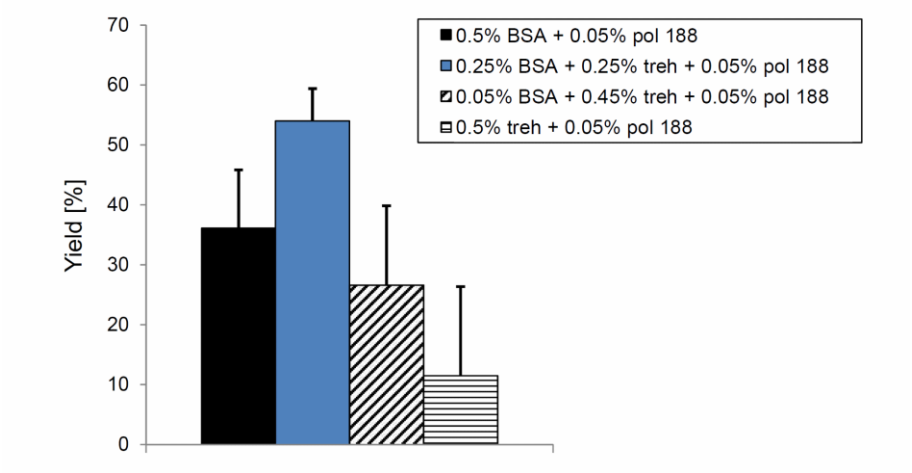

Fig. 17: Production yields of spray-dried formulations prepared with different ratios of the excipients BSA, trehalose (treh) and poloxamer 188 (pol 188). Results are presented as mean ± SD for n = 3.

For a further characterisation of the different spray solutions, the IL-10 recovery was determined by the bead-based immunoassay. Measurements were conducted for the collected particles as well as for the solutions both before the start and after the termination of the spray-drying process after 2 h. (Fig. 18). For all formulations containing BSA, only a slight reduction of IL-10 recovery to the range of 82 to 87% was observed in the remaining solution after spray drying. The recovery from the particles was slightly lower than from the solutions, ranging from 76 to 81%. For the solution containing no BSA, the recovery in the solution after spray drying and in the particles was significantly lower with 50 and 39%. The low recovery in the remaining solution indicates that the circulation of the solution through the feed system of the spray dryer or the residence time of two hours in the vessel but not the spray drying process per se may be mainly responsible for the reduced recovery.

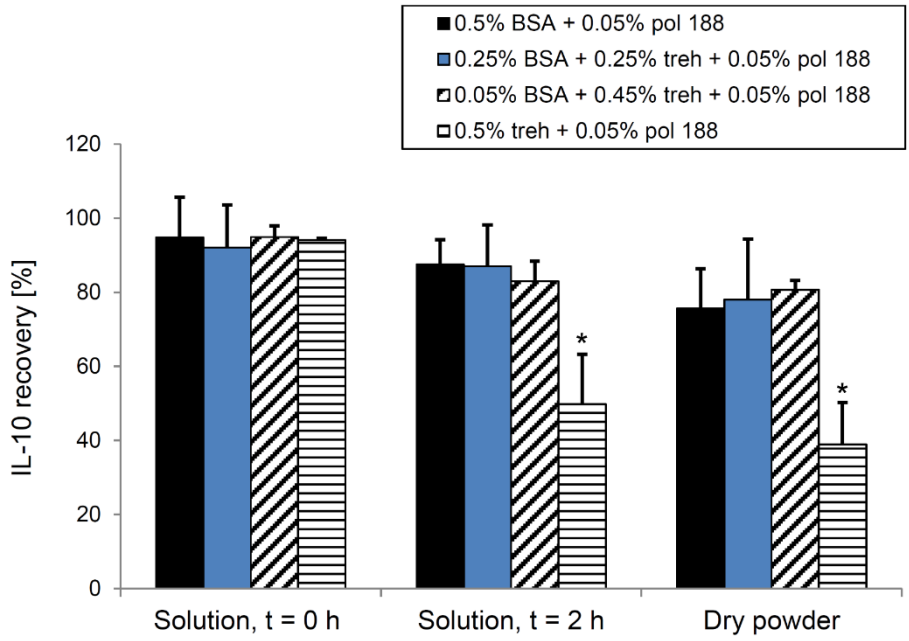

Fig. 18: IL-10 recovery from the spray drying solution before start of the spray drying, after 2 h of spray drying and from the spray-dried powder for formulations containing different ratios of the excipients BSA and trehalose (treh) and a constant concentration of poloxamer 188 (pol 188). Results are presented as mean ± SD for n = 3. One-way ANOVA, Holm-Sidak method: * $p \leq 0.05$.

Based on these results, it was decided to focus on the formulation containing 0.25% BSA, 0.25% trehalose and 0.05% poloxamer 188. During the process, the spray head reached a maximum temperature of 57°C. The outlet temperature measured in the zone where the solid particles are collected reached a maximum temperature of 34°C. Of 40 ml of initial solution, about 35 ml were spray dried before the process had to be stopped because the dead volume of the feed system was reached. The yield of the collected dry powder was 72%.

2.2.4.3 Properties of the IL-10-loaded MP

As can be seen from the SEM micrographs (Fig. 19), the spray-dried IL-10-loaded MP are spherical and have a smooth surface. The primary particles have a size in the range of 0.3 to 2.5 µm.

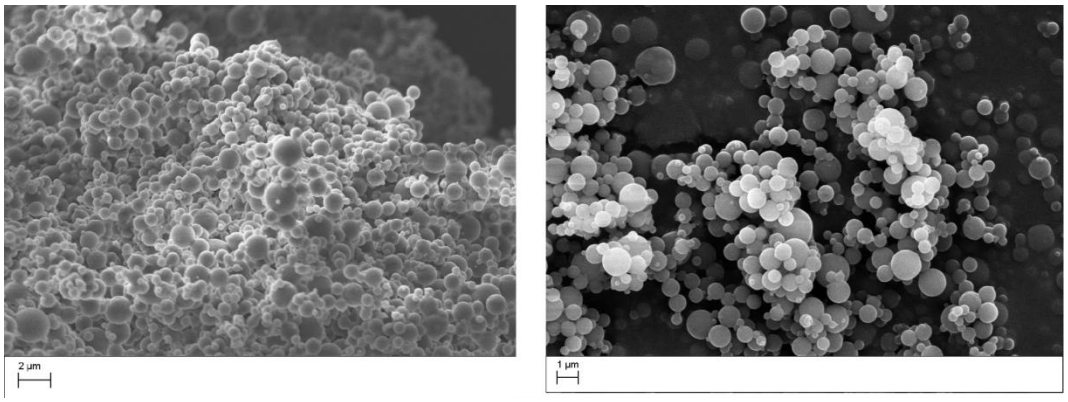

Fig. 19: SEM images of the spray-dried IL-10-loaded particles (formulation: 25 ng/ml IL-10, 0.25% BSA + 0.25% trehalose + 0.05% poloxamer). Details see text.

Other key properties of the MP are summarised in Tab. 5. The remaining IL-10 bioactivity of the particles was determined by dissolving them in cell culture medium. The solution was subsequently analysed by the MC/9 cell assay. After spray drying and collecting of the particles, the remaining IL-10 bioactivity was 69% of the initial value.

Tab. 5: Summary of the process parameters and particle properties of the formulation containing 25 ng/ml IL-10, 0.25% BSA + 0.25% trehalose + 0.05% poloxamer 188. Results are presented as mean ± SD for n = 3.

Spray-dried volume [ml]	Yield [%]	IL-10 bioactivity [%]	IL-10 recovery [%]	Mean particle size [µm]	SD of particle size distribution [µm]
34.7 ± 0.6	**72.4 ± 1.3**	**69 ± 23**	**78 ± 16**	**0.78 ± 0.03**	**0.37 ± 0.07**

The stability upon 6 months open storage at 25°C/60% RH was investigated by SEM and by measurement of the remaining bioactivity. The SEM micrographs indicate that the primary particles started to slightly coalesce after 3 months of storage (Fig. 20 D). The same effect was also visible after 6 months (Fig. 20 E).

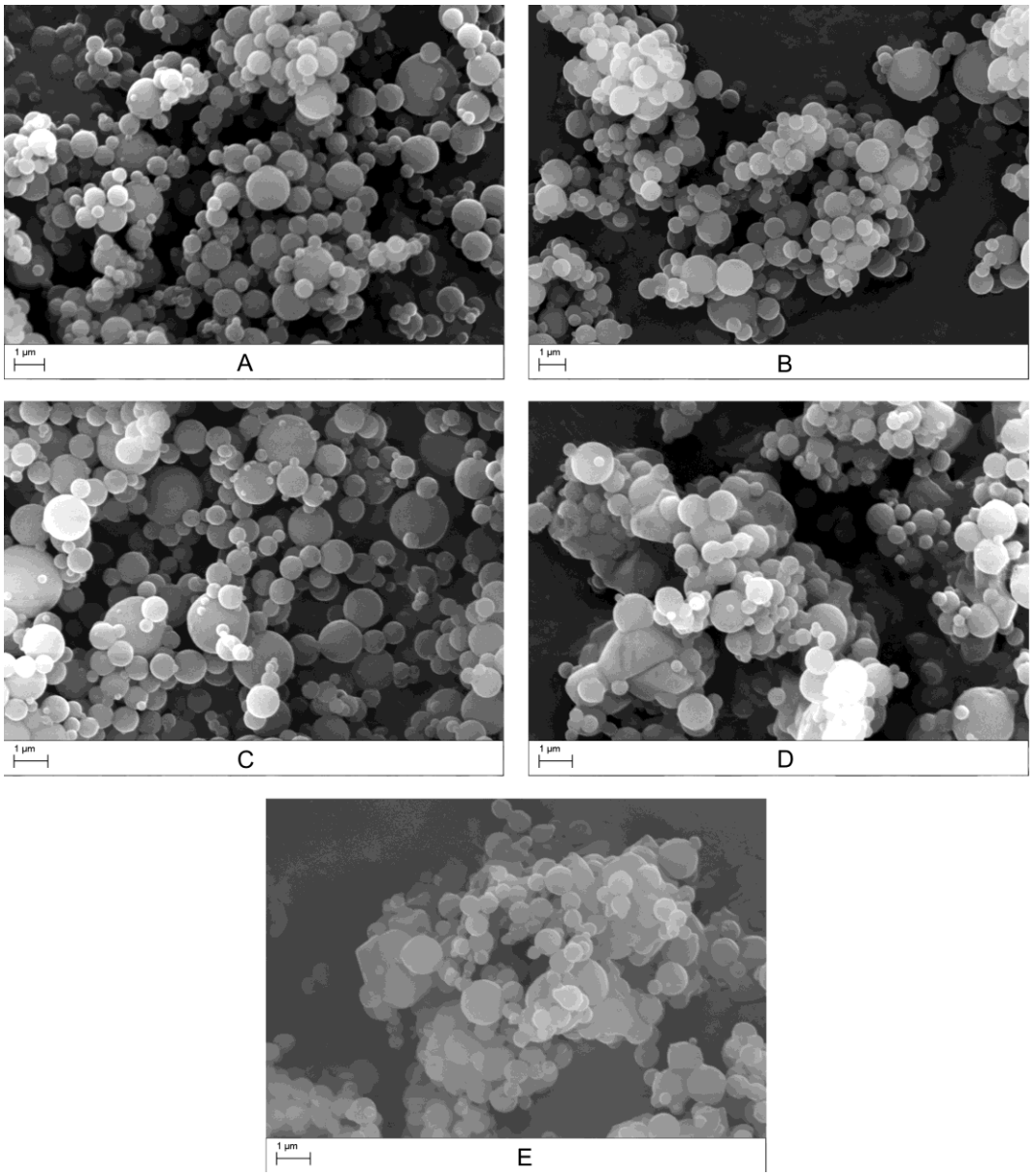

Fig. 20: SEM micrographs of spray-dried particles before (A), and after storage at 25°C/60% RH for 1 month (B), 2 months (C), 3 months (D) and 6 months (E). The particles were slightly coalesced after 3 and 6 months of storage.

The remaining IL-10 bioactivity decreased with increasing storage time (Fig. 21). After 6 months, it was reduced to 70 ± 13% of the bioactivity before storage. These results indicate that the particles are suitable for medium-term storage in the refrigerator, but not at RT. The particles also require protection from moisture.

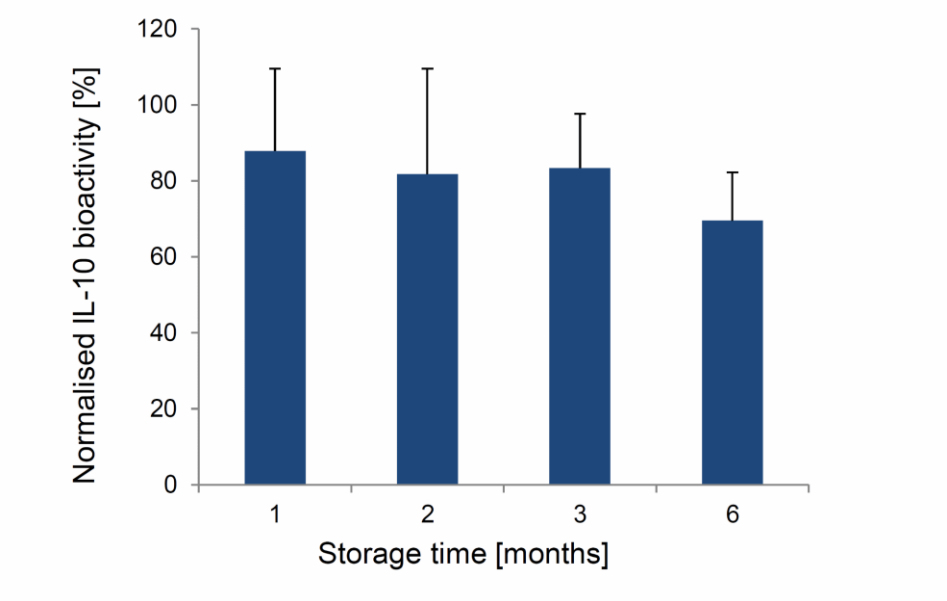

Fig. 21: Normalised remaining IL-10 bioactivity after open storage at 25°C, 60% RH over 1–6 months. Values are given as mean ± SD for n = 3. The bioactivity at the start of the storage (t = 0) was normalised to 100%.

2.2.4.4 Eudragit microspheres loaded with IL-10-loaded MP

Eudragit L 100-55 microspheres loaded with the IL-10-loaded MP were prepared by a modified S/O/O emulsion method described in section 2.2.3.4. In contrast to previously published studies, e.g. by (Chan et al., 2005) or by (Paharia et al., 2007), soy lecithin instead of sorbitan monooleate (Span® 80) or sorbitan trioleate (Span® 85) was used as oil-in-oil emulsifier. Preliminary studies showed that the surfactant soy lecithin is significantly less cytotoxic on MC/9 cells compared to the two sorbitan esters, as determined by a MTT assay. Soy lecithin was non-cytotoxic up to about 300 µg/ml as compared to Span 80 and Span 85, which both were cytotoxic at concentrations of 10 µg/ml and higher (data not shown). When a homogenisation speed of 600 rpm was applied to the spray-dried particles dispersed in the ethanolic Eudragit L 100-55 solution emulsified in viscous paraffin, spherical microspheres with a smooth surface were obtained (Fig. 22, left). The volume based mean size of the microspheres was determined to be 23.6 ± 3.4 µm and the span was 1.40 ± 0.09 (n = 3). Microspheres loaded with fluorescent FITC-BSA-labelled MP were prepared to determine the distribution of the spray-dried particles within the microspheres. CLSM images (Fig. 22, right) showed that the spray-dried particles (showing up in green) were relatively homogenously distributed within the microspheres.

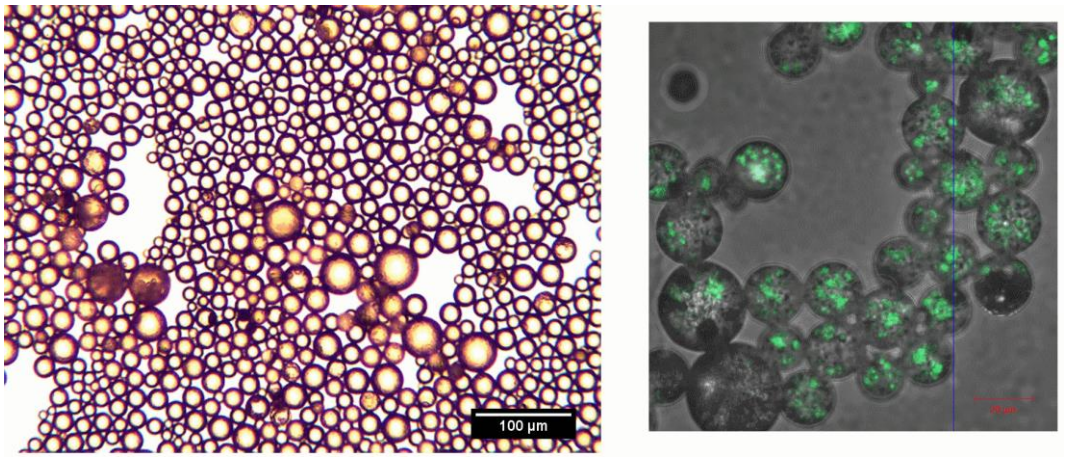

Fig. 22: Left: Light microscopic image of Eudragit L 100-55 microspheres. Right: Focus-stacked CLSM image of Eudragit L 100-55 microspheres loaded with FITC-BSA-labelled spray-dried MP showing up in green. The image was reconstructed from images taken at different focus distances (z-stack).

The *in vitro* release of the MP from the microspheres can be monitored by the release of BSA, as described in detail in chapter 2.2.3.6. As can be seen from Fig. 23, the release profile of the Eudragit L 100-55 microspheres shows a significant burst release of BSA within the first minutes after incubation in 2.5 mM HCl (pH 2.5). After 5 min, 36% of the encapsulated BSA were released. No further release occurs within the first 60 min. After the pH was increased to 7.4 at t = 60 min, the microspheres quickly dissolved and the remaining encapsulated BSA was almost completely released (93%) within 5 min.

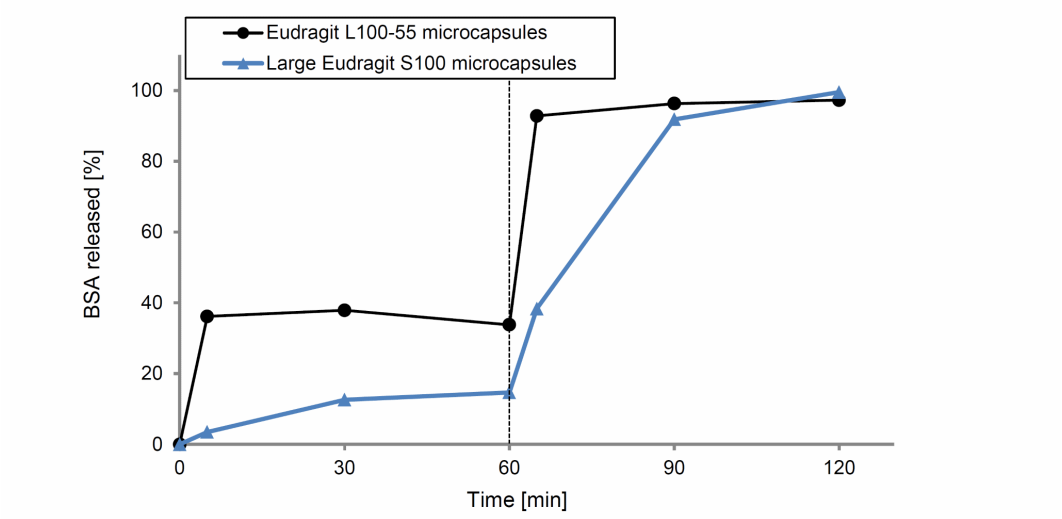

Fig. 23: BSA release from Eudragit L 100-55 microspheres and large Eudragit S 100 microspheres at 37°C, 90 rpm in 2.5 mM HCl pH 2.4 (0 to 60 min) followed by PBS pH 7.4 (60 to 120 min).

The remaining bioactivity of IL-10 after encapsulation in the microspheres and dissolution in cell culture medium was 30 ± 21% (n = 3), which is appreciably lower than the bioactivity of 69 ± 23% of the spray-dried particles (Fig. 24). When the microspheres were incubated in 2.5 mM acetic acid (final pH 4.2) or 2.5 mM HCl (final pH 2.4) for 1 h before they were dissolved in cell culture medium, the retained IL-10 bioactivity amounted to only 5% and 3%, respectively. These results clearly indicate that the Eudragit L 100-55 microspheres do not prevent acidic denaturation of the encapsulated IL-10.

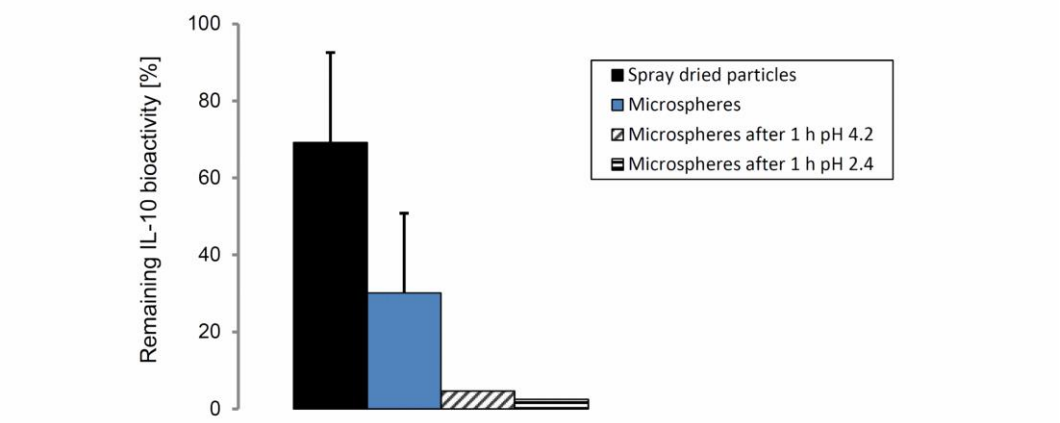

Fig. 24: Remaining IL-10 bioactivity of the spray-dried MP and in Eudragit L 100-55 microspheres loaded with the spray-dried particles after dissolution in cell culture medium without or with previous incubation at pH 4.2 (2.5 mM acetic acid) or pH 2.4 (2.5 mM HCl) for 1h. Data obtained before incubation are shown as mean ± SD (n = 3).

An improved protection against acidic degradation might be obtained with microspheres of a significantly larger size and/or a polymer with a higher pK_a. Therefore, two batches of Eudragit S 100 microspheres loaded with IL-10-loaded spray-dried particles were prepared. They had an average volume-based mean size of 301 µm and a span of 0.58. In contrast to the significantly smaller Eudragit L 100-55 microspheres (24 µm), no burst release of BSA was observed from the Eudragit S 100 microspheres in 2.5 mM HCl pH 2.4 (Fig. 23). After 60 min, only 15% of the encapsulated BSA was released. Once the pH of the release medium was increased to 7.4, the particles started to dissolve and the remaining encapsulated BSA was gradually released within the next 30 min. After dissolution of samples of the two batches in cell culture medium, a remaining bioactivity of only 7% and 2% was determined. When the microspheres were previously incubated at pH 4.2 for 1 h, a remaining bioactivity of only 1% and 0% was measured. Thus, also the large Eudragit S 100 microspheres did not protect the

encapsulated IL-10 from acidic denaturation. Furthermore, the remaining IL-10 bioactivity was significantly lower than in the Eudragit L 100-55 microspheres, even without acidic incubation. These results suggests that the IL-10 is strongly denatured during the release from the microspheres, which is prolonged in case of the Eudragit S 100 microspheres, as the higher pK_a of the polymer and the larger size of the S 100 microspheres cause a slower dissolution of these particles.

Both Eudragit polymers contain carboxylic groups that are acidic. The experimentally determined pK_a values of L 100-55 and S 100 are 6.9 and 6.8, respectively (Schmidt-Mende, 2001). Thus, it can be assumed that a slightly acidic microenvironment forms within the microspheres during dissolution. This microenvironment may cause denaturation of the encapsulated IL-10. Moreover, both polymethacrylates swell in acidic aqueous media. A weight gain of about 33% was determined for Eudragit L 100-55 films of 50 µm thickness after incubation in both 0.1 N HCl and in PBS pH 4 for 8 h. For Eudragit S 100 films, an increase in weight of about 18% was measured (Schmidt-Mende, 2001). The swelling of the polymer may cause the formation of an acidic microenvironment within the microspheres during incubation in acidic media. This in turn may trigger the denaturation of the IL-10 and result in the very low remaining IL-10 bioactivity observed after subsequent dissolution in cell culture medium.

In various studies, other proteins were successfully encapsulated in gastro-resistant polymers. Insulin was successfully encapsulated within Eudragit L 100 (Mundargi et al., 2011) and S 100 microspheres (Jain et al., 2005) and significant *in vivo* bioactivity after oral administration was shown in both rats and rabbits. (Alavi et al., 2002) encapsulated lactase powder in both Eudragit S 100 and L 100 microspheres. The optimised formulation retained a lactase activity of 89% after dissolution. However, the remaining bioactivity after acidic incubation was not determined. Furthermore, lactase is significantly more stable against acidic conditions than IL-10. No loss of bioactivity was observed in aqueous solution after incubation at pH 4.5 for 2 h. Even after incubation at pH 1.2, about 5% of the original bioactivity remained. In contrast, (Syto et al., 1998) determined for IL-10 an irreversible loss of about 50% of the original bioactivity after exposure to a pH

of 4.5. When incubated at pH 4.0, the bioactivity was even reduced by more than 90%.

Thus, IL-10 is subjected to major pH-induced degradation even at moderately acidic conditions expected to form within the Eudragit microspheres during the swelling in acidic media and presumably also during the dissolution of the polymer in pH-neutral media. In contrast, other proteins, e.g. lactase, whose bioactivity is not negatively affected by moderately acidic conditions, can be successfully protected by encapsulation in gastro-resistant polymers. The results of this study indicate that IL-10 is not suitable for an encapsulation within gastro-resistant polymers. Future studies should aim at a delivery system that protects the encapsulated IL-10 from even slightly acidic microenvironments during the gastric passage and the release.

2.2.5 Conclusion

The preceding investigations of Il-10-loaded NP revealed that IL-10 largely degraded when it came in contact with an organic solvent (EtOAc or DCM). Therefore, in this part of the thesis, it was attempted to produce IL-10-loaded particles from purely water-based solutions of IL-10 by spray drying. The solutions contained BSA and trehalose as excipients and poloxamer 188 as surfactant. The spray drying was carried out with a novel device, the B-90 nano spray dryer that uses a vibrating mesh technology. In pretrials, suitable values of the inlet temperature (50°C) and of the concentrations of the excipients were determined. The resulting spherical MP had diameters ranging from 0.3–2.5 µm. The recovery and the remaining bioactivity of IL-10 from the spray-dried particles were 78% and 68%. For the intended local targeting of the intestinal mucosa after oral administration, the MP needed protection against the gastric environment. To this end, they were encapsulated in Eudragit L 100-55 microspheres prepared by a S/O/O emulsion. After dissolution of the microspheres in cell culture medium, the IL-10 bioactivity had decreased to the still useful value of 30%. However, when the microspheres were preincubated in acidic media of pH 4.2 or pH 2.4, the remaining IL-10 bioactivity amounted to only 5% and 3%, respectively. Even worse results were obtained after encapsulation of the MP in distinctly larger microspheres (about 300 µm) made of Eudragit S 100, a polymer with a higher pK_a value. Both types of

Eudragit did not adequately protect IL-10 from denaturation under acidic conditions. This disappointing result is tentatively attributed to the swelling of the Eudragit polymers in acidic aqueous media. The swelling may result in a slightly acidic microenvironment within the microspheres that may be sufficient to cause the denaturation of the acid-sensitive IL-10.

2.3 IL-10-loaded spray-dried MP for the treatment of acute inflammation of the lung

2.3.1 Preliminary remarks

The present chapter deals with the development and characterisation of IL-10-loaded spray-dried MP and the investigation of their anti-inflammatory potential in an *in vitro* model of the inflamed deep lung. These experiments were conducted in close collaboration with M. Hittinger, who established and prepared the *in vitro* model of the uninflamed coculture in his thesis (Hittinger, 2016). The development, preparation and characterisation of the spray-dried powder formulation were performed by the present author. In collaboration, both authors conceived, designed and performed the experiments regarding the inflammation of the alveolar co-culture model and the treatment with the dry powder. A major part of these studies has been published as research paper:

Marius Hittinger[1,2,*], Nico Alexander Mell[2,3,*], Hanno Huwer[4], Brigitta Loretz[2], Nicole Schneider-Daum[2], Claus-Michael Lehr[1,2,3]. 2016. Autologous co-culture of primary human alveolar macrophages and epithelial cells for investigating aerosol medicines. Part II: evaluation of IL-10-loaded microparticles for the treatment of lung inflammation. *Alternatives to Laboratory Animals (ATLA)*, 44 (4), 349-360.

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2.3.2 Introduction

Severe mechanic trauma, haemorrhagic shock and endotoxemia are all associated with multi-organ failure (MOF) (Roumen et al., 1993), (Copeland et al., 1998). Often, the lung is severely affected by the associated systemic inflammation. The resulting medical condition is known as ARDS. The release of the pro-inflammatory cytokines IL-6, IL-1β (and TNF in case of endotoxemia and haemorrhagic shock) was shown to play a crucial role in the systemic inflammation process (Kobbe et al., 2008), (Roumen et al., 1993). Enhanced plasma levels of these cytokines are associated with increased mortality rates and the risk for the development of ARDS and MOF (Roumen et al., 1993). Therefore, modulation of the excessive pro-inflammatory cytokine release has been proposed as a promising therapeutic strategy (Finnerty et al., 2006). It was shown that an early treatment with the anti-inflammatory cytokine IL-10 following haemorrhagic shock can reduce the systemic as well as the pulmonary inflammation (Kobbe, Schmidt, et al., 2009), (Kobbe, Stoffels, et al., 2009). However, a strong systemic inhibition of inflammatory responses can cause an increase in the incidence of infection and even sepsis (Ayala et al., 1994), (Song et al., 1999), (Kobayashi et al., 2001).

To avoid susceptibility to infections caused by the systemic administration of IL-10, especially in the case of treatment of endotoxemia or sepsis, a local pulmonary application of IL-10 was proposed. Kobbe et al. treated mice previously subjected to haemorrhagic shock via the administration of nebulised recombinant murine IL-10 by inhalation. The local application decreased the pulmonary inflammation without affecting the systemic concentrations of pro-inflammatory cytokines or IL-10 (Kobbe et al., 2012). In another study, IL-10 treatment by inhalation of mice following bilateral femoral fractures attenuated the pulmonary infiltration by neutrophils and decreased the expression of the adhesion molecule ICAM-1 but had no significant effect on the systemic inflammatory response (Lichte et al., 2015).

However, all published studies on the local pulmonary application of IL-10 used nebulised aqueous solutions of the cytokine. Such solutions are not suitable for application in human patients due to the complicated preparation of the aerosol and the very limited stability of aqueous IL-10 solutions. A dry powder formulation of IL-10 could be a promising alternative. The requirements for such a formulation are

a high fine particle fraction (FPF) to reach the target cells in the deep lung (alveolar macrophages) and biodegradable compounds to ensure safety and biocompatibility.

2.3.2.1 Aim of the study

To translate the human *in vivo* situation into an *in vitro* concept treatable with a stable dry powder formulation, a suitable model of the human *alveolus* is needed. For example, Kim et al. investigated different inhalable NP *in vivo, ex vivo and in vitro* and found similar cytokine release independent from the chosen model (Kim et al., 2014). Moreover, it was found that coculture systems mimic the cross talk of cells *in vitro* and are able to address complex safety-related questions (Steiner et al., 2015). Therefore, for the present study, a modified version of the recently published setup of the human autologous coculture was used to establish a strongly inflamed human model (Haghi et al., 2015). To avoid a reduction of inflammatory effects, the cells were grown without the usual addition of hydrocortisone to the culture medium. Furthermore, LPS instead of transforming growth factor (TGF)-β was used to induce the inflammation. Spray-dried IL-10-loaded particles were prepared and characterised with focus on their aerodynamic properties. For this purpose, the particles described in chapter 2.2 were further developed to a dry powder suitable for inhalation. Safety and efficacy were studied by aerosolisation and deposition of the particles on the cell culture model with the Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOCC) (Hein et al., 2010).

2.3.3 Materials and methods

2.3.3.1 Materials

All chemicals used were of analytical or higher grade. BSA (≥ 96% purity), DMF, disodium phosphate heptahydrate, FITC-BSA, glacial acetic acid, 1 N hydrochloric acid, L-leucine, LPS (from *E. coli*), MTT, Sigmacote® (SL2), sodium chloride, SDS, 0.1 N NaOH and trehalose dihydrate were purchased from Sigma-Aldrich (St. Louis, USA). RhIL-10) and rmIL-4 were purchased from peprotech (Rocky Hill, USA). The murine mast cell line MC/9 was purchased from ATTC (Manassas, USA). For the culture of the MC/9 cells, penicillin/streptomycin and RPMI 1640

with L-glutamine but without phenol red (from PAA Laboratories, Pasching, Austria) and heat inactivated FBS (Lonza, Cologne, Germany) were used. Rat T-cell culture supplement with ConA (IL-2 culture supplement, T-STIM) was purchased from Becton, Dickinson and Company (Franklin Lakes, USA). Transparent hard gelatine capsules (model Coni-Snap®) of standard size 3 (capsule volume 0.30 ml) were obtained from Capsugel (Morristown, USA). For the pulmonary cell isolation, cell strainers with 100 µm pore size (Becton Dickinson and Company) were used. Trypsin and Percoll® were received from Sigma-Aldrich. Epithelial cell adhesion molecule (EpCAM) antibody-beads were obtained from Miltenyi (Bergisch-Gladbach, Germany). Snapwells were purchased from Corning (Schiphol-Rijk, Netherlands). For the coating, fibronectin (Becton Dickinson, Heidelberg, Germany) and collagen (Sigma-Aldrich) were applied. Saline-adenine-glucose-mannitol (SAGM) was obtained from Lonza (Verviers, Belgium). The hydrocortisone from the bullet kit was not used for the inflamed culture and the controls. The CBA Flex Sets used for the determination of the concentrations of IL-6, IL-8, IL-10 and TNF in the cell culture medium were acquired from Becton, Dickinson and Company.

2.3.3.2 Preparation of IL-10-loaded MP

The spray-dried IL-10-loaded particles were formulated as follows: An aqueous solution with a concentration of 0.25% BSA, 0.23% trehalose and 0.05% leucine (all w/v) was prepared by dissolving the constituents in deionised, sterile filtered water. The pH was set to 7.4 by addition of 0.1 N NaOH. Subsequently, 0.25 µg/ml rhIL-10 was added. Placebo particles were prepared by spray drying the same solution but without the addition of IL-10. All solutions were spray dried with a B-90 nano spray dryer (Büchi Laboratoriums Technik AG, Flawil, Switzerland). Briefly, the solution containing IL-10 was fed to the spray head by a peristaltic pump (Fig. 15). In the spray head, the solution was dispersed into fine droplets via the vibration of a mesh with a size of 4 µm. The reservoir for the solution was ice-cooled during the spay-drying process to minimise heat-induced degradation of the IL-10. The inlet air temperature of the spray dryer was set to 50°C. An air flow rate of 140 l/min was applied. The resulting outlet air temperature was in the range of 32–36°C. After the process, the dried particles were scraped off the collecting

electrode with a plastic scraper. Placebo (blank) particles were prepared in the same way by using the excipient solution without the addition of IL-10.

2.3.3.3 Characterisation of the spray-dried particles via SEM

Images of the particles were obtained by SEM. Details of the sample preparation are described in chapter 2.2.3.5. The samples were mounted on carbon sticks and sputter coated with a gold film of about 20 nm thickness. Images of the coated particles were taken with an EVO HD15 (Carl Zeiss Microscopy GmbH, Jena, Germany) at an accelerating voltage of 15 kV. The SEM was used in the secondary electron detection mode.

2.3.3.4 Characterisation of the aerodynamic properties of the spray-dried particles

To determine the aerodynamic properties of the spray-dried particles, fluorescence-labelled placebo particles were prepared. In contrast to the unlabelled placebo particles, 20% (based on the weight) of the BSA in the solution to be spray-dried was substituted by fluorescent FITC-BSA. About 20 mg of these particles were precisely weighed and filled into hard gelatine capsules of size 3. A single capsule was inserted into the chamber of a HandiHaler® device (Boehringer Ingelheim, Ingelheim am Rhein, Germany). The HandiHaler is a breath-activated dry powder inhalation system for patients with airway disease (Chodosh et al., 2001). The inserted capsule was penetrated by pushing the piercing button incorporated in the device. Subsequently, the HandiHaler® was coupled to the Next Generation Impactor (NGI), a cascade impactor, via a mouthpiece adapter made of silicone rubber (both from Copley Scientific, Nottingham, UK). The latter was in turn attached to the induction port of the NGI. The impactor itself and the underlying mechanisms of the measurements are described in detail in the literature (Marple, Roberts, et al., 2003). Briefly, the impactor has seven stages and a micro-orifice collector (MOC) to deposit particles according to their aerodynamic diameters in removable cups. The surfaces of these cups were coated with a thin layer of silicone to minimise particle bounce, which occurs especially at the upper stages. For coating, 1.5 or 0.5 ml of a solution of silicone oil in heptane (Sigmacote®, SL2)

was applied to the collection surfaces of the large and small cups, respectively (Kamiya et al., 2004). The heptane was allowed to evaporate at room temperature before the coated stages were inserted into the NGI. The preseparator of the NGI was filled with 10 ml of 10 mM PBS pH 7.4. A high capacity pump, model HCP 5 and the Critical Flow Controller (both from Copley Scientific) were coupled to the NGI to conduct the aerosolisation experiments. A flow rate of 60 l/min was applied for 4 s.

After each run, the capsule chamber of the HandiHaler® and the inner and outer wall of the gelatine capsule were carefully washed with 10 mM PBS pH 7.4 to dissolve all remaining particles. The washing solutions were pooled and filled up with PBS to a defined volume. The same procedure was repeated with the mouthpiece and the induction port, the preseparator, each of the seven collecting cups and the MOC. Standard solutions were prepared by dissolving spray-dried particles of the respective batch in PBS pH 7.4. The particle concentration of the solutions was determined by pipetting 200-µl aliquots into a 96-well microtiter plate followed by the measurement of the fluorescence intensity of the FITC-BSA (excitation wavelength 490 nm, emission wavelength 525 nm) with a microplate reader (Infinite M200, Tecan Group AG, Maennedorf, Switzerland). Each sample and each standard solution was measured in triplicate.

The aerodynamic cut-off diameter for each stage of the NGI and the MOC for the applied flow rate of 60 l/min was taken from a publication by Marple et al. (Marple, Olson, et al., 2003) and the United States Pharmacopeia (USP37, 2014). The recovery of particles in the aerosolisation experiments was calculated as the mass fraction of particles weighed into the capsule that was found in capsule, inhaler, mouth piece, induction port and all stages of the NGI including the MOC after the aerosolisation. The relative deposition was calculated as the fraction of the recovered particles that was found in mouth piece, induction port and all stages of the NGI including the MOC. The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) of the aerodynamic particle size distribution were determined by plotting the percentages of collected particle masses of the different stages against the aerodynamic diameters in a log probability graph. The two values above and below the value at which 50% of the cumulative

mass is contained were connected by a straight line. The intersection of this line with the 50% cumulative collection value was taken as MMAD. The GSD was calculated as follows:

$$GSD = (d84/d16)^{1/2} \quad \text{(eq. 6)}$$

where d84 and d16 are the diameters at which 84% and 16% of the cumulative mass are contained. The FPF was defined as the percentage of deposited particles with an aerodynamic diameter below 5 µm. This fraction was calculated from the deposited masses and the respective cut-off diameters by nonlinear regression.

2.3.3.5 Determination of the IL-10 bioactivity of the spray-dried particles

The bioactivity of the spray-dried IL-10-loaded particles was determined by its ability to induce the rmIL-4 dependent growth stimulation of MC/9 cells (Thompson-Snipes et al., 1991). The cells were grown and prepared as described in chapter 2.1.3.7.2. The IL-10-loaded particles were dissolved in CMWT and diluted to an IL-10 concentration of 0.9 ng/ml. The MC/9 cell assay was conducted on a 96-well microtiter plate. 50 µl of the diluted solution were added to 100 µl CMWT containing $2*10^5$ cells/ml and 7.5 pg/ml IL-4. For each sample, ten wells were prepared. Additionally, a solution of blank particles was prepared, and an IL-10 standard was diluted in this solution to prepare the standards. Four wells were prepared for each standard. The 96-well plates were subsequently incubated for 48 h at 37°C, 5% CO2. The IL-10-dependent proliferation of the cells was measured by a colorimetric MTT assay as described in chapter 2.1.3.7.2 (Hansen et al., 1989). The remaining IL-10 bioactivity in the particles is determined by relating the corrected absorbance values of the respective samples to those of the standards, whose activity was defined as 100%.

2.3.3.6 Characterisation of the deposited aerosolised powder

The controlled deposition of the aerosolised formulations was performed with the PADDOCC according to an established protocol (Hein et al., 2010). FITC-BSA-loaded placebo particles were deposited in the PADDOCC on SEM carbon disks. Their size distribution was subsequently investigated by correlative microscopy

combining fluorescence light microscopy and SEM as described by (Tscheka et al., 2015). The Axio Imager M1m, equipped with the LED system Colibri for excitation (Carl Zeiss Microscopy GmbH, Jena, Germany) and the scanning electron microscope (EVO HD15) were used. Afterwards, the experiment was repeated with fluorescence-labelled particles in empty Snapwell™ inserts. Particles were collected and dissolved in 500 µl 10 mM PBS buffer pH 7.4 and the fluorescence intensity was measured by a plate reader (Infinite M200) to determine the amount of particle deposition.

2.3.3.7 Mimicking the *in vivo* situation *in vitro*

In order to estimate the particle-cell interaction in the human lung, a coculture system based on human alveolar type-I-like cells and human alveolar macrophages was used (Haghi et al., 2015). Human alveolar type II pneumocytes were isolated according to a protocol described previously (Daum et al., 2012). The use of human tissue was approved by the local ethics committee (State Medical Board of Registration, Saarland, Germany). The pneumocytes were seeded on a Snapwell™ membrane in a density of $3*10^5$ cells/cm². The alveolar macrophages, treated as a waste product before, were now kept in culture within RPMI medium on petri dishes. The isolation procedure of the human alveolar macrophages was slightly modified with respect to an established protocol (Hoppstädter et al., 2010). Instead of removing the erythrocytes by a hypotonic buffer, they were simply removed by washing steps with balanced salt solution B (BSSB) pH 7.4 comprised of 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 5.5 mM glucose. After three days of culturing under submerged conditions, the pneumocytes were set under air-liquid conditions and cultured for one day in SAGM devoid of hydrocortisone. On day four, the macrophages were detached by trypsin digestion and scraped from the petri dish, washed in RPMI 1640 medium, counted and concentrated so that they reached a theoretical concentration of 10^5 cells in 10 µl cultured on a Snapwell™ membrane with a surface of 1.12 cm². Consequently, the ratio of pneumocytes to macrophages was 3:1. The 10 µl of concentrated macrophages were placed by pipetting them in the middle of the Snapwell™ on top of the pneumocytes. This procedure is necessary because of the loose adherence of the human alveolar

macrophages on the epithelial cells. The coculture was incubated overnight (day 5) and used for the deposition experiments 24 hours later (Fig. 25).

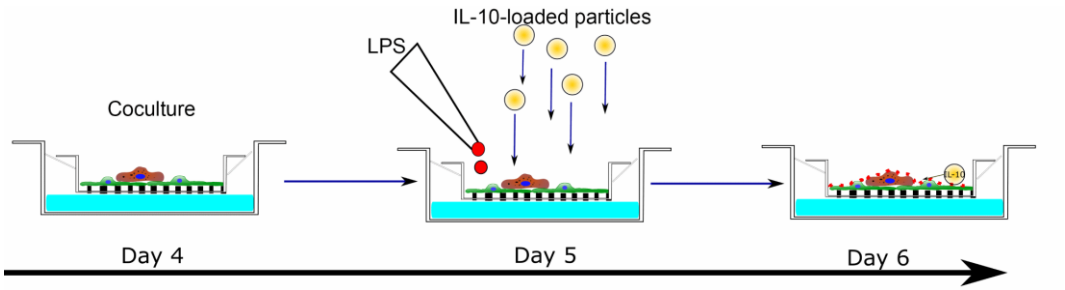

Fig. 25: Timing of the coculture experiments. Macrophages and epithelial cells were air-liquid co-cultured starting from day 4. On day 5, the cells were stimulated by LPS and subsequently incubated with IL-10-loaded particles. The cytokine release was determined 24 h after the deposition of the particles.

Finally, the coculture and the PADDOCC system were combined to determine the safety and efficacy of the IL-10-loaded spray-dried particles on primary human alveolar cells. Both IL-10-loaded and drug free placebo particles were applied to the coculture which was stimulated by adding 5 µl of 10 µg/ml LPS diluted in BSSB to the apical compartment of the coculture immediately before deposition. Overall, the number of isolated cells allowed investigating 5 groups of cocultures with 3 replicates each for three independent cell isolations, obtained from 3 different human lungs. 3 batches of IL-10-loaded particles were administered. The 5 groups were: untreated (coculture blank), LPS-stimulated, LPS-stimulated and treated with unloaded particles, LPS-stimulated and treated with IL-10-loaded particles as well as unstimulated and treated with IL-10-loaded particles. After deposition, the cells were incubated and samples from the apical and basolateral compartment were taken after 24h, respectively. 500 µl BSSB supplemented with 0.1% BSA was added to the apical compartment and 1000 µl to the basolateral compartment, resulting in a final apical and basolateral volume of 500 µl and 1500 µl. The samples were frozen and stored at -80°C until they were analysed by FACS.

2.3.3.8 Measurement of cytokine expression

To determine the cytokine concentration in the apical and basolateral compartments, the thawed culture media samples were incubated with fluorescence-labelled, antibody-coated beads (CBA Flex Sets). The samples were

prepared according to the manufacturer's instruction and subsequently analysed with a FACSCalibur™ flow cytometer (Becton, Dickinson and Company). IL-6, IL-8, IL-10 and TNFα concentrations were measured in the same run. For each sample and each standard, approximately 300 single beads were measured, and the mean fluorescence intensity of the bead population was determined from that of the standards by nonlinear regression. The 5-parameter logistics model of the software FCAP Array (version 3, Soft Flow, Pecs, Hungary) was used for the calculations. The statistical analysis of the results was performed by a one-way ANOVA in conjunction with the Turkey's range test (Tukey, 1949) using the software Origin (OriginLab Corporation, Northampton, USA).

2.3.4 Results and discussion

2.3.4.1 Properties of the IL-10-loaded MP

The IL-10-loaded particles were produced with a yield of 45 ± 7% (mean ± SD, n = 3). The remaining bioactivity of IL-10 after spray drying as determined by means of MC/9 proliferation was reduced to 8.3 ± 1.8% of the initial bioactivity.

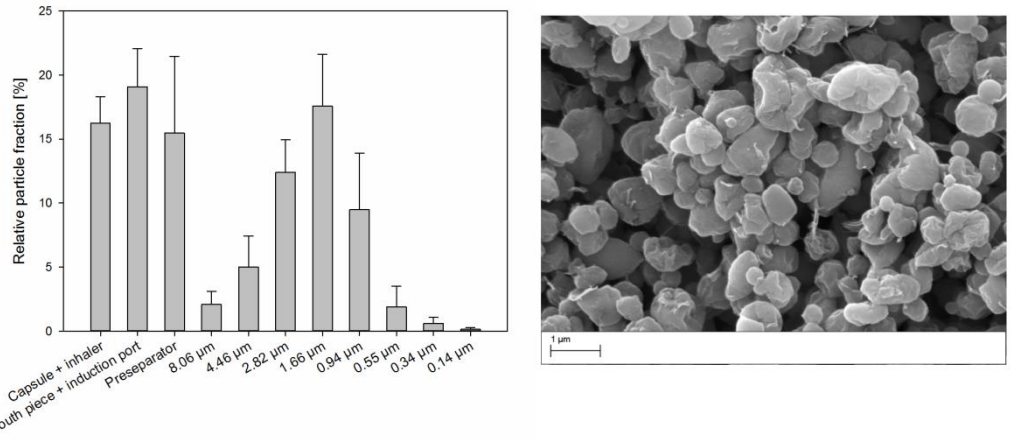

Fig. 26: Left: mass fractions of spray-dried FITC-BSA-loaded particles that remained in capsule and HandiHaler® or were deposited in the different parts of the NGI. The results for the stages 1 to 7 are labelled by their respective aerodynamic cut-off diameters (8.06–0.34 µm). Particles with an aerodynamic diameter below 0.34 µm were deposited in the MOC which has a cut-off diameter of 0.14 µm. Right: SEM image of the IL-10-loaded spray-dried particles. The primary particles have a size in the range of 0.2–2 µm. The particle surface has a corrugated structure.

SEM images of the spray-dried particles show a coarse and corrugated surface (Fig. 26, right). As the spray drying solution was dispersed via the vibration of a

membrane with a pore diameter of only 4 µm, small primary particles in a range of 0.2–2 µm were formed. From the aerosolisation experiments, the following results were obtained: 96% of the particles were recovered in capsule, inhaler and the different airway stages of the NGI (Fig. 26, left). 84% of the particles were deposited within mouth piece, induction port, preseparator and the stages (including the MOC) of the NGI. The MMAD of the particles is 2.5 µm and the GSD 1.7 µm. 57% of the deposited particles belong to the FPF, that is, they have an aerodynamic diameter below 5 µm. The aerodynamic key figures of the spray-dried particles are summarised in Tab. 6.

Tab. 6: Recovery, deposition and aerodynamic properties of the FITC-BSA-loaded spray-dried particles. Values are given as mean ± SD for n = 3.

Recovery [%]	Deposition [%]	MMAD [µm]	GSD [µm]	FPF [%]
95.8 ± 6.5	**83.8 ± 2.1**	**2.50 ± 0.05**	**1.71 ± 0.04**	**56.7 ± 11.8**

2.3.4.2 Mimicking the IL-10 treatment of the inflamed deep lung *in vitro*

Fig. 27 shows an image obtained with correlative microscopy of FITC-BSA-loaded (fluorescent) particles deposited in the PADDOCC on carbon discs using a HandiHaler®. Labelled particles in small agglomerates were seen, demonstrating a successful particle deposition in the PADDOCC. From 3 capsules loaded with 18 mg dry powder each, 86 µg/cm² ± 21 µg/cm² were detected in the Snapwell inserts, which is less than 1% of the initial amount.

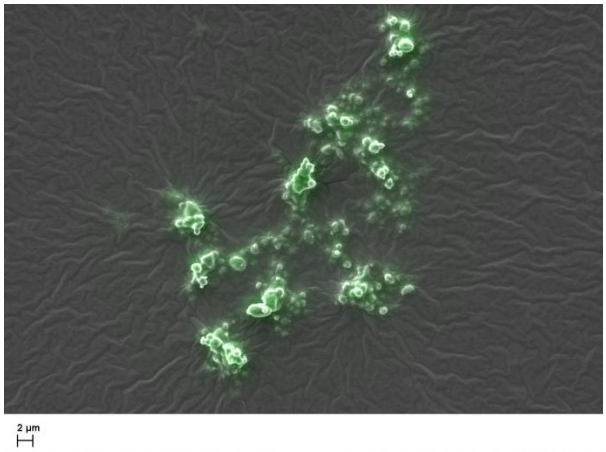

Fig. 27: FITC-BSA-loaded (fluorescent) spray-dried MP deposited in the PADDOCC as a proof of concept. This correlative image is based on fluorescence microscopy and scanning electron microscopy of the same sample area.

The cytokine concentrations expressed in the 5 groups of cocultures are displayed in Fig. 28. The untreated control (coculture blank) showed no release of TNF and only very low levels of IL-6 and IL-8 in the apical and basolateral compartments of the cocultures. This result alleviates possible concerns about a M1 or M2 preactivation of the macrophages as a possible consequence of isolation, co-cultivation or smoker donors. Surprisingly, the treatment with IL-10-loaded particles without preceding LPS stimulation (group IL-10 MP) led to a distinct IL-6 and IL-8 secretion. As expected, LPS stimulation resulted in a pronounced expression of IL-6, IL-8 and TNF (group LPS), demonstrating the successful mimicking of lung inflammation. The treatment of LPS-stimulated cocultures with IL-10-loaded particles (group LPS + IL-10 MP) resulted in a significantly reduced IL-6 and TNF expression. In contrast, IL-8 was not reduced in comparison to the untreated LPS control. Unloaded (placebo) particles had no anti-inflammatory effect on the stimulated coculture (LPS + unloaded particles). On the contrary, they caused an additional increase of the concentrations of IL-6, TNF and IL-8. Overall, cytokines were mainly detected in the apical compartment of the cocultures. The concentrations in the basolateral compartment were appreciably lower but showed a similar tendency. This also applies to the concentrations of IL-10 which amounted to 548 ± 243 pg/ml in the group IL-10 MP and to 804 ± 186 pg/ml in the group LPS + IL-10 MP, in the apical compartments. In contrast, only marginal concentrations of IL-10 (mean ≤ 6 pg/ml) were detected in the untreated and the placebo-treated cultures.

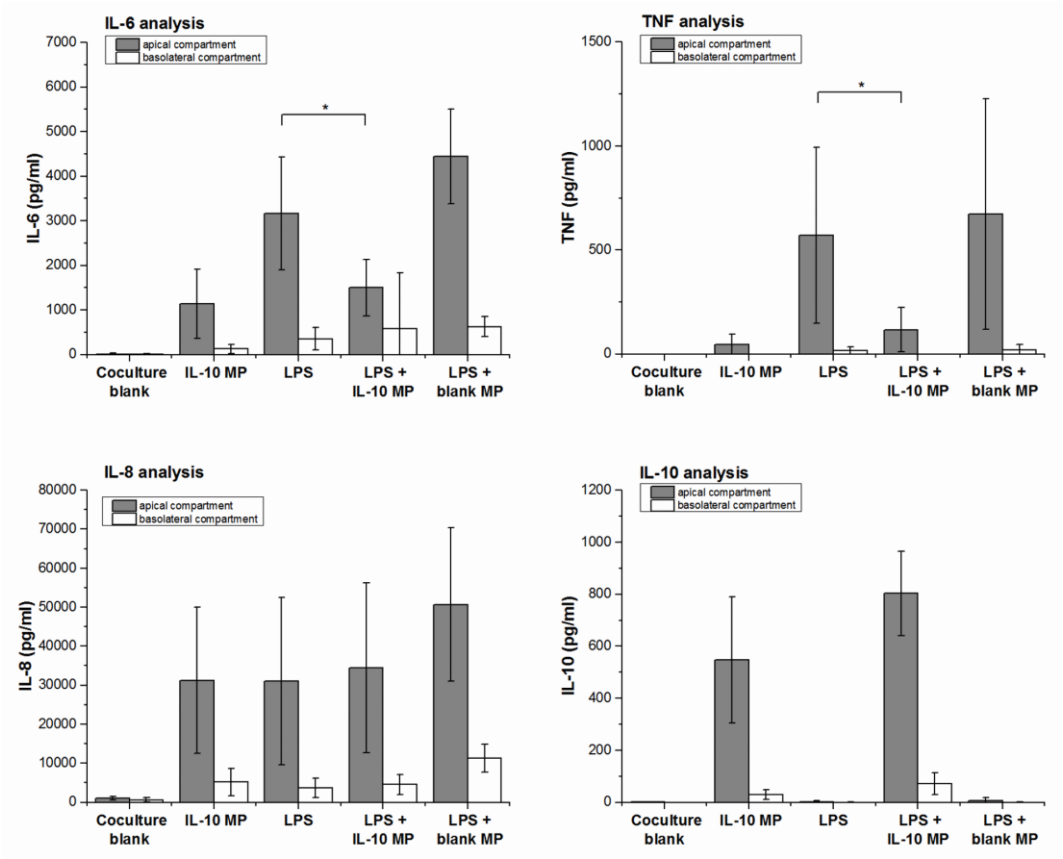

Fig. 28: Cytokine concentrations in the 5 groups of samples from the human autologous coculture based on epithelial cells and macrophages. The groups are denoted as coculture blank (unstimulated and untreated control), IL-10 MP (unstimulated and treated with IL-10-loaded particles), LPS (LPS-stimulated and untreated), LPS + IL-10 MP (LPS-stimulated and treated with IL-10 loaded MP) and LPS + blank MP (LPS-stimulated and treated with blank particles). Both the apical and the basolateral compartment of the cocultures were characterised.

In the above experiments, a dry powder formulation with the main components BSA and trehalose was successfully prepared and characterised. The preparation of aerosolisable dry powder formulations with particle sizes small enough to reach the deep lung (aerodynamic diameter below 5 µm) is technically challenging. The B-90 nano spray dryer was chosen, because the vibrating mesh technology enables the formation of very fine droplets and thus small primary particles. The main component of the excipients used in this particle formulation was albumin. Its use is well established and accepted in various drug delivery technologies intended for human application (Kratz, 2008). It has been shown that a high concentration of albumin is present in both bronchoalveolar lavage fluid (Plymoth et al., 2006) and in exhaled endogenous particles (Bredberg et al., 2012). Moreover, amongst other things, albumin is used as stabiliser for solutions of low protein concentrations. It prevents surface adsorption of bioactive proteins of low concentrations as it

unspecifically binds to surfaces itself. Furthermore, it allows the pH-buffering of the spray drying solution.

The biological activity of IL-10 is irreversibly lost below pH 6 due to irreversible changes of the protein structure under acidic conditions (Syto et al., 1998). Therefore, before the addition of the IL-10, the pH of the excipient solution was set to 7.4 by adding 0.1 N NaOH. In addition to albumin, trehalose and leucine were used as components of the excipient solution. Trehalose is often used as excipient for the spray drying of proteins as it effectively stabilises the protein during the drying process due to water replacement (Schüle et al., 2008). Leucine was added to enhance the aerodynamic properties of the particles. Spray-dried particles with leucine have a corrugated surface that reduces inter-particular adhesion and increases the respirable fraction of the powder (Prota et al., 2011).

The inlet temperature for the spray drying process was set to a moderate temperature of 50°C to reduce heat induced degradation of the IL-10. As a result, the outlet temperature during the spray drying was in the low range of 33-35°C. Overall, this strategy resulted in a remarkably high FPF of 57%. While the optimal particle size for deep lung deposition is still highly speculative, it has been frequently reported as a rule of thumb that particles in a range of approximately 1–5 µm show an excellent deposition in the deep lung (Patton and Byron, 2007), (Carvalho et al., 2011). The NGI results in Fig. 26 therefore indicate that the prepared spray-dried formulation may be well suited for an effective delivery to the deep lung using an established dry powder inhaler. Unfortunately, the remaining IL-10 bioactivity of the employed formulation was rather low (about 8%) but might be improved in future studies by further optimisation of the spray drying process and/or the excipient composition.

In this study, the human autologous coculture was successfully stimulated by LPS (group LPS in Fig. 28), resulting in a remarkably strong cytokine release in comparison to TGF-β stimulation (Haghi et al., 2015). No evidence of TNF and only marginal concentrations of IL-6 were found in the untreated control (group coculture blank). This suggests that the isolation procedure, the air-liquid culture conditions and the co-culturing of cells did not affect the inflammatory or regulatory cellular activation. The deposition of IL-10-loaded particles on the unstimulated

culture (group IL-10 MP) resulted in a small but noticeable release of inflammatory markers. As the excipients used for the development of this experimental formulation were not free from pyrogens, it appears likely that pyrogen impurities affected the system.

This suspicion is supported by the results of the cultures which were stimulated by LPS and treated with particles devoid of IL-10 (group LPS + unloaded MP in Fig. 28). In these cultures, the concentrations of the inflammatory markers were higher than in the cultures merely stimulated by LPS (group LPS), indicating that the MP themselves also caused a stimulation of the cultures. Therefore, to clarify the effect of IL-10 on the inflamed cultures, one has to compare the properties of the cultures stimulated with LPS and treated with IL-10-loaded particles (group LPS + IL-10 MP) with those of the group LPS + unloaded MP. As can be seen in Fig. 28, IL-10 significantly reduces the concentrations of IL-6 and TNF, demonstrating an anti-inflammatory effect of IL-10 on the inflamed cocultures. It is interesting to note that the concentration of IL-8, within the standard deviation, seems to be independent of the treatment of the cocultures. Further studies are needed to clarify this surprising behaviour.

The significant inflammatory response of the human autologous coculture model used in the present study underlines the relevance of cell- and tissue-based *in vitro* models of the respiratory tract. The cytokine expression of this model revealed how an autologous coculture of human primary cells responds to inflammatory stimuli. The successful deposition of particles with a size relevant for drug delivery to the respiratory tract allowed to investigate the therapeutic efficacy of a novel formulation on human primary cells.

Other cellular models might be bases on cell lines or use cell types which are not directly linked to lung cells. However, many of these models show a similar behaviour as compared to the primary model. For example, the recently published paper of (Kasper et al., 2015) compared different coculture models addressing different macrophage phenotypes (Kasper et al., 2015). They also observed an increase in IL-8 expression by LPS stimulation. The work of (Herzog et al., 2014) investigated the triple coculture model, which is based on A549 epithelial cells, human peripheral blood monocyte-derived dendritic cells and macrophages.

Stimulation by LPS also caused an increase in the TNF and IL-8 expression. Even if the released amounts of cytokines are different between the cellular models, a standardised protocol and a comparison to primary cells might reveal that the usage of cellular models, independent of their origin, is qualified to replace animal experiments.

Differences between *in vitro* models and the human *in vivo* situation might be bridged in further studies by using improved primary *in vitro* models. The human primary autologous coculture used in this study has several limitations related to its costs and to the quantity of isolated cells, leading to problems when applied in large screening experiments. However, this coculture can be employed to improve the understanding of cellular interactions and responses and of interactions in the alveolar space. Furthermore, it can help to identify useful cell line-based models for the respiratory tract. Stimulated by LPS and combined with a suitable deposition system such as the PADDOCC, the model can serve as a powerful tool to identify new drugs for the treatment of pulmonary inflammatory diseases.

2.3.5 Conclusion

The IL-10-loaded MP prepared in the preceding part of this thesis provided an excellent bioactivity of the Il-10 (68%). Thus, it appeared reasonable to employ such particles for a dry-powder formulation for the treatment of the deep lung. To this end, the formulation was modified by the substitution of the surfactant poloxamer 188 with leucine. The resulting MP showed excellent aerodynamic properties with a FPF of 57% and a MMAD of 2.5 µm. Unfortunately, the remaining IL-10 bioactivity reached only about 8%. Nevertheless, this dry powder formulation was tentatively applied to test a human alveolar co-culture model based on epithelial cells and macrophages. For the deposition of the MP on the coculture, a special aerosolisation system was used, the PADDOCC. Before the deposition of the particles, the co-culture was stimulated with LPS. This gave rise to a pronounced expression of the inflammatory markers IL-6, IL-8 and TNF, demonstrating the successful mimicking of lung inflammation. The treatment with the IL-10-loaded MP caused a significant reduction of the IL-6 and TNF secretion as compared to the deposition of placebo (blank) particles, demonstrating the anti-inflammatory effect of the IL-10-loaded particles on the inflamed co-culture. This

indicates the potential of IL-10-based dry powder formulations for application to both *in vitro* and *in vivo* models of inflammatory lung diseases. The present results also suggest that the human alveolar co-culture model, in combination with a deposition system such as the PADDOCC, may serve as a powerful tool to test the safety and efficacy of dry powder formulations for pulmonary delivery. A surprising result was obtained after the deposition of blank MP, devoid of IL-10, on the co-culture. These particles caused an inflammation similar to that induced by LPS. This results is tentatively attributed to the action of pyrogens which are presumably contained in the excipient BSA.

2.4 IL-10-homologous peptides

2.4.1 Preliminary remarks

All experiments presented in this chapter were exclusively planned, conducted and evaluated by the present author. The primary human macrophages used for the determination of the anti-inflammatory IL-10-mimetic activity of the peptides were kindly extracted from the donor tissue and cultured by Marius Hittinger until they were used in the assay.

2.4.2 Introduction

It is known from the literature (see chapter 1.1) and further confirmed by the results presented in chapter 2.1.4 that IL-10 is highly sensitive to denaturation, namely under acidic conditions, even moderate heat stress or O/W interfaces. This susceptibility severely limits the options for the development of innovative drug delivery systems. Thus, a more stable molecule mimicing the biological activity of IL-10, that is, binds to and activates the IL-10 receptor complex, would be highly desirable.

(Gesser et al., 1997) describe the identification of the nonapeptide IT9302 with the amino acid sequence **Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn (**AYMTMKIRN). This peptide is completely homologous to a fragment of the C-terminal part of hIL-10 (residues 152-160). IT9302 was found to exhibit several biological activities that were previously observed for IL-10. Among other things, IT9302 inhibited the synthesis of IL-8 by human CD8+ T lymphocytes and inhibited spontaneous IL-8 production in human monocytes in a concentration-dependent manner (Gesser et al., 1997). Furthermore, IT9302 stimulated mast cell proliferation in MC/9 cells costimulated with IL-4, an effect that is well known for IL-10 (Thompson-Snipes et al., 1991). The peptide was patented in 2005 (Larsen and Gesser, 2005). Several other studies on IL-10-mimetic effects of IT9302 were published. Osman et al. showed that preventative parenteral application of the peptide annihilated increased IL-1β, IL-8 and TNF serum levels in a rabbit model of acute necrotising pancreatitis. Furthermore, the development of acute lung injury was prevented and the mortality rate was reduced in comparison to untreated animals (Osman et al.,

1998), (Osman et al., 2002). Matching results had been obtained by prophylaxis with IL-10 in mouse and rat models in earlier studies (Van Laethem et al., 1995), (Kusske et al., 1996), (Rongione et al., 1997). In another study, (Kurte et al., 2004) demonstrated the downregulation of MHC class 1 and an inhibition of the expression of TAP1 and TAP2 proteins in melanoma cells by IT9302. These effects had previously been reported for IL-10 as well (Matsuda et al., 1994). Furthermore, it was shown that IT9302, like IL-10, enhances the IFN-γ- and TNF-induced expression of the thymus- and activation-regulated chemokine (TARC) in HaCaT cells (Vestergaard et al., 2001).

A second nonapeptide, homologous to a part of the N-terminus (residues 8-16) of hIL-10, was investigated by (Gesser et al., 1997) as well and named IT9403. It has the sequence **Ser-Glu-Asn-Ser-Cys-Thr-His-Phe-Pro** (SENSCTHFP). In contrast to IT9302, IT9403 was found to not inhibit cytokine synthesis but to stimulate IL-4 dependent proliferation of murine MC/9 mast cells in a concentration-dependent manner. Both human and murine IL-10 are well known for this effect (Thompson-Snipes et al., 1991).

Thus, IT9302 seems to have a selective, strong immunosuppressive effect, while IT9403 appears to have only immunostimulatory properties. Based on these findings, (Gesser et al., 1997) proposed the existence of two different functional domains of IL-10, an immunostimulatory domain at the C-terminus and an immunosuppressive domain at the N-terminus.

It is obvious that the use of nonapeptides as IL-10-mimetic drugs would potentially have two major advantages over the application of IL-10 itself. Firstly, oligopeptides can be conveniently and relatively inexpensively synthesised in large quantities nowadays, while the chemical synthesis of larger proteins is still complicated and expensive and does not guarantee the correct tertiary and quaternary structure. Thus, bioactive proteins are usually expressed in cell cultures, a cumbersome and expensive process that provides only relatively low yields. Secondly, the chemical and structural stability of oligopeptides is usually higher than that of proteins because the former ones lack a tertiary or quaternary structure that is usually prone to denaturation.

2.4.2.1 Aim of the study

The aim of this part of the present thesis was to proof the IL-10-mimetic activities of newly synthesised batches of the IL-10-homologous peptides IT9302 and IT9403 in direct comparison to hIL-10. In the case of positive results, it was further aimed to use IT9302 as drug for the development of delivery systems suitable for the local treatment of the intestinal mucosa, like PLGA NP. With its reported bioactivity in the MC/9 cell assay, IT9403 could be used as model drug for IT9302 during the development of a delivery system, because the release of IT9403 could be quantitatively characterised by the established MC/9 cell proliferation assay. In the context of the above studies, the potential bioactivity of two nonapeptides homologous to the residues 152–160 and 8–16 of murine IL-10 was investigated, too.

2.4.3 Materials and methods

2.4.3.1 Materials

BSA (≥ 96% purity), DMF, disodium phosphate heptahydrate, glacial acid, 1 N HCl, LPS (extracted from *E. coli*) and MTT were purchased from Sigma-Aldrich (St. Louis, USA). RhIL-10 and rmIL-4 were purchased from peprotech (Rocky Hill, USA). The murine mast cell line MC/9 (CRL-8306™) was purchased from ATTC (Manassas, USA). For the culture of the MC/9 cells, penicillin/streptomycin and RPMI 1640 with L-glutamine, without phenol red (PAA Laboratories, Pasching, Austria) and heat inactivated FBS (Lonza, Cologne, Germany) were used. Rat T-cell culture supplement with ConA (IL-2 culture supplement, T-STIM) was purchased from Becton, Dickinson and Company (Franklin Lakes, USA). For the pulmonary cell isolation, cell strainers with 100 µm pore size were used (Becton Dickinson and Company). Trypsin was received from Sigma-Aldrich, RPMI 1640 from Thermo Fisher Scientific (Waltham, USA). The CBA Flex Sets used for the determination of the cytokine concentrations in the cell culture samples were acquired from Becton, Dickinson and Company.

2.4.3.2 Custom-synthesised peptides

All peptides were synthesised by single-step solid-phase peptide synthesis in a quantity of 50–60 mg per peptide by GenScript (Piscataway, USA). No terminal capping was applied. The peptides were delivered as salts with trifluoroacetic acid. All peptides had a purity of ≥ 98% as determined by HPLC and confirmed by the certificates of analysis. The theoretical molecular weights of the peptides were consistent with the mass spectra that were obtained by electrospray ionisation. An overview on the peptides and their analysis is presented in Tab. 7. After delivery, the peptides were stored at -80°C until use. Directly before cell experiments, standard solutions of the peptides were prepared. IT9302, IT9403 and MP2 were each dissolved at 10 mg/ml in deionised sterile-filtered. For peptide MP1, 2% glacial acid had to be added to the water to enhance the solubility to 10 mg/ml.

Tab. 7: Overview of the custom-synthesised peptides and the analytical results obtained by GenScript. The $MW_{observed}$ was obtained by mass spectrometry.

Name used in this study	GenScript ID	Amino acid sequence	$MW_{theoretical}$	$MW_{observed}$
IT9302	**220678_1**	**AYMTMKIRN**	**1127.39**	**1127.00**
MP1	**220678_2**	**AYMMIKMKS**	**1102.44**	**1102.20**
IT9403	**220678_3**	**SENSCTHFP**	**1021.07**	**1020.70**
MP2	**220678_4**	**EDNNCTHFP**	**1076.10**	**1075.70**

2.4.3.3 IL-10 bioactivity test on MC/9 cells

The IL-10-mimetic bioactivity of the peptides was determined by their ability to induce the proliferation of murine mast cells (cell line MC/9) in a concentration-dependent manner when costimulated with murine IL-4 (Thompson-Snipes et al., 1991). The cells were grown and prepared as described in chapter 2.1.3.7.2. Frozen aliquots (-80°C) of IL-10 and the peptides were thawed and diluted to the respective concentrations in CMWT. The MC/9 cell assay was conducted on a 96-well plate. 50 µl of the diluted samples were each added to 100 µl CMWT containing $2*10^5$ cells/ml and a defined concentration of IL-4. Additional wells were prepared in the same way for each sample but without cells to generate background samples for the absorbance measurement. The 96-well plates were subsequently incubated for 48h

at 37°C, 5% CO_2. The IL-10-dependent proliferation of the cells was measured by a colorimetric MTT assay (Hansen et al., 1989) as described in chapter 2.1.3.7.2.

2.4.3.4 IL-10 bioactivity test on activated macrophages

In addition to the MC/9 cell assay, human macrophages were used to examine the IL-10-mimetic bioactivity of the peptides in comparison to IL-10. Lung tissue for the isolation of macrophages was received from patients undergoing lung resection. The macrophages used were surpluses from isolations performed for the development of an alveolar coculture model by Marius Hittinger (see section 2.3.3.7). The isolation procedure was based on an established protocol (Daum et al., 2012). Briefly, the lung tissue was chopped into cubes with an edge length of 5 µm and washed three times in a buffered salt solution (pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 10 mM HEPES, 5 mM glucose) with a cell strainer. For the extraction of the macrophages, the filtrate of the first washing step containing macrophages and erythrocytes was further processed. The cells were washed with RPMI 1640 containing 5% FBS and 100 I.U./ml penicillin + 100 µg/ml streptomycin and subsequently distributed to petri dishes and incubated for one hour at 37°C, 5% CO_2. The erythrocytes were removed from the macrophages by washing the petri dishes three times with buffered salt solution. The macrophages were further cultured in RPMI until day 4 after isolation and the medium was exchanged after 24 h. After 96 h, the macrophages were trypsinised for 10 min, then detached from the petri dish with a cell scraper, washed with RPMI and counted with a haemocytometer. Subsequently, the cell concentration was set to $1.65*10^5$ cells/ml. The cells were then cultured on a 96-well plate. 200 µl of cell suspension were added to each well (resulting in a cell density of 10^5 cells/cm^2) and the cells were incubated at 37°C, 5% CO_2 for 24 h. Afterwards, 50 µl of IL-10 or peptide solution, each diluted in RPMI 1640 + FBS, were added to each well. Untreated controls were prepared as well and supplemented with 50 µl of blank medium.

The macrophages were stimulated by the addition of 2.5 µl of 1 mg/ml LPS solution per well and further incubated. 24 h after treatment and stimulation, media samples were drawn from the wells and frozen at -80°C. The cytokine concentration was

measured by incubation of the thawed culture media samples with fluorescence-labelled, antibody-coated beads (CBA Flex Set system). The concentrations of IL-6, IL-8, and TNF were measured in the same run. The samples were prepared according to the manufacturer's instruction and analysed with a FACSCalibur™ flow cytometer (Becton, Dickinson and Company). For each sample and standard, approximately 300 single beads were measured, and the mean fluorescence intensity of the bead population was determined by nonlinear regression. The 5-parameter logistics model of the software FCAP Array (version 3, Soft Flow, Pecs, Hungary) was used for the calculations.

2.4.4 Results and discussion

Four peptides, namely IT9302, IT9403, MP1 and MP2 (Tab. 7) were tested in comparison with hIL-10 on their proliferation-inducing properties in MC/9 cells, with 5 pg/ml of rmIL-4 as co-stimulant. As can be seen from Fig. 29, hIL-10 showed the expected concentration-dependent induction of cell proliferation. The cell viability, represented by the measured absorbance, increased markedly in the concentration range of 0.1 to 1 ng/ml and saturated at higher concentrations. Unexpectedly, no comparable effect was observed for IT9403. Neither did the other peptides reveal any bioactivity up to the highest tested concentration of 33 ng/ml.

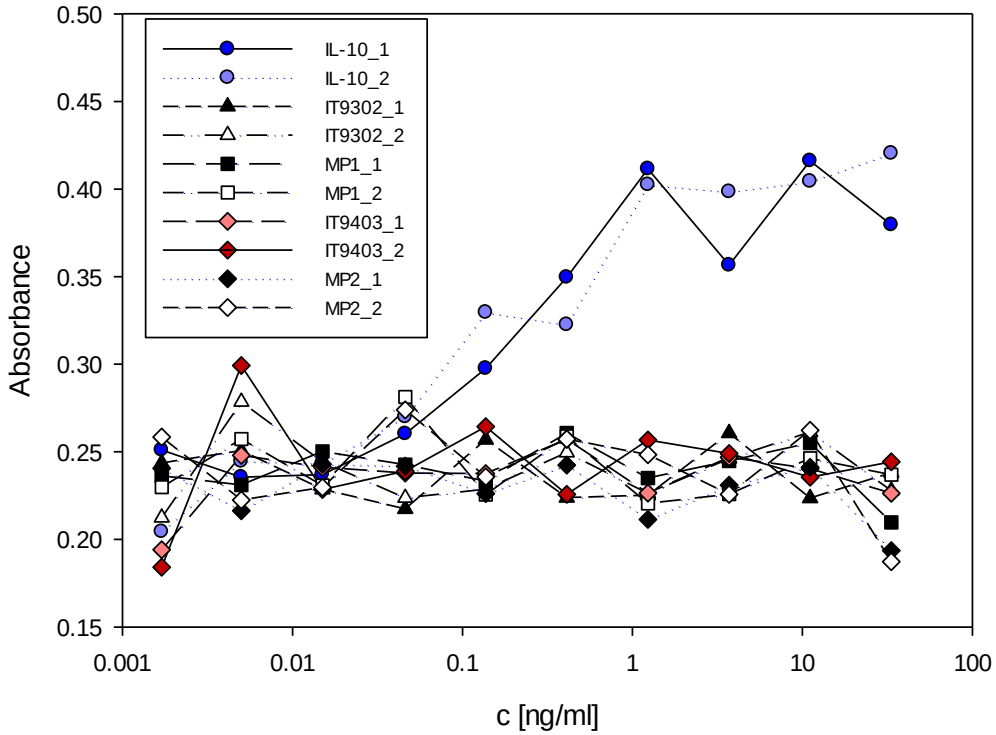

Fig. 29: Concentration-dependent proliferation of MC/9 cells as determined by absorbance after stimulation with various concentrations of rhIL-10 or the homologous peptides IT9302, MP1, IT9403 and MP2. The cells were costimulated with 5 pg/ml rmIL-4. The samples 1 and 2 of each peptide and IL-10 were each prepared independently.

To exclude the hypothetical possibility that the co-stimulation with the specific concentration 5 pg/ml IL-4 does not produce the IL-10-mimetic effect of IT9403, a mix with equivalent concentrations (w/v) of all four peptides was tested in combination with three different concentrations of IL-4, namely 5 pg/ml, 50 pg/ml and 500 pg/ml (Fig. 30). Moreover, higher concentrations, up to 1 µg/ml, of each peptide were tested to reveal even very weak bioactivity. But again, no concentration-dependent stimulation of the MC/9 cells was observed for the peptides, even at the highest tested concentration of 1µg/ml. On the other hand, a profound concentration-dependent stimulation of the cells by IL-4 was revealed by the marked increase of the absorbance levels with increasing IL-4 concentration from 5 pg/ml to 50 pg/ml and further to 500 pg/ml IL-4. For comparison, the concertation-dependent effect of IL-10 together with 5 pg/ml IL-4 was shown again, confirming the corresponding result in Fig. 29.

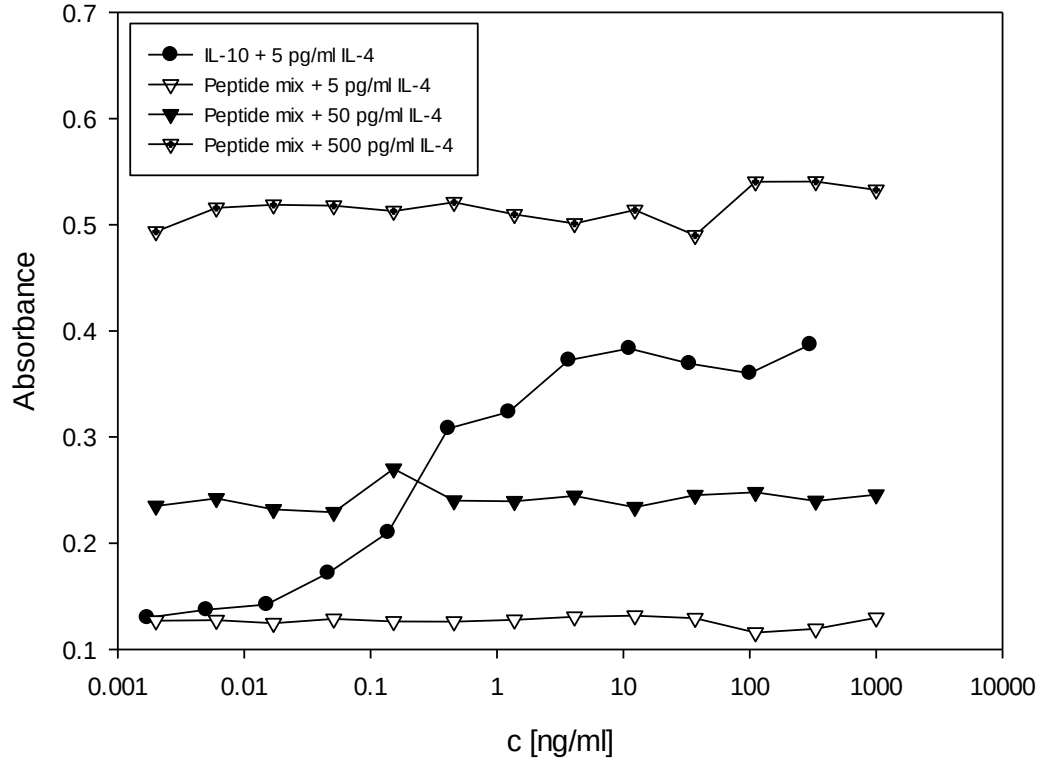

Fig. 30: Concentration-dependent proliferation of MC/9 cells after stimulation with various concentrations of rhIL-10 or of a mix of the equi-concentrated peptides IT9302, MP1, IT9403 and MP2. The indicated concentrations are those of each single peptide in the mix. The cells were costimulated with three different concentrations of rmIL-4 (5 pg/ml, 50 pg/ml or 500 pg/ml, respectively).

In the present study, no evidence for an IL-4-dependent, IL-10-like induction of murine mast cell proliferation of IT9302 and IT9403 could be found in two independent experiments. To the author's knowledge, the finding of (Gesser et al., 1997) that both peptides stimulate MC/9 mast cell proliferation have not been confirmed by others so far. (Imlach et al., 2002) questioned whether IT9403 really stimulates mast cell proliferation, as the assumption that the IL-10 residues 8–16 are responsible for this specific effect is not consistent with neither their own findings on the mast cell-stimulatory effect of ORF virus IL-10 nor the finding of (Ding et al., 2000) that the amino acid leucine in position 87 of the IL-10 sequence is required for its immunostimulatory activity.

Additionally, the potential IL-10-mimetic immunosuppressive activity of the peptides was examined on human primary lung macrophages. The cells were stimulated with 10 µg/ml LPS and treated with either one of the peptides or with

IL-10 in a concentration of 100 ng/ml. After treatment with IL-10, a strong inhibition of IL-6, IL-8 and TNF secretion by the LPS-stimulated macrophages could be observed (Fig. 31) as previously reported for monocytes and macrophages (Fiorentino et al., 1991), (de Waal Malefyt, Abrams, et al., 1991). In contrast to IL-10, none of the tested peptides showed an inhibitory effect on the LPS-stimulated cells.

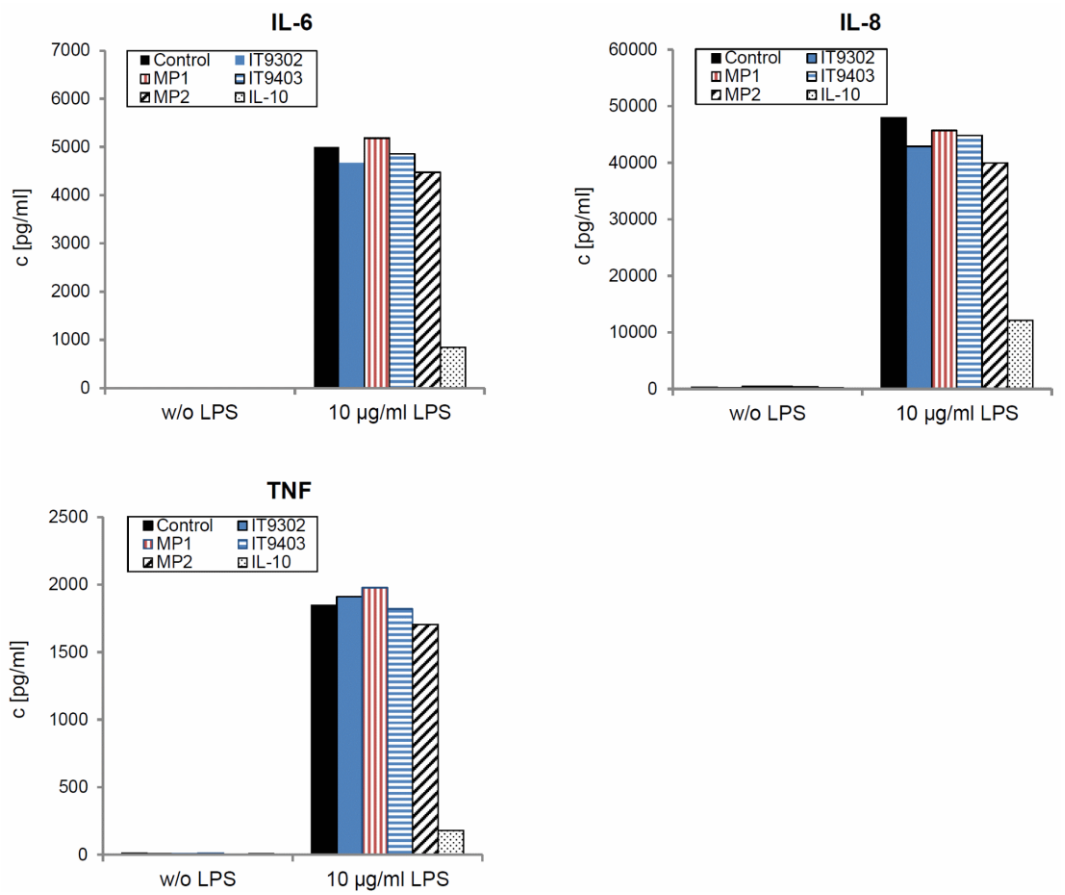

Fig. 31: Effect of the treatment with IL-10 and IL-10-homologous peptides on the secretion of pro-inflammatory cytokines by macrophages stimulated with LPS. The cells were treated with either IL-10 or one of the peptides (IT9302, MP1, IT9403, MP2) in a concentration of 100 ng/ml. Unstimulated (w/o LPS) and untreated (control) macrophages were analysed for comparison. Cytokine concentrations were measured in the culture medium 24 h after treatment and LPS stimulation. n = 1, 3 replicates per group. Results are given as mean.

Subsequently, a second experiment with macrophages of an independent isolation was performed to control these results. This experiment was conducted in the same manner as the previous one except for one difference: instead of testing each single peptide, a peptide mix containing each peptide in the same concentration (1000 ng/ml) was used. As can be seen in Fig. 32, IL-10 clearly inhibited the secretion of IL-6, IL-8 and TNF by the LPS-stimulated macrophages once again, while the

peptide mix showed no inhibitory effect in comparison to the untreated control. This and the previous results suggest that the four peptides IT9302, IT9403, MP1 and MP2 do not exhibit relevant IL-10-mimetic properties.

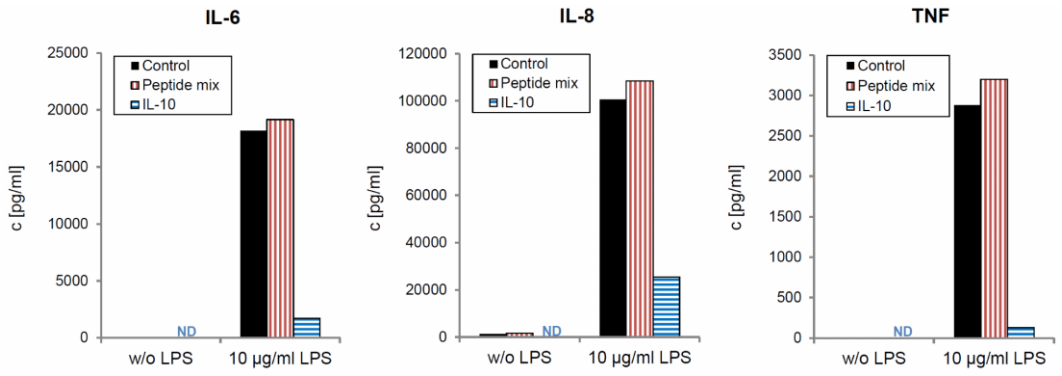

Fig. 32: Effect of the treatment with IL-10 and IL-10-homologous peptides on the secretion of pro-inflammatory cytokines by macrophages stimulated with LPS. Cells were treated with either IL-10 in a concentration of 100 ng/ml or a mix of the peptides IT9302, MP1, IT9403, MP2, each peptide in a concentration of 1000 ng/ml. Unstimulated (w/o LPS) and untreated (control) macrophages were also analysed. No untreated, unstimulated control for IL-10 was included in the experiment (ND: not determined). Cytokine concentrations were measured in the culture medium 24 h after treatment and LPS stimulation. n = 1, 4 replicates per group. Results are given as mean.

In case of IT9302, these negative results are in contradiction with findings in the literature. In addition to the original publication by (Gesser et al., 1997), the immunosuppressive IL-10-mimetic bioactivity of IT9302 was shown in several further studies, using both *in vitro* and *in vivo* models (Osman et al., 1998), (Kurte et al., 2004), (Vestergaard et al., 2001). Specifically, the inhibition of the IL-1β-induced expression of the pro-inflammatory cytokine IL-8 in monocytes was shown by (Gesser et al., 1997). It is therefore very surprising that no reduction of the LPS-induced IL-8 secretion in macrophages could be achieved in the present experiments. Mass spectrometry measurements confirmed the amino acid compositions of the synthesised peptides, but the specific order of the sequences was not checked. Thus, a possible reason for the negative results could be an incorrect amino acid sequence. Another possible reason for the negative results in the macrophage experiments might be a differing activation of signal cascades after interaction of IL-10 and IT9302 with the IL-10 receptor complex. It was shown that IT9302 inhibits the STAT3 activation in dendritic cells, whereas IL-10 activates this transcription factor (López et al., 2011). In future studies, the IL-10-mimetic bioactivity on both murine mast cells and human macrophages should be examined again with newly synthesised IT9302 and IT9403, including the verification of the

order of sequences in the peptides. Furthermore, the molecular mode of action of IT9302 in comparison to IL-10 and the receptor downstream should be investigated in detail. If IL-10 could be substituted by a nonapeptide, this would enhance the possibilities for drug delivery approaches and make anti-inflammatory therapies based on an agonistic activity on the IL-10 receptor more probable.

2.4.5 Conclusion

In view of the limited stability of IL-10 confirmed in the preceding chapters, the development of new drugs mimicking IL-10 bioactivity by activation of the IL-10 receptor would be highly desirable. Therefore, four nonapeptides homologous to human or murine IL-10, namely IT 9302, IT9403, MP1 and MP2 were tested in comparison with hIL-10 on their proliferation-inducing properties on MC/9 cells. However, none of the tested peptides showed a measurable IL-10-mimetic bioactivity. The same result was obtained in a second assay, in which primary human lung macrophages were stimulated with LPS and thereafter treated with a mix of the peptides or with IL-10. In case of IT9302, the negative results conflict with positive reports in the literature. A possible reason may be a differing amino acid sequence of the peptides, which was not characterised in the present study.

3. Summary and outlook

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine and a promising potential drug for the treatment of inflammatory diseases, such as inflammatory bowel disease (IBD) or the acute respiratory distress syndrome (ARDS). In this thesis, three strategies for the preparation of particulate drug delivery systems for the local delivery of IL-10 were investigated, with focus on the remaining bioactivity of the protein.

In a first attempt, IL-10-loaded poly(lactic-co-glycolic) acid (PLGA) nanoparticles (NP) for the local delivery of IL-10 to the inflamed intestinal mucosa were prepared by a water-in-oil-in-water (W/O/W) double emulsion solvent evaporation method. Ethyl acetate (EtOAc) was used as solvent for PLGA and bovine serum albumin (BSA) as the carrier/stabiliser of IL-10. However, no bioactivity of the IL-10 released from the particles was observed. Therefore, a design of experiment (DoE) was conducted to investigate the dependence of the IL-10 denaturation on the preparation parameters of the primary emulsion process. Even without homogenisation, the addition of EtOAc or dichloromethane (DCM) to the aqueous solution of IL-10 and BSA caused a reduction of the initial bioactivity by more than 95%. Moreover, the addition of poloxamer 188 as well as of enhanced concentrations of BSA had only negligible protective effects. (2-Hydroxypropyl)-β-cyclodextrin (HP-β-CD) and trehalose did not significantly protect IL-10 either. Hence, water-in-oil (W/O) emulsion methods using EtOAc or DCM do not seem to be suitable for the preparation of polymeric particles loaded with low concentrations of IL-10 (5 µg/ml in the aqueous solution). The present study was limited to this concentration due to the high costs of commercially available IL-10 and therefore could not benefit from a possible self-stabilization of IL-10 at higher concentrations.

As a second approach, spray-dried IL-10-loaded microparticles (MP) were prepared from an aqueous solution with a B-90 nano spray dryer. Based on the excipients bovine serum albumin (BSA) and trehalose and the surfactant poloxamer 188, particles with a suitable remaining bioactivity of 69% and a yield of 72% were produced. To protect the IL-10 against the gastric environment, these particles were encapsulated in Eudragit L 100-55 microspheres by a solid-in-oil-in-oil (S/O/O)

emulsion method. While 30% of the initial IL-10 bioactivity remained after dissolution of the microspheres in cell culture medium, incubation at pH 2.4 caused almost complete denaturation of the IL-10. Even worse results were obtained by encapsulating the IL-10-loaded MP in distinctly larger microspheres of Eudragit S 100. Both types of Eudragit microspheres did not adequately protect IL-10 from denaturation under acidic conditions that are expected after oral application. A possible reason may be the swelling of the Eudragit polymers in acidic aqueous media. This may give rise to an acidic microenvironment inside the microspheres that may be sufficient to cause the denaturation of the sensitive IL-10. Possibly, a more suitable system for the protection of IL-10-loaded spray-dried MP against the gastric environment can be found in future studies.

The third strategy aimed at developing an IL-10 formulation suitable for the local targeting of the deep lung. To this this end, the preparation of spray-dried IL-10-loaded MP was modified by the substitution of the surfactant poloxamer 188 with L-leucine. The resulting particles showed excellent aerodynamic properties with a fine particle fraction (FPF) of 57% and a mass median aerodynamic diameter (MMAD) of 2.5 µm. However, the remaining IL-10 bioactivity was only about 8%. Nevertheless, this dry powder formulation was applied to an inflamed human alveolar co-culture model based on epithelial cells and macrophages. The deposition of the MP on the coculture was achieved with a special deposition system, the Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOCC), which was complemented by an established dry powder inhaler, the HandiHaler®. The treatment with the IL-10-loaded particles caused a significant reduction of the secretion of the inflammatory markers IL-6 and TNF as compared to the deposition of placebo (blank) particles. This result demonstrates the anti-inflammatory effect of the IL-10-loaded particles on the inflamed coculture and shows the relevance of a further development of IL-10-based dry powder formulations for application to both *in vitro* and *in vivo* models of inflammatory lung diseases. In future studies, the excipient composition of the spray-dried MP should be optimised to enhance the remaining IL-10 bioactivity, while preserving the excellent aerodynamic properties of the particles. Furthermore, pyrogen-free qualities of the excipients, namely of BSA should be used to reduce the pro-inflammatory properties of the blank particles.

An important factor that severely impaired this study was the very limited availability of IL-10. Commercial IL-10 was very expensive (more than 4000 € for 1 mg) and no non-commercial source was available. Thus, all experiments had to be conducted with relatively small quantities of IL-10 (usually in the low microgram range). In other studies, much larger quantities of IL-10 were used. For instance, (Egilmez and Sikora, 2005) patented the preparation of IL-10-loaded PLGA MP by a double emulsion method. They used 1 mg of IL-10 for the preparation of a single particle batch, resulting in a high concentration of 5 mg/ml IL-10 in the aqueous phase and 0.5% (w/w) IL-10 in the final formulation. These concentrations were significantly higher than those in the present experiments. It was shown that a high concentration of a protein in solution or emulsion is of vital importance for its stability, as it gives rise to a self-protecting effect (Bilati et al., 2005d), (van de Weert, Hennink, et al., 2000).

As it became evident during the present investigations that IL-10 is a very challenging protein for the development of drug delivery systems, four nonapeptides homologous to human or murine IL-10, IT 9302, IT9403, MP1 and MP2, were investigated for a possible IL-10-mimetic bioactivity in the MC/9 cell assay and on inflamed human alveolar macrophages. However, none of the peptides showed any IL-10-mimetic bioactivity. In case of IT9302, this result is in conflict with reports in the literature , e.g. (Gesser et al., 1997), (Osman et al., 2002). A possible reason may be a differing order of the amino acid sequence in the peptides, which was not characterised in the present study.

In future studies on the formulation of particulate carrier systems for IL-10, two main issues of this work should be addressed. Firstly, a larger quantity of IL-10 should be available to enable the preparation of formulations with higher concentrations in the mg/ml range, which should benefit from the self-protecting effect of proteins at higher concentrations. Furthermore, the formulation of higher concentrations of IL-10 could possibly also eliminate the need for using albumin (e.g. BSA) as an excipient and would thus allow the use of additional analytical methods for both the quantification (e.g. size exclusion chromatography, reversed phase HPLC) and the structural analysis of IL-10 (e.g. circular dichroism, Fourier transform infrared spectroscopy). Secondly, the commercially available immunoassays for IL-10 should be thoroughly screened to identify a system that is

only sensitive to native IL-10 and shows a good correlation to bioassays. This would enable the development of rapid and robust extensive screenings of multiple process factors and excipient compositions to optimise the formulation conditions.

Considering the limited stability of IL-10, the development of new drugs mimicking the IL-10 bioactivity by activation of the IL-10 receptor would be highly preferable to the formulation of IL-10 itself. According to studies in the literature (Gesser et al., 1997), (Osman et al., 1998), the use of IL-10-mimetic peptides like IT 9302 seems to be a highly promising approach. Although no IL-10-mimetic activity could be observed in this study, such peptides should be further examined with focus on an improved characterisation of the order of sequences in the peptides, the detailed investigation of the molecular mode of action as well as on the receptor downstream.

Additional promising alternatives are modifications of IL-10 that provide enhanced stability of the structure elements responsible for receptor activation. A monomeric isomer of wild-type IL-10 was engineered by insertion of amino acids in the original sequence (Josephson et al., 2000). This monomer showed an enhanced thermal stability compared to wild-type IL-10 and exhibited significant bioactivity. Moreover, an IgG Fc IL-10 fusion protein was recently engineered and expressed in the plant *Pichia pastoris* (Guo et al., 2012). It showed a higher *ex vivo* bioactivity in human peripheral blood mononuclear cells (PBMCs) than native IL-10. Very recently, a fusion protein of an IgG antibody with two IL-10 monomers was designed that showed high bioactivity (Duerner et al., 2014). Such fusion proteins can be expected to exhibit higher stability than native IL-10. It is very desirable that these and other potential IL-10-mimetic drugs are further investigated with regard to their stability and suitability as drug candidates for the development of local drug delivery systems, as the local activation of the IL-10 receptor is a highly promising approach for the treatment of various inflammatory diseases.

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Poster presentations

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