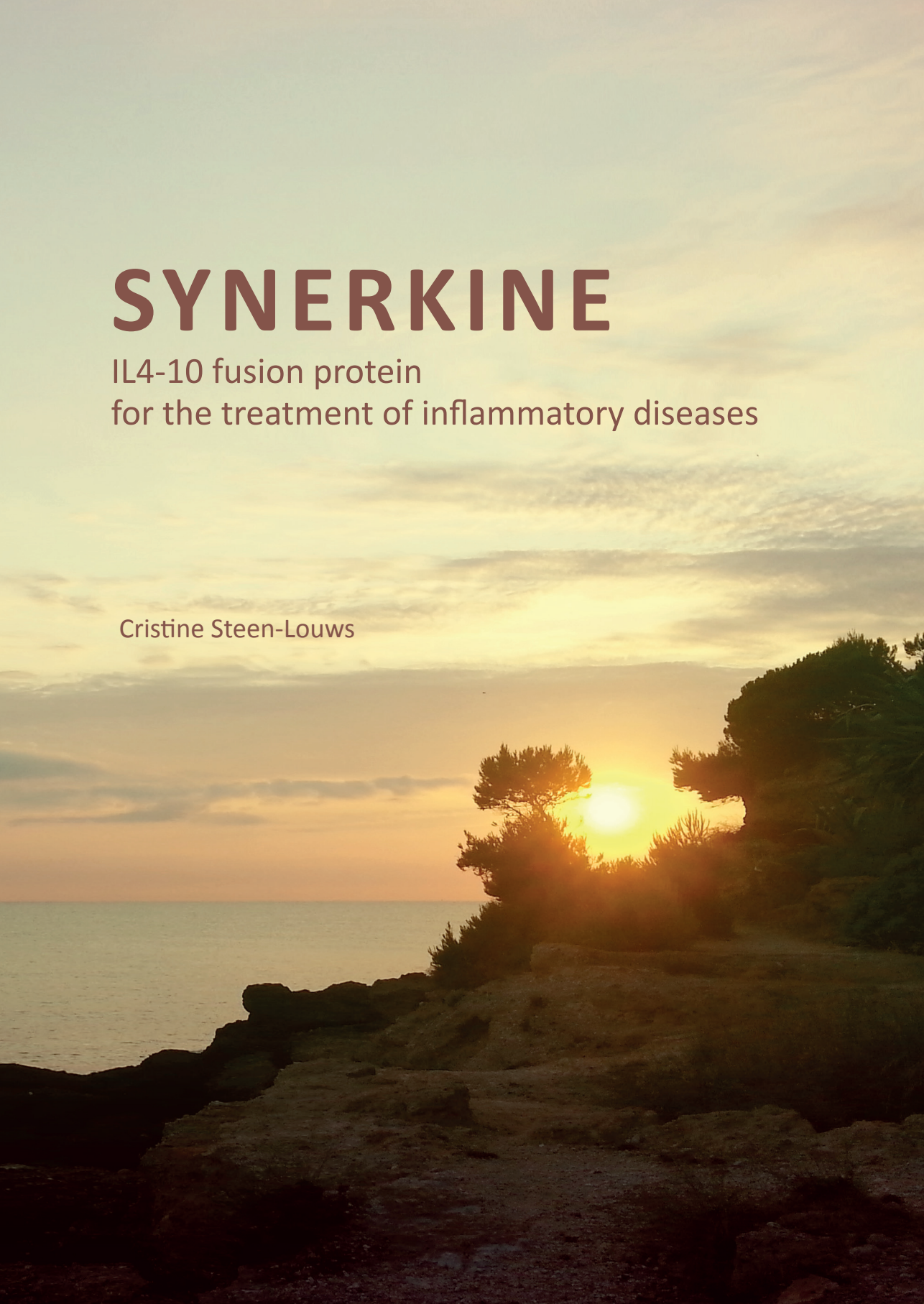
SYNERKINE

IL4-10 fusion protein
for the treatment of inflammatory diseases

Cristine Steen-Louws



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IL4-10 fusion protein

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SYNERKINE

IL4-10 fusie-eiwit voor de behandeling van ontstekingsziekten

(met een samenvatting in het Nederlands)

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	Dr. J. Popov-Celeketic

Hoop, wat is hoop?

Dis die wens dat die son weer opkom

Hoop, wat is hoop?

Dis dat vrede by ons uit kom

Bring die hoop weer terug

| Jo Black |

Leescommissie:	Prof. dr. T.W.J. Huizinga
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	Prof. dr. J.M. van Laar
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Paranimfen:	Peter Boross
	Rowena Melchers

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1 General Introduction

Therapeutic potential of regulatory cytokines in inflammatory diseases and chronic pain

Steen-Louws, C

This introduction is based on a review written by:
Steen-Louws C, Preusting LC, Hack CE, Popov-Celeketic J, and Eijkelkamp N

To be submitted

Inflammation is an important response of the human body to harmful stimuli. The course of inflammation is orchestrated amongst others by regulatory proteins called cytokines (Stumpo et al., 2010). Cytokines are small (15-25kDa), hormone-like proteins involved in communication between primarily immune cells (Schooltink & Rose-John, 2002). Cytokines include interleukins (IL) (which are mainly produced by leukocytes), chemokines (which have chemotaxic properties), lymphokines (which are produced by lymphocytes), and monokines (which are produced by monocytes) (Zhang & An, 2007). Usually, cytokines released by cells interact and communicate with other cells through paracrine or endocrine signaling, and sometimes autocrine signaling (Zhang & An, 2007). Generally, three major groups of cytokines can be distinguished: pro-inflammatory cytokines, which initiate and enhance inflammation, anti-inflammatory cytokines, which dampen inflammation, and hematopoietic growth factors which regulate differentiation of blood progenitor cells. Over the past years, it became clear that the term anti-inflammatory cytokines is somewhat incomplete, as their function is not solely 'anti-inflammatory' and their role is broader than dampening inflammation. The term regulatory cytokines seems more appropriate, as they are considered more 'inflammation-controlling', skewing inflammatory reactions into a certain type of inflammation, i.e. into Th2-inflammation in case of interleukin-4 (IL-4), or Th17-inflammation in case of transforming growth factor-β (TGF-β). Cytokines are pleiotropic and regulate cell differentiation, growth, survival, and responsiveness. Furthermore, different cytokines have overlapping activities, a feature referred to as redundancy (Schooltink & Rose-John, 2002). Moreover, cytokines influence their own production at multiple levels including transcription, translation and mRNA degradation (Stumpo et al., 2010). Importantly, characteristics as pleiotropy and redundancy indicate that cytokines act in a network and interact with each other (Schmitz et al., 2011; Schooltink & Rose-John, 2002).

As illustrated in Figure 1, acute inflammation is a rapid response of the body to harmful stimuli, such as injuries, pathogens and toxic compounds, to eliminate these stimuli and start a healing process (Laveti et al., 2013). Pro-inflammatory cytokines such as IL-1β, TNF, IL-6, IL-12, and others are key mediators in starting inflammation (Dinarello, 2000), whereas regulatory cytokines such as IL-4, IL-10, IL-13 and TGF-β, prevent overshoot of Th1- and Th17-driven inflammatory response and skew immune responses to target specific classes of pathogens such as parasites (Chen et al., 2017; Pavlov & Tracey, 2006; Opal & DePalo, 2000). Resolution of acute inflammation requires elimination of the stimulus, appropriate fine-tuning of the

inflammatory response to induce an appropriate attack towards the type of pathogen, as well as a balance between pro-inflammatory and regulatory cytokines (the "on and off signals"). In case of an inappropriate response type, or a disbalance between inflammatory and regulatory cytokines, inflammation can become chronic, self-perpetuating, causing lasting tissue damage. Chronic inflammation contributes to a variety of human diseases, generally called chronic inflammatory diseases (Pavlov & Tracey, 2006; Chen et al., 2017). Currently, 5-7% of the western society is affected by some form of chronic inflammatory disease (El-Gabalawy et al., 2010). Inflammatory bowel disease (IBD) for example, is characterized by chronic inflammation of the intestine and affects 10 million people worldwide (Sanchez-Muñoz et al., 2008). Rheumatoid arthritis (RA) is another example of a disease associated with chronic inflammation and it is characterized by chronically inflamed joints (Williams et al., 2007). Nowadays even osteoarthritis (OA) is considered to be driven by a chronic inflammation (Nees et al., 2019). Uncontrolled inflammatory responses also play an important role in chronic pain (Graeber and Christie, 2012; Ji et al., 2013). In IBD, RA and OA, persisting pain is an important clinical symptom. Moreover, chronic neuro-inflammatory processes maintain pain and may underlie various chronic pain conditions such as persistent inflammatory pain or neuropathic pain (Breivik et al., 2008; Zhang & An 2007).

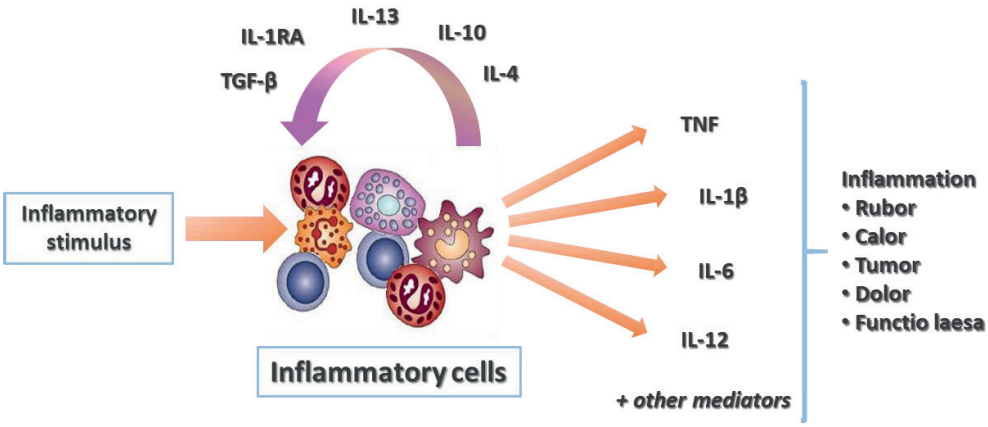

Figure 1: Schematic representation of an inflammatory response upon inflammatory stimulus. In healthy individuals, both Th1/Th17 and Th2 cytokines will be produced, enabling a balanced immune response, whereas in chronic inflammation these responses are out of balance, with a trend towards Th1/Th17 cytokine release. *Adapted from an image by Hack, C.E.*

Traditionally, nonsteroidal anti-inflammatory drugs (NSAIDS) are prescribed as a treatment for inflammatory diseases, because they exert anti-inflammatory, anti-pyretic and analgesic effects. Although these drugs are taken by many patients, their beneficial effects are limited due to a wide range of adverse effects such as gastrointestinal symptoms including ulcerative colitis and bleeding, cardiovascular, and renal side effects (Pereira-Leite et al., 2017). Glucocorticoids are also widely used to dampen inflammation and have effect in a wide variety of inflammatory diseases. However, glucocorticoids come with a severity of side effects, including Cushing's syndrome, hypertension, diabetes, osteoporosis, and gastrointestinal damage (Barnes & Adcock, 2009). It is therefore desirable to replace NSAIDS and glucocorticoids with more specifically acting drugs.

Specific targeting of key mediators that drive inflammation in chronic inflammatory diseases will reduce the risk of side effects and infections. One of the first successful attempts was anti-TNF therapy, preventing TNF-receptor signaling. Although TNF inhibitors are successfully used in diseases such as RA, psoriasis, and IBD, part of the patients does not respond to these powerful drugs or becomes resistant over time. For example, in IBD patients, TNF inhibitors have a temporary effect in half of the patients, whereas in ~30% they are not effective at all (Rutgeerts et al., 2004). Also in OA patients, they seemed insufficiently effective (Persson et al., 2018). Redundancy of inflammatory mediators may explain failure of a therapy targeting only a single inflammatory cytokine. Therefore, regulatory cytokines are attractive candidates for therapeutic drugs to treat chronic inflammatory diseases as they potentially inhibit the formation and release of multiple inflammatory mediators (Schooltink & Rose-John, 2002).

In this introduction, the effects and limitations of regulatory cytokines as a therapeutic tool in inflammatory diseases like IBD, RA, and chronic pain are reviewed. In addition, cytokine fusion proteins are discussed as these may overcome some of the limitations of wild-type regulatory cytokines. The introduction is followed by an outline of this thesis, presenting a novel fusion protein of IL-4 and IL-10 and its potential use in the treatment of inflammatory disorders.

1.1 | Levels of regulatory cytokines in inflammatory diseases

In chronic inflammatory diseases like RA, IBD, and chronic pain, concentrations of inflammatory cytokines are often high compared to levels of regulatory cytokines, which may shift the balance to Th1- and Th17-inflammatory reactions. Indeed, in synovial fluid of most RA patients, levels of the IL-4 cytokine are undetectable (Miossec et al.,1990; Vervoordeldonk

& Tak, 2002). Furthermore, it is suggested that the -1082A/G polymorphism in the IL-10 gene, resulting in increased IL-10 expression, is positively associated with development of RA. Meta-analysis of a large cohort of patients and controls showed that carriers of the G allele have a 25% decreased risk to develop RA compared to the AA homozygote carriers (Zhang et al., 2011). Similarly, lower expression of TGF-β1 due to a polymorphism has been associated with increased development of RA (Mattey et al., 2005). IL-13 levels are lower in synovial fluid and peripheral blood of active RA patients compared to healthy controls (Woods et al, 1997). Finally, a genetically driven imbalance between IL-1 and IL-1RA in favor of IL-1 is observed in RA. (Firestein et al., 1993; Buchs et al., 2002).

Low levels of regulatory cytokines and genetic associations with the IL-4 and IL-10 gene in humans have been proven a hallmark for the development of IBD as well (Klein et al., 2001). IL-10 deficient mice develop chronic enterocolitis (Kühn et al., 1993), in combination with a strong increase in pro-inflammatory cytokine levels like IL-12 and IFN-γ (Gazzinelli et al., 1996). Administration of IL-4 in active Crohn's disease (CD) and ulcerative colitis (UC) patients leads to a significant reduction of the vascular endothelial growth factor (VEGF) production by peripheral blood mononuclear cells (Sanchez-Muñoz et al., 2008). Increased evidence points towards a protective role of IL-13 in the development of IBD. The risk of IBD is associated with the IL-13Rα2, a decoy receptor that binds the cytokine IL-13 with high affinity and diminishes its STAT6-mediated effector functions. Human subjects with a R130Q polymorphism in the IL-13 gene, leading to expression of a more active IL-13 with low affinity for IL-13Rα2, reported a lower risk for IBD development (Karmele et al., 2019). In intestinal mucosal cells of Crohn's disease and ulcerative colitis patients the IL-1RA:IL-1 ratio is reduced compared to healthy controls (Arend, 2002; Casini-Raggi et al., 1995). Furthermore, decreased TGF-β activity due to decreased levels or improperly functioning TGF-β is related to the development of IBD (Sanchez-Muñoz et al., 2008; Zotto et al., 2003).

Also in chronic pain, serum protein concentrations of IL-4, IL-10, and IL-13 are significantly lower compared to healthy individuals and are associated with the magnitude of pain (Üçeyler et al., 2006; Bollettino et al., 2005). A relation of low serum cytokine concentrations to development of chronic pain has been confirmed by experiments in mice. IL-4 deficient mice, for example, have increased neuronal hyperreactivity upon mechanical stimulation compared to wild-type mice (Lemmer et al., 2015). Next, IL-4, IL-10, and IL-13 inhibit neuropathic pain through various mechanisms - either due to direct effect on sensory neurons, or through

inhibition of spinal microglia (Balasingam & Yong, 1996; Cunha et al., 1999), or by exerting suppressive effects on M1 macrophage-related inflammation via a common receptor, IL-4Rα (Pepe et al., 2014; Kiguchi et al., 2017). In addition, IL-1RA has a protective role in chronic pain. IL-1RA, released at sites of inflammation, limits inflammatory hyperalgesia induced by several inflammation inducing agents such as carrageenan and LPS. (Cunha et al., 2000). In line with this, injection of IL-1β causes hyperalgesia indicating a key role of IL-1 in development of chronic pain (Zhang & An, 2007). It has even been suggested that IL-1β, in addition to producing inflammation and inducing synthesis of several nociceptor sensitizers, rapidly and directly activates nociceptors to generate action potentials and induce pain hypersensitivity (Binshtok et al., 2008). TGF-β is also involved in the regulation of pain; the isoform TGF-β1 controls proliferation of neurons, blocks microglial proliferation and has anti-inflammatory effects on astroglia (Krieglstein et al., 1998; Suzumura et al., 1993; Bottner et al., 2000). TGF-β1 treatment of neuropathic pain in rats not only prevents development of neuropathic pain following nerve injury, but also reverses previously established neuropathic pain conditions (Echeverry et al., 2009).

1.2 | Regulatory cytokines and their potential as therapeutics

Cytokines with immune-regulatory properties include IL-4, IL-10, IL-13, IL-27, IL-35, IL-37, and TGF-β (Opal & DePalo, 2000; Banchereau et al., 2012). In the 90's of the last century, regulatory cytokines, in particular IL-10, and to a lesser extent IL-4, have been evaluated as stand-alone therapy treatment for RA and other inflammatory diseases.

1.2.1 | Interleukin 4 (IL-4). IL-4 is multifunctional cytokine involved in the regulation of immune responses. Together with IL-13, it contributes to the development of allergic diseases by stimulating Th2-type cell responses (Gour & Wills-Karp, 2015). However, in Th1 driven autoimmune diseases, IL-4 acts as an anti-inflammatory cytokine. It stimulates differentiation of naïve Th cells into Th2 cells that produce additional IL-4 triggering a positive feedback loop (Opal & DePalo, 2000). IL-4 also stimulates IL-10 production by Th1 cells, preventing further development of Th1 driven pathology (Mitchell et al., 2017). Additionally, IL-4 suppresses secretion of inflammatory cytokines TNF, IL-6, and IL-1β by monocytes and macrophages while stimulating production of anti-inflammatory mediators like IL-1RA (te Velde et al., 1990; Allen et al., 1993).

In vitro IL-4 slightly reduces production of inflammatory cytokines by activated mononuclear cells isolated from RA synovial fluid and synovial tissue, and reverses cartilage destruction (van Roon et al., 1996). Yet, in preclinical arthritis models such as a collagen-induced arthritis in mice, treatment with IL-4 did not affect clinical activity scores (Joosten et al., 1997). Interestingly, gene therapy with IL-4 in the same model, did not affect inflammation, but prevented bone erosions (Lubberts et al., 2000), indicating IL-4 may at least have beneficial effects on bone. Human IL-4 has been evaluated in clinical studies and is well tolerated. Three daily subcutaneous administrations of 0.2-0.5 μg/kg recombinant human IL-4 (rhuIL-4) to patients with psoriasis decreased the psoriasis affected area, reduced the diseases severity index (PASI), and increased Th2 cell responses in skin biopsies of psoriasis patients (Ghoreschi et al., 2003). In cancer patients, IL-4 administration (10 mg/kg 3 times/day for a period of 10 days), significantly increased circulating levels of IL-1RA, whereas IgG, IgA, and IgM levels were decreased. Despite the high dose of IL-4, levels of soluble CD23 (type II IgE receptor) were increased and were accompanied by a decrease in IgE levels, without allergic manifestations (Atkins et al., 1992). Treatment of RA patients with recombinant human IL-4 (subcutaneous administration, up to 2 mg/kg 3 times per week for 6 weeks) in a phase I study did not result in significant improvement of disease activity, although IL-4 was well tolerated (van den Bosch et al., 1998). Recombinant IL-4 was not administered to patients with chronic pain, though results with this cytokine in preclinical models are encouraging. Local administration of IL-4 or using HSV IL-4 gene therapy in mice with neuropathic pain inhibited inflammation by skewing macrophage polarization towards M2, suggesting a potential in suppressing allodynia and neuropathic pain (Hao et al., 2006; Pepe et al., 2014; Kiguchi et al., 2015).

1.2.2 | Interleukin 10 (IL-10). IL-10 is considered to be the most potent cytokine to inhibit Th1- and Th17-driven inflammation in the human immune response (Moore, 1994). IL-10 is produced by a wide range of immune cells of both the innate and the adaptive immune system, indicating its important role as regulator of the inflammatory response (Saraiva & O'Garra, 2010). IL-10 possesses some similar actions as IL-4, like the inhibition of IL-1β, IL-6, IL-8, and TNF and stimulation of IL-1RA synthesis (de Waal Malefyt, 1991). However, in contrast to IL-4, IL-10 down-regulates the expression of MHC class II molecules on monocytes and macrophages, resulting in lower immune activation. In addition, IL-10 causes up-

regulation of FcγRI on monocytes, which is considered a pro-inflammatory process (te Velde et al., 1992).

Treatment of stimulated RA mononuclear cells (MNC's) with IL-10 alone had a more potent effect on reducing pro-inflammatory cytokine production than IL-4. In cartilage cultures with conditioned medium from these stimulated MNC's, IL-10 potently reversed cartilage destruction (van Roon et al., 1996). IL-10 also modestly suppressed collagen-induced arthritis in mice where IL-4 did not have an effect (Joosten et al., 1997). Recombinant human IL-10 (rhuIL-10) has been evaluated in multiple RA clinical trials and was well tolerated. In a placebo-controlled trial, patients received daily subcutaneous doses of 0.5, 1, 5, 10 or 20 μg/kg rhuIL-10 for 28 days. Minor clinical improvement and an increase in serum IL-1RA levels were observed in the 5 μg/kg group, while toxicity was only observed in patients receiving the highest dose (Maini et al., 1997; St Clair, 1999). The disappointing results with IL-10 in clinical trials might in part be caused by its potential pro-inflammatory effects (van Roon et al., 2003). Administration of rhuIL-10 to 6 patients with active RA demonstrated little efficacy in reducing inflammation and did not cause clinical improvement based on ACR criteria. Indeed, an up-regulation of FcγRI and IIa by IL-10 on macrophages and monocytes of the RA patients was observed, which may have counteracted the otherwise anti-inflammatory effects of IL-10.

In IBD the efficacy of IL-10, but not of other regulatory cytokines, has been evaluated. Culture of intestinal biopsies of patients with active Crohn's disease or ulcerative colitis in the presence of IL-10, reduced the production of pro-inflammatory cytokines like TNF and IL-1 and increased the production of IL-1RA in intestinal lamina propria mononuclear cells (Schreiber et al., 1995). In addition, IL-10 treatment showed efficacy in an animal model for IBD (Nakase, 2002). Schreiber and colleagues were the first to evaluate IL-10 therapy in steroid refractory ulcerative colitis patients. Subjects that received 100 μg IL-10 per day had lower production of inflammatory cytokines and reduced severity of the intestinal mucosal lesions, indicating that IBD patients benefit from IL-10 treatment. In a double-blinded study, Crohn's disease patients received daily different subcutaneous doses (1, 5, 10 or 20 μg/kg) of rhuIL-10 for 28 days. The dose of 5 μg/kg rhuIL-10 appeared to be optimal. In 23,5% of the patients receiving 5 μg/kg rhuIL-10, endoscopic improvements and reduced symptoms were observed, resulting in a complete remission (Fedorak et al., 2000).

Although there is evidence for the role of IL-10 to inhibit chronic pain, this cytokine has not been evaluated in clinical studies for that indication. In patients with, for example, painful non-

diabetic polyneuropathy or post-traumatic neuralgia, low IL-10 levels were detected in blood and cerebrospinal fluid samples (Backonja et al., 2008). In preclinical animal models, intrathecal administration of IL-10 reduced neuropathic pain in rats (Milligan et al., 2005; 2006). However, as reviewed by Kwilasz et al. (2015), despite the potential of IL-10 for the treatment of chronic pain, the effects seen are minimal. The rapid clearance of IL-10 from the intrathecal space is thought to be the cause of these minimal effects, and therefore attempts like gene therapy have been suggested.

1.2.3 | Interleukin-13 (IL-13). IL-13 is mainly produced by activated T lymphocytes and is structurally and functionally related to IL-4. IL-13, like IL-4, uses the IL-4 type 1 receptor explaining the similar activities of these cytokines (de Waal Malefyt et al., 1993; Opal & DePalo, 2000). Similar to IL-4, IL-13 inhibits the macrophage activity and production of inflammatory cytokines IL-1, IL-6, IL-8, IL-12, INF-α, and TNF. Furthermore, IL-13 enhances the production of IL-1RA by stimulated monocytes. IL-13 has been suggested as a therapeutic target, because of its association with inflammatory disorders like RA and ulcerative colitis (Mao et al., 2019). Preclinical studies suggest the neuroprotective role and potency of IL-13 to reverse neuropathic pain (Kiguchi et al., 2017). However, a safety concern is the potential of IL-13 to stimulate Th2 inflammatory reactions and to enhance allergy, atopic dermatitis and asthma. The human monoclonal antibody Dupilumab that inhibits the IL-4Rα subunit of the IL-4- and IL-13 receptor, is successfully used to target IL-4- and IL-13-mediated inflammation in atopic dermatitis and asthma (Thaçi et al., 2016).

1.2.4 | IL-1 receptor antagonist (IL-1RA). IL-1RA is an anti-inflammatory cytokine that belongs to the IL-1 family. IL-1 is the only cytokine that has its natural antagonist: IL-1RA (Schreuder et al., 1997). IL-1RA solely exerts anti-inflammatory actions while all other anti-inflammatory cytokines have at least a few pro-inflammatory properties (Dinarello, 1998). The main function of IL-1RA is competitive inhibition of the pro-inflammatory IL-1 cytokine by binding to the IL-1 receptor with a similar or higher affinity than IL-1. The balance between IL-1RA and IL-1 (the IL-1RA:IL-1 ratio) determines a pro- or anti-inflammatory outcome (Opal & DePalo, 2000). To obtain a 50% inhibition of IL-1, a 10 to 100 fold higher concentration of IL-1RA is required (Arend et al., 1990). The first clinical study evaluating the therapeutic effect of IL-1RA in RA showed promising results and were the basis of FDA approval for Anakinra, under the trade

name Kineret® (Bresnihan et al., 1998). Although the FDA label does not recommend use in pediatrics, Anakinra is also widely prescribed for the treatment of juvenile idiopathic arthritis (Nigrovic et al., 2011).

1.2.5 | Transforming growth factor-β (TGF-β). The TGF-β (25 kDa as dimer) subfamily consists of six isoforms, of which three are expressed in mammalians. TGF-β1 is most abundant and has an important regulatory role in the immune system (Banchereau et al., 2012). TGF-β1 exerts both pro- and anti-inflammatory actions. TGF-β1 inhibits the activation of macrophages (Li et al., 2006), inhibits pro-inflammatory cytokines and stimulates IL-1RA production by macrophages (Opal & DePalo, 2000). Furthermore, TGF-β1 down-regulates FCγR expression on myeloid cells and like IL-10, TGF-β1 inhibits MHC class II molecule expression on macrophages leading to reduced inflammatory response (Li et al., 2006). However, some members of the TGF-β family stimulate the development of Th17 inflammatory reactions (Zhang et al., 2018).

1.2.6 | Clinical results of regulatory cytokines as stand-alone therapy

Although regulatory cytokines showed some therapeutic efficacy in clinical studies, the effects are small and do not compare to the effects seen in some preclinical models. These disappointing results reflect some limitations of therapy with wild-type cytokines. A major drawback of cytokines as a therapeutic agent is their short plasma half-life *in vivo*. Cytokines have a molecular weight of ~25 kDa or less, and are therefore rapidly (5-50 min) cleared by the kidneys (Eliason, 2001). In addition to renal clearance, half-life of cytokines is further reduced by proteolytic degradation or receptor-mediated endocytosis (Jazayeri & Carroll, 2008). Therefore, wild-type cytokines must be administered frequently in order to obtain a meaningful therapeutic effect, like in the case of rhuIL-4 in psoriasis. In addition, the clinical effects of stand-alone regulatory cytokines may have been disappointing because of some pro-inflammatory activities such as upregulation of Fc-receptors in the case of IL-10. One may also speculate that cytokines in general don't have their main biologic effects as stand-alone mediators but rather work in a network of multiple cytokines. Adding or eliminating only one cytokine may be bypassed in the complexed network of cytokines by others.

1.3 | Strategies to overcome limitations of stand-alone cytokine therapy

1.3.1 | Increasing the half-life of clearance from the plasma. Renal clearance of proteins is, amongst others, dependent on their molecular mass and negative charge. A well-known and successful strategy to increase the molecular size of cytokines is the covalent conjugation to polyethylene glycol (PEG) (Eliason, 2001). PEGylation changes the chemical and physical characteristics of molecules to improve their pharmacokinetics. In addition to the increased retention time in the blood and a decreased renal clearance, other advantages of conjugation with PEG include improved drug stability, decreased proteolysis, and less immunogenicity (Veronese & Mero, 2008). A disadvantage of PEGylation is that it may decrease the functional activity of therapeutic proteins by interfering with receptor binding. In addition, product heterogeneity may sometimes pose a challenge. However, site-directed PEGylation can largely overcome these problems. PEGylation has been successfully used to improve the pharmacokinetics of cytokines such as G-CSF, interferon-α, IL-10 and others, allowing less frequent injections in patients.

PEGylated IL-10 was initially developed for cancer and neuropathic pain treatment (Mumm et al., 2011; Soderquist et al., 2010). The molecular weight of PEGylated IL-10 was increased with 20 kDa and the molecule showed prolonged analgesic effect compared to wild type IL-10 when administered intravenously (Soderquist et al., 2010).

Another method to increase the half-life of cytokines is to fuse a cytokine to the constant heavy chain region of an immunoglobulin molecule (often IgG): the Fc fragment. The fusion of cytokines to an Fc fragment increases the molecular weight several-fold (Jazayeri & Carroll, 2008), resulting in a prolonged half-life. Furthermore, adding Fc tails also improves half-life due to the binding to the neonatal Fc receptor (FcRn), which enables recycling of the Fc fusion protein in the circulation (van Witteloostuijn et al., 2016). One example is murine IL-10/Fc, consisting of two IL-10 monomers fused to a mutated, noncytolytic Fcγ2a fragment with two hinge regions. IL-10/Fc showed a prolonged circulating half-life compared to rhuIL-10. Furthermore, IL-10/Fc was as effective as rhuIL-10 in preventing septic shock in LPS-induced sepsis in mice (Zheng et al., 1995). So far, efforts using Fc-coupling or PEGylation in combination with regulatory cytokines have only been investigated in the preclinical phase.

Finally, yet another approach to increase molecular size to prolong the half-life of a cytokine is glycoengineering, which provides the additional advantage of introducing more negative

charges through sialic acids, further decreasing renal clearance of the protein. This approach has been successfully used for erythropoietin (Kiss et al., 2010).

1.3.2 | Combination therapy with regulatory cytokines. Cytokines have complementary and synergistic effects and preferably act in networks to achieve optimal effects (Opal & DePalo, 2000; Schmitz et al., 2011; Schooltink & Rose-John, 2002). It is, therefore, attractive to combine cytokines to increase their therapeutic effect (van Roon et al., 2002). For example, the combination of IL-4 and IL-10 is superior to the stand-alone cytokines to inhibit pro-inflammatory cytokine production by cultured synovial tissue of RA patients (van Roon et al., 1996). Moreover, in a murine collagen-induced arthritis model, combination therapy with IL-4 and IL-10 to inhibit cartilage destruction is more effectively than the sum of the effects of IL-4 and IL-10 alone, suggesting synergistic effects (Joosten et al., 1997). This synergistic effect might be explained by the fact that these cytokines mutually inhibit each other's pro-inflammatory effects (van Roon et al., 2001). For example, the increased Fc-receptor expression on human monocytes by IL-10 (te Velde et al., 1992; van Roon et al., 2003), is neutralized by IL-4, which reduces expression of FcγRI/II/III on LPS-stimulated monocytes. Conversely, whilst IL-4 induces expression of FcεR on monocytes (Vercelli et al., 1988), IL-10 leaves FcεR unaltered. Synergy of IL-4 and IL-10 is also observed in cell-mediated immunity. *In vivo*, the combination of IL-4 and IL-10 more effectively inhibits a Th1 response to Leishmania parasites than IL-4 and IL-10 alone (Powrie et al., 1993 Bhowmick et al., 2014). The drawback of a combination therapy with several cytokines, however, is its high development costs. Furthermore, the short half-life of cytokines, an important limitation of therapy with stand-alone cytokines, is not addressed by a combination therapy.

1.4 | Cytokine fusion protein of IL-4 and IL-10

Strategies to combine regulatory cytokines and to increase their half-life have been explored in order to improve therapeutic efficacy. In this thesis, we present IL4-10 fusion protein, in which these strategies are combined. IL4-10 fusion protein (IL4-10 FP) consists of the regulatory cytokines IL-4 and IL-10. In the fusion protein, these cytokines are covalently linked to each other using a linker sequence rich in serine and glycine residues to allow maximal flexibility between both cytokine moieties (Figure 2). This thesis describes the production and characterization of IL4-10 FP, as well as its therapeutic potential in inflammatory diseases.

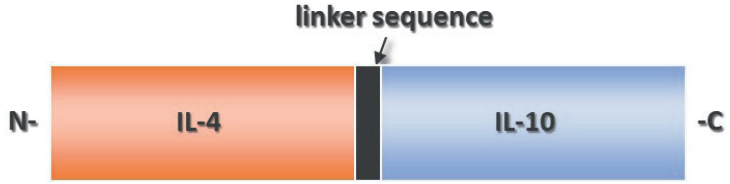

Figure 2: Schematic overview of the IL4-10 fusion protein and its amino acid sequence.

In **Chapter 2** we describe the capacity of IL4-10 FP to shift multiple pro-inflammatory pathways towards immune regulation in several *in vitro* and *ex vivo* systems, and to inhibit pro-inflammatory activity in an *in vivo* arthritis model.

In **Chapter 3** we evaluate the effects of IL4-10 FP in osteoarthritis (OA) models. OA is a rheumatic disease in which inflammation, cartilage degeneration and pain contribute to disease severity. We studied the effectiveness of IL4-10 FP on each of these three pathophysiological phenomena in *in vitro, ex vivo* and *in vivo* models.

In **Chapter 4** we describe the ability of IL4-10 FP to attenuate inflammatory pain in several *in vivo* models. The effect of IL4-10 fusion protein was studied in a carrageenan-induced inflammatory pain and in a neuropathic pain model, in comparison to IL-4, IL-10 and a combination therapy of IL-4 and IL-10.

The promising initial results of IL4-10 FP described in the previous chapters, tempted us to modulate IL4-10 FP to increase its bioavailability.

In **Chapter 5** we study the effect of differential sialylation of the glycans of IL4-10 fusion protein on *in vitro* and *in vivo* bioactivity and pharmacokinetic parameters.

Next, glycoengineering as strategy to increase the plasma half-life of our IL4-10 FP was studied in **Chapter 6**. Additional N-linked glycosylation sites were introduced in the human IL4-10 FP sequence, based on comparison of the homology with several animal species.

Batches of human IL4-10 FP contain monomers and non-covalently linked dimers. **In chapter 7** we describe whether the functional activity of the IL-10 moiety of IL4-10 FP is dependent on the non-covalent dimerization of the fusion protein, by isolating and testing both monomeric and dimeric IL4-10 FP preparations.

Finally, in **Chapter 8** I summarize and discuss the above findings and provide suggestions for future studies.

1.5 | References

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Chapter 2

IL4-10 fusion protein: a novel immunoregulatory drug combining activities of IL-4 and IL-10

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Abstract

Objective. To test the capacity of a newly developed fusion protein of IL-4 and IL-10 (IL4-10 FP) to shift multiple pro-inflammatory pathways towards immune regulation, and to inhibit pro-inflammatory activity in arthritis models.

Methods. The effects of IL4-10 FP in comparison with IL-4, IL-10 and IL-4 plus IL-10 on pro- and anti-inflammatory mediators, T-cells, and Ig receptors in favour of immunoregulatory activity were studied. In addition, the capacity of IL4-10 FP to inhibit pro-inflammatory activity in *ex vivo* and *in vivo* arthritis models was investigated.

Results. IL4-10 FP robustly inhibited pro-inflammatory cytokine (IL-1β, TNFα, IL-6, and IL-8) production in whole blood cultures, mediated by both the IL-10 and the IL-4 moiety. IL4-10 fusion protein induced IL-1RA production, and preserved sTNFR levels, strongly increasing IL-1RA/IL-1β and sTNFR/TNFα ratios. In addition, IL4-10 FP strongly inhibited Th1/Th17 cytokine secretion, while maintaining FoxP3 expression and up-regulating Th2 activity. In addition, while largely leaving expression of activating FcγRI, III and FcεR receptors unaffected it significantly shifted the FcγRIIa/FcγRIIb ratio in favour of the inhibitory FcγRIIb. Moreover, IL4-10 FP robustly inhibited secretion of pro-inflammatory cytokines by rheumatoid arthritis synovial tissue and suppressed experimental arthritis in mice, without inducing B-cell hyperactivity.

Conclusion. IL4-10 fusion protein is a novel drug signalling cells to induce immunoregulatory activity, that overcomes limitations of IL-4 and IL-10 stand-alone therapy, and therefore has therapeutic potential for inflammatory diseases such as rheumatoid arthritis.

2.1 I Introduction

Lack of efficacy by some biologics may reflect that targeting one mediator of inflammation is insufficient to overcome redundancy of the immune system. Therefore, a multi-target approach constitutes an attractive therapeutic option. Since Interleukin-4 (IL-4) and Interleukin-10 (IL-10) attenuate multiple inflammatory processes via distinct, overlapping, and complementary mechanisms, both cytokines are promising candidates for treatment of inflammatory diseases [1,2]. However, clinical trials with human IL-4 or IL-10 as stand-alone drugs did not fulfil the promise raised in preclinical studies. Patients with rheumatoid arthritis (RA) and psoriasis only modestly improved upon administration of IL-4 or IL-10 in phase-I/II studies, although both cytokines were well tolerated [3,4].

Disappointing results of IL-4 and IL-10 as stand-alone drugs in clinical trials may reflect their poor bioavailability or lack of synergy with other anti-inflammatory cytokines. Indeed, IL-4 and IL-10 synergistically inhibit many inflammatory activities and induce anti-inflammatory mechanisms such as IL-1RA induction [2,5,6]. IL-4 and IL-10 also synergize in experimental models for arthritis, and - importantly - also mutually neutralize their pro-inflammatory effects, such as upregulation of activating Fc receptors [2,7,8,9]. Thus, a combination therapy of IL-4 and IL-10 is potentially superior to stand-alone therapy. However, high costs virtually exclude development of a combination therapy. Moreover, a combination therapy does not address the limited bioavailability of cytokines due to their relatively low molecular weight.

To solve these limitations we designed a prototype of a novel class of cytokines: fusion-proteins of two anti-inflammatory and/or regulatory cytokines, combining activities of these cytokines into one molecule. We generated a glycosylated IL4-10 fusion protein (IL4-10 FP, patent application No 61/556,843 [10]), in its dimeric form ~70 kDa, and here demonstrate preserved additive function and strong immunosuppressive activity *in vitro*, *ex vivo* and *in vivo*.

2.2 I Methods

2.2.1 I Human IL4-10 fusion protein. The construction and production of human IL4-10 fusion protein has been described previously [10]. Briefly, IL4-10 fusion protein was produced by transient co-transfection of HEK293-cells with a vector encoding the peptide-sequence and a vector containing a sialyltransferase transgene, ensuring optimized sialylation of the fusion protein (U-ProteinExpress BV, Utrecht, the Netherlands).

2.2.2 I Mouse IL4-10 fusion protein. Mouse IL4-10 FP was produced via transient transfection of HEK293E cells with a pUPE-expression vector containing a cystatin signal sequence and synthetic cDNA coding for the peptide sequence (U-ProteinExpress BV). Glycan-capping with sialic acid residues was optimized by co-transfection with a vector carrying the beta-galactoside-alpha2,3-sialyltransferase5 (SIAT9, homo sapiens) transgene. Cells were cultured in FreeStyle medium (Invitrogen, Carlsbad, CA, USA) with 0.9% primatone, ~0.04% FCS, total volume 1L, for 5 days. Supernatant was collected, concentrated 10-fold (10kDa QuixStand hollowfiber cartridge, GE-Healthcare, Waukesha, US), and diafiltrated against PBS pH7.4. Precipitates were removed by passing over a glass-syringe filter. Supernatant was stored in aliquots at -80˚C. Mouse IL4-10 fusion protein was purified using a 2-steps purification method. First, a cation exchange chromatography (HiTrap SP FF 1ml column; GE-Healthcare) on an AKTAprime Plus (GE-Healthcare) was performed. A low-salt buffer (bufferA: 10mM phosphate, pH6.5) was used as running buffer, a high-salt buffer (bufferB: bufferA with 1M NaCl) was used as elution buffer. Before injection 50ml supernatant was diluted 1:5 in bufferA. After loading the supernatant on the column, IL4-10 fusion protein was eluted using a linear gradient to 100% bufferB (in 20min, flow rate 1ml/min). 1ml fractions were collected and analysed for IL4-10 fusion protein content using IL-4 and IL-10 ELISA. Fractions containing IL4-10 fusion protein were pooled, diafiltrated against PBS pH7.4 and concentrated a factor 10 to yield a ~400 µg/ml (total volume 500 µl) mIL4-10 fusion protein batch. Thereafter, a size exclusion chromatography (SEC) on a high-performance liquid chromatography (HPLC) system (Shimadzu) was done. For this purpose, cation-exchange fractions containing IL4-10 fusion protein were pooled and re-buffered in SEC running buffer (50mM phosphate, pH7.0, 0.5M NaCl). The column (BioSuite125 4µm UHR SEC; Waters) was calibrated with reference proteins. Semi-pure IL4-10 FP was loaded in several runs (10x 50 µl) with a flowrate of 0.35ml/min (35bar). 175µl fractions were collected and fractions of all runs were pooled on basis of the A280 chromatogram. Upon analysis of fractions in ELISA, IL4-10 FP containing

fractions were pooled, diafiltrated against PBS pH7.4 and concentrated to yield a ~80 µg/ml mIL4-10 fusion protein batch.

2.2.3 I IL4-10 fusion protein detection. Both human and mouse IL4-10 fusion protein were detected in both the IL-4 and the IL-10 ELISA to determine the batch concentration. The ELISA's were performed according to manufacturer's instructions (hIL4 and hIL10 Pelipair ELISA; Sanquin; mIL4 and mIL10 DuoSet ELISA; R&D Systems). Regarding the hIL-4 and hIL-10 ELISA, however, a sample buffer of PBS containing 0,1% Tween-20 was used instead. Results were compared with recombinant IL-4 and IL-10 (standard) provided by the manufacturer.

2.2.4 I Cell and tissue cultures. Blood from healthy human controls was obtained from our in-house donor service. Synovial tissue was obtained from RA patients that underwent total knee arthroplasty. The study was approved by the medical ethics review board of the UMCU and all patients and healthy volunteers gave informed consent.

*LPS-stimulated whole blood culture***.** Heparinized blood obtained from healthy volunteers was diluted 1:10 in RPMI1640 medium (Thermo Fisher Scientific), supplemented with penicillin/streptomycin (PAA Laboratories). Lipopolysaccharide (LPS; Sigma) was added at 10 ng/ml. IL4-10 fusion protein and controls recombinant human IL-4 and IL-10 (eBioScience) were simultaneously added and titrated at equimolar concentrations, ranging from 0.01-3 nM. The concentration of 1.47 nM of IL4-10 fusion protein (50 ng/ml) was chosen for further experiments. Receptor blocking antibodies against IL-4 receptor (anti-IL4R; R&D Systems) or IL-10 receptor (anti-IL10R; BioLegend) were added at 10 µg/ml and 20 µg/ml, respectively. After 18h incubation at 37°C, 5%CO_2, supernatant was collected and stored at -80°C. The mouse whole blood assay was performed in the same way, using mouse blood from sacrificed BALB/c control-mice.

SEB-stimulated PBMC culture. Peripheral blood mononuclear cells (PBMCs) from blood of healthy donors were isolated with Ficoll-paque (Stem Cell Technologies), cultured in RPMI GlutaMAX (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% P/S (PAA Laboratories), in addition of Staphylococcal enterotoxin-B (SEB; Sigma, 0.1 ng/ml) and 1.47 nM IL-4, IL-10, the combination or IL4-10 fusion protein. Final culture conditions: $1*10^6$ cells/ml, 0.5ml/well, 37°C, 5%CO_2, 42h. Supernatant was collected and stored at -80°C.

Monocyte Culture. Monocytes were isolated from healthy donor PBMCs using CD14+ AutoMACS (Miltenyi Biotech) purification, cultured in RPMI GlutaMAX with 10% FBS and 1%

P/S. To study Fc receptor expression, cells were cultured unstimulated, in addition of 1.47 nM IL-4, IL-10, the combination or IL4-10 fusion protein. Dose-dependent inhibition of TNFα by IL4-10 FP (0.01-3 nM) was studied using LPS (10 ng/ml) stimulated cells. Final culture conditions: 1*106 cells/ml, 0.5ml/well, 37°C, 5%CO_2, 42h (and additional 18h for titration curves of IL4-10 FP). For flow cytometry (to study FcR expression), cells were put on ice 30min before harvesting. For qPCR (FcR expression), cell lysates were prepared using RLTplus lysis buffer. Supernatants were collected and stored at -80°C.

Synovial tissue culture. Weighed macroscopically identical synovial tissue pieces obtained from knee joints of RA patients were cultured for 72h at 37°C, 5%CO_2 in the presence or absence of 1.47 nM IL4-10 fusion protein in RPMI GlutaMAX, with 10% human AB-serum and 1% P/S. Supernatant was collected and stored at -80°C.

2.2.5 I Proliferation assay. PBMCs were stimulated with SEB (0.1 ng/ml) and cultured (1*10^6 cells/ml) in the presence or absence of IL4-10 fusion protein; total volume 200µl/well, 37°C, 5%CO_2, 42h. ^{3}H-thymidine was added (5mCi/ml; NEN Life Science Products) during the last 18h. Cells were harvested and ^{3}H-thymidine incorporation measured by liquid scintillation counting. Incorporated tritium was counted and expressed in counts per minute.

2.2.6 I Cytokine & antibody assessments. Supernatants were analysed for TNFα, IL-1β, IFNγ, IL-5, IL-6, IL-8,IL-17, IL-1RA and sTNFR2 by Multiplex (Luminex) and/or ELISA, according to manufacturer's instructions (Multiplex kits: hIFNγ (BD Biosciences), hIL5, hIL17 (R&D Systems); ELISA kits: hTNFα Pelipair ELISA (Sanquin), IL6, IL8 CytoSet ELISA (Invitrogen (Thermo Fisher Scientific), IL1RA, sTNFR2, DuoSet ELISA (R&D Systems). Mice sera were analyzed for IgG and IgE, using ELISA kits (DuoSet; R&D Systems), according to manufacturer's instructions.

2.2.7 I Flow cytometry. Viable monocytes were gated based on forward-sideward scatter and a viability dye. Fcγ receptor (FcγR) and Fcε receptor (FcεR) expression was evaluated using labelled antibodies against FcγRI (anti-CD64-PECy7, Clone 10.1; BD Biosciences), FcγRIIa (anti-CD32a-FITC, Clone IV.3; Stem Cell Technologies), FcγRIIb (anti-CD32b-FITC, Clone 2B6; kind gift from Genmab B.V.), FcγRIII (anti-CD16-BV510, Clone 3G8; BD Biosciences), and FcεRII (anti-CD23-PE, Clone EBVCS-5; Biolegend). FoxP3 and Ki-67 staining was performed using an intracellular staining kit (eBioscience). Percentages positive/negative cells were based on set markers using isotype controls. Cell acquisition was done using the FACSCanto (BD Biosciences), analysis using FlowJo (Tree Star Inc).

2.2.8 I Gene expression by quantitative PCR. Expression of FcγRIIa and FcγRIIb2 genes in relation to GUSB as housekeeping gene was evaluated. Total RNA extraction from the cultured monocytes (as described above) was performed with the AllPrep Kit according to manufacturer's instructions (Qiagen). Qubit (Thermo Fisher Scientific) was used to quantify the amount of total RNA extracted. RNA was reverse transcribed with the Superscript IV kit (Thermo Fisher Scientific), according to manufacturer's instructions. Duplicate real-time PCR reactions were performed in the QuantStudio 12k Flex system (Invitrogen (Thermo Fisher Scientific)), using specific FcγRIIa, FcγRIIb2 and GUSB primers with SyBR Select Master Mix (Thermo Fisher Scientific), according to manufacturer's instructions. Reactions were performed with the following thermal cycling profile: 2min at 50°C followed by 5min at 95°C, plus 40 cycles of 15s at 95°C and 1min at 60°C. Real-time qPCR data were normalized to the expression of GUSB and the relative gene expression of mRNA was calculated by using the formula: $2^{-\Delta Ct}$ where $\Delta Ct = Ct_{mean}$ sample – Ct_{mean} GUSB.

Primers used for qPCR:

FCγRIIa (FW: ACCTGTGGCTGCTTCAACCATTG; RV: ATCCACGGGGGCTCAAGTTTCA)

FCγRIIb2 (FW: AAAGCGGATTTCAGCCAATC; RV: CAAGACAATGGAGACTAAATACGGT)

GUSB (FW: CACCAGGGACCATCCAATACC; RV: GCAGTCCAGCGTAGTTGAAAAA)

2.2.9 I Proteoglycan-induced arthritis (PGIA). Human proteoglycan (PG) was dissolved at a concentration of 2.5 mg/ml in PBS and emulsified in an equal volume of the synthetic adjuvant dymethyl-dioctadecylammoniumbromide (DDA; Sigma) in PBS. 24- week old female BALB/c wild type mice (Charles River Laboratories Inc.) were used for immunization. The experiment was performed in accordance with the guidelines of the animal ethical committee. All mice were immunized intraperitoneal (i.p.) with 200 µl of the emulsion (250 µg PG and 2 mg DDA), on day 21 mice were boosted with 200 µl i.p. injection of the same emulsion. Mice were examined for onset and severity of disease in a blinded manner. Arthritis symptoms were graded by the following scoring system: grade 0, normal appearance; grade 1, slight erythema/edema (1-3 digits); grade 2, erythema/edema in more than 3 digits, or mild swelling in ankle/wrist joint; grade 3, erythema/edema in entire paw; grade 4, massive erythema/edema of entire paw extending into proximal joints, ankylosis, loss of function. Each limb was graded, giving a maximum possible score of 16 per mouse. Arthritis incidence and severity was assessed from day 21 onwards and was defined by a single affected paw of at least 1 point. Only upon onset of arthritis at day 28, mice were included in the study and were

divided in 2 groups on basis of arthritis scores. Mice were treated with intravenous injections of 200 ng mouse IL4-10 fusion protein or PBS as a control. Injections were given every day for a period of 4 days, starting at day 28. Mice were sacrificed on day 35. Blood was retrieved and processed for measurement of IgG and IgE in sera of all mice.

2.2.10 I Statistical analysis. Cytokine concentrations in supernatants, Fc receptor expression, proliferation and expression of FoxP3 were analysed by the Wilcoxon signed rank test, comparing the (stimulated) medium condition to the IL4-10 FP condition. Statistical analysis of the mIL4-10 FP effect on PGIA was analysed by an unpaired t-test, since data were normally distributed (D'Agostino-Pearson normality test).

2.3 I Results

2.3.1 I IL4-10 fusion protein robustly suppresses pro-inflammatory cytokine production and alters the balance between pro-inflammatory cytokines and their naturally occurring inhibitors. Recombinant human IL4-10 fusion protein dose-dependently inhibited TNFα, production in LPS-stimulated whole blood cultures (Figure 1A). Monocytes are assumed to be the main targets in our LPS-stimulated whole blood assay. Here we demonstrate that IL4-10 FP equally effective inhibited TNFα production by isolated monocytes as in whole blood (Figure 1B). In addition, prolonged exposure of LPS-stimulated whole blood to IL4-10 FP (18 vs 42 hours) resulted in stronger inhibition of TNFα production (Figure 1B). The effect of IL4-10 fusion protein on TNFα production was abolished by receptor-blocking antibodies against the IL-4-receptor (anti-IL-4R) or the IL-10 receptor (anti-IL-10R), indicating that the effect of IL4-10 FP is mediated by both moieties of the protein (Figure 1C). IL4-10 FP also strongly inhibited IL-1β production (Figure 1D). Importantly, IL4-10 fusion protein left activation-induced production of IL-1RA and sTNFR, naturally produced antagonists of IL-1β and TNFα, largely unaffected, strongly increasing IL-1RA/IL-1β and sTNFR/TNFα ratios (Figure 1D).

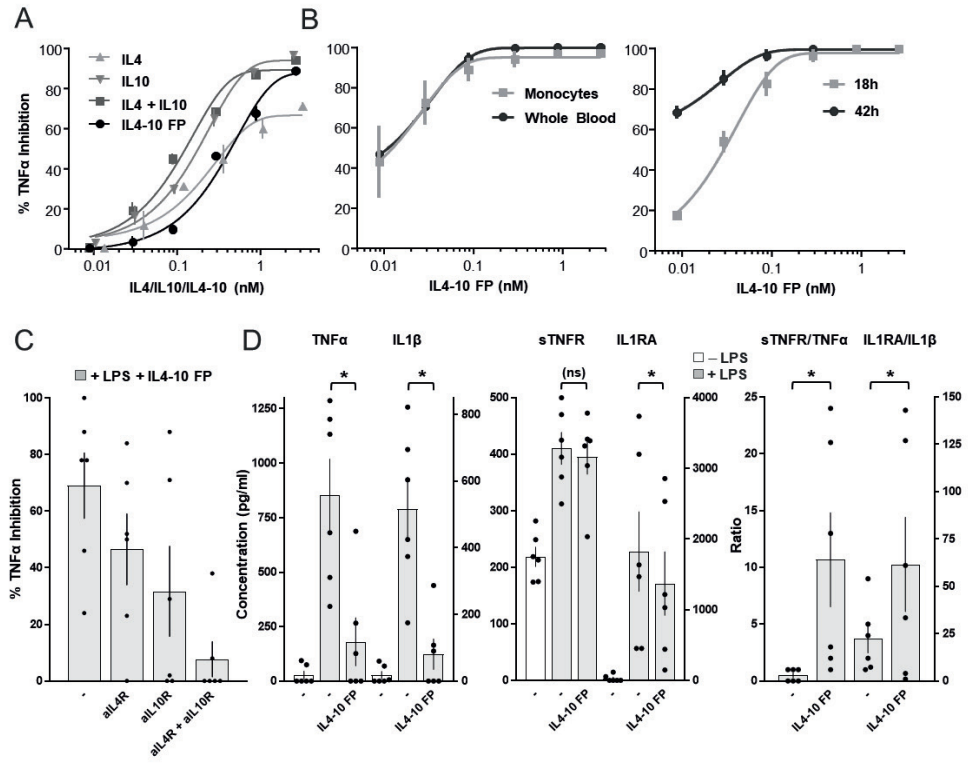

Figure 1. IL4-10 fusion protein alters the balance of TNFα and IL-1β and their naturally occurring inhibitors *in vitro*. A) Diluted (1:10 v/v) heparinized blood from healthy volunteers was incubated 18 hours with LPS to induce TNFα release. The capacity of different concentrations of human IL4-10 fusion protein (FP) to inhibit TNFα production in LPS-stimulated whole blood cell cultures was compared to IL-4 and IL-10 (n=4). **B)** The potency of IL4-10 FP to inhibit LPS-induced TNFα production by whole blood and isolated monocytes (n=4) was compared. In addition, the capacity of prolonged incubation (42 hours) to enhance the potency of IL4-10 FP to inhibit LPS-induced TNFα production in whole blood was studied (n=2). **C)** To indicate the requirement of both the IL-4 and IL-10 moiety IL4-10 FP-mediated inhibition (1.47 nM) of TNFα production in whole blood was tested upon blockade of the IL-4 and IL-10 receptor (IL-4R and IL-10R) using monoclonal antibodies. **D)** Next to TNFα, the potency of IL4-10 FP to regulate LPS-induced IL-1β production as well as naturally occurring inhibitors IL-1RA and soluble TNFR in whole blood was studied. In addition, the potency to skew the balance of IL-1RA/IL-1β and sTNFR/TNFα was studied. Bars (containing individual data points) and graphs represent mean and SEM. * indicates a statistically significant difference of $p<0.05$.

2.3.2 I IL4-10 fusion protein skews Th1/Th17 activity towards Th2 activity and sustains regulatory T-cells. Superantigen SEB induced IFNγ, IL-17 and IL-5 production, lymphocyte (^{3}H Thymidin incorporation) and CD4 T-cell proliferation (Ki-67 expression) in PBMC cultures, which was associated with increased numbers of FoxP3-expressing regulatory T-cells (Figure 2). Production of the pro-inflammatory cytokines IFNγ, and IL17, indicative of Th1 and Th17 T-cell activity, respectively, were reduced by IL4-10 FP, which effect was comparable to that of IL-10 alone or the combination of IL-4 and IL-10 (Figure 2A). Opposite to this down regulation, IL-5 production, indicative of Th2 activity, was upregulated by IL-4 alone and by IL4-10 FP. Pooled data demonstrated that IL4-10 FP significantly inhibited antigen-induced Th1 and Th17 cytokine secretion (IFNγ, IL-17) and upregulated Th2 activity (IL-5) (Figure 2B). In addition, IL4-10 FP sustained proliferation and the number of FoxP3-expressing regulatory T-cells (Figure 2C).

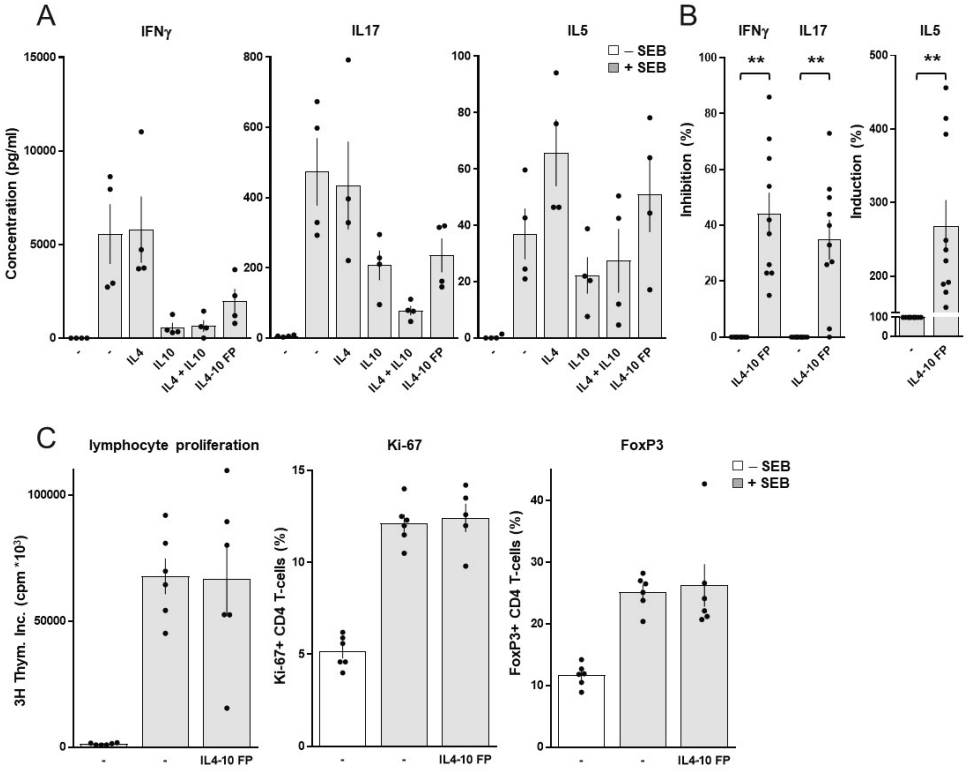

Figure 2. IL4-10 FP skews Th1/Th17 activity towards Th2 activity and sustains regulatory T-cells. Blood mononuclear cells were cultured with superantigen SEB (42 hours) in the presence of IL-4, IL-10, the combination, or IL4-10 FP (all 1.47 nM). **A)** IL4-10 FP-induced regulation of Th1-, Th2- and Th17-associated cytokines (IFNγ, IL-5 and IL-17, respectively) was studied (n=4). **B)** T-cell cytokine production by SEB-activated PBMCs upon IL4-10 FP was measured in additional six donors. Pooled data (n=10), expressed as % change vs medium-only cultures are shown. **C)** In addition, the effect of IL4-10 FP on proliferation of lymphocytes (^{3}H Thymidine incorporation) and CD4 T-cells (percentage of Ki-67 expressing cells) and on the percentage of regulatory T-cells (percentage of FoxP3 expressing cells, n=6) was studied. Bars (containing individual datapoints) represent mean and SEM. ** indicates a statistically significant difference of $p<0.01$.

2.3.3 I IL4-10 fusion protein minimally affects IgG and IgE receptor levels. Consistent with literature we found that IL-4 and IL-10 differentially regulate Fcγ and Fcε receptor expression on monocytes. IL-4 inhibited expression of activating Fcγ receptors I, IIa and III and upregulated FcεR, whereas IL-10 increased FcγRI, IIa, IIb and III, not affecting FcεR (Figure 3A and C). The combination of IL-4 and IL-10 largely normalized Fcγ and FcεRs, confirming previous literature showing that IL-4 controls IL-10 upregulated FcRs and vice versa. Pooled data revealed that IL4-10 FP did not significantly alter the expression of monocyte activating FcγRI, IIa and IIb and minimally increased FcγRIII and FcεR expression as compared to control cultures (Figure 3B,D). Interestingly, IL4-10 FP significantly changed the ratio of FcγRIIb/IIa in favour of the inhibitory FcγRIIb (Figure 3D). This was confirmed by measuring mRNA level of either receptor (Figure 3E).

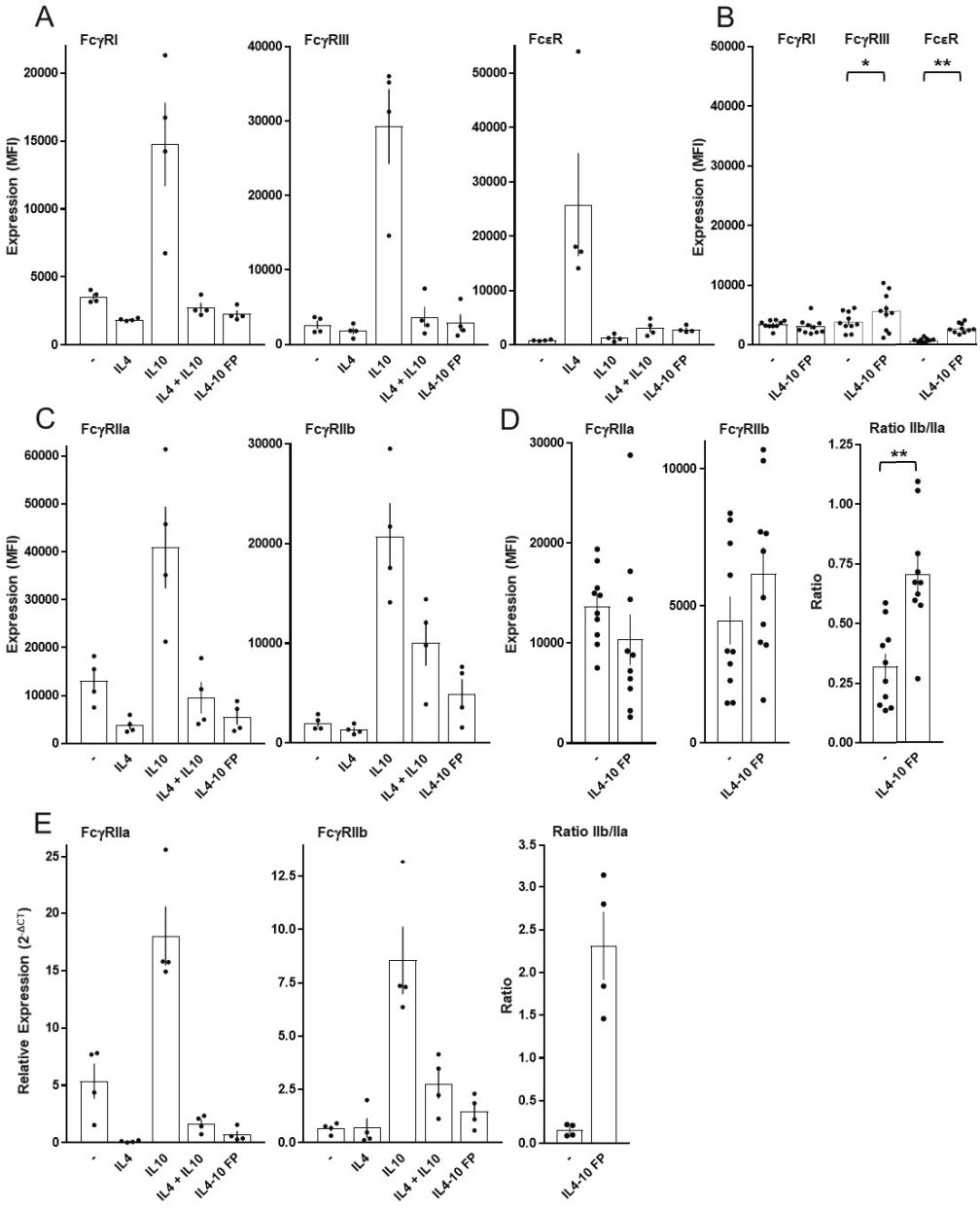
A
FcγRI
FcγRIII
FcεR
Expression (MFI)
-
IL4
IL10
IL4 + IL10
IL4-10 FP
B
FcγRI
FcγRIII
FcεR
*
**
Expression (MFI)
-
IL4-10 FP
C
FcγRIIa
FcγRIIb
Expression (MFI)
D
FcγRIIa
FcγRIIb
Ratio IIb/IIa
Expression (MFI)
Ratio
E
FcγRIIa
FcγRIIb
Ratio IIb/IIa
Relative Expression (2-ΔCT)
Ratio

Figure 3. IL4-10 fusion protein sustains normal expression of immunoglobulin receptors on monocytes. Isolated monocytes were cultured (42 hours) in the presence of IL-4, IL-10, the combination, or IL4-10 FP (all 1.47 nM). **A)** The capacity of IL4-10 FP and the individual and combined cytokines to (differentially) regulate different subclasses of FcγRs (I and III) and FcεR expression was measured using flow cytometry (n=4). **B)** Expression of the FcγR subclasses and FcεR on monocytes upon IL4-10 FP was measured in an additional six donors. Pooled data are shown (n=10). **C)** The effect of IL4-10 FP, the individual and combined cytokines on the activating FcγRIIa and inhibitory FcγRIIb was also studied using flow cytometry (n=4). **D)** In an additional six donors, regulation of IL4-10 FP on FcγRIIa and FcγRIIb expression as well as their ratio was measured. Pooled data are shown (n=10). **E)** The effect of IL4-10 FP on FcγRIIa and FcγRIIb gene expression as well as their ratio was additionally measured using quantitative PCR (n=4). Bars (visualizing individual data points) represent mean and SEM. * and ** indicate statistical significant differences of $p<0.05$ and 0.01, respectively.

2.3.4 I IL4-10 fusion protein inhibits *ex vivo* cytokine production by synovial tissue of arthritic patients and inhibits experimental arthritis. In line with the inhibition of IL-1β and TNFα (Figure 1), human IL4-10 FP strongly inhibited secretion of pro-inflammatory cytokines IL-6 and IL-8 in whole blood cultures triggered by LPS (Figure 4A). In addition, spontaneous production of IL-6 and IL-8 by RA synovial tissue *ex vivo* was markedly inhibited by IL4-10 FP (Figure 4B). Finally, to further explore the therapeutic potential of IL4-10 fusion protein we assessed its potency to inhibit experimental proteoglycan-induced arthritis. For *in vivo* mouse studies, a mouse recombinant IL4-10 fusion protein (mIL4-10 FP) was developed to avoid induction of neutralizing antibodies against the human fusion protein. Mouse recombinant IL4-10 FP dose-dependently inhibited TNFα production in mouse whole blood assays, and turned out to be more potent than human IL4-10 FP (Figure 4C). mIL4-10 fusion protein significantly suppressed disease severity in established experimental arthritis in mice (Figure 4D), without inducing B-cell hyperactivity, as measured by IgG and IgE serum levels that were not changed upon IL4-10 FP treatment (Figure 4E).

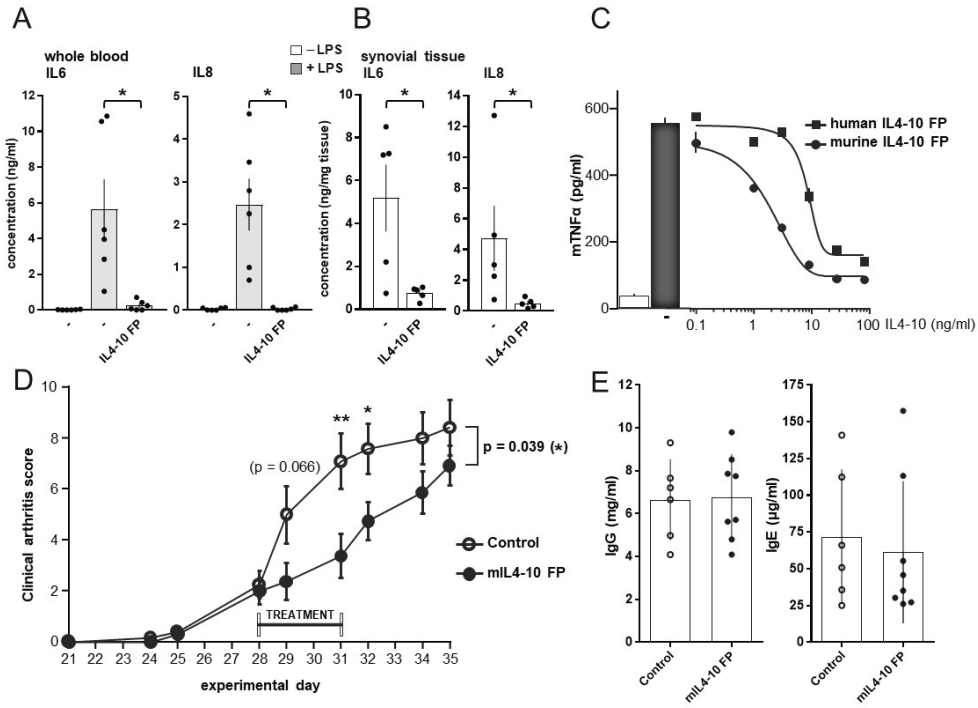

Figure 4. IL4-10 fusion protein inhibits inflammatory activity of *ex vivo* cultured RA synovial tissue and suppresses established experimental arthritis. A) LPS-induced secretion of IL-6 and IL-8 in whole blood cultures was measured upon treatment with IL4-10 FP (n=6). **B)** Furthermore, IL4-10 FP effects on spontaneous release of IL-6 and IL-8 from RA synovial tissue explants were studied (n=5). **C)** For *in vivo* mouse studies, a mouse recombinant IL4-10 fusion protein (mIL4-10 FP) was developed. The potency of mIL4-10 FP and hIL4-10 FP to inhibit TNFα release in a LPS-stimulated mouse whole blood assay was investigated. **D)** The ability of mIL4-10 FP upon intravenous injection to inhibit arthritis severity in established proteoglycan-induced arthritis in mice was studied (mIL4-10 FP was administered on 4 subsequent days, starting from day 28). **E)** mIL4-10 FP effects on B cell activity were studied by measuring serum IgG or IgE levels using ELISA (n=6 for control/PBS and n=8 for mIL4-10 FP group). Bars (visualizing individual datapoints) and graphs represent mean and SEM. * and ** indicate statistically significant differences of $p<0.05$ and 0.01, respectively.

2.4 I Discussion

We here show that IL4-10 fusion protein inhibits the production of a number of key pro-inflammatory cytokines in LPS-stimulated whole blood cultures. It also inhibits production of superantigen-induced pro-inflammatory Th1/Th17 activity, while preserving regulatory T-cell activities. IL4-10 FP also shifts the balance of IL1β and TNFα towards their naturally occurring antagonists IL-1RA and sTNFR, and largely preserves normal Fcγ receptor expression by monocytes. Moreover, IL4-10 FP decreases pro-inflammatory cytokine production from *ex vivo* synovium cultures of RA patients and inhibits established experimental arthritis.

While inhibiting production of multiple monokines and Th1/Th17 cytokines, IL4-10 fusion protein *in vitro* clearly skewed T-cell activity towards Th2 activity as witnessed by increased levels of IL-5 (and IL-13, data not shown) and FcεR expression. Similar polarization towards Th2 activity due to atopic diseases is associated with good clinical outcome of diseases such as RA [11]. It is unclear whether these beneficial *in vitro* effects of IL4-10 FP can be extrapolated to *in vivo* conditions. Although obvious care should be taken with respect to induction of atopic responses, apparently the generation of allergic reactions requires additional triggers, since in clinical trials with IL-4 or IL-10, no allergic-type responses were induced [11], not even upon high doses of IL-4 [12]. In line herewith, mIL4-10 FP inhibits experimental arthritis without inducing B-cell hyperactivity, and in particular IgE concentrations are not significantly altered. The exact mechanisms behind this lack of B-cell stimulation is unclear, but might be related to the activity of IL-10 to inhibit IgG and IgE production by (IL-4/T- cell activated) B-cells if in the context of antigen-presenting cells such as monocytes, as has previously been demonstrated [16].

IL4-10 fusion protein may be used to target Th1/Th17 driven inflammatory diseases like RA and spondyloarthropathies, but also to target inflammation in conditions associated with monokine production (IL-1β, TNFα, IL-6) such as osteoarthritis, and many more. Essentially, all diseases considered for IL-10 treatment in the past may be targets for IL4-10 fusion protein. Compared with IL-10, IL4-10 FP has several advantages. First, some adverse effects of IL-10 are prevented by the IL-4 moiety of the cytokine. For example, activating FcγRs are upregulated by IL-10, which enhances immune complex-mediated events in IL-10 treated RA patients [9,13]. IL-4 counteracts the increased expression of FcγRs by IL-10 [2]. This counteracting effect results in constant levels of FcγRs [2] as we also observed for IL4-10 FP.

In addition, IL4-10 FP skews the balance of activating FcγRIIa to inhibitory FcγRIIb. Second, IL4-10 fusion protein combines the activities of two different anti-inflammatory cytokines in a single molecule, including synergistic activities that have previously been demonstrated [2,5]. Therefore, IL4-10 FP may have more potential than stand-alone IL-4 or IL-10 in some of these diseases, for example in arthritis [2,5,8,14]. Third, IL4-10 fusion protein has an improved bioavailability as compared to their natural counterparts due to a larger molecular weight. Proteins with a molecular weight <60 kDa are filtered through the basal membrane in the glomeruli and are cleared by the kidney rapidly. Following intravenous administration, recombinant human IL-10 has a terminal phase half-life of 2.7 to 4.5 h [15]. Due to its longer peptide chain the molecular weight of the IL4-10 fusion protein is larger than that of IL-10 (~70kDa). Glycosylation of IL4-10 FP further adds to its molecular weight. We recently performed a pharmacokinetics study in rats. hIL4-10 FP indeed was shown to have an increased half-life as compared to hIL-10 (C. Steen-Louws, ms in preparation), demonstrating an increased bioavailability. To demonstrate the feasibility of IL4-10 fusion protein, we recently tested its potential in animal models for inflammatory- and neuropathic chronic pain. IL4-10 FP strongly relieved hyperalgesia in all models, and inhibited pain superior to IL-4 or IL-10 mono or combination therapy [10]. This emphasizes the potential of IL4-10 fusion protein to inhibit immunopathology and pain in inflammatory and degenerative diseases.

IL4-10 fusion protein is a new drug that signals cells to induce immunoregulatory activity and that overcomes limitations of IL-4 and IL-10 stand-alone therapy, and therefore has therapeutic potential for inflammatory diseases such as rheumatoid arthritis. Recent data suggest an even broader potential as, next to prevention of pain [10], IL4-10 FP suppresses articular cartilage damage in models for osteoarthritis [17].

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C. Steen-Louws performed the experiments, analysed the data, wrote the paper; S.A.Y. Hartgring performed the experiments, wrote the paper; J. Popov-Celeketic performed the experiments; A.P. Lopes performed the experiments; M.B.M. de Smet performed the experiments; N. Eijkelkamp performed the experiments; F.P.J.G. Lafeber designed the study; C.E. Hack MD designed the study, wrote the paper; J.A.G. van Roon PhD designed the study, analysed the data, wrote the paper.

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2.6 I Conflict of Interest

The authors declare no conflicts of interest.

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Chapter 3

IL4-10 fusion protein has chondroprotective, anti-inflammatory and potentially analgesic effects in the treatment of osteoarthritis

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Abstract

Objective. Effective disease-modifying drugs for osteoarthritis (DMOAD) should preferably have chondroprotective, anti-inflammatory, and analgesic activity combined in a single molecule. We developed a fusion protein of IL4 and IL10 (IL4-10 FP), in which the biological activity of both cytokines is preserved. The present study evaluates the chondroprotective, anti-inflammatory, and analgesic activity of IL4-10 FP in *in vitro* and *in vivo* models of osteoarthritis.

Methods. Human osteoarthritic cartilage tissue and synovial tissue were cultured with IL4-10 FP. Cartilage proteoglycan turnover and release of pro-inflammatory, catabolic, and pain mediators by cartilage and synovial tissue were measured. The analgesic effect of intra-articularly injected IL4-10 FP was evaluated in a canine model of osteoarthritis by force-plate analysis.

Results. IL4-10 FP increased synthesis ($p=0.018$) and decreased release ($p=0.018$) of proteoglycans by osteoarthritic cartilage. Release of pro-inflammatory IL6 and IL8 by cartilage and synovial tissue was reduced in the presence of IL4-10 FP (all $p<0.05$). The release of MMP3 by osteoarthritic cartilage and synovial tissue was decreased ($p=0.018$ and 0.028) whereas TIMP1 production was not significantly changed. Furthermore, IL4-10 FP protected cartilage against destructive properties of synovial tissue mediators shown by the increased cartilage proteoglycan synthesis ($p=0.036$) and reduced proteoglycan release ($p=0.043$). Finally, intra-articular injection of IL4-10 FP improved the deficient joint loading in dogs with experimentally induced osteoarthritis.

Conclusion. IL4-10 FP is potentially new DMOAD since it shows chondroprotective and anti-inflammatory effects *in vitro*, as well as analgesic effect in canine *in vivo* model of osteoarthritis. Further study on its therapeutic potential in osteoarthritis is warranted.

3.1 I Introduction

Osteoarthritis (OA) is a highly prevalent chronic degenerative joint disease and a growing socioeconomic problem, imposing enormous costs and challenges to healthcare. It is clinically characterized by pain and functional disability of the affected joints. Clinical management of OA is mainly symptomatic and includes non-pharmacological, pharmacological, and surgical approaches [1, 2]. Currently available treatments are inefficient to slow down OA progression, leaving a clear need for development of disease modifying therapy [3, 4].

OA is characterized by structural changes that include the damage and the loss of articular cartilage, peri-articular bone changes, and synovial inflammation. Importantly, these three tissues are functionally interconnected and their interplay influences the pathogenesis of OA. These structural changes result from multiple anabolic and catabolic factors that act upon several joint tissues. For example, cartilage and synovial tissue release multiple mediators and breakdown products that mutually affect these tissues [5]; subchondral bone affects the (calcified) cartilage in OA through neurovascular invasion [6, 7]; synovial inflammation contributes to osteophyte formation [8] and subchondral bone marrow lesions [9]. However, the exact sequence of pathological events in these tissues and their exact relation to pain remains elusive, and may even differ among distinct OA subtypes [1, 10, 11].

The ideal Disease-Modifying OA Drug (DMOAD) presumably should combine three basic pharmacological effects: relief of pain, reduction of synovial inflammation, and protection against, and repair of, cartilage damage. None of the existing pharmacological therapies for OA combines these effects; neither do the various novel therapeutic approaches of OA. In principle, approved and experimental novel pharmacological treatments of OA target only one of the disease components, such as mediators of cartilage tissue turnover (e.g. MMP inhibitors [12]), bone turnover (e.g. bisphosphonates and strontium ranelate [13]), synovial inflammation (e.g. the more recent attempts with methotrexate or anti-TNF [14]), or pain (like the debated anti-NGF studies [15, 16]). Therefore, there is still an unmet medical need for DMOAD.

Interleukin 4 (IL4) and in particular IL10, have shown impressive results in preclinical models for inflammatory disease [17, 18]. However, clinical evaluation of their anti-inflammatory potential in inflammatory conditions like rheumatoid arthritis, inflammatory bowel disease, and psoriasis has been disappointing [19-22]. These disappointing results may reflect poor bioavailability of the wild-type cytokines as well as their use as stand-alone drug. Various

studies suggest a therapeutic potential of IL4 and IL10 in OA also: IL4 and IL10 receptors (IL4R, IL10R) are expressed on synovial tissue cells as well as on chondrocytes [23-25]. IL4R signalling alters mechano-transduction in chondrocytes linked to matrix turnover in OA [26] and genetic variants of the IL4R gene increase susceptibility to OA [27]. IL10 and IL4 inhibit chondrocyte apoptosis and cartilage breakdown [28, 29]. IL4 and IL10 also reduce synovial inflammation as they reverse TNFα-induced production of prostaglandin E2 by OA synovial fibroblasts [30]. Moreover, IL4 and IL10 have partially overlapping and complementary activities 17. In fact, combined administration of both cytokines showed promising, synergistic effects in experimental models of arthritis [31, 32]. However, such a combination therapy of IL4 and IL10 has never been evaluated in clinical trials.

We have designed a IL4-10 fusion protein, IL4-10 FP (patent application No 61/556,843), which combines the activity of IL4 and IL10 in a single molecule. In several *in vitro* models IL4-10 FP inhibits the production of multiple pro-inflammatory cytokines while preserving the production of cytokine inhibitors and antagonists [33]. Furthermore, recently published studies show the chondroprotective and analgesic activity of IL4-10 FP: the fusion protein limits blood induced cartilage damage *in vitro* and *in vivo* in haemophilic-arthropathy models [34] and it attenuates pain in mouse models of chronic inflammatory and neuropathic pain [35]. So far, data in experimental models of OA is lacking. In the present study, we evaluated potential chondroprotective, anti-inflammatory, and analgesic effects of this novel molecule in various human *in vitro* models and in a canine *in vivo* model [36-38] of OA.

3.2 I Methods

3.2.1 I Production and purification of IL4-10 FP. Human IL4-10 FP was produced and purified according to the protocol published by Eijkelkamp et al [35]. In short, IL4-10 FP was produced by transient transfection of HEK293 cells (U-Protein Express BV, Utrecht, the Netherlands) with pUPE expression vector carrying cDNA sequence coding the IL4-10 FP. To optimize glycan capping with sialic acid, cells were co-transfected with beta-galactoside-2,3-sialyl-transferase construct. IL4-10 FP was purified by cation exchange chromatography. Purified protein was stored in sterile PBS, pH 7.4, at -80^{o}C until use.

3.2.2 I *Ex vivo* Immunohistochemistry. Cartilage samples obtained from OA knee joints (n=8; 62 ±5 years) and healthy shoulder joints (n=8; 53 ±4 years) were frozen in Tissue-Tec. In general, tissue with a Mankin grade 4-6 is collected representing full thickness clearly

fibrillated cartilage 39. Cryo-sections (6µm) were incubated with the primary antibody overnight at 4˚C (mouse anti-human IL4Rα, R&D systems or rabbit anti-human IL10Rα, LifeSpan BioSciences) (no primary antibody for the negative control). Subsequently, sections were incubated for 30 minutes at room temperature with the secondary antibody (Powervision Ready-to-use HRP-Anti-Mo/Ra/Rb-IgG, Immunologic). The tissue sections were counterstained with 0.1% kernechtrot (Sigma Aldrich) in 5% Al2(SO4)3. The number of cells expressing IL4R or IL10R over the total number of cells at three different locations (superficial, middle, and deep zone) per section was microscopically determined. The percentage of positive cells for either IL4R or IL10R was calculated. A cell was regarded positive if a positively stained nucleus was identified in association with appropriate receptor staining.

3.2.3 I *In vitro* Cartilage Cultures. Human OA cartilage tissue was harvested from patients (n=7, 60 ±4 years) undergoing total knee arthroplasty. Healthy human shoulder cartilage tissue as a control (n=7 donors, 58 ±13 years) was harvested post-mortem. Full-thickness cartilage pieces were cut aseptically and sliced into square pieces, weighted aseptically (range 5-15mg, accuracy ±0.1mg) and cultured individually in 96-well round bottomed microtiter plates in 200µl medium with or without addition of IL4-10 FP. Cartilage culture medium consisted of Dulbecco's modified Eagle's medium, supplemented with glutamine (2mmol/l), penicillin (100UI/ml), and 10% heat inactivated pooled human male AB serum. IL4-10 FP was added at 20ng/ml final concentration. The concentration of 20ng/ml was chosen as optimum concentration based on previous dose response studies [32, 34]. Samples were cultured for four days at 37°C, 5% CO_2 in air. Proteoglycan synthesis rate and release as a measure of cartilage matrix turnover were determined. Part of the cartilage culture supernatants were collected and stored at -80^{o}C for further analysis.

3.2.4 I *In vitro* Synovial tissue cultures. Synovial tissue was obtained from OA patients (n=6, age 66 ±11 years) undergoing total knee arthroplasty. Ten pieces of about 30mg wet weight (range 20-40mg, accuracy ±0.1mg) were cut aseptically and cultured individually in 24-wells flat bottom plates in culture medium with or without addition of 20ng/ml IL4-10 FP. Synovial tissue culture medium consisted of RPMI Glutamax (Invitrogen, Life Technologies), supplemented with 10%, v/v, human AB serum and 1%, w/v, penicillin/streptomycin. Samples were cultured for 24h at 37°C, 5% CO_2 in air. Culture supernatants were collected and stored in aliquots at -80^{o}C for further analysis.

3.2.5 I *In vitro* evaluation of cartilage destructive properties of OA synovial tissue. Human OA synovial tissue explants (n=8 donors, age 67 ±11 years) were cultured in presence or absence of IL4-10 FP (20ng/ml) for a fixed culture time (varying between 1 and 3 days between experiments). Culture supernatants were harvested and subsequently from each experiment added (25% v/v) to fresh healthy human cartilage cultures. After four days of culture proteoglycan turnover of the cartilage samples was analysed. For glycosaminoglycan (GAG) release, one outliner data point was removed as the value was above (Mean + 2*SD).

3.2.6 I Proteoglycan turnover. As measure of proteoglycan synthesis, the rate of sulphate incorporation was determined by use of $^{35}SO_4^{2-}$ - pulse labelling of GAGs [40]. After the four-day culture period, newly formed GAGs were labelled for 4h with $^{35}SO_4^{2-}$. Subsequently cartilage explants were washed with cold phosphate-buffered saline and digested for 2h at 65°C with papain buffer. GAGs were precipitated with cetylpyridium chloride (CPC) and the $^{35}SO_4^{2-}$ - labelled GAGs were measured by liquid scintillation analysis. Proteoglycan synthesis is expressed as nanomoles of sulphate incorporated per hour per gram wet weight of cartilage tissue (nmol/h*g). To measure release of GAGs from the explants as a measure of proteoglycan release, GAGs in the culture medium were precipitated and stained with Alcian blue dye solution, as described previously [39]. Alcian blue staining of the medium was quantified photometrically by the change in absorbance at 620nm with chondroitin sulphate (Sigma C4384) as a reference. GAG release is expressed as mg GAGs/g wet weight of cartilage tissue released in four days (mg/g). To minimize the influence of biological (focal) variation between cartilage samples, at least 8 cartilage specimens were randomly collected from each donor and tested individually for proteoglycan release and turnover. Results of these 8 specimens were averaged, and the average value was used as the outcome of an individual donor. Values of different donors were used for statistical analysis.

3.2.7 I Cytokine and other mediator assessments. Supernatants of cartilage and synovium cultures were analysed for matrix-metalloproteinase (MMP) 1 and MMP3, tissue-inhibitor-of-metalloproteinases (TIMP1), and pain-related mediators nerve-growth-factor (NGF) and vascular-endothelial-growth-factor (VEGF) using multiplex assays [41]. Some MMP3 concentrations measured were above the highest concentration of the standard. As the reproducibility of the assay for these high concentrations is good (coefficient of variation <3% for the 3 highest concentrations of the standard curve), these data could still be used in the analyses. The cytokines IL6 and IL8 were measured by ELISA (CytoSet ELISA Kit's; Invitrogen),

according to manufacturer's instructions. All values were corrected for tissue weight and culture volume.

3.2.8 I *In vivo* canine Groove model. In an exploratory set-up (n=4 only), analgesic effects of the IL4-10 FP were tested in an *in vivo* canine model of OA. Four skeletally mature mixed breed dogs (mongrels, females, 0.8 ±0.1 years, 20.5 ±0.7kg; animal laboratory Utrecht University) were housed in cages (approximately 3x3m2) in groups of two dogs (randomly divided), with at least one hour a day on a patio (approximately 7x6m2) to move freely. They were fed a standard diet and water ad libitum. Joint degeneration was induced in the right knee according to the Groove model [36-38]. This model is characterized by its limited inflammatory component as compared to other models of OA. Ten longitudinal and diagonal grooves, depth 0.5mm, were made on the weight-bearing parts of the femoral condyles, while preventing bleeding and soft tissue damage as much as possible to avoid induction of inflammation. After surgery, synovium, fasciae and skin were sutured. The contralateral healthy knee served as a control. In order to obtain information on potential dose dependency, two doses of IL4-10 FP were tested - 1ug and 10ug per knee with a washout period of two weeks in-between. Five weeks after induction of joint damage, when surgery related pain is resolved and OA related pain presents, IL4-10 FP (1µg in 1ml) was injected intra-articularly in the OA joint. A second injection of IL4-10 FP (10µg in 1ml) was given in week 7. The contralateral healthy knee was injected with 1ml PBS at both time points. Joint loading was measured as a surrogate measure of pain, using force plate analysis (FPA) [42]. Longitudinal changes in vertical stance ground reaction forces (GRFs) were evaluated for each leg by FPA (each dog served as its own control). A force-plate (FP), flush-mounted with the surface of an 11-meter walkway, sampled (100 Hz) peak GRFs. Forces were normalized by body weight and time, and expressed in N/kg. A dedicated technician guided the dogs by leash over the FP, at a walking pace of constant speed (1 ±0.2m/s). A successful run consisted of sequential, distinct paw strikes of the right front and hind paw or the left front and hind paw, respectively. Ten valid runs were collected for each side of the dog and GRFs were averaged for each of the four legs. FPA was performed at several time points: data at baseline (pre-surgery) and subsequently prior and for three days after injection with IL4-10 FP, twice, starting at week 5 and week 7 are presented. The data is expressed as the ratio of OA/ contralateral knee joint of each animal. For all procedures (surgery, intra-articular injections and pain measurements) dogs were treated in a random

order. The experiment was approved by animal ethical committee of the University Utrecht (DEC 2011.III.12.123).

3.2.9 I Statistical analysis. For all data analysis SPSS statistical software was used (SPSS Statistics 21, IL, USA). Since *in vitro* data did not always follow normal distribution, statistics were performed using Wilcoxon signed rank test for the related data. Statistics of receptor expression between OA and healthy cartilage were performed with Mann-Whitney test for non-paired data. Data are presented as mean values with 95% confidence interval. For the *in vivo* data, paired T test (exploratory) was performed. For each animal, the post-treatment values were compared to baseline values. Data are presented as mean values ± standard deviation. Exact p-values are indicated in the text and figures.

3.3 I Results

3.3.1 I The expression of IL4R and IL10R is higher in human knee OA cartilage compared to healthy human shoulder cartilage. The number of chondrocytes expressing IL4R and IL10R was higher in OA cartilage compared to healthy cartilage. The percentage of IL4R positive cells in superficial, middle and deep zone of OA cartilage was 68±10%, 72±15%, and 65±18%, respectively. This was significantly higher as compared to 41±12%, 36±11%, and 50±10% positive cells in healthy cartilage (p=0.0006, p=0.0003 and p=0.038, respectively) (Figure 1A). The percentage of IL10R positive chondrocytes was significantly higher in the superficial and middle zone of OA cartilage with 70±7% and 70±12% positive cells in each zone compared to 48±17% positive cells in superficial and 50±18% in middle zone of healthy cartilage (p=0.021 and p=0.038, respectively). In the deep zone difference was less pronounced with 64±14% positive cells in OA vs 60±18% positive cells in healthy cartilage (p=0.959) (Figure 1B).

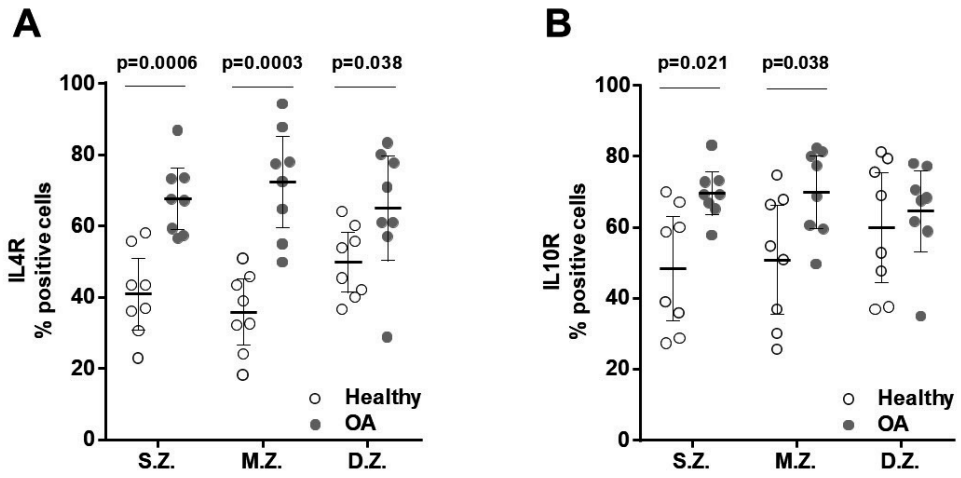

Figure 1: Expression of IL4 receptor (IL4R) (A), and IL10 receptor (IL10R) (B) on chondrocytes in healthy shoulder and OA knee cartilage. Data are expressed as percentage positive cells in the superficial (S.Z.), middle (M.Z.) and deep zone (D.Z.) of the cartilage. Mean values with 95% confidence interval are shown for healthy cartilage (n=8) and OA cartilage (n=8).

3.3.2 I IL4-10 FP beneficially affects proteoglycan turnover in OA cartilage *in vitro*. Proteoglycan synthesis rate of OA cartilage tissue explants was increased when cultured in the presence of IL4-10 FP. The increase of 45±39% by IL4-10 FP was statistically significant compared to control culture (p=0.018) (Figure 2A). IL4-10 FP had reduced the proteoglycan release measured by release of GAGs by 11±7% compared to control (p=0.018) (Figure 2B). In the case of healthy cartilage tissue explants no significant effect of IL4-10 FP on proteoglycan turnover was seen *in vitro* (Figures 2A and 2B).

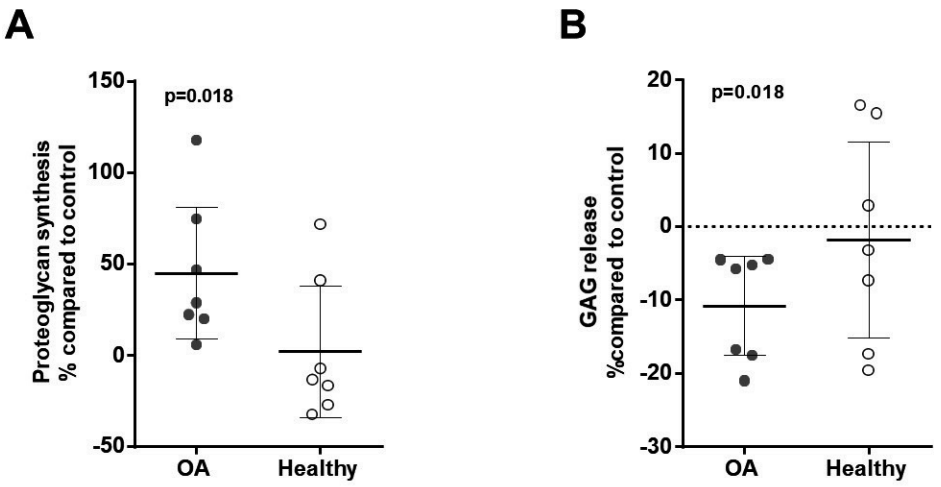

Figure 2: **The effect of IL4-10 FP on proteoglycan synthesis rate (A) and GAG release (B) in OA and healthy cartilage.** Healthy and OA cartilage pieces were cultured individually in the medium with or without addition of IL4-10 FP. After four days of culture, proteoglycan synthesis rate and release as a measure of cartilage matrix turnover were determined. Data are expressed as percentage change compared to control cultures. Mean values with 95% confidence interval (n=7 for OA cartilage**,** n=7 for healthy cartilage) are shown. Dotted lines (proteoglycan synthesis rate of control cultures was on average 2.1nmol/h.g wet weight of tissue; GAG release of control cultures was on average 5.5mg/g wet weight of tissue released in four days).

3.3.3 I IL4-10 FP reduces release of inflammatory, catabolic and pain-related mediators from human OA cartilage and synovial tissue *in vitro*. In the presence of IL4-10 FP, release of the inflammatory cytokines IL6 and IL8 by OA cartilage explants was reduced by 84±18% and 76±18%, respectively (both p=0.018) compared to controls (Figure 3A). The release of MMP3, a protease involved in degradation of extracellular matrix proteins, was significantly reduced (p=0.018) while release of MMP1, also involved in degradation of extracellular matrix, tended to increase but was not statistically significantly changed (p=0.063). The release of TIMP1, an inhibitor of MMPs, by OA cartilage tissue was not significantly changed by IL4-10 FP (p=0.176; Figure 3B).

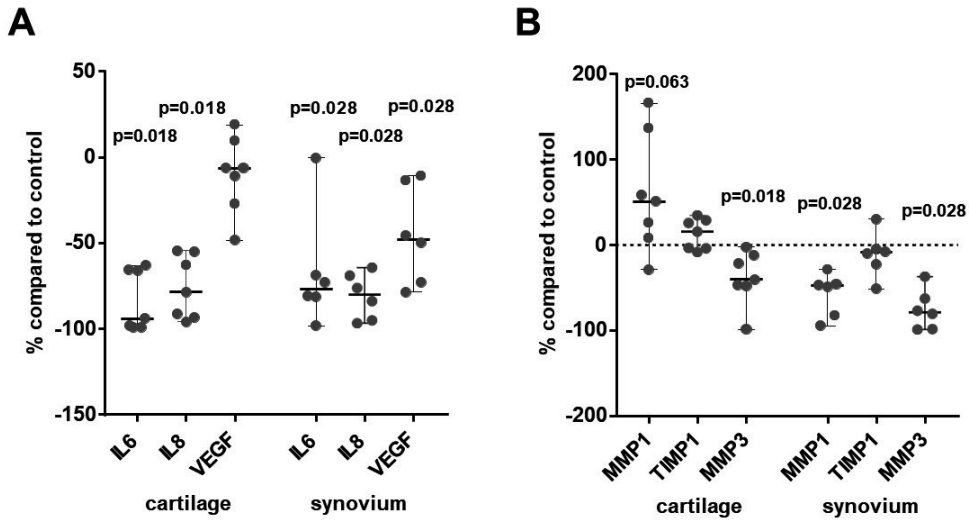

Figure 3: IL4-10 FP influences the release of pro-inflammatory (A) and catabolic (B) mediators from OA cartilage and synovial tissue *in vitro*. All parameters were measured in culture supernatants of cartilage and synovial tissue samples without IL4-10 FP addition (control cultures) or with addition of IL4-10 FP. Data are expressed as percentage change compared to control cultures. Median values with 95% confidence interval (n=7 for cartilage, n=6 for synovial tissue) are shown. Control cultures mean values of the mediators released from cartilage: IL6 IL8 VEGF MMP1 – 8.05ng/ml, TIMP1 – 25.15ng/ml, MMP3 - 1.34µg/ml. Mean baseline values of mediators released from synovial tissue: IL6 – 10.89ng/ml, IL8 – 32.02ng/ml, VEGF -, MMP1 – 140,57ng/ml, TIMP1 - 30,81ng/ml, MMP3 – 8.41µg/ml.

IL4-10 FP also influenced the release of different mediators by human OA synovial tissue. The amount of inflammatory cytokines IL6 and IL8 released into the culture medium was significantly reduced (68±34%, p=0.028 and 81±13%, p=0.028, respectively) compared to control (Figure 3A). Moreover, the release of MMP1 and MMP3 by OA synovial tissue was reduced by 58±25% (p=0.028) and 76±23% (p=0.028), respectively. The release of TIMP1 was not significantly changed (p=0.249; Figure 3B). No significant effect of IL4-10 FP was seen on IL1RA or TNFRII release by synovial tissue (data not shown). The absence of the effect on IL1Ra is intriguing, however similar to the effect observed in LPS stimulated whole blood culture where IL4-10 FP only minimally altered IL-1Ra concentrations while strongly reducing IL1beta levels [33].

The release of vascular-endothelial-growth-factor (VEGF) and nerve-growth-factor (NGF), pain-related mediators associated with neurovascular invasion, by OA synovial tissue samples

was significantly inhibited by IL4-10 FP *in vitro* as well. The release of VEGF was decreased by 45% (p=0.028) compared to control culture. NGF was measurable in five out of six donors and in those was significantly reduced in the presence of IL4-10 (61%; p=0.031, data not shown). The decrease in release of VEGF from cartilage tissue explants was also observed although it was less pronounced (10%; p=0.310) (Figure 3A). NGF was not detectable in OA cartilage culture supernatants.

3.3.4 I IL4-10 FP reduces cartilage destructive properties of OA synovial tissue *in vitro*. Exposure of healthy cartilage explants to culture supernatant of OA synovial tissue reduced proteoglycan synthesis rate by 45±41% and increased the GAG release by 15±41% compared to control (healthy cartilage cultured in medium only) (Figure 4B). In contrast, medium from synovial tissue (25% v/v) cultured with of IL4-10 FP when added to healthy cartilage (not susceptible to IL4-10 FP at a fourfold concertation itself) did not affect proteoglycan turnover of the cartilage. The proteoglycan synthesis rate was comparable to control and significantly higher than that of cartilage samples incubated with medium from synovial tissue cultured without IL4-10 FP (p=0.036). Similarly, GAG release by healthy cartilage explants exposed to medium from synovial tissue cultured with IL4-10 FP was 21% significantly lower compared to those exposed to conditioned medium without IL4-10 FP (6±26% and 15±41% respectively, p=0.043).

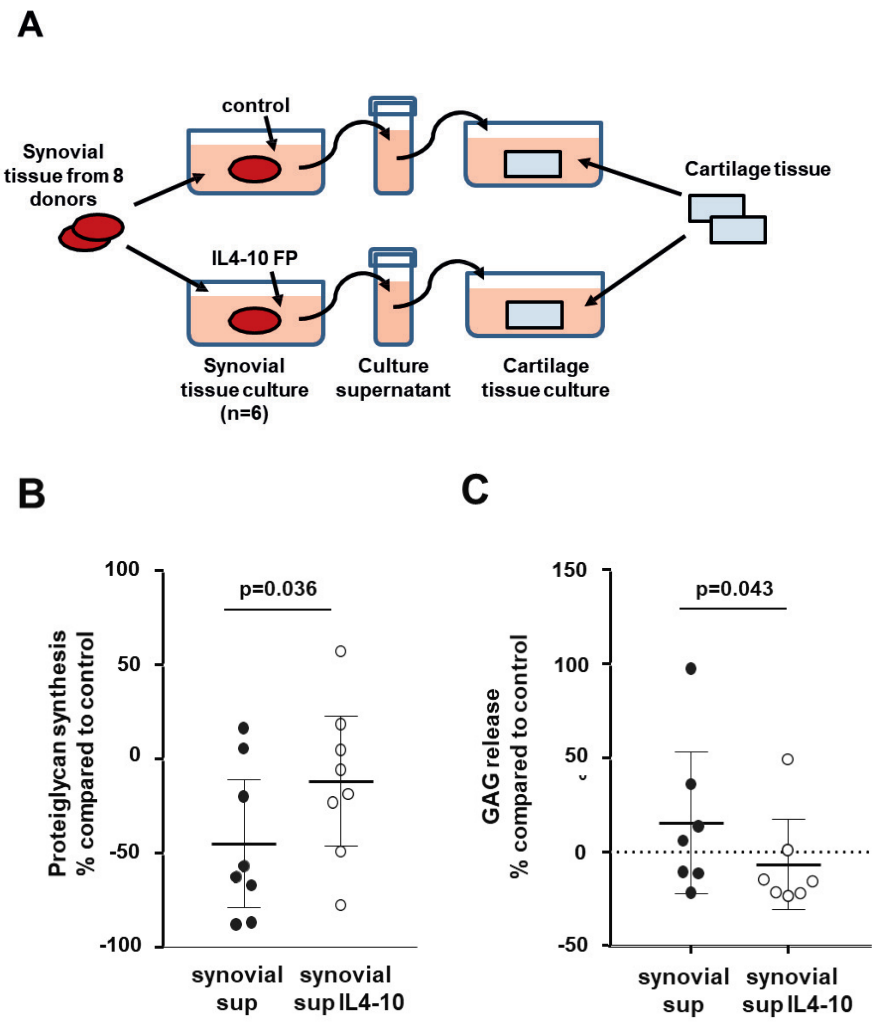

Figure 4: IL4-10 FP reduces cartilage destructive properties of OA synovial tissue *in vitro*. Schematic representation of the experimental design: For each synovial tissue donor, synovial tissue was cultured without (control) and with IL4-10 FP for a fixed time period. Subsequent, culture supernatants were harvested and tested on healthy cartilage tissue **(A)**. The effect of synovial tissue culture supernatant (with and without IL4-10 FP) on proteoglycan synthesis rate **(B)** and GAG release by healthy cartilage samples **(C)**. Healthy human cartilage tissue cultured in culture medium only was used as control (dotted lines). Data are expressed as percentage change compared to control cultures (dotted lines). Mean values with 95% confidence interval are shown. PG synthesis (n=8), GAG release (n=7).

3.3.5 I IL4-10 FP reduces OA pain *in vivo*. The analgesic effect of IL4-10 FP was measured in the canine Groove model of OA where significant decrease in stance force, a surrogate measure of pain, continues for at least 12 weeks after OA induction [42]. Also in the present experiment, stance force decreased after induction of OA (ratio OA/control knee joint) (week 5: 88±8% of baseline, p=0.054), indicating pain of the affected joint (Figure 5). Upon intra-articular injection of 1µg of IL4-10 FP, stance force returned towards baseline in the experimental joint reaching the maximum effect at 3 days after the injection (93±7% of baseline). At 2 and 3 days after injection stance force of the affected joint was not significantly different from baseline anymore (p=0.051 and p=0.118 respectively). Before the second injection unloading had increased again (increase in pain), demonstrated by a statistical significant level compared to baseline (week 7: 89±6% of baseline; p=0.037), demonstrating the transient effect of the injection. The analgesic effect of IL4-10 FP could be repeated upon a second injection, with a 10 fold concentration resulting again in an increase in joint loading (decrease in pain) as compared to pre-treatment values, increasing up to 93±5% already at the first day after injection. At 1 and 2 days after injection stance force of the affected joint was not statistically significant different from baseline anymore. At day 3 after injection stance force of the affected joint decreased again demonstrating the transient effect of the injection (p=0.009 compared to pre-OA baseline conditions).

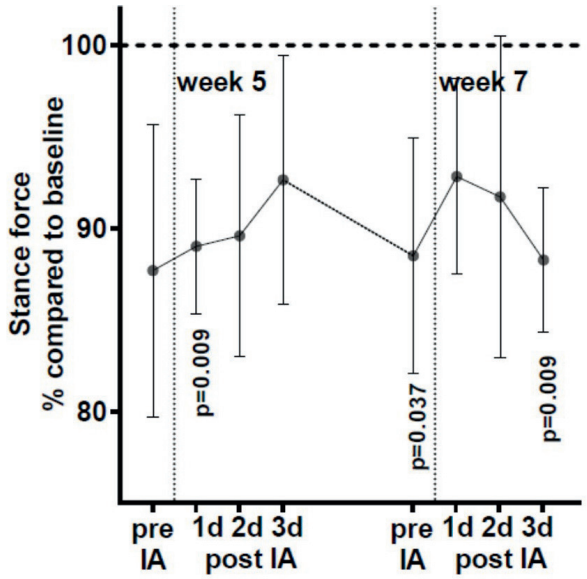

Figure 5: The effect of intra-articular (IA) injection of IL4-10 FP on stance force in the canine Groove model for OA. These exploratory data are expressed as percentage change in ratio osteoarthritic over control knee joint compared to baseline before OA induction (horizontal dashed line). IL4-10 FP was injected at week 5 and week 7 after OA induction, indicated with vertical dotted lines. Mean values ± standard deviation are shown, n=4. IA – intra-articular, d – days.

3.4 I Discussion

IL4 and IL10, as stand-alone or combination therapy, showed chondroprotective effects in models of inflammatory joint disease like rheumatoid arthritis (RA) [17, 32] and more degenerative joint disease like hemophilic arthropathy [25, 43]. Moreover, these cytokines modulate inflammation, and contribute to remodeling of articular cartilage extracellular matrix [44, 45]. Cytokines in essence exert their effects in a network of other cytokines, which probably explains why combination therapy of IL4 and IL10 is more efficient than stand-alone treatment in diseases like arthritis. For that reason, we have designed a fusion protein of IL4 and IL10 (IL4-10 FP), which can be developed as a single biologic with combined activity of two cytokines. In the present study we evaluated IL4-10 FP as a potential Disease-Modifying Drug for the treatment of OA (DMOAD). We show that IL4-10 FP combines chondroprotective, anti-inflammatory, and analgesic effects in human OA tissue *in vitro* and in canine OA *in vivo*.

Remodeling of articular cartilage extracellular matrix by IL4-10 FP requires the presence of receptors for either cytokine on chondrocytes. Chondrocytes produce IL4 and IL10 and the receptors for both cytokines are expressed on their cellular surface [24, 46]. Several studies support a role of IL4 and IL10 in OA. For example, reduced IL4 expression in OA cartilage is associated with decreased overall anti-catabolic activity in OA cartilage [47]. Secretion of IL10 by T regulatory cells isolated from of OA patients is reduced compared to non-OA controls [48] while the production of IL10 by OA chondrocytes is not changed. We here show that expression of both IL4- and IL10-receptors is higher in knee OA cartilage compared to healthy shoulder cartilage. This extends findings on enhanced gene expression of both receptors in models of blood induced cartilage damage where an increase in expression of IL4R and IL10R was induced in healthy shoulder cartilage *in vitro* [25]. Although source differences for receptor expression cannot be ruled out, this observation supports that damage of articular cartilage is accompanied by increased expression of IL4- and IL10-receptors. The mechanism causing increased expression remains elusive. As up-regulation of the receptors was predominantly

found on chondrocytes in the superficial layer, mediators from the synovial fluid are likely involved. We also show in our study that the fusion protein composed of IL4 and IL10 beneficially affects proteoglycan turnover in human OA cartilage *in vitro* while proteoglycan turnover in healthy cartilage was not affected. The differential effect on OA versus healthy cartilage reflects the increased receptor expression by OA cartilage.

Even though OA is not predominantly driven by inflammation, it can be accompanied by synovial inflammation in subsets of OA patients. IL4-10 FP not only has direct beneficial effect on cartilage proteoglycan turnover, but also silences synovial inflammation. The anti-inflammatory properties of IL4 and IL10 have extensively been discussed in literature and studies have shown their additive and/or synergistic effect when applied in combination. Here we show that IL4-10 FP significantly decreases release of inflammatory markers from human OA synovial and cartilage tissue *in vitro*. Additionally, cartilage destructive proprieties of OA synovial tissue were inhibited when synovium was pre-treated with IL4-10 FP *in vitro*, suggesting that IL4-10 FP has also indirect chondroprotective effects.

Pain is a dominant clinical feature of OA. Therefore, a DMOAD should have analgesic activity in addition to chondroprotective and anti-inflammatory activity. OA related pain is not well understood. In general, pain perception in OA does not reflect the extent of cartilage damage. Only severely damaged cartilage in OA becomes innervated at the bone cartilage interface [6, 7]. Hence, synovial inflammation and bone damage more likely are involved in pain sensation. Here, we show that in dogs with induced OA, intra-articular injection of human IL4-10 FP increases joint loading. Although the effect with a higher dose was reached quicker, based on only two injections in this limited number of animals it can only be concluded that the fusion protein has analgesic properties. To determine degree and time dependency of the effect, further studies are needed.

IL4-10 FP reduces synovial inflammation, which is known to contribute to increased responsiveness of peripheral nociceptive neurons and therefore increased pain sensitivity in OA [49, 50]. As such the analgesic effect may (in part) be dependent on its anti-inflammatory effect. Additionally, the analgesic effects of intra-articular IL4-10 FP may result from its inhibitory effects on the release of pain promoting mediators VEGF and NGF by OA synovial tissue, as was observed in the human tissues. Such, and other, mechanisms may explain the analgesic effect of IL4-10 FP in the Groove model.

Local application of IL4-10 FP via intra-articular injection is a treatment option for larger joints affected by OAas it provides high concentration of IL4-10 FP in treated joints with limited or no systemic exposure. Although in the present study the analgesic effect with its maximum between 1 and 3 days seems to lag behind an anticipated bioavailability of a few hours [51,52] effects are relatively short and demand repeated injections over time. We are currently investigating various approaches to prolong bioavailability of IL4-10 FP in the joint cavity, including administration via controlled release systems.

In summary, we show that IL4-10 has DMOAD potentials since it shows cartilage protective, and anti-inflammatory effects *in vitro*, as well as analgesic activity in a canine OA model. This combined activity in a single molecule has to our knowledge never been described before. Further studies on its therapeutic potential in osteoarthritis seem justified and needed.

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3.6 I Contributors

All authors provided substantial contributions to conception and design, or analysis and interpretation of data and drafting the article or revising it critically for important intellectual content and all gave final approval of the version to be published.

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3.8 I Competing interests

The authors declare that there is no conflict of interest.

3.9 I Patient consent

The University Medical Utrecht medical ethical committee approved the use of human post-mortem materials and of residual tissue upon joint replacement surgery upon informed consent which was obtained.

3.10 I Ethics approval

The Utrecht University Committee for Experiments on Animals approved the study according to Dutch law (DEC nr: 2011.III.12.123).

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Chapter 4

IL4-10 fusion protein is a novel drug to treat persistent inflammatory pain

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Abstract

Chronic pain is a major clinical problem that is difficult to treat and requires novel therapies. Although most pain therapies primarily target neurons, neuro-inflammatory processes characterized by spinal cord and dorsal root ganglion production of pro-inflammatory cytokines play an important role in persistent pain states and represent potential therapeutic targets. Anti-inflammatory cytokines are attractive candidates to regulate aberrant neuro-inflammatory processes, but the therapeutic potential of these cytokines as stand-alone drugs is limited. Their optimal function requires concerted actions with other regulatory cytokines and their relatively small size causes rapid clearance. To overcome these limitations, we developed a fusion protein of the anti-inflammatory cytokines interleukin (IL)4 and IL10. The IL4-10 fusion protein is a 70 kDa glycosylated dimeric protein that retains the functional activity of both cytokine moieties. Intrathecal administration of IL4-10 dose-dependently inhibited persistent inflammatory pain in mice: three IL4-10 injections induced full resolution of inflammatory pain in two different mouse models of persistent inflammatory pain. Both cytokine moieties were required for optimal effects. IL4-10 fusion protein was more effective than the individual cytokines or IL4+IL10 combination therapy and also inhibited allodynia in a mouse model of neuropathic pain. Mechanistically, IL4-10 inhibited the activity of glial cells and reduced spinal cord and dorsal root ganglion cytokine levels without affecting paw inflammation. In conclusion, we developed a novel fusion protein with improved efficacy to treat pain, compared with wild-type anti-inflammatory cytokines. IL4-10 fusion protein has potential as treatment for persistent inflammatory pain.

4.1 I Introduction

Chronic pain is a major clinical problem affecting more than 20% of the world population, resulting in severe reduction in the quality of life (Institute of Medicine (US) Committee on Advancing Pain Research, 2011;Breivik et al., 2006). Chronic pain is a major complaint of many patients suffering from chronic inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease. In these conditions, pain may even persist after successful treatment of the inflammation (Lee et al., 2011;Lomholt et al., 2013;Bielefeldt et al., 2009). Current treatments provide modest pain relief at best, due to lack of effectiveness or because treatment has to be suspended due to severe side effects (Borsook et al., 2014). Therefore, development of novel therapies is required.

Most chronic-pain therapies primarily target neurons or peripheral inflammation. However, persistent inflammatory pain is the result of neuronal plasticity characterized by peripheral sensitization of primary sensory neurons and central sensitization in the spinal dorsal horn that involves local neuro-immune interactions. Persistent pain states have been associated with activation of glial cells in the spinal cord and dorsal root ganglia (DRG) in rodent models for chronic pain (Graeber and Christie, 2012a;Ji et al., 2013a). Human data support a role for glial cells in different chronic pain states (Del et al., 2009;Brisby et al., 1999;Shi et al., 2012;Banati et al., 2001;Loggia et al., 2015). Activated glial cells in the dorsal horn of the spinal cord and infiltrating macrophages or activated satellite glial cells in the DRG produce pro-inflammatory cytokines that sensitize the sensory system (Ren and Dubner, 2010). Therefore, these neuro-inflammatory pathways, including spinal-cord and DRG cytokine production and glial-cell activation, represent potential therapeutic targets for treating persistent pain states (Graeber and Christie, 2012a;Ji et al., 2014;Ji et al., 2013a;Ren and Dubner, 2010).

Anti-inflammatory cytokines are attractive candidates to dampen spinal-cord and DRG cytokine production and glial activation (Milligan et al., 2005). The mechanism of action of regulatory anti-inflammatory cytokines, such as interleukin (IL)4 and IL10, differs significantly from that of biologics that inhibit single (pro)-inflammatory mediators (e.g., anti-tumor necrosis factor (TNF) therapy). These regulatory cytokines downregulate inflammatory responses by turning off release and production of multiple mediators, including cytokines, chemokines, proteases, and reactive oxygen species. In addition, anti-inflammatory cytokines shift cells towards an inhibitory/regulatory profile. Importantly, glial cells and sensory neurons respond to anti-inflammatory cytokines. The regulatory cytokines IL4 and IL10 inhibit glial cell

proliferation (Kloss et al., 1997). IL4 skews microglia towards an M2a regulatory phenotype (Pepe et al., 2014), and IL10 administration reduces astrocyte activation (Balasingam and Yong, 1996) and strongly reduces lipopolysaccharide (LPS)-induced cytokine production by microglia (Sawada et al., 1999;Lodge and Sriram, 1996;Balasingam and Yong, 1996).

Strong evidence exists for a therapeutic potential of IL10 to inhibit pain, whereas a limited number of studies have shown a therapeutic potential for IL4 (Vale et al., 2003a;Kwilasz et al., 2015;Hao et al., 2006a;Cunha et al., 1999a). Nonetheless, the therapeutic potential of the native unmodified anti-inflammatory cytokines as stand-alone drugs is limited. First, the optimal function of these cytokines requires them to act in concert with other immunoregulatory cytokines (Joosten et al., 1997;van Roon et al., 1996;van Roon et al., 2001). Second, their relatively small size causes rapid clearance, thereby reducing their bioavailability.

To overcome these limitations, we designed a novel strategy to fuse regulatory cytokines into one molecule to promote efficacy in treating pain by facilitating synergy and bioavailability. We here developed a fusion protein of IL4 and IL10 that combines the anti-inflammatory actions of the two regulatory cytokines into one molecule. We demonstrate that the functional activity of both cytokine moieties was maintained in IL4-10 and that it reversed persistent inflammatory pain in mouse models through the regulation of neuro-inflammatory responses in the spinal cord and DRG.

4.2 I Methods

4.2.1 I Construction, production, and purification of IL4-10 fusion protein. IL4-10 fusion protein was produced by transient transfection of HEK293-cells (tested negative for mycoplasma contamination) with a pUPE-expression vector containing a cystatin signal sequence and synthetic cDNA coding for the peptide sequence (U-ProteinExpress BV, Utrecht, the Netherlands) (Durocher et al., 2002). Glycan-capping with sialic acid residues was optimized by co-transfection with a vector carrying the beta-galactoside-alpha2,3-sialyltransferase5 (SIAT9, homo sapiens) transgene. Cells were cultured in FreeStyle medium (Invitrogen, Carlsbad, CA, USA) with 0.9% primatone, ~0.04% fetal calf serum, total volume 1 L, for 5 days. Supernatant was collected, concentrated 10-fold (10 kDa QuixStand hollowfiber cartridge, GE-Healthcare, Waukesha, WI, USA), and diafiltrated against phosphate-buffered saline (PBS) pH 7.4. Precipitates were removed by passing over a glass-syringe filter. Supernatant was stored in aliquots at –80˚C.

4.2.2 I Ion exchange chromatography (IEC). IL4-10 fusion protein was purified using cation-IEC (HiTrap SP FF 1 mL column; GE-Healthcare) on an AKTAprime Plus (GE-Healthcare). Low-salt buffer (bufferA): 10 mM phosphate, pH 6.5. High-salt buffer (bufferB): bufferA with 1 M NaCl. Before injection, 50 mL supernatant was diluted 1:5 in bufferA. A linear gradient to 100% bufferB (in 20 min, flow rate 1 mL/min) was used to elute IL4-10 fusion protein. 1 mL fractions were collected and analyzed for IL4-10 fusion protein content using IL4 and IL10 enzyme-linked immunosorbent assay (ELISA). Fractions containing IL4-10 fusion protein were pooled, diafiltrated against PBS pH7.4, and concentrated to yield a ~20 µg/mL IL4-10 fusion protein batch.

4.2.3 I Size exclusion chromatography (SEC). Molecular weight was determined by high-performance-SEC on a high-performance liquid chromatography system (Shimadzu, Kyoto 604-8511, Japan). Running buffer: 50 mM phosphate, pH 7.0, 0.5 M NaCl (flow rate: 0.35 mL/min, 35 bar). The column (BioSuite125 4 µm UHR SEC; Waters) was calibrated with reference proteins. 50 µL of 20 µg/mL (IEC) purified IL4-10 fusion protein was analyzed. 175 µL fractions were collected, and IL4 and IL10 content was measured with ELISA (in 1/500 dilution). Similar runs with IL4 and IL10 (Sigma-Aldrich, St. Louis, MO, USA) were performed.

4.2.4 I IL4-10 fusion protein detection. IL4 and IL10 content was measured with ELISA (Pelipair ELISA kits, Sanquin, Amsterdam, the Netherlands) according to the manufacturer's instructions. Results were compared with those for recombinant IL4 and IL10 provided by the

manufacturer.

A cross-ELISA specific for IL4-10 fusion protein was generated by modification of the IL4 and IL10 ELISA. Anti-IL4 coated plates were combined with biotinylated anti-IL10 monoclonal antibody, and vice versa. Antibodies from IL4 and IL10 Pelipair ELISA (Sanquin) were used, and further performed according to manufacturer's instructions. An amount of IL4-10 fusion protein equivalent to 75 pg/mL recombinant IL10 and IL4-10 was tested. As there is no standard for this assay, results are given as optical density at 450 nm.

4.2.5 I Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Page) and Western-blotting. Samples were diluted 1:1 in sample buffer (Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol-blue; BioRad, Richmond, VA, USA) containing 100 mM DL-dithiotreitol (Sigma-Aldrich), incubated 10 min at 100˚C, and loaded on a 12% polyacrylamide gel (Mini-PROTEAN-TGX; BioRad). Markers (WesternC Standard, 250-10 kD; BioRad) were run in a separate lane. Electrophoresis: 150 V, 1 h, reducing conditions (Tris/glycine/SDS buffer; BioRad). After electrophoresis, proteins were transferred (100 V, 1 h) to a polyvinylidene difluoride (PVDF) membrane (BioRad). Thereafter, the PVDF membrane was incubated in PBS 0.1% Tween-20 (Merck; Whitehouse Station, NJ, USA) containing 4% milkpowder (Elk; Campina, Zaltbommel, the Netherlands). Membrane was incubated with mIgG1 anti-human IL4 or mIgG1 anti-human IL10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz) and WesternC-marker detecting antibody (StrepTactin-HRP; BioRad). Some experiments required deglycosylation of IL4-10 fusion protein prior to electrophoresis by PNGaseF (Sigma-Aldrich), according to manufacturer's instructions.

4.2.6 I Animals. All animal experiments were performed in accordance with international guidelines and with prior approval from the University Medical Center Utrecht experimental animal committee. Prior to ethical permissions approval, power calculations were performed to determine sample size to detect a minimal pre-defined effect size. All animals were allocated to a group prior to the start of any measurements/treatment. Experiments were conducted using both male and female C57Bl/6 mice aged 8–12 weeks. Observers performing behavioral experiments were blinded to treatment.

4.2.7 I Persistent inflammatory pain and assessment of hypersensitivity. Carrageenan (2%, 20 µL; Sigma-Aldrich) was injected in both hind paws to induce persistent inflammatory pain. 20 µL Complete Freund's Adjuvant (CFA, 1mg of *Mycobacterium tuberculosis* (H37Ra, ATCC

25177), heat killed and dried, 0.85 mL paraffin oil and 0.15 mL mannide monooleate; Sigma-Aldrich) was injected in one hind paw to induce persistent inflammatory pain, while the other paw received 20 µL vehicle (saline). Heat-withdrawal latency times were determined using the Hargreaves test (IITC Life Science, Woodland Hills, CA, USA), as described (Hargreaves et al., 1988). Mechanical hypersensitivity was measured using von Frey hairs (Stoelting, Wood Dale, IL, USA), and the 50% paw-withdrawal threshold was calculated using the up-and-down method (Chaplan et al., 1994).

IL4-10 fusion protein and recombinant human IL4 and IL10 were produced in HEK293 cells (Sigma-Aldrich) and intrathecally injected (Eijkelkamp et al., 2013). Each mouse received 5 µL, and concentration was adjusted to appropriate dosing. Receptor blocking antibodies against mouse IL4-receptor or IL10-receptor (BD Pharmingen) were administered together with IL4-10 fusion protein at a dose of 6 µg/mouse.

Paw thickness was measured using a Digimatic Micrometer (Mitutoyo, Veenendaal, the Netherlands).

Spared nerve injury surgery was performed as described (Willemen et al., 2012;Zhou et al., 2015). Briefly, the sural common peroneal and tibial branches of the left sciatic nerve were exposed under isoflurane anesthesia. The tibial and common peroneal nerves were transected, while the sural nerve was kept intact. IL4-10 was injected intrathecal at days 6 and 7 after transection and pain behaviors were measured 3 and 6 hours after administration.

To assess motor function, mice were individually placed in a clean cage identical to the home cage but without beddings and were permitted to freely explore the whole cage for 10 min. A new cage was used for each mouse. The cage was divided virtually into four quadrants. Locomotor activity was quantified by counting the number of quadrant entries during the last 5 min. The number of full rears also was counted during the same time interval. Scoring was conducted by a well-trained observer who was blind to treatments.

4.2.8 I Immunohistochemistry. Spinal cord and DRGs were excised from mice perfused with 4% paraformaldehyde in PBS. Tissues were postfixed, cryoprotected in sucrose, embedded in Optimal Cutting Temperature (OCT) compound, and frozen at –80°C. Frozen sections of DRG and spinal cord (lumbar L3–L5 section) were stained with rabbit anti-Iba1 (1:1000; cat nr. 019-19741; Wako Pure Chemical Industries) or mouse anti-glial fibrillary acidic protein (GFAP) (cat nr. bm2287; Cymbus, Acris) or rabbit-anti-GFAP (cat. nr. 04-1062; Millipore) followed by alexafluor 488-conjugated or 594-conjugated secondary donkey antibodies (Invitrogen).

Photographs were taken with a Zeiss Axio Observer microscope (Zeiss, Oberkochen, Germany) using identical exposure times for all slides. Iba1 and GFAP-positive area was analyzed with the US National Institutes of Health ImageJ program using identical thresholds to identify area positive for staining. At least three slides per animals were analyzed in a blinded fashion.

4.2.9 I Primary spinal microglia and astrocyte culture. Microglia were isolated from spinal cord using enzymatic digestion with papain (Worthington Biochemical), as described previously (Yip et al., 2009). Adult spinal-cord astrocytes were isolated using enzymatic digestion with papain followed by Optiprep (Accurate Chemical) gradient, as described previously (Kerstetter and Miller, 2012). Neonatal spinal cord astrocytes were isolated using 0.25% trypsin, as described earlier (Nijboer et al., 2013). Cells were cultured in poly-L-Lysine coated in a 96-well plate in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (Invitrogen).

LPS (Sigma-Aldrich) was added to the culture medium at 100 ng/mL for astrocytes and 1 µg/mL for microglia in combination with human IL4-10 fusion protein or controls recombinant human IL4 and IL10 (Sigma-Aldrich). Receptor-blocking antibodies against mouse IL4-receptor or IL10-receptor (both BD Pharmingen) were added at 2 µg/mL. After 18 h incubation at 37°C, supernatant was collected and stored at –80°C. Glutamate uptake by spinal astrocytes was measured by incubating astrocytes in Hanks' balanced salt solution (HBSS) supplemented with 5 mM Hepes for 15 min at 37°C. Uptake was started by adding 0.33 µCi/mL of L-glutamate (GE Healthcare, Eindhoven, The Netherlands) mixed with unlabeled L-glutamate to a final concentration of 100 µM (Sawada et al., 1999). After incubation of 0, 3, and 10 minutes at 37°C, uptake was terminated by two washes with ice-cold HBSS, immediately followed by cell lysis with 0.1 N NaOH/0.01% SDS for 15 minutes on ice.

4.2.10 I mRNA Isolation and real-time polymerase chain reaction (PCR). Whole lumbar DRGs and spinal cord (L3-L5) were homogenized in Trizol (Invitrogen, Paisley, UK). Total RNA was isolated with RNeasy Mini Kit (Qiagen) and reverse-transcribed using an iScript™ Select cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was performed with an iQ™ SYBR® Green Supermix (Invitrogen). Primer pairs used were as follows:

IL1β: CAACCAACAAGTGATATTCTCCATG (forward), GATCCACACTCTCCAGCTGCA (reverse)

TNFα: GCGGTGCCTATGTCTCAG(forward), GCCATTTGGGAACTTCTCATC (reverse)

CCL2: GGTCCCTGTCATGCTTCTG (forward),CATCTTGCTGGTGAATGAGTAG (reverse)

BDNF: CACATTACCTTCCAGCATCTGTTG (forward), ACCATAGTAAGGAAAAGGATGGTCAT (reverse)
IL6: TCTAATTCATATCTTCAACCAAGAGG (forward), TGGTCCTTAGCCACTCCTTC(reverse)
COX2: GGTCTGGTGCCTGGTCTG (forward), CTCTCCTATGAGTATGAGTCTGC (reverse)
CCR2: ACCTGTAAATGCCATGCAAGT (forward), TGTCTTCCATTTCCTTTGATTTG (reverse)
CX3CR1: TGTCCACCTCCTTCCCTGAA (forward), TCGCCCAAATAACAGGCC (reverse)
KC: AAAAGGTGTCCCCAAGTAACG (forward), GTCAGAAGCCAGCGTTCAC (reverse)
TGFβ: CAGAGCTGCGCTTGCAGAG (forward), GTCAGCAGCCGGTTACCAAG (reverse)
GAPDH: TGCGA CTTCA ACAGC AACTC (forward), CTTGC TCAGT GTCCT TGCTG (reverse).
HPRT: TCCTCCTCAGACCGCTTTT (forward), CCTGGTTCATCATCGCTAATC (reverse)
β-actin: AGAGGGAAATCGTGCGTGAC (forward), CAATAGTGATGACCTGGCCGT (reverse).
Expression was normalized against GAPDH and HPRT (spinal cord and DRG) or GAPDH and β-actin (hind paws).

4.2.11 I Cytokine assessments. Supernatants were analyzed for TNFα by ELISA. Mouse TNFα production was measured using ELISA kits (mouse: TNFα DuoSet ELISA Kit; R&D), according to manufacturer's instructions and using the standards provided.

4.2.12 I Statistical analysis. For all measurements**,** data are expressed as mean with standard error of the mean. Investigators performing the behavioral assays or assessing outcome were blinded for treatment. Data were analyzed for statistical significance by one-way or two-way analysis of variance with Bonferroni post-tests where appropriate. A p value less than 0.05 was considered significant.

4.3 I Results

4.3.1 I IL4-10 fusion protein characteristics. Human IL4-10 fusion protein was produced by transient transfection of human embryonic kidney (HEK) 293 cells (Durocher et al., 2002) and co-transfected with β-galactoside-α2,3-sialyltransferase to optimize glycan-capping with sialic acid (**fig 1a**). In the supernatant of HEK293 cells expressing IL4-10 fusion protein, both IL4 and IL10 were detected (**fig 1b-e**). Wild type (wt) IL4 and wt IL10 migrated with a molecular mass of ~15 kDa on SDS-PAGE, whereas IL4-10 migrated as two protein bands with a molecular mass of ~30-35 kDa that was detected by anti-IL4 and anti-IL10 (**fig 1f-g**). The two bands represent glycoforms of IL4-10 fusion protein as they migrated as one band upon deglycosylation of the supernatant with PNGaseF (**fig 1f-g**). IL4-10 fusion protein was purified by cation exchange chromatography and analyzed by high-pressure size-exclusion chromatography. Wild-type IL4 eluted as a monomer with an apparent mass of ~15 kDa and wt IL10 as a dimer of ~40 kDa, consistent with the notion that the active form of wt IL10 is a non-covalently linked dimer. IL4-10 fusion protein predominantly eluted with an apparent mass of ~70 kD, consistent with a non-covalently linked dimer (**fig 1h**).

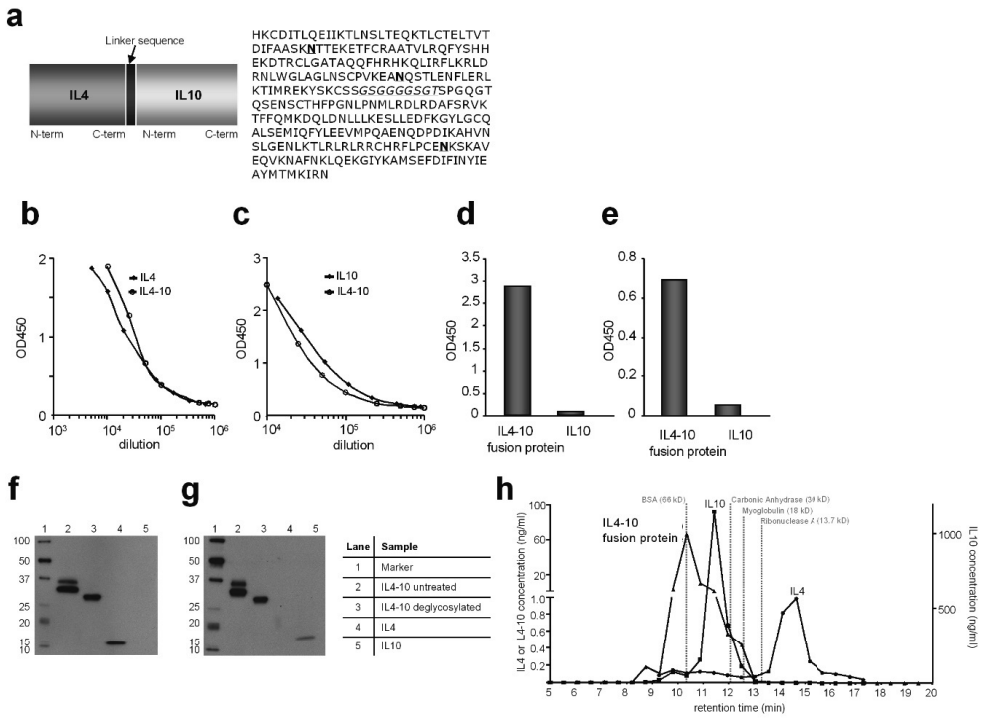

Figure 1: Molecular and functional characterization of IL4-10 fusion protein. (a) Schematic overview of the IL4-10 fusion protein and its amino acid sequence. The linker sequence is indicated in italic and potential N-linked glycosylation sites are indicated in bold. **(b-c)** Supernatant of HEK293 cells expressing IL4-10 fusion protein was tested in sandwich ELISAs for IL4 **(b)** and IL10 **(c)** and compared with the wild-type cytokines. **(d)** A cross-ELISA with anti-IL4 as capture antibody and biotinylated anti-IL10 as detecting antibody or **(e)** vice versa (capture: anti-IL10, detection anti-IL4) indicated presence of IL4-10 fusion protein. Recombinant IL10 was used as a negative control. (f-g) Western blot analysis of untreated and deglycosylated purified supernatants containing IL4-10 fusion protein was detected with **(f)** anti-IL4 or **(g)** anti-IL10. Untreated IL4-10 fusion protein contains both IL4 and IL10 and migrates as a double band (lane 2). Upon deglycosylation with pNGaseF, the IL4-10 fusion protein migrated as one band with a molecular weight of 34 kDa (lane 3). **(h)** The molecular weights of IL4-10 fusion protein, IL4, and IL10 were estimated based on the retention time of standard proteins (positions by dotted grey lines) in a size-exclusion chromatography (SEC). IL4 eluted as a monomer (~15 kDa), whereas the molecular size of IL10 (~36 kDa) and the IL4-10 fusion protein (~70 kDa) were consistent with a dimer. Figure shows representative data. ** $p<0.01$ and *** $p<0.001$.

4.3.2 I IL4-10 fusion protein inhibits persistent inflammatory pain. The potential of IL4-10 fusion protein to inhibit persistent hyperalgesia was evaluated in the well-established murine model of carrageenan-induced persistent inflammatory pain (Ren and Dubner, 1999a;Willemen et al., 2012;Ren and Dubner, 1999b;Eijkelkamp et al., 2010;Clapper et al., 2010). Intrathecal injection of 40 ng IL4-10 fusion protein during persistent inflammatory pain significantly inhibited mechanical and thermal hyperalgesia during the first 24 h after administration. Increasing the dose of IL4-10 fusion protein increased the magnitude and duration of inhibition of persistent inflammatory hyperalgesia. At a dose of 200 ng, IL4-10 inhibited persistent inflammatory hyperalgesia for at least 2 (mechanical) to 4 (thermal) days (**fig 2a/b**). Intrathecal IL4-10 injection did not affect baseline thermal thresholds (vehicle: 7.9±0.2 s; IL4-10 fusion protein: 8.3±0.2 s, n=10, 24 h after injection) or mechanical thresholds (vehicle: 0.51±0.08; IL4-10 fusion protein: 0.67±0.07, n=6, 24 h after injection). Injection of 100 ng IL4-10 into the inflamed paw, a dose that effectively inhibited persistent hyperalgesia when injected intrathecally, did not affect persistent inflammatory hyperalgesia (**fig 2c**). In contrast, expression of TNFα and keratinocyte chemoattractant (KC, CXCL1) but not IL1β in the inflamed paw was reduced 48 hours after intraplantar IL4-10 (**fig 2d**). These data indicate

that intrathecal injections that target spinal cord and DRG are required to inhibit persistent inflammatory pain.

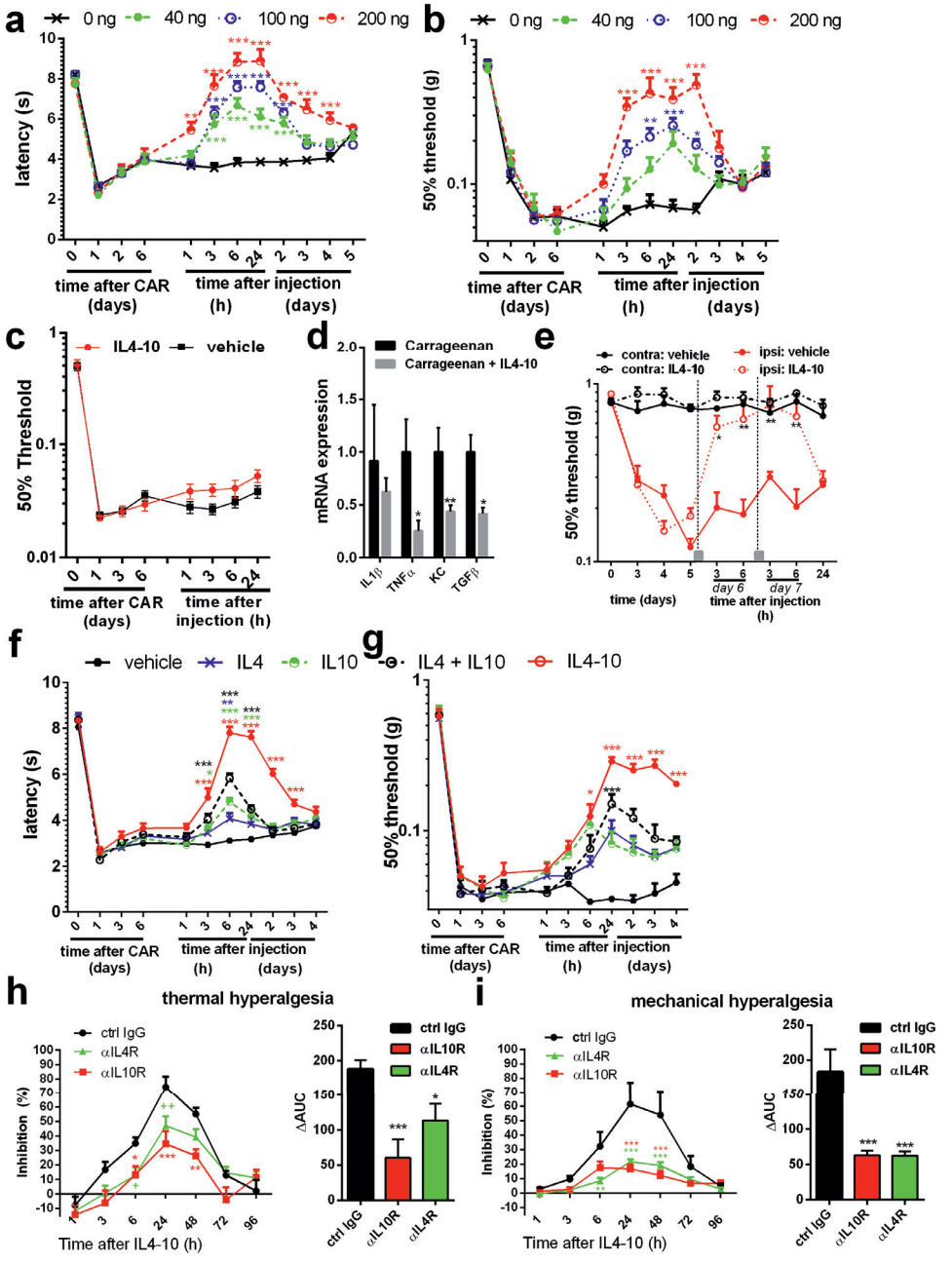

Figure 2: IL4-10 suppresses persistent inflammatory pain. Inflammatory pain was induced by an intraplantar injection of 20 µL of 2% carrageenan in mice. **(a,f,h)** Thermal sensitivity was measured over time using the Hargreaves (HG) test and **(b, c, e, g, i)** mechanical hypersensitivity was measured using the von Frey (vF) test. **(a-b)** Six days after intraplantar injection, mice received an intrathecal injection of different concentrations of IL4-10 fusion protein (n=6, 14, 6, 12 for 0, 40, 100, 200 ng). **(c)** Six days after intraplantar injection, mice received an intraplantar injection of the IL4-10 fusion protein in one paw or vehicle in the other (n=10) and **(d)** mechanical hyper sensitivity was followed. **(d)** The inflamed hind paws were collected 2 days later and analyzed for mRNA expression for TNFα, IL1 β and KC and were normalized for GAPDH and β-actin. **(e)** Mice were exposed to an unilateral spared nerve injury (SNI) and mechanical sensitivity was analyzed. IL4-10 fusion protein was intrathecally injected (gray square) at days 6 and 7 after SNI (n=4). **(f-g)** The effectiveness of the IL4-10 fusion protein (100 ng/mouse) to block inflammatory pain after intrathecal administration was compared with IL4 and IL10 alone (50 ng/mouse) or IL4 and IL10 (50 + 50 ng/mouse) combined (HG: n=16, vF: n=8 per group). The IL4-10 fusion protein blocks inflammatory pain more efficiently compared to the individual cytokines or the combination of IL4 and IL10. Asterisks mark statistical differences compared to vehicle treated animals. **(h-i)** The inhibitory effect of the IL4-10 fusion protein (100 ng/mouse) on inflammatory pain was attenuated in presence of receptor blocking antibodies (6 µg/mouse) against the IL4 receptor (αIL4R) and the IL10 receptor (αIL10R), demonstrating functional activity of both cytokine moieties and requirement of both moieties for optimal effect (n=8). Right: Area under curve (ΔAUC) for percentage inhibition between 1 and 48 hours after intrathecal injection. Asterisks marks statistical differences compared to vehicle treated mice (a-d) or control IgG (e-f). All data are expressed as mean and SEM. *, **, *** indicate statistical significant difference of $p<0.05$, 0.01 and 0.001, respectively.

To test whether IL4-10 fusion protein is also efficacious in a murine model of persistent neuropathic pain, we assessed its effect in the spared nerve injury model. Intrathecal administration of the IL4-10 fusion protein completely but transiently restored mechanical thresholds to control levels (**fig 2e**). IL4-10 did not affect the mechanical threshold in the unaffected contralateral paw (**fig 2e**).

To determine whether IL4-10 fusion protein has increased efficacy in inhibiting persistent inflammatory pain as compared with the native cytokines, we compared it with native cytokines either as stand-alone or combination therapy. Intrathecal administration of IL4 or IL10 alone had a statistically significant but small transient inhibitory effect on persistent hyperalgesia (**fig 2f/g**). An equimolar dose of IL4-10 was significantly more potent in inhibiting persistent inflammatory hyperalgesia, compared with IL4 or IL10 alone. In addition, the IL4-10

fusion protein was still more effective in treating pain than a double concentration of IL4+IL10 (data not shown). The combination of IL4 and IL10 partially inhibited persistent hyperalgesia and had a larger effect than the stand-alone cytokines, indicating a synergistic or additive effect of the two regulatory cytokines in inhibiting persistent pain (**fig 2f/g**). However, the fusion protein IL4-10 completely inhibited persistent inflammatory hyperalgesia for at least 2 days, whereas the combination of IL4 and IL10 only partially inhibited this hyperalgesia (~45%) and for a much shorter period of time (<1 day), indicating that IL4-10 has superior efficacy over IL4 and IL10 combination therapy. Co-injection of the fusion protein IL4-10 with antibodies blocking either IL10 or IL4 receptor antibodies reduced the beneficial effect of IL4-10 on persistent pain (**fig 2h/i**), indicating that both cytokine moieties of IL4-10 are required for maximal efficacy.

4.3.3 I IL4-10 fusion protein inhibits neuroinflammation. Rodent models for chronic pain and human data support a role for glial cells in different chronic pain states (Del et al., 2009;Ji et al., 2013b;Graeber and Christie, 2012b;Brisby et al., 1999;Shi et al., 2012;Banati et al., 2001;Loggia et al., 2015). Activated glial cells in the dorsal horn of the spinal cord and infiltrating macrophages or activated satellite glial cells in the DRG produce pro-inflammatory cytokines that sensitize the sensory system (Ren and Dubner, 2010). IL4-10 fusion protein dose-dependently inhibited LPS-induced TNFα release by isolated primary spinal cord microglia (**fig 3a**). IL4-10 was equally efficient as compared to IL10 alone, or IL10 and IL4 combined. IL4 only moderately inhibited LPS-induced TNFα production by spinal microglia. In spinal microglia, inhibition of LPS-induced TNFα release by IL4-10 was completely reversed by a blocking antibody against the IL10R but not by blocking the IL4R (**fig 3b**). Combining IL4 and IL10 receptor-blocking antibodies did not further prevent the inhibitory effect of IL4-10 compared with only IL10R blocking antibodies, indicating that IL4-10-mediated inhibition of microglial activity mainly depended on the IL10 moiety (**fig 3b**). Spinal cord astrocytes also produced TNFα after LPS stimulation that was dose-dependently inhibited by IL4-10 fusion protein (**fig 3c**). IL4-10 inhibited TNFα release to a similar extent as IL10, or IL4 and IL10 combined (**fig 3c**), whereas IL4 only moderately reduced this release.

Impaired astrocyte glutamate transporter function leads to excessive glutamate receptor stimulation and is implicated in pathological pain states (Yan et al., 2014;Nie and Weng, 2010;Weng et al., 2014). LPS reduced glutamate uptake by primary spinal astrocytes, but this

was not affected by IL4-10 (**fig 3d**). Thus, IL4-10 effectively attenuated inflammatory responses of glial cells, without affecting their capacity to take up glutamate. In astrocytes, the IL10 receptor blocking antibody partially reduced the inhibitory effect of IL4-10 on LPS-induced TNFα release. Blocking IL4R was insufficient to attenuate the inhibitory effect of IL4-10, but blocking both IL4 and IL10 receptors prevented IL4-10-mediated inhibition to a greater extent, versus blocking the IL10R alone (**fig 3e**). Thus, inhibition of astrocytes by IL4-10 is dependent of the biological activity of both cytokine moieties.

To define whether inhibition of persistent inflammatory pain is also associated with a reduction in glial cell activation *in vivo*, Iba1 and GFAP expression were quantified as measures of microglia and astrocyte/satellite cell activation in spinal cord or satellite glial cells and macrophage activation in the DRG (Ji et al., 2013a). Carrageenan-induced persistent inflammation increased Iba1 mRNA expression (**fig 3f**) and Iba1-positive area (**fig 3g,i**) in the spinal cord. Importantly, intrathecal IL4-10 treatment attenuated the carrageenan-induced increase in spinal cord Iba1 mRNA expression (**fig 3f**) and Iba1-positive area in the dorsal horn of the spinal cord (**fig 3g,i**). Iba1 mRNA expression or Iba1-positive area in the DRG was not affected after intraplantar carrageenan injection or IL4-10 treatment (**fig 3k-l**). GFAP expression in the dorsal horn of the spinal cord and DRG was increased after intraplantar carrageenan injection at the level of mRNA (**fig 3f,k)** and GFAP-positive area **(fig 3h,j,m,n)**. IL4-10 potently reduced GFAP expression in both in spinal cord (**fig 3f,h,j**) and DRG (**fig 3k,m,n**).

We verified whether the inhibition of glial cell activity in spinal cord and DRG was associated with reduced cytokine expression. IL4-10 fusion protein attenuated carrageenan-induced mRNA for TNFα, CCL2, and BDNF in the DRG (**fig 3o**). In the spinal cord, IL4-10 inhibited the carrageenan-induced expression of IL1β, TNFα and CCL2 (**fig 3p**). These data indicate that IL4-10 fusion protein inhibits inflammatory response in the spinal cord and DRG during persistent inflammatory pain.

To verify whether IL4-10 fusion protein treatment affected the local inflammatory response in the hind paw, we performed qPCR on paw tissue 24 h after intrathecal IL4-10 treatment. Carrageenan-induced expression of IL1β, TNFα, IL6, CCL2, COX2, and the chemokine receptors CX3CR1 and CCR2, which are highly expressed on macrophages, were not affected by intrathecal administration of IL4-10 fusion protein (**fig 3q**). These data show that IL4-10 fusion protein inhibits spinal/DRG neuro-inflammation and inflammatory pain without modifying the peripheral inflammatory response.

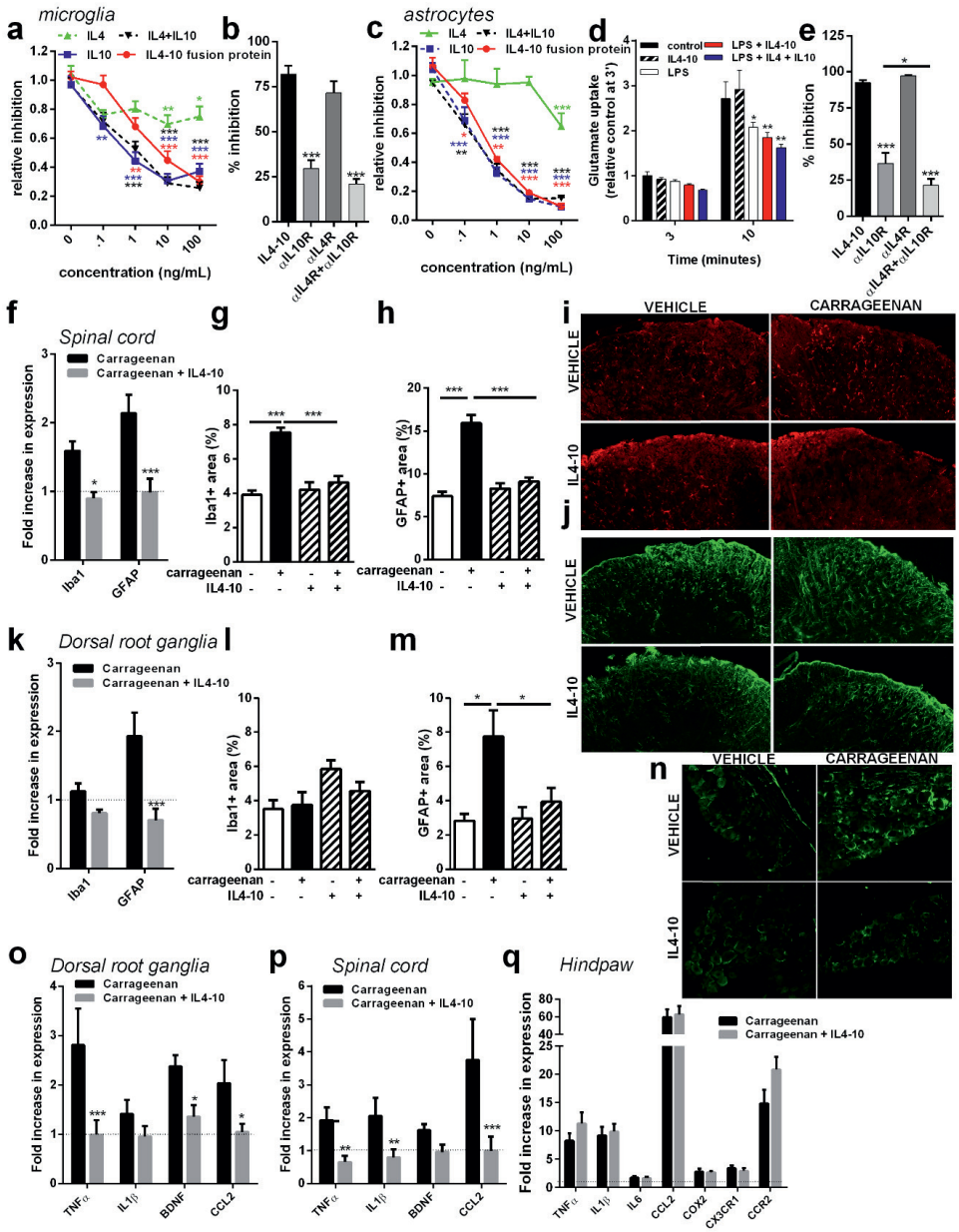

Figure 3: IL4-10 fusion protein inhibits neuroinflammation in spinal cord and dorsal root ganglia. (a) IL4-10 fusion protein dose-dependently inhibited LPS-induced (1 µg/mL) TNFα release by microglia (n=17). **(b)** Requirement of the cytokine moieties of the IL4-10 fusion protein on LPS-induced TNFα release by spinal cord microglia (n=10) was tested by adding receptor blocking antibodies (2 µg/ml) against the IL4 receptor (αIL4R), the IL10 receptor (αIL10R), or both. **(c)** IL4-10 fusion protein dose-

dependently inhibited LPS-induced (100 ng/ml) TNFα release by spinal astrocytes (n=6-17 per condition). **(d)** IL4-10 fusion protein did not affect glutamate uptake by spinal cord astrocytes nor prevent the LPS-induced reduction in glutamate uptake (n=9). **(e)** Requirement of the cytokine moieties to inhibit LPS-induced TNFα release by spinal astrocytes (n=8) **(f-p)**. Persistent inflammatory pain was induced by an intraplantar injection of carrageenan (20 µL, 2%) and 6 days later mice were treated intrathecal with vehicle (PBS) or fusion protein (100 ng/mouse). One day after fusion protein application glial cells activation in the **(f-j)** spinal cord and **(k-n)** dorsal root ganglia was assessed by analyses of **(f)** spinal cord mRNA encoding for Iba1 (microglia) and GFAP (astrocytes). Data are expressed as fold-induction compared to the saline-treated groups (n = 10). **(g-j)** One day after fusion protein application the extent of glial cell activation was quantified by determining **(g)** Iba1 or **(h)** GFAP positive area in the dorsal horn of the lumbar spinal cord for respectively microglia/macrophages and astrocytes (no carrageenan, n=3/group, carrageenan=7). Exemplar images of the **(i)** Iba1 and **(j)** GFAP staining quantified in g/h. **(k)** Fold-induction of mRNA for Iba1 and GFAP in the DRG compared to the saline-treated groups (without carrageenan). **(i-m)** Quantification of the **(i)** Iba1 and (m) GFAP positive area in the DRG. **(n)** Exemplar images of GFAP expression in the DRG quantified in m. **(o-p)** Mice received intraplantar injection of carrageenan or saline and 6 days later mice either PBS or IL4-10 fusion protein (100 ng/mouse, n=10 per group). Lumbar **(o)** DRGs, lumbar **(p)** spinal cord, and inflamed hind paws were collected 1 day later and analyzed for mRNA expression for several cytokines, chemokines, growth factors, and/or chemokine receptors and normalized for GAPDH and β-actin or HPRT. Data are expressed as fold-induction compared with the saline-treated groups. All data are expressed as mean and SEM. *, **, *** indicate statistical significant difference of $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

4.3.4 I Multiple IL4-10 fusion protein administration induces full resolution of inflammatory pain. To further evaluate the therapeutic potential of IL4-10 fusion protein, we tested whether multiple intrathecal injections with IL4-10 induced full resolution of inflammatory pain in two different models of persistent inflammatory pain. Three intrathecal injections of the IL4-10 fusion protein every other day completely reversed carrageenan-induced hyperalgesia, which persists for weeks without treatment. No hyperalgesia was observed for the complete observation period of 2 weeks after the first IL4-10 treatment (**fig 4a-b**).

A comparable effect of IL4-10 fusion protein was observed in another well-established persistent inflammatory pain model, i.e. mice receiving an intraplantar injection of CFA (Ren and Dubner, 1999b). Intrathecal injection of IL4-10 fusion protein 7, 9, and 11 days after the intraplantar CFA injection completely and persistently resolved the inflammatory thermal and

mechanical hyperalgesia (**fig 4c-d**), whereas the inflammatory response in the affected paw was unaltered (**fig 4e/f**). Notably, the multiple intrathecal injections of the human IL4-10 fusion protein did not induce detectable antibodies against IL4-10 fusion protein as examined 3 weeks after the last intrathecal injections (data not shown). Multiple intrathecal injections of IL4-10 fusion protein or vehicle did not affect spontaneous locomotor activity (**fig 4g**). Overall, these data illustrate the potential of the IL4-10 fusion protein to fully resolve persistent inflammatory pain.

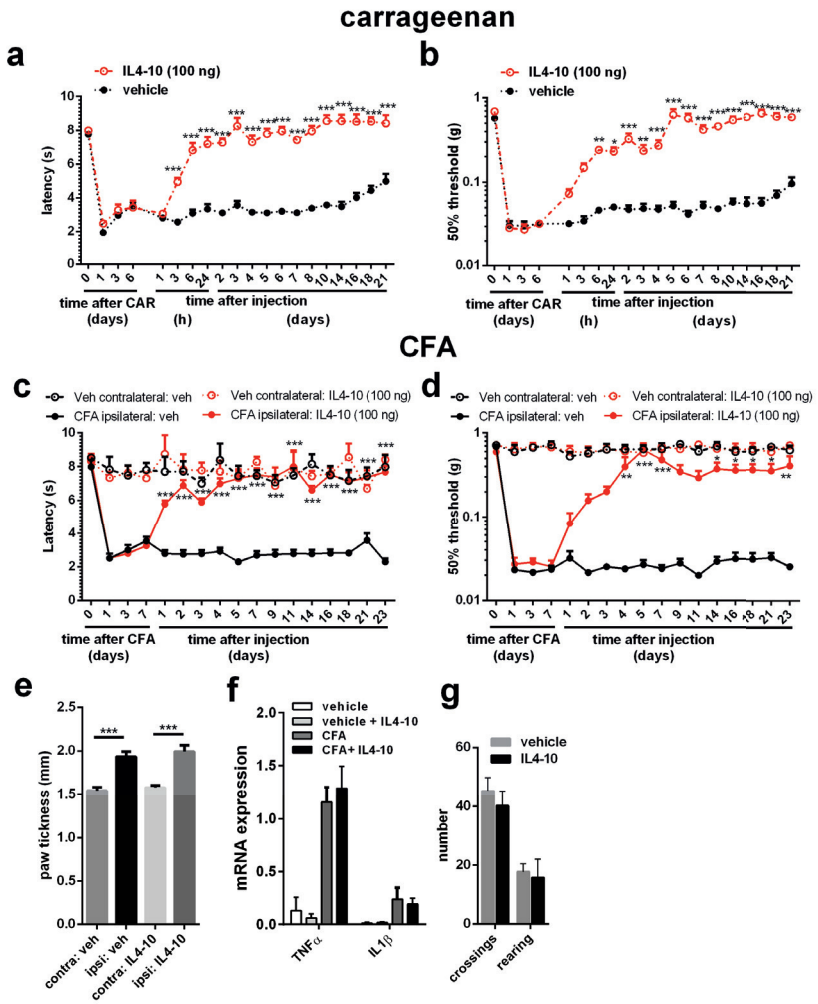

Figure 4: Repeated IL4-10 fusion protein injections completely resolves inflammatory pain. Inflammatory pain was induced by **(a-b)** an intraplantar injection of 20 µL, 2% carrageenan, or **(c-d)** a

unilateral injection of 20 µL CFA in mice, and **(a, c)** thermal sensitivity and **(b, d)** mechanical hypersensitivity was followed over time. At 6, 8, and 10 days (carrageenan; n=8) or 7, 9, 11 days (CFA, n=5) after intraplantar injection, mice received intrathecal injections of IL4-10 fusion protein (100 ng) or vehicle (veh). **(e)** Paw edema in CFA-injected and vehicle-injected hind paws was measured using a digital micrometer 5 weeks after intraplantar injection and **(f)** subsequently collected and analyzed for mRNA expression for TNFα and IL1 β and normalized for GAPDH and β-actin. **(g)** To assess whether multiple injection of IL4-10 fusion protein affect motor behavior, mice were assessed in an open field after 3 intrathecal injection, and the number of crossing and rearing were counted in a 5-min interval. All data are expressed as mean and SEM. *, ** and *** indicate statistical significant differences versus vehicle treated mice with $p<0.05$, 0.01 and 0.001, respectively.

4.4 I Discussion

Here we describe a new approach to treat persistent inflammatory pain using a fusion protein of the regulatory cytokines IL4 and IL10. We found that three intrathecal doses of this IL4-10 fusion protein completely resolved persistent inflammatory pain in two mouse models and inhibited neuro-inflammatory response in the dorsal root ganglia and spinal cord, without affecting paw inflammation. The IL4-10 fusion protein also inhibited neuropathic pain. Biochemical and functional characterization of the IL4-10 fusion protein revealed that the full biological activity of its two cytokine moieties are retained, as assessed *in vitro* and *in vivo*.

Thus far, there is very limited evidence for sustained therapeutic effects of intrathecal administration of cytokines like IL10 (Dengler et al., 2014a). Here we show that the IL4-10 fusion protein overcomes this limitation. The efficacy of IL4-10 to reverse persistent inflammatory pain was superior in duration and in magnitude compared with the individual cytokines or even the combination of free IL4 and free IL10. Three intrathecal injections of IL4-10 fully and permanently reversed persistent inflammatory hyperalgesia in two models without affecting inflammatory response in the hind paws.

The IL4-10 fusion protein has multiple advantages over individual cytokines to treat pain. IL4 and IL10 act through different mechanisms: IL4 primarily increases degradation of pro-inflammatory cytokine mRNA, whereas IL10 primarily inhibits transcription (Wang et al., 1995). These complementary mechanisms of action explain why IL4 and IL10 can act synergistically (van Roon et al., 2001;van Roon et al., 1996;Joosten et al., 1997). Indeed, *in vivo,* combination therapy with IL4 and IL10 was more effective than treatment with the

individual cytokines in attenuating persistent inflammatory pain, suggesting additive or synergistic activities. Consistently, maximal efficacy to reduce pain with IL4-10 required the activity of both cytokine moieties; blocking antibodies against either the IL4 or the IL10 receptor reduced the beneficial effects of the IL4-10 fusion protein.

Incorporation of the two cytokines into one fusion protein increases the molecular size without impairing the tendency of the IL10 moiety to dimerize. Because of its larger molecular size, IL4-10 fusion protein likely has a better bioavailability than the separate cytokines. It is likely that increased bioavailability contributes to the increased efficacy of the fusion protein versus the mixture of free IL4 and IL10 in reducing pain. Thus, the strategy of incorporating IL4 and IL10 into one protein overcomes some of the limitations associated with the use of IL4 and IL10 as stand-alone therapies. Moreover, by using the cytokine fusion approach, we avoid problems linked to viral gene therapy and the non-viral transduction vectors used to induce prolonged production of native cytokines (Milligan et al., 2005;Dengler et al., 2014b;Soderquist et al., 2010;Hao et al., 2006b;Cunha et al., 1999b;Vale et al., 2003b). In addition, direct protein administration intrathecally is advantageous over viral therapy because it is effective immediately and avoids potential unwanted systemic effects.

Chronic pain in inflammatory disease, such as rheumatoid arthritis, is frequently disconnected from peripheral inflammation. For example, disease-modifying anti-rheumatic drugs reduce disease activity, yet a large proportion of patients continue to report moderate to severe pain (American College of Rheumatology Pain Management Task Force, 2010;Kojima et al., 2009). We here describe that a single or multiple injections of the IL4-10 fusion protein into the spinal compartment inhibited persistent pain without affecting the local peripheral inflammatory response that induced the pain. Conversely, intraplantar injection of IL4-10 into the inflamed hind paw reduced inflammatory responses but without affecting the ongoing hyperalgesia. These are important findings because they highlight that persistent pain induced by peripheral inflammation can continue independently of ongoing peripheral inflammation. More than 60% of patients with rheumatoid arthritis report unacceptable pain levels, indicating the pain is insufficiently controlled by the anti-rheumatic drugs and anti-pain medications these patient take. Interestingly, even when the response rates to the anti-rheumatic drug on the basis of European League Against Rheumatism criteria is good, patients can still complain of pain (Taylor et al., 2010). In these situations, targeting the inflammation in the central nervous

system rather than in the joints may contribute to resolution of pain, because it directly targets pathological pain at the core of its driving mechanism.

We showed that both cytokines moieties of the IL4-10 fusion protein contribute to optimal pain inhibition *in vivo*. *In vitro* experiments revealed that the effect of the fusion protein on primary cultures of spinal-cord microglia is mainly mediated via the IL10 moiety, whereas optimal inhibition of cytokine production by spinal astrocytes required both cytokine moieties. Probably some effects of IL4-10 *in vivo* are mediated via cells other than glial cells. For example, immune cells that migrate to DRG and contribute to persistent pain states are strongly regulated by both IL4 and IL10 (Massier et al., 2015;Sorge et al., 2015;Willemen et al., 2014;Kiguchi et al., 2015). Other studies have shown that IL10 controls sensory neuron activity, and IL4 deficient mice show enhanced spinal neuron excitability (Lemmer et al., 2015;Shen et al., 2013). Thus, IL4-10 could have its effect through neurons, in addition to its inhibitory effects on glial cells. The potential neuronal effects of IL4-10 are currently subject of future studies.

In conclusion, we have developed a novel neuro-immune regulatory drug consisting of IL4 and IL10 that overcomes some of the limitations of IL4 and IL10 as stand-alone therapies. Its remarkable potency in inhibiting inflammatory pain in multiple models supports the potential of an IL4-10 fusion protein for treatment of pain and possibly other symptoms of chronic inflammatory disease in humans.

4.5 I Acknowledgments

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4.6 I Financial Disclosure

The authors declare no competing financial interests.

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Chapter 5

Sialic acid-engineered IL4-10 fusion protein is bioactive and rapidly cleared from the circulation

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Abstract

Purpose. Modulating sialylation of therapeutic glycoproteins may be used to influence their clearance and systemic exposure. We studied the effect of low and high sialylated IL4-10 fusion protein (IL4-10 FP) on *in vitro* and *in vivo* bioactivity and pharmacokinetic parameters.

Methods. CHO cell lines producing low (IL4-10 FP lowSA) and high sialylated (IL4-10 FP highSA) fusion protein were generated. Bioactivity of the proteins was evaluated in an LPS-stimulated whole blood assay. Pharmacokinetics were studied in rats, analyzing plasma levels of IL4-10 FP upon intravenous injection. *In vivo* activity was assessed in an inflammatory pain mice model upon intrathecal injection.

Results. IL4-10 FP lowSA and IL4-10 FP highSA had similar potency *in vitro*. The pharmacokinetics study showed a 4-fold higher initial systemic clearance of IL4-10 FP lowSA, whereas the calculated half-life of both IL4-10 FP lowSA and IL4-10 FP highSA was 20.7 minutes. Finally, both IL4-10 FP glycoforms inhibited persistent inflammatory pain in mice to the same extent.

Conclusions. Differential sialylation of IL4-10 fusion protein does not affect the *in vitro* and *in vivo* activity, but clearly results in a difference in systemic exposure. The rapid systemic clearance of low sialylated IL4-10 FP could be a favorable characteristic to minimize systemic exposure after administration in a local compartment.

5.1 I Introduction

Interleukin-4 (IL-4) and IL-10, well-known anti-inflammatory cytokines, have potential for the treatment of inflammatory diseases. However, clinical studies with IL-4 or IL-10 have been disappointing[1-4]. Several explanations for these results have been put forward including pharmacokinetic limitations, and lack of synergism with other cytokines when used as stand-alone drugs[5, 6]. IL4-10 FP, a fusion protein of IL-4 and IL-10, combines the effects of both cytokines in a single molecule[7, 8], and overcomes some of the limitations of stand-alone IL-4 or IL-10.

Previously, we published the immunoregulatory activity of IL4-10 fusion protein *in vitro*. In whole blood and peripheral blood mononuclear cell (PBMC) cultures IL4-10 FP strongly inhibits the production of LPS-induced pro-inflammatory cytokines such as IL-1β, TNF, IL-6, and IL-8, while having minimal, if any, effect on the production of IL-1RA and sTNFR[8]. In addition, the therapeutic potential of IL4-10 FP was demonstrated in several disease models both *ex vivo* and *in vivo*. The fusion protein inhibits pro-inflammatory cytokine production by rheumatoid arthritis (RA) and osteoarthritis (OA) synovial tissue and cartilage cultures[8, 9]. Systemic administration of IL4-10 FP attenuates experimental arthritis in mice[8]. Intrathecal administration of IL4-10 FP remarkably reduces pain in various mouse models of inflammatory and neuropathic pain, as well as intra-articular administration in dogs with experimental OA[8, 9]. These findings suggest a potential of IL4-10 FP for the treatment of diseases, such as inflammatory pain and OA, where local application would be preferred over systemic administration.

Cytokines are potent modulators of immune and inflammatory reactions and other biological processes. Systemic administration of cytokines therefore may induce side effects resulting in severe morbidity or even death[10-12]. Although systemic administration of IL-4 and IL-10 was well tolerated[1, 4], systemic exposure to IL4-10 FP upon leakage of the local compartment may result in undesirable side effects, such as an increased risk of developing allergy. Therefore, we evaluated the feasibility to generate a modified IL4-10 FP that is active upon local administration but rapidly cleared from the circulation upon entering the bloodstream. IL4-10 FP has three potential N-linked glycosylation sites that can be occupied with complex glycans, which may be capped with a terminal sialic acid. Glycoproteins with glycans insufficiently capped with sialic acids are rapidly removed from the circulation by asialoglycoprotein receptors (ASGPR) in the liver[13]. Thus, engineering of sialylation can be used to modulate the

systemic exposure of a glycoprotein. To what extent sialylation impacts the bioactivity of IL4-10 FP is, however, not known. We hypothesized that selection of cell lines that produce IL4-10 FP with glycans insufficiently capped by sialic acids, may yield a glycoform of IL4-10 FP that is bioactive upon local administration, but is rapidly removed from the circulation when leaking from the local compartment. To test this hypothesis, we generated Chinese hamster ovary (CHO) cell lines producing IL4-10 FP. CHO cells provide a popular mammalian host for large-scale commercial production of therapeutic proteins as these cells are safe and allow high volumetric yields[14, 15]. We generated CHO cells lines producing high- and low-sialylated IL4-10 FP (IL4-10 FP highSA and IL4-10 FP lowSA, respectively). Recombinant IL4-10 FP glycoforms were tested for functional activity *in vitro*, for pharmacokinetics in a rat model, and for efficacy upon intrathecal injection in a mouse model of persistent inflammatory pain.

5.2 I Methods

5.2.1 I Generation of CHO cell lines. CHOBC® cell lines producing IL4-10 FP were generated in the facilities of Bioceros, Utrecht, the Netherlands. Unless indicated otherwise, CHOBC® cells, thawed from a working cell bank, were cultured and expanded in serum-free ProCHOTM-5 medium (Lonza) supplemented with 4 mM L-glutamine (Gibco) with pluronic F-68 (Life Technologies), at 0.1 or 0.2%, w/v (culture medium) in a humidified CO2 incubator. Cell number and viability were assessed with a CASY counter (Roche Innovatis AG). Levels of IL4-10 FP produced by the cells were measured in the supernatant with a human IL-10 ELISA kit (Sanquin, Cat# M9310) according to manufacturer's instructions.

To generate IL4-10 FP producing cell lines, CHOBC® cells were transfected with a proprietary expression plasmid containing cDNA encoding IL4-10 FP, and with a plasmid with cDNA encoding α2,3-sialyltransferase (ST3GAL_IV) using an AMAXA Nucleofector II device (Lonza). After transfection, cells were pooled and seeded at 1.0 x 106 viable cells (vc) per ml culture medium in T-flasks. Cells were incubated for 48 hours and seeded in 96-well plates at 300 to 3,000 vc/well in culture medium supplemented with Zeocin (Invitrogen) and Blasticidin (Invitrogen) (selection medium). The cells were incubated for ~3 weeks. Visible cell clones were then transferred into 24-well plates containing 2 ml fresh selection medium and screened for IL4-10 FP levels (IL-10 ELISA). Best producing clones were selected, cultured in 6-well plates and expanded into 25 ml T-flasks (4-day culture). Subsequently, they were adapted to culture under shaking conditions and cultured for 7 days while cell density, viability and

production were monitored at various intervals (7-day culture). In addition, sialylation of IL4-10 FP produced by different clones was analyzed using lectin-based ELISAs (see below). Finally, two cell lines producing IL4-10 FP with respectively low and high sialylation were selected.

5.2.2 I Glycan analysis with lectin-based ELISAs. Sialylation of IL4-10 FP produced by the CHOBC® clones was evaluated using ELISA-type assays with the galactose-binding Erythrina Cristagalli Lectin (ECL), or the α2,3 sialic acid binding Maackia Amurensis Lectin II (MAA). CHOBC® cell supernatant to be tested was diluted in PBS to yield a concentration of 10 µg/ml IL4-10 FP. 100 µl of diluted supernatant was coated in duplicate in the wells of maxisorp plates (Nunc). After O/N incubation at 4°C, plates were washed 3 times with PBS containing 0.05%, w/v, Tween-20 (Merck), followed by incubation with 200 µl/well PBS 0.1%, w/v, Tween-20 (PBS-T), 1 hour at room temperature (RT), while gently shaking (300 rpm). 100 µl of biotinylated ECL or MAA (Vector Laboratories), diluted 1 to 10.000 and 1 to 3000 in PBS-T, respectively, were added to the plates, which were subsequently incubated for 1 hour at RT. Bound biotinylated lectins were detected by incubation with Streptavidin poly-HRP (1:10,000, Sanquin) in PBS-T for 30 minutes at RT, followed by incubation with TMB (Invitrogen). The reaction was stopped with 1 M H2SO4 and the absorbance at 450 nm was measured. The ratio MAA/ECL, calculated using the optical density values at 450 nm (OD450) of bound MAA and bound ECL, is indicative for the sialylation ratio of the IL4-10 fusion protein.

5.2.3 I Purification of recombinant IL4-10 FP. IL4-10 FP was purified from conditioned medium using affinity chromatography according to a previously published protocol[16]. In short, protein G-purified, in-house made mouse anti-IL4 monoclonal antibody (mAb) was coupled to Cyanogen Bromide-activated Sepharose 4B (GE Healthcare Life sciences), according to manufacturer's instructions, and packed into a column. To prevent non-specific binding of proteins the column was flushed with phosphate buffered saline (PBS, pH 7.4), containing 1%, w/v, Bovine Serum Albumin (BSA), and then equilibrated with PBS without BSA. Concentrated conditioned medium containing IL4-10 FP was loaded on the column, which was then washed with PBS. Bound IL4-10 FP was eluted with 0.1 M Glycine, pH 2.25. Fractions were immediately neutralized with 1 M Tris, pH 9.0 and dialyzed in PBS. Fractions containing IL4-10 FP were pooled, assessed for purity and protein content by running a protein gel (see below), a Bicinchoninic Acid (BCA) protein assay (Thermo Scientific) and the IL-10 ELISA, aliquoted and stored at -80°C until further analysis.

5.2.4 I Protein Electrophoresis, Protein Stain & Western Blot. Cell supernatants were diluted 1 to 1.33 in Laemmli sample buffer (BioRad), containing 100 mM Dithiotreitol (Sigma-Aldrich), incubated for 10 minutes at 100˚C, whereafter 10 ul was loaded on a 12% polyacrylamide Gel (Mini-PROTEAN-TGX, BioRad). After electrophoresis, the gel was either stained with Instant Blue (Expedeon) for visualization of total protein content, or prepared for Western blotting in order to specifically detect IL4-10 FP. Blotting was performed using the Trans-Blot system (BioRad) in combination with a 0.2 µm nitrocellulose-membrane Transfer Pack (BioRad), according to manufacturer's instructions. After transfer, the membranes were blocked in 4% milk (Elk, Campina) in PBS 0.1%, w/v, Tween-20 (PBS-T). The membrane was incubated with the primary Ab mouse anti-human IL-4 (1:400, Santa Cruz Biotechnology, Clone 13Z07) or mouse anti-human IL-10 (1:100, Santa Cruz, Clone 3C12C12), in 1% milk in PBS-T, followed by HRP-conjugated goat anti-mouse IgG (1:2000, Santa Cruz). To visualize the bands enhanced chemiluminescence (ECL) Western blotting substrate was added according to manufacturer's protocol (Pierce, Thermo Scientific). Stained protein gels and Western blots were imaged using the ChemiDoc MP system (BioRad).

5.2.5 I Functional activity of IL4-10 FP. Heparinized human blood obtained from healthy volunteers was diluted 1:10 in RPMI1640 medium (Invitrogen), supplemented with 1% penicillin/streptomycin (P/S, PAA Laboratories) in 48-well culture plates (Nunc). Lipopolysaccharide (LPS, Sigma-Aldrich) was added at 10 ng/ml, as well as low and high sialylated IL4-10 fusion protein at 0.01 – 3 nM (0.3 - 270 ng/ml), final concentrations. As control, HEK293 produced IL4-10 fusion protein was taken along 16. After 18 hours incubation at 37°C, 5% CO2, culture supernatant was collected and tested for TNF concentration with ELISA (DiaClone, Cat# 851 570 020). The percentage inhibition was calculated relative to TNF production by LPS in absence of IL4-10 FP.

5.2.6 I Deglycosylation of IL4-10 FP. IL4-10 FP was enzymatically deglycosylated using PNGaseF (New England BioLabs) according to manufacturer's protocol. Effectiveness of deglycosylation was monitored by Western blotting.

5.2.7 I Rat model to evaluate the pharmacokinetics of IL4-10 FP. Clearance of IL4-10 FP from the circulation was studied in Wistar Crl:WI female rats (mean body weight 205 ± 15 grams; Charles River Laboratories). The study was approved by the animal ethical committee (project number AVD11500201744) and performed at the animal facility of the Utrecht University. Rats were acclimatized for 1 week prior to the experiment. Rats were intravenously injected via

the tail vein with 5 µg IL4-10 FP lowSA or IL4-10 FP highSA, or with a mixture of 5 µg IL-4 (eBioScience) and 5 µg IL-10 (Invitrogen) in a volume of 200 µl PBS. Some rats were injected intravenously with 30 mg asialofetuin (Sigma-Aldrich) in 0.5 ml PBS 10 minutes prior to injection of low sialylated IL4-10 FP, to saturate the asialoglycoprotein receptor (ASGPR). Blood was collected from the tail using a winged infusion system (150 ul per timepoint) in lithium-heparin tubes (Sarstedt) either at 5, 15, 30 and 120 minutes, or 10, 20, 60 and 120 minutes after injection of IL4-10 FP and plasma was prepared by centrifugation for 5 minutes at 2000g. IL4-10 FP and IL-10 levels were measured with the IL-10 ELISA. IL-4 levels were measured with a human IL-4 ELISA (Sanquin, Cat# M9314) according to manufacturer's instructions. Pharmacokinetics were evaluated using non-linear mixed effects modeling (with NONMEM v.7.3). Models were parameterized in terms of fixed effects (population mean) and two levels of random effects; per subject (inter-individual variability [IIV]) and per measurement (residual variability). A one-compartmental model with linear elimination was used as a structural model. Herein, clearance was assumed to be different for IL-10, IL-4, and IL4-10 FP. For IL4-10 FP, the clearance was further differentiated into a slow and fast route, where the fast route represented rapid ASGPR-mediated clearance. The fraction of the dose administered that eliminated via the fast route was estimated separately for high and low sialylated IL4-10 FP. For the rats that received asialofetuin prior to injection with IL4-10 FP, the proportion fast clearance was assumed to be zero. The 95% confidence interval of parameter estimates were calculated by sampling-importance-resampling (SIR). Half-life was calculated using the formula: T1/2 = 0.693*(V/CL), where V is the distribution volume (ml) and CL is the clearance rate (ml/min).

5.2.8 I Mouse model for inflammatory pain. The efficacy of IL4-10 FP to reduce persistent inflammatory pain was tested in the carrageenan-induced inflammatory pain model as described before[7]. Briefly, carrageenan (2%, 20 µL; Sigma-Aldrich) was injected in both hind paws of 8-12 weeks old C57Bl/6 mice to induce persistent inflammatory pain. Six days after carrageenan injection, 1 ug low or high sialylated IL4-10 FP (in 5 µl PBS; n=9 for both groups) or vehicle (saline; n=7) was injected intrathecally. Mechanical hypersensitivity was measured using von Frey hairs (Stoelting), and the 50% paw withdrawal threshold was calculated using the up-and-down method[17]. Investigators performing the behavioral assays or assessing outcome were blinded for treatment. Experiment was performed in two sessions and results (group mean) were analyzed with a repeated measures 2-way ANOVA with the Geisser-

Greenhouse correction, followed by a Tukey's post hoc test, with individual variances computed for each comparison.

5.3 I Results

5.3.1 I CHO cell lines producing IL4-10 FP. CHO cell lines producing IL4-10 FP were generated according to the described procedures. During screening for production and sialylation, lead cell lines were selected using a combination of IL-10 and lectin-based ELISAs. The sialylation of recombinant IL4-10 FP, produced by 16 finally selected cell lines is shown in **Fig 1B**. In the lectin-based ELISA, bound recombinant protein is fixed to an ELISA plate and assessed for sialylation using MAA and ECL lectins, which are specific for end-standing α2,3-sialic acid or galactose, respectively. The ratio of the OD450 values obtained with both lectins, was taken as a measure for sialylation of IL4-10 FP (**Fig 1A**). Moderate sialylation, with a MAA/ECL ratio of 2, was observed for 8 of the clones. One clone, CHO38, had a much lower MAA/ECL ratio (0.7), indicating that this cell line produced low sialylated IL4-10 FP (IL4-10 FP lowSA), while a relative high MAA/ECL ratio (3.8) was observed for clone CHO372, indicative for the production of high sialylated IL4-10 FP (IL4-10 FP highSA). Indeed, in case of clone CHO38, preferential ECL binding suggested the presence of IL4-10 FP with glycans containing end-standing galactose, produced by this cell line. Conversely, IL4-10 FP produced by the cell line CHO372 had most glycans capped with α2,3 sialic acid, as shown by the high MAA-lectin OD450 (**Fig 1C**).

Assessment of the productivity during cell line generation indicated specific IL4-10 FP production by cell line CHO38 of 3.1 pg/cell/day (production (in picogram) per cell per day; pcd) as a result of the 4-day culture and 4.2 pcd during the 7-day culture. For CHO372, the specific IL4-10 FP productivity was 3.9 and 3.7 pcd during 4-day culture and 7-day culture, respectively (**Fig 2A**). These results demonstrate that IL4-10 FP production during different cultures and conditions is consistent and sufficiently high for further development. Cumulative growth curve and volumetric productivity over time, during the 7-days culture of both cell lines are presented in **Fig 2B** and **2C** and confirm, together with the data in Fig 2A that cell viability and productivity of the selected clones were as expected.

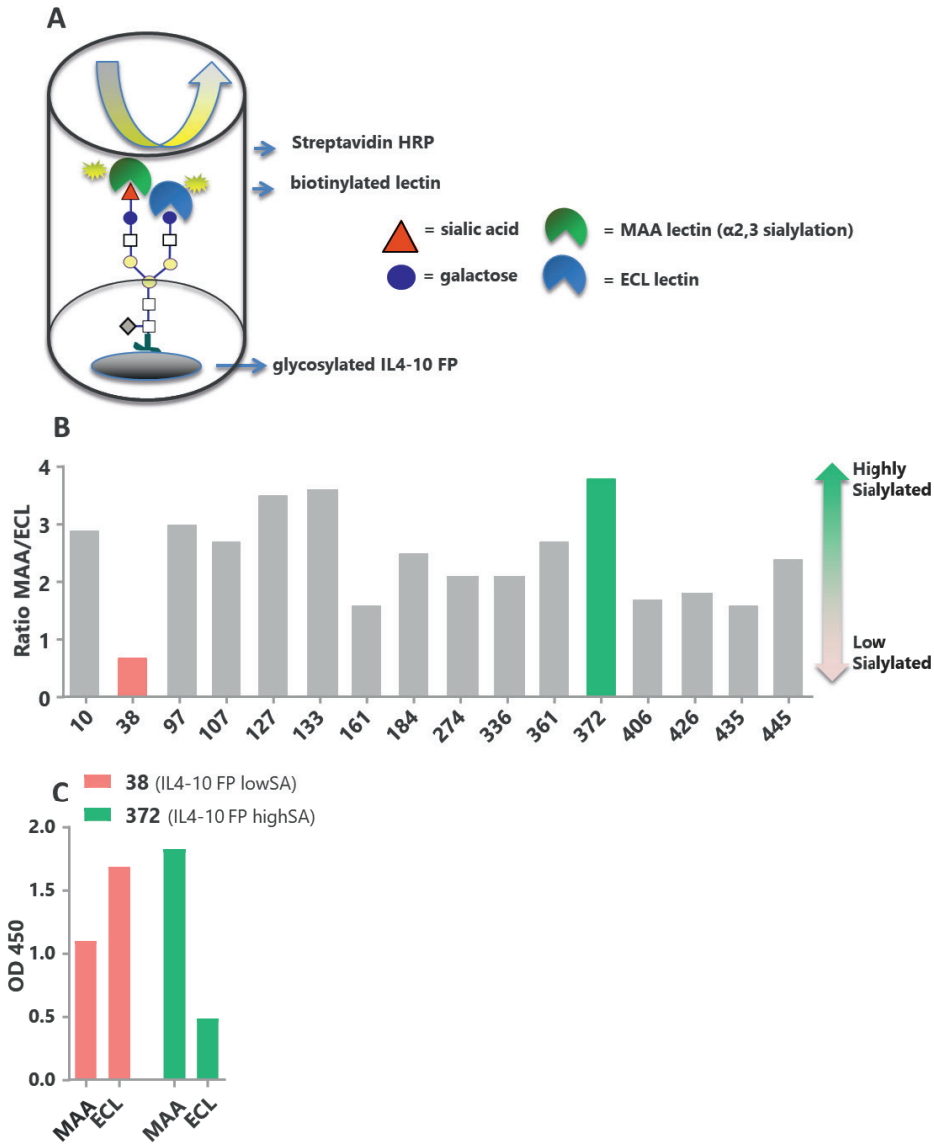


Figure 1. Differential sialylation of IL4-10 FP produced by various CHO cell lines. IL4-10 FP was produced by various CHO cell lines, and evaluated for sialylation using lectin-based analysis. Conditioned medium containing IL4-10 FP was coated on ELISA plates, biotinylated MAA-lectin and biotinylated ECL-lectin were used to detect end-standing sialic acids or end-standing galactose residues, respectively (**A**). The ratio of 450 nm optical densities between MAA and ECL reflects the degree of sialylation of the cell lines (**B**). IL4-10 FP produced by the cell lines CHO38 and CHO372 has least (IL4-10 FP lowSA) and most (IL4-10 FP highSA) end-standing sialic acids, respectively. The raw

OD450 values show that IL4-10 FP lowSA contains some glycans capped with sialic acids, while IL4-10 FP highSA still contains some end-standing galactose residues (**C**).

A

		IL4-10 FP lowSA (CHO38)	IL4-10 FP highSA (CHO372)
1st screening	µg/ml	2.383	1.650
4-day culture	µg/ml	8.615	7.699
	pcd (pg)	3.116	3.849
7-day culture	µg/ml	49.09	20.27
	pcd (pg)	4.234	3.739

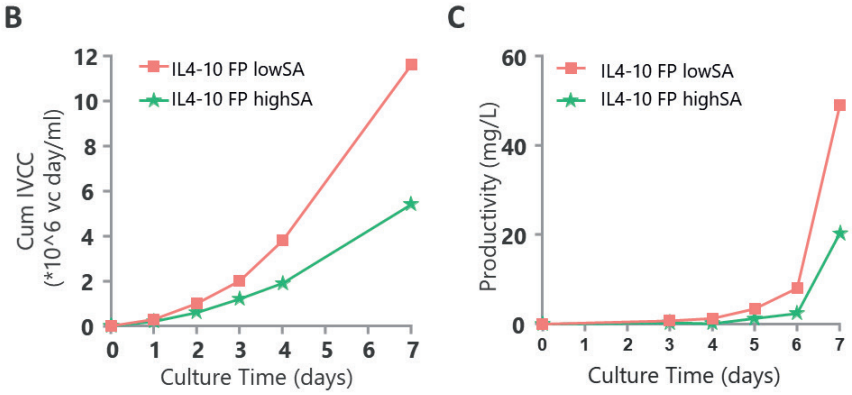


Figure 2. Viability and productivity of CHO cell lines 38 and 372. Productivity of the cell lines CHO38 and CHO372 producing respectively IL4-10 FP lowSA and IL4-10 FP highSA, during cell-line development (**A**). Cumulative integral viable cell concentration (**B**) and volumetric yield during 7-days culture are plotted (**C**). Production of IL4-10 FP was measured using a human IL-10 sandwich ELISA.

5.3.2 I Characterization of IL4-10 FP produced by CHO38 and CHO372 cell lines. Both IL4-10 FP lowSA and IL4-10 FP highSA appeared as protein bands with an apparent mass of ~34 kDa on a protein-stained SDS PAGE gel (**Fig 3A**) as well as on Western blot, where both recombinant proteins were identified with an anti-IL4 and anti-IL10 antibody (**Fig 3B**). Purification of IL4-10 FP lowSA and IL4-10 FP highSA from culture medium using affinity chromatography via an anti-IL4 antibody recovered ~50% of the recombinant protein and yielded ~90-95% purity (data not shown). Both IL4-10 FPs inhibited LPS-induced TNF production in whole blood in a concentration dependent manner, with maximal inhibition at

3 nM (**Fig 3C**). The inhibitory activity was comparable between IL4-10 FP lowSA and IL4-10 FP highSA. Furthermore, both glycoforms inhibited LPS-induced TNF production similar to that of HEK293-produced IL4-10 FP (used in our previous *in vitro* and *in vivo* studies) 16. These data indicate that the cellular platform (HEK293 or CHO cells) used for production, nor differential sialylation affect the potency of IL4-10 FP. Next, we further evaluated whether glycosylation affects the functional ability of IL4-10 FP by deglycosylation of IL4-10 FP produced by both clones with PNGaseF. Importantly, deglycosylation did not affect the dose-dependent inhibition of LPS-induced TNF production by both IL4-10 FP lowSA and IL4-10 FP highSA. (**Fig 3D**). These results indicate that neither glycosylation nor sialylation affect the inhibitory capacity of human IL4-10 FP *in vitro*.

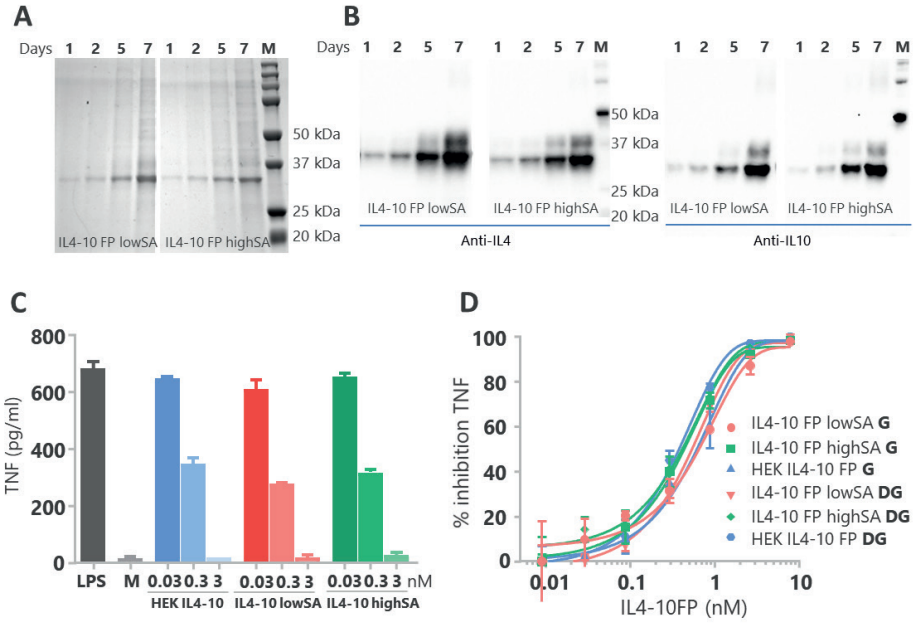


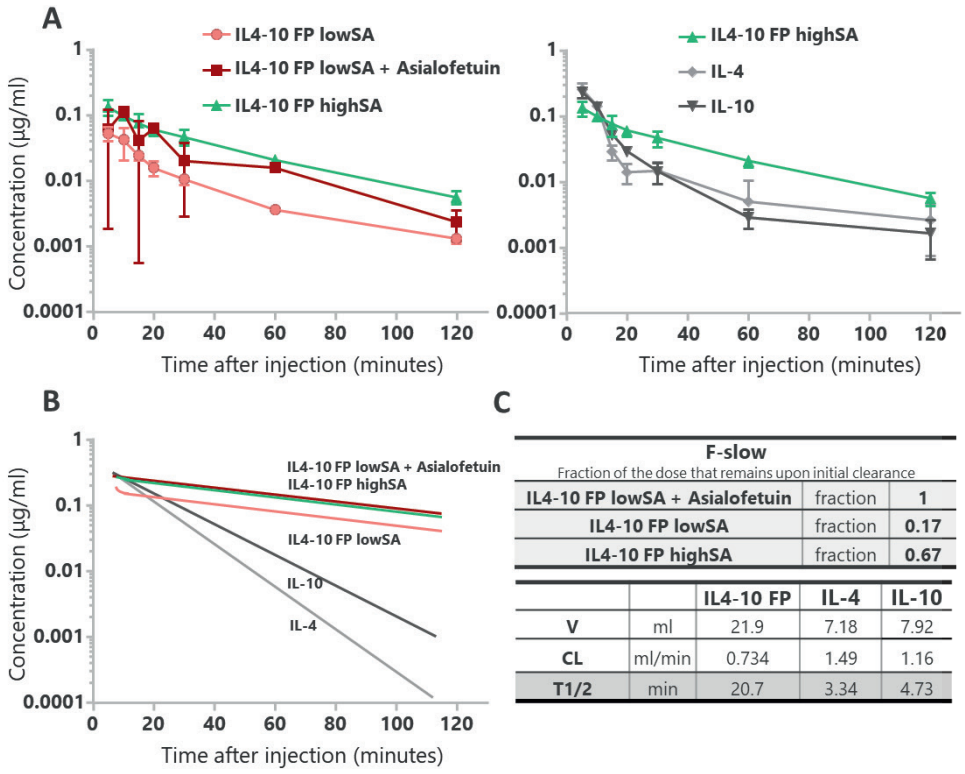
Figure 3. Characterization of IL4-10 FP produced by the cell lines CHO38 and CHO372.

Production of IL4-10 FP during 7 days culture by CHO38 (IL4-10 FP lowSA) and CHO372 (IL4-10 FP highSA), as shown on protein-stained SDS-PAGE (**A**; IL4-10 FP migrates at ~33kD) and immunoblot with anti-IL4 (**B**; left) and anti-IL10 (**B**; right). IL4-10 FP was purified from culture medium of each cell line using affinity chromatography with anti-IL4 mAb, and incubated overnight in whole blood together with LPS, as was purified IL4-10 FP produced by HEK293 cells. Cultures were measured for TNF production. The activity of IL4-10 FP is shown by means of TNF inhibition compared to production with LPS only (**C**). The effect of deglycosylation ("DG") of IL4-10 FP on the functional activity in the whole

blood assay was tested upon PNGaseF treatment of IL4-10 FP highSA, IL4-10 FP lowSA and HEK293 produced IL4-10 FP. As controls, glycosylated ("G") IL4-10 FP incubated under equal conditions but without PGNaseF, was also tested (**D**). Data in C and D represent mean and SD of duplicates.

5.3.3 I Clearance of IL4-10 FP is dependent on sialylation. In a pharmacokinetics study we investigated whether the differential sialylation of IL4-10 FP affects its clearance from the circulation. Each IL4-10 FP preparation was injected in rats intravenously (5 µg via the tail vein), and the circulating concentration of IL4-10 FP in serial plasma samples was assessed by ELISA.

Concentrations of IL4-10 FP showed an initial rapid decrease upon administration of IL4-10 FP lowSA presumably reflecting fast hepatic clearance (**Fig 4A and B**). Administration of asialofetuin in animals prior to injection with IL4-10 FP lowSA largely prevented the fast elimination of IL4-10 FP, indicating that the rapid clearance of IL4-10 FP lowSA in part is mediated by the ASGPR. However, a small fraction of the administered dose shows a slow clearance, similar to the clearance of IL4-10 FP lowSA in combination with asialofetuin (**Fig 4C**; estimated fraction "F-slow" is 0.17, 95% confidence interval 0.14-0.22). This indicates that the elimination route of that fraction of IL4-10 FP lowSA is not sialylation-dependent, which can be explained by the presence of a small fraction of sialylated IL4-10 FP in the batch of IL4-10 FP lowSA. The slow elimination pattern was also observed for IL4-10 FP highSA. However, the fraction of highly sialylated IL4-10 FP in this product was much higher, as expected (**Fig 4C**; estimated fraction "F-slow" is 0.67, 95% confidence interval 0.5-0.86). The slow elimination of IL4-10 FP (most likely representing the elimination of the highSA fraction of both products) resulted in a half-life of 20.7 minutes (**Fig 4B and C**). In contrast, half-lives of wild-type IL-10 and IL-4 were much shorter and were estimated at 4.73 minutes and 3.34 minutes, respectively (**Fig 4B and C**).



C

F-slow Fraction of the dose that remains upon initial clearance		
IL4-10 FP lowSA + Asialofetuin	fraction	1
IL4-10 FP lowSA	fraction	0.17
IL4-10 FP highSA	fraction	0.67

		IL4-10 FP	IL-4	IL-10
V	ml	21.9	7.18	7.92
CL	ml/min	0.734	1.49	1.16
T1/2	min	20.7	3.34	4.73

Figure 4. Clearance of high and low sialylated IL4-10 FP upon intravenous injection in rats. Wistar rats were injected with IL4-10 FP highSA or IL4-10 FP lowSA (20 µg each) or with a combination of IL-4 and IL-10 (10 µg each) via the tail vein. Some rats injected with IL4-10 FP lowSA were pretreated with asialofetuin, to block the ASGPR in the liver. Levels of IL4-10 FP, IL-4 and IL-10, were then measured in plasma samples collected at various time intervals (**A**). Curves represent mean and SD of 6 animals in total, composed of samples taken at alternating time-points (3 animals per time-point). The alternating sampling gives some variation in the IL4-10 FP lowSA group, pre-injected with Asialofetuin. Pharmacokinetic parameters were calculated using a one-compartmental model with linear elimination in NONMEM (**B, C**).

5.3.4 I IL4-10 inhibits persistent inflammatory pain in mice. Next, we tested whether low or high sialylation of IL4-10 FP affects its therapeutic potential to suppress inflammatory pain *in vivo*. Intrathecal injection of IL4-10 FP produced in HEK293 cells reduces pain in various mouse models, including models for persistent inflammatory pain7. For the current study we evaluated the efficacy of CHO produced IL4-10 FP lowSA and IL4-10 FP highSA to reduce

carrageenan-induced inflammatory pain. Intraplantar injection of carrageenan increased sensitivity to mechanical stimuli in mice, indicating development of mechanical hyperalgesia. Intrathecal injection of IL4-10 FP at day 6 after carrageenan injection inhibited established mechanical hyperalgesia for 3-4 days. Importantly, IL4-10 FP highSA and IL4-10 FP lowSA suppressed persistent inflammatory hyperalgesia to the same extent and duration **(Fig 5)**. These results indicate that the efficacy of IL4-10 FP upon intrathecal injection is not affected by sialylation in this inflammatory pain model.

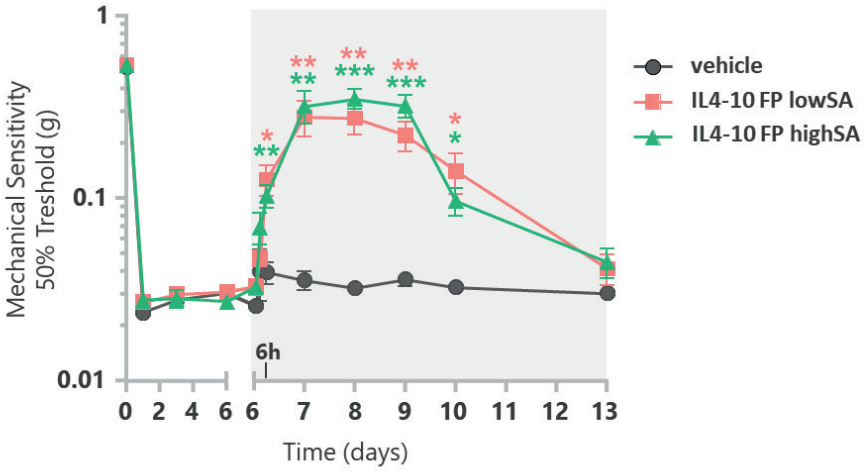


Figure 5. Differential sialylation does not affect the efficacy of intrathecally injected IL4-10 FP to inhibit established persistent inflammatory pain in mice. Persistent inflammatory pain was induced by an intraplantar injection of 20 µl of 2% carrageenan. Six days (d) after carrageenan injection, mice received an intrathecal injection of 1 µg IL4-10 FP highSA (n=9), IL4-10 FP lowSA (n=9), or vehicle (n=7). Mechanical hypersensitivity was measured over time using the von Frey test. Results were expressed as 50% threshold (g) and represent mean and standard error of two combined experiments. Colored asterisks mark statistical differences compared to vehicle-treated mice. The grey asterisk marks a statistical difference between IL4-10 FP highSA and IL4-10 FP lowSA on day 3. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

5.4 I Discussion

Intrathecal administration of IL4-10 FP inhibits hyperalgesia and allodynia in mouse models of chronic pain in a superior fashion compared to stand-alone wild-type IL-4 or IL-10, or a combination therapy of both cytokines[7]. For clinical application of IL4-10 FP to treat pain by local therapy it might be important to limit systemic exposure to avoid potential side effects. Here we describe the development of two IL4-10 FP glycoforms expressed by CHO, one with glycans poorly capped with sialic acids (IL4-10 FP lowSA) and one with glycans that are mainly capped with sialic acids (IL4-10 FP highSA). The poorly sialylated IL4-10 FP lowSA had a faster clearance from the circulation compared to the highly sialylated IL4-10 FP highSA, whilst retaining its functional ability to inhibit inflammatory responses *in vitro* and to inhibit persistent inflammatory pain *in vivo*.

Depending on culture conditions, expression of therapeutic glycoproteins in CHO and other mammalian cell lines often yields proteins with incomplete capping of glycans by sialic acids18. Incomplete sialylation of their glycan residues may lead to the rapid removal of therapeutic proteins from the circulation through the uptake by the asialoglycoprotein receptor (ASGPR) in the liver[19, 20]. This mechanism may account for a serum half-life of only a few minutes. As biologics are nowadays mostly administered systemically, improving sialylation in order to increase systemic exposure is a well-known tool that is applied to improve the pharmacokinetic characteristics of therapeutic proteins. On the other hand, if the treatment with therapeutic proteins needs to be local, an under-sialylated form of a therapeutic protein would be a drug of choice. To evaluate the effect of differential sialylation of IL4-10 FP, we engineered the CHO cells by introducing co-transfection with α2,3-sialyltransferase. Most proteins obtained from cell lines using this strategy were indeed significantly sialylated. Cell line CHO372 showed most extensive sialylation of IL4-10 FP and this cell line was selected to produce a research batch of IL4-10 FP highSA. However, co-transfection of cells with α2,3 sialyltransferase does not guarantee cell lines that produce high-sialylated glycoprotein. Rather, this strategy may result in a variable number of cell lines with poor sialylation capacity[28]. We indeed identified a cell line, CHO38, that produced IL4-10 FP which predominantly bound ECL lectin, suggesting the presence of glycans with mainly end-standing galactose. This cell line was used to produce a research batch of IL4-10 FP lowSA.

Although glycosylation of proteins affects their systemic exposure *in vivo*, the different capping of glycans did not affect the anti-inflammatory properties of IL4-10 FP *in vitro* and *in*

vivo. In the whole blood assay, the potency of both glycoforms of IL4-10 FP appeared to be similar. This is in line with the findings that wild-type IL-4 and IL-10, expressed in E.coli and therefore non glycosylated, are functionally active[21, 22]. Furthermore, both IL4-10 FP lowSA and IL4-10 FP highSA were equally able to inhibit chronic inflammatory pain in mice.

The contribution of differential glycosylation to the systemic exposure was shown in the pharmacokinetics study by the rapid initial elimination of IL4-10 FP lowSA (upon injection, only 17% was left in circulation). Blockade of the ASGPR with asialofetuin[23] in rats that were injected with IL4-10 FP lowSA, yielded a similar clearance curve as compared to IL4-10 FP highSA, indicating that the differences in initial clearance between both glycoforms were mostly due to interaction of IL4-10 FP lowSA with the ASGPR in the liver. For IL4-10 FP highSA, the initial elimination was much lower, resulting in 67% IL4-10 FP in circulation upon injection. The fraction of the dose that remained in circulation was however lower than expected, since IL4-10 FP highSA is highly sialylated and should therefore be protected against clearance via the ASGPR. Blockade of the ASGPR in rats injected with IL4-10 FP highSA did not affect the clearance (data not shown), suggesting other mechanisms that contribute to the initial clearance of IL4-10 FP, such as tissue distribution, or binding to other receptors, such as IL10R or IL4R on PBMCs. In addition, insufficiently capped glycoproteins can be cleared via other receptors, like the mannose receptor that binds end standing n-acetylglucosamine glycans, too. Based on our data in which the ASGPR is specifically blocked by asialofetuin, it seems that the mannose receptor has a minor role in initial clearance, if any.

Although the initial clearance of IL4-10 FP lowSA differed from that of IL4-10 FP highSA, the terminal serum half-life for both IL4-10 FP lowSA and IL4-10 FP highSA were similar. This result is likely due to the presence of a fraction of IL4-10 FP with sialylated galactose-residues in the batch of IL4-10 FP lowSA. Indeed, glycan analysis of IL4-10 FP lowSA, though predominantly showing ECL binding, revealed significant MAA binding too, pointing to the presence of galactose-residues capped with sialic acids.

Pharmacokinetics in humans of both IL-4 and IL-10 have been studied and indicated a relative short half-life for both cytokines. The apparent serum half-life of recombinant human IL-4 is 19 minutes[24], while the serum half-life of recombinant human IL-10 is much longer, 2.7 to 4.5 h[25]. Indeed, we also observed short half-lives for both cytokines in rats, with IL-4 being cleared more rapidly than IL-10. Homodimerization of IL-10 increases the size of IL-10, compared to that of IL-4 (36 kDa vs 15 kDa). This difference in size impacts the clearance via the kidneys

that is mainly affected by the size and the charge of a protein. IL4-10 FP highSA is expressed as a dimer with a molecular weight of ~70 kDa[7]. Indeed, we observed a considerably increased serum half-life of IL4-10 FP highSA (approximately 21 minutes), which underlines the contribution of the molecular weight to the clearance rate.

IL-4 and IL-10 are potent anti-inflammatory cytokines that in preclinical studies yielded promising results[26, 27]. Yet clinical results with recombinant IL-4 and IL-10 were disappointing, possibly because these cytokines have limitations when used as stand-alone therapeutic molecules. These limitations include poor pharmacokinetics due to rapid renal clearance, failure to suppress multiple pro-inflammatory mediators in inflammatory diseases in absence of other anti-inflammatory cytokines, and counterbalance of anti-inflammatory effects by their immune-stimulating activities. We postulate that a fusion protein of IL-4 and IL-10 may in part overcome the limitations of stand-alone IL-4 or IL-10 since a) the IL-4 moiety of IL4-10 FP neutralizes the immune-stimulating activities of IL-10 such as enhanced expression of activating Fc-receptors[8]; b) IL4-10 FP has synergistic effects, combining the functional activities of two anti-inflammatory cytokines; and c) improved half-life of IL4-10 FP by reduced renal clearance compared to wild-type IL-10 or IL-4. Indeed, superior efficacy of IL4-10 FP compared to (a combination of) wild-type IL-4 and IL-10 was observed in inflammatory pain models[7]. Here we report two different glycoforms of IL4-10 FP with similar *in vitro* potency and different clearance from the circulation. Importantly, both forms effectively reduce inflammatory pain upon local, intrathecal administration. Thus, IL4-10 FP lowSA is an attractive option for local administration as the risk for systemic effects in case of leakage from the local compartment will be minimal due to a rapid clearance by the liver. Conversely, IL4-10 FP highSA might be suitable for systemic therapy since it provides improvements compared to stand-alone IL-10 and IL-4 such as a longer serum half-life and synergistic therapeutic effects. Future studies are warranted to evaluate the clinical potential of either molecule.

5.5 I Key words

Inflammation, cytokines, therapeutic protein, sialylation, pharmacokinetics

5.6 I Acknowledgements

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5.7 I Disclosures

Cristine Steen-Louws, Judith Prado, Erik Hack, Niels Eijkelkamp and Jelena Popov-Celeketic are shareholders of Synerkine Pharma BV, a recently founded company in which in which preclinical development and evaluation of IL4-10 fusion protein is enrolled. Cristine Steen-Louws, Peter Boross, Judith Prado, Alwin Huitema, Floris Lafeber, Erik Hack, Niels Eijkelkamp and Jelena Popov-Celeketic are co-inventors of the patent “Modified therapeutic glycoprotein for local administration”. Erik Hack and Floris Lafeber are consultants for Synerkine Pharma BV. Jelena Popov-Celeketic is partly employed by Synerkine Pharma BV.

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Chapter 6

Glycoengineering prolongs plasma half-life of IL4-10 fusion protein

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Submitted

Abstract

Introduction. Glycoengineering as strategy to increase the plasma half-life of therapeutic proteins was applied to a potential novel biologic, IL4-10 fusion protein (IL4-10 FP).

Strategy. Three additional glycosylation sites were introduced in the IL4-10 FP sequence creating IL4-10 FP Glyc3+. Sites were chosen on basis of homology between species and the change of a single amino acid.

Results. IL4-10 FP Glyc3+ was expressed by HEK293 cells as a properly folded and bioactive protein, although the *in vitro* activity of IL4-10 FP Glyc3+ was somewhat impaired as compared to wt IL4-10 FP. An increased molecular size of 33 kDa and a two-fold extended plasma half-life was determined for IL4-10 FP Glyc3+.

Conclusion. Glycoengineering improves the plasma half-life of human IL4-10 FP.

6.1 I Introduction

Biologics constitute the fastest growing class of therapeutics in the pharmaceutical industry, with on average 43 new therapeutics approved by the FDA on a yearly basis, and a record of 59 biologics approved in 2018 (Mullard, 2018; Walsh, 2018). The majority of these biologics are still monoclonal antibodies, but effort is also made in the field of other protein drugs like hormones, growth factors, cytokines, et cetera. A common challenge in non-antibody therapeutic protein development is the short plasma half-life due to a relatively small molecular mass. Effective treatment with such a biologic may require frequent administration, which impacts the treatment burden. Several strategies have been developed to improve the plasma half-life of biologics, including pegylation, glycoengineering, Fc-coupling and coupling to serum albumin (Kontermann, 2011). These different approaches all have their specific advantages and disadvantages.

Previously, our group developed a fusion protein of the human cytokines interleukin 4 (IL-4) and interleukin 10 (IL-10), named IL4-10 FP (Eijkelkamp et al., 2016). The rationale of IL4-10 FP is to combine the immunoregulatory activities of both cytokines for the treatment of inflammatory disorders like rheumatoid arthritis, osteoarthritis and inflammatory pain. And, additionally, to create a therapeutic with a larger molecular mass compared to the wild-type cytokines, to improve the plasma half-life and systemic exposure. Human IL4-10 FP showed improved efficacy in several *in vitro* and *in vivo* disease models, as compared to the effect of the individual cytokines IL-4 or IL-10, or a combination of both cytokines (Eijkelkamp et al., 2016; Steen-Louws et al., 2019). Moreover, human IL4-10 FP has a fourfold longer plasma half-life as compared to wild-type IL-10 (Steen-Louws, 2020). Further improvement of the systemic exposure promises a superior therapeutic effect of IL4-10 FP.

Glycoengineering is a proven strategy to manipulate the serum half-life of biologics. Previously, we have shown that engineering low sialylation of IL4-10 FP is an effective approach to reduce its systemic exposure upon local administration (Steen-Louws, 2020). In contrast, Darbepoetin alfa (Aranesp), human erythropoietin with two additional N-linked glycans has a threefold increased plasma half-life in humans (Kiss, Elliott, Jedynasty, Tesar, & Szegedi, 2010), illustrating that glycoengineering can also be used to prolong the plasma half-life of biologics. Notably, darbepoetin alfa had ~ twofold lower specific activity *in vitro* than wild-type erythropoietin due to decreased affinity for its receptor. Yet, the *in vivo* effect of

darbepoetin alfa was markedly increased compared to wild-type erythropoietin: threefold less glycoengineered erythropoietin as compared to the wild-type variant was needed to achieve a similar erythropoietic effect when administered thrice-weekly, whereas given as a single weekly injection thirteenfold less darbepoetin alfa was needed (Egrie, Dwyer, Browne, Hitz, & Lykos, 2003; Sinclair, & Elliott, 2005). The example of darbepoetin alfa illustrates that a lower *in vitro* activity, can be readily compensated by a longer half-life *in vivo*.

Here we explored the feasibility to prolong the plasma half-life of human IL4-10 FP by introducing additional glycans in the molecule. We identified three sites in human IL4-10 FP, comparing its sequence with that of other animal species, that could be used to introduce additional glycosylation sites. We carried out a one-by-one point mutation strategy, in which the effect of each additional glycan on the bioactivity of IL4-10 FP was studied separately. Various glycoengineered variants were expressed in HEK293 cells, and functionally characterized *in vitro* and in a *in vivo* pharmacokinetics study in rats.

6.2 I Methods

6.2.1 I Introduction of point mutations to add glycosylation sites. To identify potential sites for insertion of additional glycans, the amino acid sequence (aa-sequence) of human IL4-10 FP was aligned with the sequence of canine, mouse and rat IL4-10 FP (**Supplementary Figure 1**). Animal sequences were then screened for the presence of N-X-S/T consensus motifs and compared to the human sequence. In this way two sites were identified in the human sequence, in which the mutation of a single amino acid into an asparagine (N), would generate a glycosylation site at the same position as occurring in at least one of the animal sequences. Additionally, a third site was identified in which the mutation of a single amino acid into an asparagine (N) would generate a glycosylation site. The three amino acids to be replaced in the human sequence were aspartic acid at position 4 (D4); which would generate a potential glycosylation site as in canine IL4-10 FP, glutamine at position 142 (Q142); which would generate a potential glycosylation site N-terminally of IL-10, and serine at position 149 (S149); which would generate a potential glycosylation site in the human sequence, comparable with those found in mouse and rat IL4-10 FP. To evaluate the possible effect of each of the point mutations D4N, Q142N and S149N, a total of seven constructs with single mutations and all possible combinations were generated (table 1).

Construct Name	Position of point mutations	Number of PM(s)	Location of PM(s)
Clone 1	D4N	1	IL4
Clone 2	Q142N	1	IL10
Clone 3	S149N	1	IL10
Clone 4	Q142N, S149N	2	IL10
Clone 5	D4N, Q142N	2	IL4/IL10
Clone 6	D4N, S149N	2	IL4/IL10
Clone 7	D4N, Q142N, S149N	3	IL4/IL10
wt IL4-10 FP	- - -	0	- - -

Table 1: Overview of the glycoengineered constructs with the locations of the point mutations (PMs).

6.2.2 I Production and Purification. Wild-type human, codon optimized, IL4-10 FP (wt IL4-10 FP) was obtained from GeneArt (Thermo Scientific). Mutations were inserted using PCR techniques with specific primers. In addition, a polyhistidine tag was introduced N-terminally of IL4-10 FP sequence to enable purification. Mutants and wt IL4-10 FP were cloned in a dual CMV-promoter expression vector (Meeldijk, & Hack, unpublished), containing an α2,3 sialyltransferase gene to enhance sialylation of the proteins. The inserts of all final constructs were confirmed by Sanger sequencing. Proteins were expressed in HEK293 cells by transient transfection as described previously (Prado et al., 2018). Recombinant protein was isolated from the culture supernatant using a nickel-affinity chromatography (Ni-NTA, Thermo Scientific), according to manufacturer's instructions. The elution fraction was dialyzed in PBS, assessed for purity and protein content with SDS-PAGE (see below) and a BCA protein assay (Thermo Scientific), aliquoted and stored at -80°C until further analysis.

6.2.3 I Protein Electrophoresis and Western Blotting. Purified IL4-10 FP was pre-diluted in PBS and further diluted 1:1.33 in Laemmli sample buffer (BioRad) containing 100 mM dithiothreitol (Sigma-Aldrich), to obtain a final concentration of 50 µg/ml for total protein stain, or 1 µg/ml for Western blotting. Samples were incubated for 10 minutes at 100˚C, whereafter 10 µl was loaded on a 12% polyacrylamide gel (Mini-PROTEAN-TGX, BioRad). After electrophoresis, the gel was either stained with Instant Blue (Expedeon) for visualization of total protein content, or prepared for Western blotting to specifically identify IL4-10 FP.

Blotting was done with the TurboBlot system (BioRad) in combination with pre-fab packages containing a nitrocellulose-membrane, according to manufacturer's instructions. After transfer, the membranes were blocked in 4%, w/v, milk (Elk, Campina) in PBS 0.1%, v/v, Tween-20 (PBS-T). The membrane was then incubated with the primary Ab (mIgG1 anti-human IL-4 (1:400) or mIgG1 anti-human IL-10 (1:100), Santa Cruz Biotechnology), in 1% milk in PBS-T, followed by HRP-conjugated goat anti-mouse IgG (1:2000, Santa Cruz). In the case of canine IL4-10 FP, membranes were incubated with monoclonal mouse anti-canine IL-10 (1 µg/ml, R&D systems), in 1% milk in PBS-T, and subsequently with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). To visualize the bands ECL Western blotting substrate was added according to manufacturer's protocol (Pierce, Thermo Scientific). Stained protein gels and Western blots were analyzed using the Chemidoc Image Station (BioRad).

6.2.4 I Whole blood assay and TNF ELISA. The potency assay was performed as published before (Prado et al., 2018). Briefly, heparinized human blood obtained from healthy volunteers was diluted 1:10 in RPMI1640 medium (Invitrogen), supplemented with 1% penicillin/streptomycin (P/S, PAA Laboratories) in 48-well culture plates (Nunc). Lipopolysaccharide (LPS, Sigma-Aldrich) was added at 10 ng/ml, as well as IL4-10 FP or mutants thereof, at 0.003 - 8 nM (0.1 - 270 ng/ml), final concentrations. After 18 hours incubation at 37°C, 5% CO_2, supernatant was collected and tested for TNF concentration with ELISA (DiaClone). The percentage inhibition was calculated relative to TNF production by LPS in absence of IL4-10 FP.

6.2.5 I Deglycosylation. IL4-10 FP was deglycosylated using PNGaseF (New England BioLabs) according to manufacturer's protocol. Effectiveness of deglycosylation was monitored by Western blotting.

6.2.6 I Size exclusion chromatography (SEC). Molecular size was determined by high-performance-SEC (HP-SEC) on a high-performance liquid chromatography (HPLC) system (Shimadzu), using a 3 µm SEC-2000 column (150x7.8 mm; Phenomenex). As a mobile phase, 100mM phosphate containing 150mM NaCl, pH6.8 was used, at a flow rate of 1 ml/min. The column was calibrated with reference proteins (Column performance check; ALO-3042; Phenomenex), according to manufacturer's instructions. Thereafter, 40 µl of 1 mg/ml affinity-purified and filtered (0.22 µm Ultrafree-MC Centrifugal Filter Units; Millipore) IL4-10 FP preparations were loaded on the column. Using the elution profiles of the reference proteins, the molecular weight of the IL4-10 FP peaks was estimated.

6.2.7 I Pharmacokinetics study in rats. Clearance of IL4-10 FP from the circulation was studied in Wistar Crl:WI female rats (Charles River Laboratories). The study was approved by the animal ethical committee (approval number Netherlands Food and Consumer Product Safety Authority 11500, projectnumber AVD11500201744), and performed at the animal facility of the Utrecht University. Rats were acclimatized for one week prior to the experiment. On the experiment day, the animals were intravenously (IV) injected via the tail vein with 5 µg IL4-10 FP mutant Glyc3+ or with wt IL4-10 FP, in a volume of 200 µl PBS, using a winged infusion system. In a control group, rats were pre-injected with 30 mg asialofetuin (Sigma-Aldrich) in 0.5 ml PBS, 10 minutes prior to injection of IL4-10 FP Glyc3+, to block clearance via the asialoglycoprotein receptor (ASGPR). Blood was collected at 5, 10, 20, 30 and 60 minutes (150 µl per timepoint) in lithium-heparin tubes (Sarstedt), and centrifuged for 5 minutes at 2000g. Plasma samples were stored at -80°C until measurement. IL4-10 FP levels were measured with an IL-10 ELISA (Sanquin), according to manufacturer's instructions. Relative plasma levels were calculated as percentage in circulation (recovery), compared to the injected amount, assuming a plasma distribution volume of 10 ml per rat (Lee, & Blaufox, 1985). Half-life was calculated using the formula $T1/2\ (min) = (0{,}693 / k) * 60$, where k is the elimination rate constant, calculated by the formula $k = (\ln conc_{t=60} - \ln conc_{t=20}) / 0.67$.

6.3 I Results

6.3.1 I Generation of IL4-10 FP mutants with additional glycans. Human wt IL4-10 fusion protein (schematically presented in **Figure 1A**) contains three potential glycosylation sites, of which two are *in silico* predicted to be glycosylated (*www.cbs.dtu.dk/services/NetNGlyc/*) (**Figure 1B**). Under reducing conditions, human wt IL4-10 FP, has an apparent molecular weight of about 35 kDa (**Figure 1C**). However, under equal conditions, canine wt IL4-10 FP has an apparent molecular weight of 45 kDa. Canine IL4-10 FP possesses seven potential glycosylation sites, five in addition to human IL4-10 FP, likely explaining the increased molecular weight of canine IL4-10 FP. Deglycosylated human and canine IL4-10 FP indeed have a similar apparent molecular weight, as expected based on their calculated molecular weight; 34 kDa and 32 kDa for human and canine protein respectively (*www.expasy.org*) (**Figure 1C)**. Alignment of the human IL4-10 FP sequence with that of canine, as well as of those of mouse and rat, revealed that three potential N-linked glycosylation sites could be created in the human sequence by mutation of three amino acids, D4N, Q142N and S149N (**Supplementary**

Figure 1). By inserting these three mutations, the engineered protein, IL4-10 FP Glyc3+, contains six potential glycosylation sites in total, of which five sites are predicted to be glycosylated (**Figure 1D**).

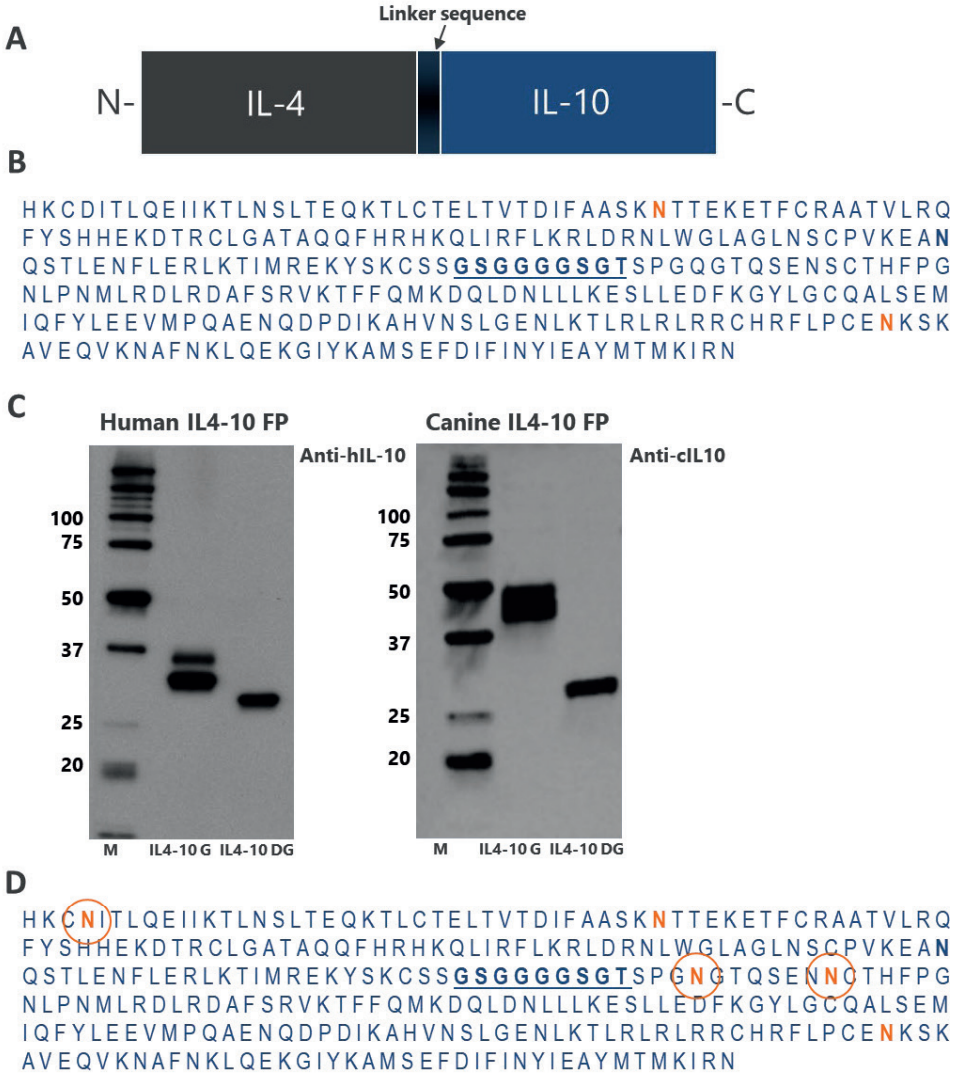


Figure 1. Glycosylation of wt IL4-10 FP and glycoengineered IL4-10 FP. (A) Schematic presentation of human IL4-10 FP. **(B)** Amino acid sequence of human IL4-10 FP contains three potential glycosylation sites, of which two (orange) likely are glycosylated. The linker sequence is underlined and in bold. **(C)** Immunoblotting under reducing conditions reveals that canine IL4-10 FP has a higher apparent molecular mass than human IL4-10 FP, which difference is lost upon deglycosylation (DG). **(D)** Amino

acid sequence of glycoengineered human IL4-10 FP which contains three additional glycosylation sites upon introduction of three amino acid point mutations (circled).

6.3.2 I *In vitro* bioactivity of glycoengineered IL4-10 FP. The effect of the single or multiple mutations on the potency of IL4-10 FP was evaluated by determining the ability of the glycoengineered proteins to inhibit LPS-induced TNF production in the whole blood culture assay, in comparison to the potency of wt IL4-10 FP (**Figure 2A**). Results showed that each single additional glycosylation site had no effect on the IL4-10 FP-mediated inhibition of LPS-induced TNF release. However, mutants carrying more than one additional glycosylation site exhibited a somewhat lower functional activity compared to wt IL4-10 FP, the lowest activity displayed by IL4-10 FP Glyc3+. To evaluate if the decrease in activity was caused by the additional glycans, both IL4-10 FP Glyc3+ and wt IL4-10 FP were deglycosylated using PNGaseF. As a control, both proteins were incubated under similar conditions but without the addition of PNGaseF. Effectiveness of the deglycosylation was proven on Western Blot by showing that both deglycosylated preparations migrated at about 34 kDa. The evaluation of the four preparations in the LPS-induced whole blood assay, confirmed that deglycosylated IL4-10 FP Glyc3+ inhibits the TNF production to a similar extent as both glycosylated and deglycosylated wt IL4-10 FP (**Figure 2B**). This finding not only confirms that the natural glycosylation of IL4-10 FP does not contribute to the bioactivity of the protein, but also showed that the introduction of more than one additional glycan, and not the mutations themselves, impacts the *in vitro* bioactivity. We additionally checked if one of the two moieties in IL4-10 FP was more affected by glycoengineering using receptor blocking antibodies. Ability of 0.3 nM IL4-10 Glyc3+ to inhibit LPS-induced TNF production upon blockade of the IL-10 receptor was shown to be lower compared to wt IL4-10 FP under equal conditions, whereas the activity of both preparations was not significantly altered upon blockade of the IL-4 receptor (**Supplementary Figure 2**), suggesting that the IL-4 moiety is affected by the glycoengineering of IL4-10 FP.

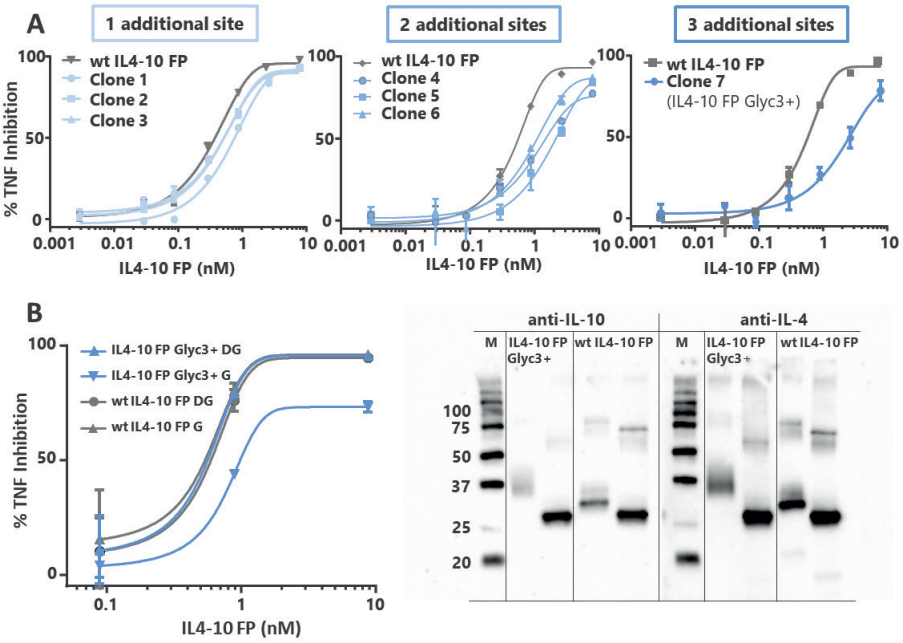
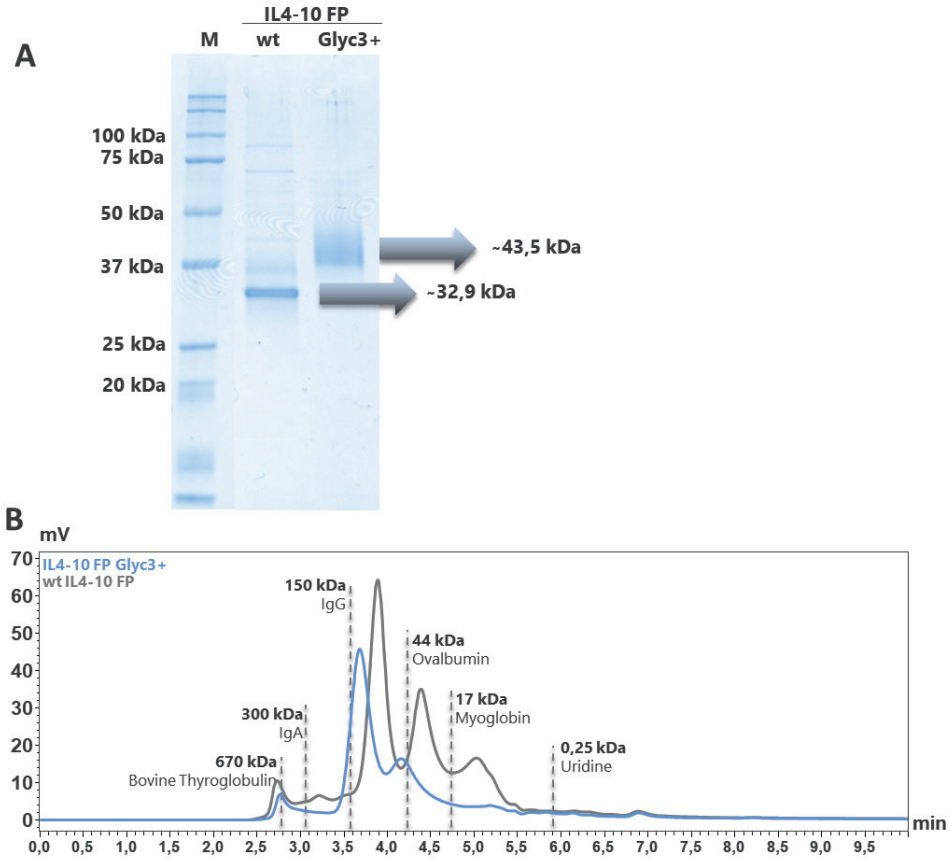


Figure 2. Glycoengineering of IL4-10 FP gradually decreases the *in vitro* bioactivity. (A) The introduction of more than one glycosylation site decreases the functional activity of IL4-10 FP to inhibit LPS-induced TNF production in the whole blood assay. Data represent duplo's from a representative experiment (n=3). **(B)** Deglycosylation of IL4-10 FP Glyc3+ fully restores its functional activity in the whole blood assay. Wild-type and IL4-10 FP Glyc3+ were deglycosylated with PNGaseF and tested in the whole blood assay (left) and checked for deglycosylation by Western Blotting (right). G=glycosylated; DG= deglycosylated (n=1).

6.3.3 I Glycoengineered IL4-10 FP Glyc3+ has an increased size compared to wt IL4-10 FP. On SDS-PAGE under reducing conditions IL4-10 FP Glyc3+ migrated higher than wtIL4-10 FP, confirming that the introduction of additional glycosylation sites increases the relative mass of the monomeric form of IL4-10 FP with ≥ 10 kDa (**Figure 3A**). Since IL4-10 FP is mainly expressed as a homodimer, we next evaluated the size-shift under native conditions, by HP-SEC analysis. Indeed, a shift in retention time between the dimer peak of IL4-10 FP Glyc3+ and wt IL4-10 FP can be seen (**Figure 3B**). Approximate molecular size of IL4-10 FP Glyc3+ and wtIL4-10 FP was calculated based on the retention times of reference proteins. The approximate size of the dimer peak was 107 kDa and 74 kDa for IL4-10 FP Glyc3+ and wt IL4-10 FP respectively, suggesting a total size shift of 37 kDa (**Figure 3C**). As expected, the

monomer peak had a size shift of 17 kDa, explained by the fact that the monomer possesses half of the glycosylation sites compared to the dimer.



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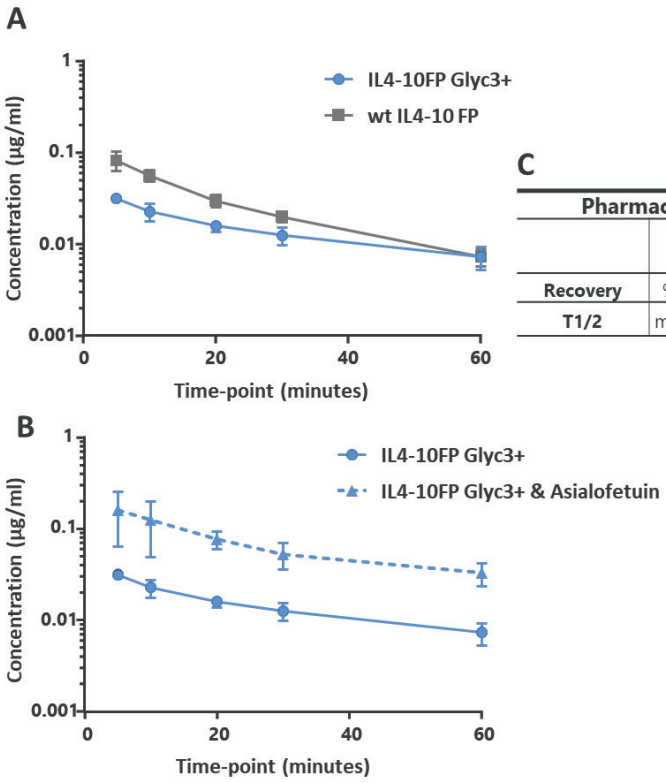
			IL4-10 FP Glyc3+	wt IL4-10 FP
Dimer Peak	Rt	min	3,678	3,884
	MW	kDa	117	80
Monomer Peak	Rt	min	4,154	4,382
	MW	kDa	49	32

Figure 3. Size-shift of IL4-10 FP Glyc3+ compared to wt IL4-10 FP. (A) Migration pattern of IL4-10 FP Glyc3+ and wt IL4-10 FP on SDS-PAGE under reducing conditions. Proteins are visualized with Instant Blue staining. **(B)** UV (A280) elution profile of IL4-10FP Glyc3+ and wt IL4-10 FP on HP-SEC. Position of molecular size markers are indicated as dotted lines. **(C)** Summary of retention time (RT), and calculated molecular weight (MW) of the dimer and monomer peak respectively.

6.3.4 I Improved plasma half-life of IL4-10 FP upon glycoengineering. In a pharmacokinetics study in rats we investigated the effect of the three additional glycosylation sites on the plasma half-life of IL4-10 FP. Both IL4-10 FP Glyc3+ and wt IL4-10 FP were injected intravenously at 5 µg via the tail vein of the rats, and the circulating levels of IL4-10 FP in serial plasma samples was assessed by ELISA. A relatively high first order elimination for both proteins was seen, determined by low plasma concentrations of both proteins at 5 minutes after injection, as compared to the expected plasma concentration based on the distribution volume; 13% for IL4-10 FP Glyc3+ and 20% for wt IL4-10 FP (**Figure 4A, 4C**). However, the second elimination phase of wt IL4-10 FP was more rapid than for IL4-10 FP Glyc3+, leading to a half-life of clearance from the plasma compartment of 20 minutes for wt IL4-10 FP and 38 minutes for IL4-10 FP Glyc3+. The high first order elimination is likely explained by incomplete sialylation of both proteins, leading to increased uptake by the ASGPR in the liver. Both proteins are heterogeneously sialylated due to the HEK293 expression system (Croset et al., 2012), affecting IL4-10 FP Glyc3+ more substantially than wt IL4-10 FP due to the presence of three additional glycans. This was confirmed by pretreating rats with asialofetuin, which saturates the ASGPR, increasing the plasma concentration compared to the distribution volume to 53% at 5 minutes after injection (**Figure 4B**). The phenomenon that under-sialylation in part accounts for high first order elimination was previously shown for wt IL4-10 FP (Steen-Louws, 2020). The fact that blockade of the ASGPR does not gain full recovery of first order elimination suggests that other mechanisms also contribute to the initial clearance of IL4-10 FP, such as tissue distribution, or binding to other receptors, such as IL-10R or IL-4R on PBMCs.



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Pharmacokinetic Parameters			
		IL4-10 FP Glyc3+	IL4-10 FP wt
Recovery	%	13	20
T1/2	min	38	20

Figure 4. Plasma clearance of IL4-10 FP Glyc3+ and wt IL4-10 FP upon intravenous injection in rats. Wistar rats were injected with IL4-10 FP Glyc3+ or wt IL4-10 FP (5 µg each) via the tail vein. Plasma samples were collected at several time points and analyzed for IL4-10 FP levels by ELISA. Plasma levels were calculated to percentage in circulation, compared to the injected amount. **(A)** Course of plasma levels of IL4-10 FP Glyc3+ (n=5) and of wt IL4-10 FP (n=4). **(B)** Effect of blocking the asialoglycoprotein receptors in the rats by pretreatment with asialofetuin on the course of plasma levels of IL4-10 FP Glyc3+ (n=3). Note that the recovery at 5 minutes, but not the half-life, is affected blocking these receptors. **(C)** Summary of Recovery (percentage in circulation) and calculated plasma half-life data. Graphs represent mean + SEM per treatment group.

6.4 I Discussion

A limitation of cytokines as therapeutic proteins is their short plasma half-life due to the relatively low molecular weight, which enhances renal clearance. Therefore, several strategies have been proposed to increase the plasma half-life of cytokine therapeutics, including Fc-coupling, pegylation and albumin binding (Kontermann, 2011). Another promising strategy is glycoengineering, particularly the introduction of extra glycans. Through addition of glycans molecular mass and negative charge (via sialic acid) of glycoproteins are increased, both being very important for decreased renal clearance (Byrne, Donohoe, & O'Kennedy, 2007; Solá, & Griebenow, 2009; Solá, & Griebenow, 2010). For example, glycoengineering has been successfully used to increase the plasma half-life of erythropoietin (Egrie et al., 2003; Sinclair et al., 2005; Kiss et al., 2010). In this study, we show the feasibility of glycoengineering the IL4-10 fusion protein, resulting in up to three additional glycosylation sites (IL4-10 FP Glyc3+). Though somewhat less active than wt IL4-10 FP, IL4-10 FP Glyc3+ is substantially bioactive in the whole blood assay, pointing to proper overall folding of the glycosylated protein. Importantly, a significant shift in size of approximately 37 kDa, resulted in almost a doubling in plasma half-life of IL4-10 FP Glyc3+ compared to wt IL4-10 FP.

The position of additional glycosylation sites should be carefully chosen, since even a slight change in amino acid composition can affect the folding and stability, and impact the characteristics of a therapeutic protein (Solá, & Griebenow, 2009). Glycoengineering can also change the affinity of the glycoprotein for the receptor, affecting its functional activity, as has been shown for darbepoetin alfa and hyperglycosylated IFN-α (Egrie et al., 2003; Ceaglio et al., 2010). To minimize the risk of additional glycans affecting the integrity or functional activity of IL4-10 FP, we identified potential additional glycosylation sites in the human protein, based on naturally occurring glycosylation sites in mouse, rat and canine IL4-10 FP. Furthermore, we selected the sites where only one amino acid mutation was required to create a potential glycosylation site. We identified three such sites in the human IL4-10 FP sequence. In all cases the original amino acid was changed into asparagine, and two of the three mutations (D4N, Q142N) are conservative, i.e. the original amino acid has similar biochemical properties as asparagine. We anticipated that using this strategy, additional glycans would have minimal, if any, impact on the integrity of human IL4-10 FP, its receptor binding and functional activity. Indeed, the introduction of a single glycosylation site had no effect on the functional activity of IL4-10 FP *in vitro*. However, the ability of IL4-10 FP to inhibit

LPS-induced TNF in whole blood was impaired by mutants possessing more than one additional glycosylation site. Upon deglycosylation, the difference in functional activity between wt IL4-10 FP and IL4-10 FP Glyc3+ was lost, confirming that the impaired functional activity was related to the incorporation of two or three glycans, and not due to the amino acid mutations themselves. However, a lower *in vitro* activity of a protein upon hyperglycosylation, may be compensated *in vivo* by a significant increase in plasma half-life, resulting in an overall increased *in vivo* activity, as has been demonstrated for darbepoetin alfa (Egrie et al., 2003).

The HP-SEC pattern of IL4-10 FP Glyc3+ showed an increase in size compared to wt IL4-10 FP. But it also revealed that the folding of IL4-10 FP Glyc3+ is not disturbed by the additional glycans. Part of human IL4-10 FP is a non-covalently linked dimer, which dimerization is mediated by the IL-10 moiety of the molecule (Zdanov et al., 1995). However, we have noticed before that part of IL4-10 FP is folded as a monomer, which is biologically active (manuscript in preparation). Interestingly, IL4-10 FP Glyc3+ tends to form more dimers than wt IL4-10 FP, suggesting that introduction of additional glycans supports dimerization. This phenomenon was seen for wt canine IL4-10 FP, naturally possessing seven glycosylation sites, too (**Supplementary Figure 3**).

Pharmacokinetic analysis of IL4-10 FP Glyc3+ in rats revealed almost two-fold longer plasma half-life as compared to wt IL4-10 FP. It has to be noted that the first order elimination of both IL4-10 FP Glyc3+ and wt IL4-10 FP was high due to the low sialylation of proteins in batches produced in HEK293 cells, known for the heterogenous sialylation of glycans (Croset et al., 2012).

We have published a series of studies on the treatment with IL4-10 FP administered intrathecally or intra-articularly in preclinical models for (osteo)arthritis and pain (Eijkelkamp et al., 2016; Steen-Louws et al., 2018). We are currently evaluating the intravenous administration of IL4-10 FP in various inflammatory disease models. Our results presented here as well as previously published studies (Steen-Louws et al., 2020), show that glycoengineering allows the plasma half-life of IL4-10 FP to be manipulated. Future studies should reveal if the prolonged plasma half-life of IL4-10 FP also results in improved *in vivo* efficacy.

6.5 I Authorship

CSL, PB, JM, AR, JPC have had substantial contribution to conception and design, acquisition of data, analysis and interpretation of data. CSL, JPC and EH prepared a draft of the manuscript. All authors have been involved in revising the manuscript critically for important intellectual content. All authors have given final approval of the version to be published and agreed to be accountable for the content of the manuscript.

6.6 I Acknowledgments

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6.7 I Funding

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6.8 I Conflict of Interest

Cristine Steen-Louws, Erik Hack, Niels Eijkelkamp and Jelena Popov- Celeketic are shareholders of Synerkine Pharma BV, a recently founded company in which preclinical development and evaluation of IL4-10 fusion protein is enrolled. Erik Hack, Floris Lafeber and Niels Eijkelkamp are consultants for Synerkine Pharma BV. Jelena Popov-Celeketic is partly employed by Synerkine Pharma BV. Cristine Steen-Louws, Floris Lafeber, and Erik Hack are co-inventors of the patent “Fusion protein comprising an Interleukin 4 and Interleukin 10”.

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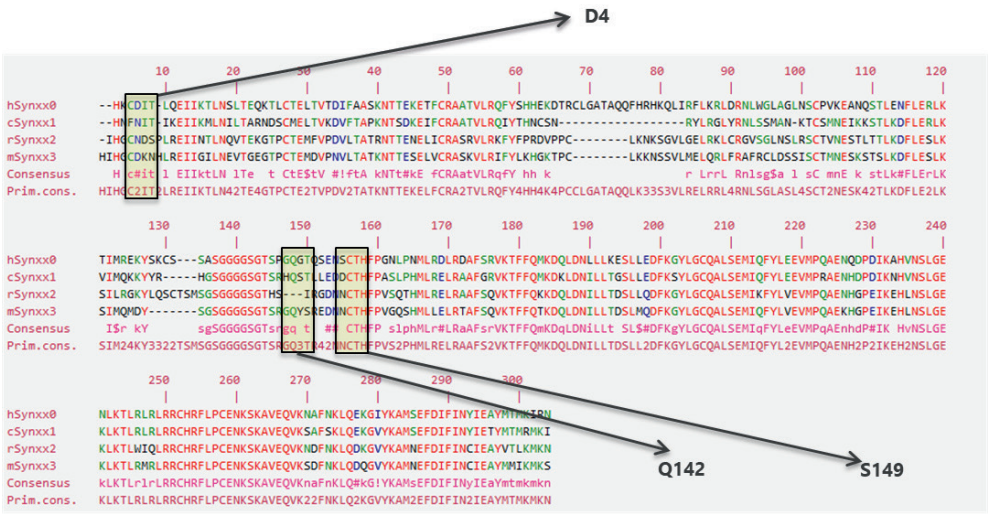
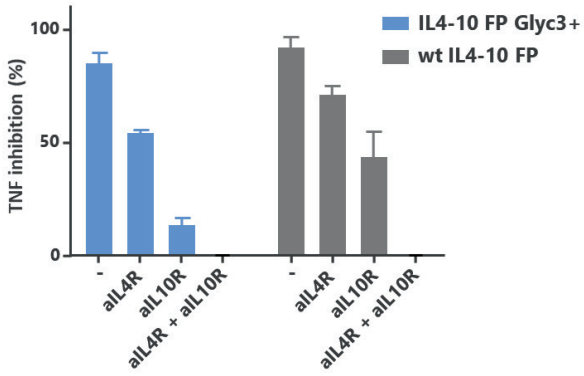
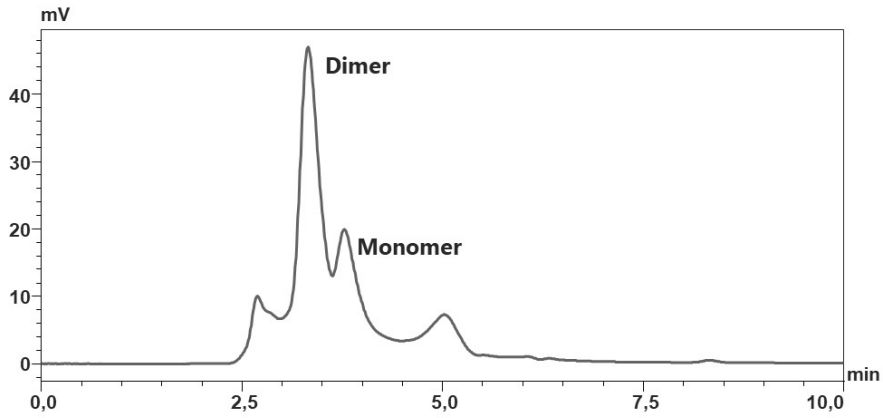
6.10 I Supplementary Figures



Supplementary Figure 1. Alignment of the amino acid sequence (aa-sequence) of human IL4-10 FP with the aa-sequence of canine, mouse and rat IL4-10 FP. The three sites chosen for point mutation of human glycoengineered IL4-10 FP Glyc3+, D4N, Q142N and S149N, are marked.



Supplementary Figure 2. *In vitro* bioactivity of the IL-4 and IL-10 moiety within IL4-10 FP Glyc3+ and wt IL4-10 FP compared. Activity of either moiety of IL4-10 FP Glyc3+ was evaluated by the addition of IL-4 and IL-10 receptor blocking antibodies in the LPS-induced whole blood assay. Both the IL-4 and the IL-10 moiety within IL4-10 FP Glyc3+ contribute to the inhibition of LPS-induced TNF production. However, the IL-4 activity of IL4-10 FP Glyc3+ seems slightly impaired compared to the IL-4 activity of wt IL4-10 FP, as shown upon addition of anti-IL-10R. Data represent mean + SEM (n=2).



Supplementary Figure 3. HP-SEC Elution pattern of canine IL4-10 FP. UV (A280) elution profile of canine IL4-10 FP, showing that canine IL4-10 FP mainly consists of the dimeric form.

7 Chapter

IL-10 is functionally active in both monomeric and dimeric human IL4-10 fusion protein

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To be submitted

Abstract

Background & Objective: Active IL-10 forms a domain swopped homodimer. Here we studied whether the functional activity of the IL-10 moiety of the recently developed IL4-10 FP is dependent on the non-covalent dimerization of the fusion protein.

Methods. IL4-10 FP was produced in HEK293 cells, affinity purified, and fractionated on HP-SEC, yielding monomeric and dimeric IL4-10 FP preparations. Functional activity of the IL4-10 FP preparations was assessed using LPS-induced TNF production in whole blood assays.

Results. All IL4-10 FP batches tested contained dimeric and monomeric IL4-10 FP. Both forms were isolated on basis of molecular size and appeared stable *in vitro*. Isolated monomeric and dimeric IL4-10 FP dose-dependently inhibited LPS-induced TNF release in whole blood with comparable potency. Functional activity of monomeric IL4-10 FP was dependent on both the IL-4 and the IL-10 moiety. The IL-10 moiety of dimeric and monomeric fusion protein had similar activity in the presence of an IL-4 receptor blocking antibody.

Conclusion. This study shows that human IL4-10 FP consists of monomers and non-covalently linked dimers, which have similar activity in a whole blood assay. In contrast to wild-type IL-10, monomeric IL-10 as part of IL4-10 FP and consisting of the wild-type sequence, is as active as dimeric IL-10. These data suggest that dimerization of wild-type human IL-10 is not mandatory for its functional activity.

7.1 | Introduction

Interleukin 10 (IL-10) was discovered in the early 90's of the previous century and its structure and function have been extensively studied since then [1]. IL-10 was first named "cytokine synthesis inhibitory factor" (CSIF), reflecting its capacity, as a co-factor of Th2 cells, to potently inhibit production of multiple cytokines by Th1 cells. Indeed, IL-10 is mainly known as an immunoregulatory cytokine, dampening Th1- and Th17-mediated inflammatory effects in various ways. Next to the inhibition of IFNγ, other proinflammatory cytokines like IL-1β, TNF, IL-6, IL-8 and IL-17 are also strongly inhibited by IL-10 [2]. IL-10 is produced by Th2 cells, but during inflammation and immune activation, it is also produced by monocytes, macrophages, B-cells, naive T cells and other cells [2-5].

Interleukin 10 is the founding member of the IL-10 cytokine family, furthermore including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29. Together with the interferon family, the IL-10 family forms the Class II cytokine family, which are characterized by a common structure consisting of six α-helices, a similar genomic organization, and have 20–30% amino-acid identity, including conserved cysteine positions in their receptor sequences [6, 7]. Interestingly, though most members of this cytokine family have a monomeric structure, some members are expressed as non-covalently linked homo-dimers. Indeed, IL-10 forms a domain swopped homodimer. IL-10 consists of six α-helices (A-F) and dimerization is achieved via the exchange of the adjacent helices E and F of two IL-10 molecules [8]. Dimerization is caused and stabilized by the strong hydrophobic core that helices A, C, D and F' form, of which the latter is strongly embedded in the middle. Furthermore, helices A, B and F' are involved in receptor binding [8, 9]. A dimeric form of IL-10 will first bind the high-affinity IL-10 receptor 1 (IL-10R1, also referred to as IL-10Rα) in a 1:2 ratio, where after the low-affinity IL-10 receptor 2 (IL-10R2, also referred to as IL-10Rβ) will be bound through a common binding site, resulting in a 1:2:2 complex [6, 10, 11].

Under physiological conditions mostly all IL-10 is present as a dimer and is functionally active as such. However, dimerization of IL-10 is not obligatory for functional activity of the cytokine. Introduction of six amino acid mutations in the DE loop yields a stable IL-10 monomer (IL-10M1). This monomeric IL-10 interacts with the IL-10 receptor in a 1:1:1 stoichiometry and can elicit cellular responses though with reduced activity [12]. Notably, IL-10 monomers with the wild-type sequence have not been identified. It remains unclear whether the decreased

functional activity of the monomeric IL-10 mutant is due to the mutations per se or reflect an inherent reduced functional activity of monomeric IL-10.

We have generated a fusion protein of IL-4 and IL-10 and have observed that under physiologic conditions the IL4-10 fusion protein (IL4-10 FP) forms a monomer or a non-covalently linked homo-dimer [13, 14]. The IL-10 sequence of IL4-10 FP is identical to that of wild-type IL-10. In order to establish the functional activity of monomeric IL-10 consisting of the wild-type sequence, we purified the monomeric fusion protein and assessed its functional activity, while blocking the activity of the IL-4 moiety.

7.2 | Methods

7.2.1 | Production and purification of IL4-10 FP. Briefly, cDNA encoding for IL4-10 FP was cloned in a dual CMV-promoter expression vector (Meeldijk, & Hack, unpublished), containing an α2,3-sialyltransferase gene to enhance sialylation of the proteins. Plasmids were transiently transfected in HEK293 cells, and IL4-10 FP was isolated from cell culture supernatant via IL-4 specific affinity chromatography, as described previously [15]. The elution fraction was dialyzed in PBS, assessed for purity and protein content using SDS-PAGE followed by Instant Blue total protein staining (Expedeon), and a BCA protein assay (Thermo Scientific), respectively. Finally, the preparation was aliquoted and stored at -80°C until further analysis.

7.2.2 | Isolation of the IL4-10 FP monomer and dimer. Dimeric and monomeric IL4-10 FP were separated using size exclusion chromatography. A high-performance liquid chromatography (HPLC) system (Shimadzu) was equipped with a SEC-2000 column (3 µm; 150x7.8 mm; 00F-4512-K0; Phenomenex). As a running buffer 100 mM phosphate with 150 mM NaCl, pH 6.8, was used. All runs were performed at a flowrate of 1 ml/min and at a maximal pressure of 70 bar. The column was calibrated with reference proteins (ALO-3042; Phenomenex), according to manufacturer's instructions. Affinity-purified IL4-10 FP was concentrated to 1 mg/ml (Vivaspin 500 centrifugation tubes; Sartorius) and filter-sterilized through a GV Durapore 0.22 µm filter (Millipore). 50 µl of the filtrate was injected onto the column. Fractions of peaks were collected in ± 400 µl portions. The fractions of respectively the dimer and monomer peak were concentrated to 50 µl, filter-sterilized and injected in two separate runs to analyze stability of dimeric and monomeric IL4-10 FP. For the analysis of dimeric and monomeric IL4-10 FP in the whole blood assay, two subsequent runs of IL4-10 FP were performed as described above, except that smaller portions (± 100 µl) were collected to optimize collection of dimeric or

monomeric IL4-10 FP. Peak fractions containing solely dimeric or monomeric IL4-10 FP were pooled, yielding ± 500 µl pooled fractions for both dimeric and monomeric IL4-10 FP. Pooled fractions were dialyzed in PBS, aliquoted in 50 µl portions and stored at -80 °C until further analysis. Concentration of IL4-10 FP in fractions and in starting material was determined with an IL-10 ELISA (DuoSet; R&D Systems, according to manufacturer's instructions). This ELISA equally detects the monomeric and dimeric form of IL4-10 FP. To confirm concentrations measured in the ELISA, protein content was determined with a BCA protein assay, and gel electrophoresis followed by total protein staining.

7.2.3 | Gel electrophoresis and immunoblotting of dimeric and monomeric IL4-10 FP. Starting material, dimeric, and monomeric IL4-10 FP were diluted in PBS and mixed with Laemmli sample buffer (BioRad), containing 100mM Dithiotreitol (Sigma-Aldrich), to obtain a concentration of ~0.5 ug/ml. Samples were incubated for 10 minutes at 100˚C, and loaded (20 µl) on a 12% polyacrylamide Gel (Mini-PROTEAN-TGX, BioRad). After electrophoresis, proteins were transferred to a pre-fab nitrocellulose-membrane (TurboBlot system; BioRad) according to manufacturer's instructions. The membranes were then blocked in 4%, w/v, milk (Elk, Campina) in PBS 0.1%, v/v, Tween-20 (PBS-T). The membrane was incubated with the primary Ab mIgG1 anti-human IL-10 (1:100; Santa Cruz Biotechnology; Clone 3C12C12), in PBS-T containing 1% milk, followed by HRP-conjugated goat anti-mouse IgG (1:2000; Santa Cruz). To visualize the bands ECL Western blotting substrate was added according to manufacturer's protocol (Pierce, Thermo Scientific) and the blot was analyzed using the ChemiDoc Image Station (BioRad).

7.2.4 | Whole blood assay to test the functional activity of IL4-10 FP. Heparinized human blood obtained from healthy volunteers was diluted 1:10 in RPMI1640 medium (Invitrogen), supplemented with 1% penicillin/streptomycin (P/S, PAA Laboratories) in 48-well culture plates (Nunc). Lipopolysaccharide (LPS, Sigma-Aldrich) was added at 10 ng/ml. IL4-10 fusion protein (starting material, dimer or monomer) and recombinant human IL-10 (*E.* coli; eBioScience), were added at concentrations ranging from 0.01 to 3 nM. In follow-up experiments, fixed concentrations of 3 nM (90 ng/ml) and 1.5 nM (45 ng/ml) of the IL4-10 fusion protein preparations were added. In these experiments, receptor blocking antibodies against IL-4 receptor (anti-IL-4R; R&D Systems, Clone 25463) or IL-10 receptor (anti-IL-10R; BioLegend, Clone 3F9) were added at a final concentration of 10 ug/ml and 20 ug/ml, respectively. After 18h incubation at 37°C, 5% CO2, supernatant was collected and tested for

TNF concentration with ELISA (DiaClone). The percentage inhibition was calculated relative to TNF production by LPS in absence of IL4-10 FP. The significance of the differences in activity of monomeric and dimeric IL4-10 FP was analyzed with multiple t-tests (one per dataset).

7.3 | Results

7.3.1 | HP-SEC characterization of dimeric and monomeric human IL4-10 fusion protein. Analysis of a commercial, *E. coli* produced IL-10 preparation on HP-SEC revealed no detectable monomers of IL-10 (unpublished observation). In contrast, purified human IL4-10 FP contains both the dimeric and monomeric form of the fusion protein (**Figure 1A**). The dimeric form of IL4-10 FP appeared as the second peak, which by reference to the standards was estimated to have a molecular weight of ~70 kDa, while the third peak has a molecular weight of ~35 kDa, reflecting the predicted molecular weight of the monomeric form of IL4-10 FP. The first peak probably represents high molecular weight contaminants since it hardly contained IL-4 or IL-10 as analyzed by immunoblotting. Dimeric and monomeric IL4-10 FP were collected **(Figure 1B**) and re-run on HP-SEC to assess stability of either form. Upon re-run, dimeric and monomeric IL4-10 FP eluted at the same position as compared to the initial run, indicating that both forms are stable over time and are not in a dynamic equilibrium with each other (**Figures 1C and D**;). Re-runs of the dimeric and monomeric fraction were also performed upon overnight incubation at 37°C and revealed a similar peak pattern (data not shown). Analysis of the peaks using Western Blot with an anti-IL-10 antibody confirmed the presence of intact IL4-10 FP in all preparations and showed that both the dimer and monomer are comparably glycosylated (**Figure 1E**).

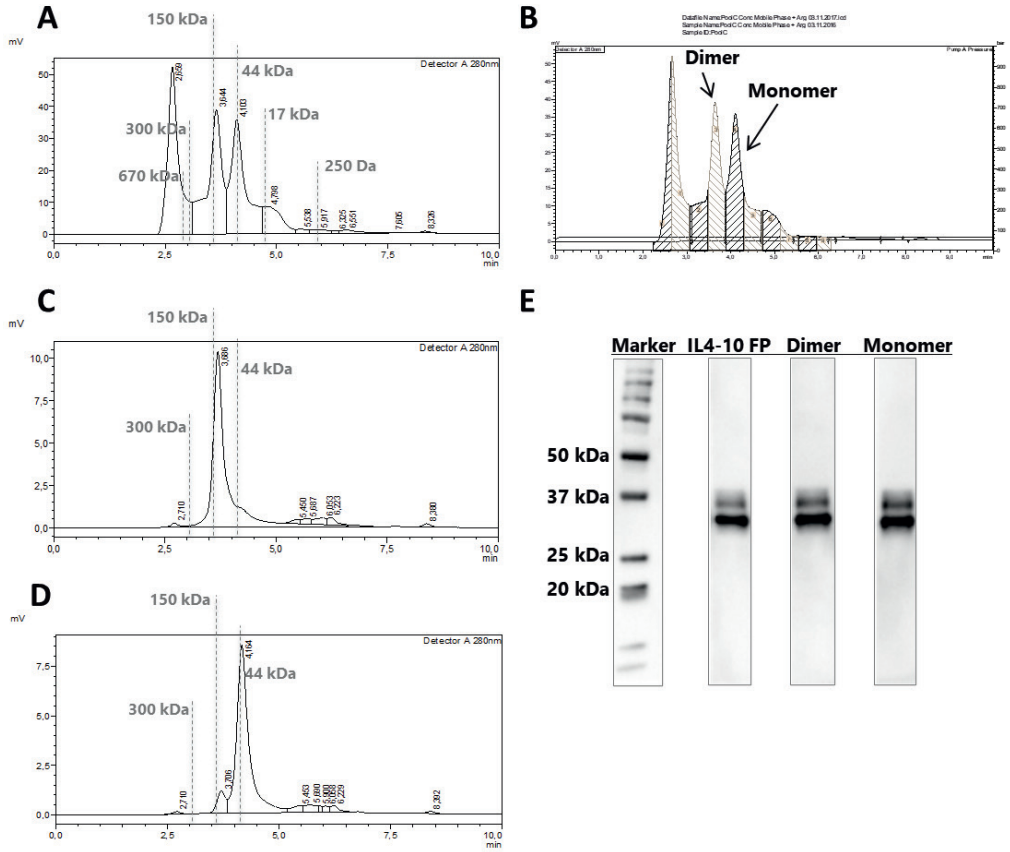

Figure 1. HP-SEC characterization of dimeric and monomeric human IL4-10 fusion protein.

A) 50 ug of human IL4-10 FP was analyzed using HP-SEC as described in methods. Position of the molecular weight markers (670 kDa Bovine Thyroglobulin; 300 kDa IgA; 150 kDa IgG; 44 kDa Ovalbumin; 17 kDa Myoglobin; 250 Da Uridine) is indicated. The peak at MW >670 kD represents high molecular weight contaminants and aggregated IL4-10 FP. The second peak has an estimated molecular weight of ~70 kDa, whereas the third peak has an apparent molecular weight of ~35 kDa. **B)** The dimer and monomer peaks of IL4-10 FP were collected as indicated and re-run. **C/D)** Both dimeric and monomeric IL4-10 FP appear to be stable. **E)** Immunoblotting with an anti-IL10 antibody shows that both the dimer and monomer migrate at the same position, and have a similar glycosylation.

7.3.2 | Monomeric and dimeric human IL4-10 fusion protein have similar IL-10 activity. To assess the functional activity of dimeric and monomeric IL4-10 FP, we collected the respective peak fractions upon HP-SEC and determined the concentration of the preparations as described in the methods section. The initial batch containing a mixture of monomeric and dimeric IL4-10 FP, dose-dependently inhibited LPS-induced TNF release in whole blood assay, however, as shown before to a lesser extent than wild-type IL-10 [14]. Importantly, isolated monomeric and dimeric IL4-10 FP showed an identical dose-dependent TNF inhibition, indicating that the functional activity of dimeric and monomeric IL4-10 FP is equal (**Figure 2A**). Addition of receptor blocking antibodies showed that both the IL-4 and the IL-10 moiety contributed to the inhibition of LPS-induced TNF by dimeric and by monomeric IL4-10 FP (**Figure 2B**). Adding neutralizing IL4R antibodies even revealed that, at 90 ng/ml IL4-10 FP, the IL-10 moiety of monomeric IL4-10 FP was as active as that of dimeric IL4-10 FP. Upon addition of neutralizing IL4R antibodies at 45 ng/ml IL4-10, ~45% of the inhibitory capacity of dimeric IL4-10 FP was retained whilst that of monomeric IL4-10 FP was ~25%. This difference, however, was not significant.

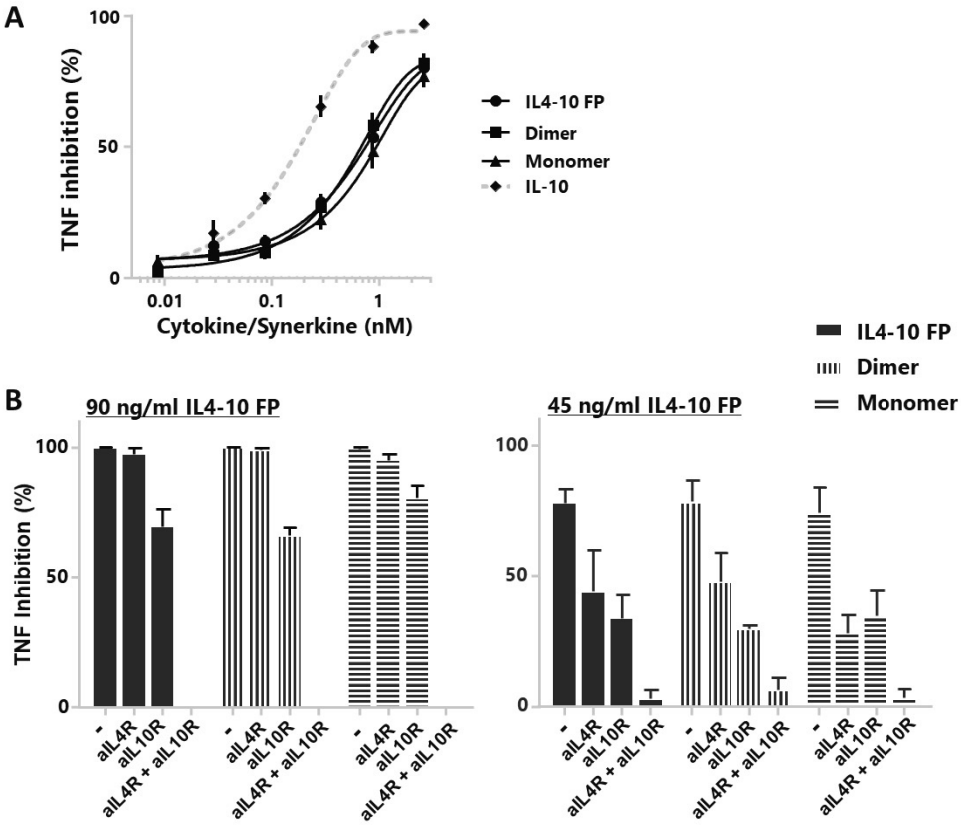

Figure 2. Functional activity of the IL-10 subunit of monomeric and dimeric human IL4-10 fusion protein. Monomeric and dimeric IL4-10 FP were purified by HP-SEC and tested in the whole blood assay. In this assay inhibition of LPS-induced TNF production during overnight culture is measured. Results (mean + SEM) are expressed as % inhibition (see Methods for details). IL4-10 FP is the starting material before HP-SEC. **A)** Shows dose response curves of the IL4-10 FP preparations in comparison to that of wild-type IL-10 (n=4). **B)** Shows activity of IL4-10 FP preparations at 90 ng/ml (left) and 45 ng/ml (right) in presence of blocking antibodies against IL-4R or IL-10R (n=3). Note that in presence of blocking anti-IL-4R antibodies, dimeric and monomeric IL4-10 FP have similar inhibitory concentration at 90 ng/ml (left), whereas at 45 ng/ml (right graph in B) monomeric IL4-10 FP is somewhat less active than dimeric IL4-10 FP. This difference is not significant (as analyzed with multiple t-tests).

7.4 | Discussion

The existence of IL-10 as a domain swopped homodimer has been extensively documented in the past years [8-11]. A monomeric form of IL-10 consisting of the wild-type sequence has not been described in the literature. We demonstrate this by making use of the HP-SEC based observation that IL4-10 FP is expressed in dimeric and in monomeric form, and assess the function of monomeric and dimeric wild-type sequence IL-10 within IL4-10 FP. Remarkably, isolated dimeric and monomeric IL4-10 FP showed similar ability to inhibit LPS-induced TNF production in the whole blood assay, indicating that monomeric IL-10 consisting of its wild-type sequence is as active as its dimeric counterpart.

An obvious explanation for the similar functional activity of dimeric and monomeric IL4-10 FP would be that these forms are not stable but rather form a dynamic equilibrium with a constant ratio of monomer to dimer. However, our data argue against this explanation. A re-run of monomers and dimers on HP-SEC revealed that both forms are stable (Figure 1), even after overnight incubation at 37 °C. This makes it unlikely that the observed similar functional activity resulted from a dynamic equilibrium yielding comparable concentrations of monomers and dimers in both preparations after HP-SEC purification.

It should be noted that the activity of the IL-10 moiety of IL4-10 FP is slightly less compared to wild-type IL-10, as was shown previously [14]. It is not clear how this impaired activity of the IL-10 moiety is mediated. Possible explanations include that the presence of a linker sequence and the IL-4 moiety interfere with signaling through IL-10R1. Another, likely explanation is that through its IL-4 moiety, part of IL4-10 FP is removed from the reaction by cells only having IL-4-, and no IL-10 receptors. In other words, the presence of cells with only IL-4R, and no IL-10R will decrease the amount of IL4-10 FP available to inhibit TNF production by monocytes.

Although IL-10 was discovered as one of the first cytokines, its exact binding and signaling mechanism took years to unravel. A crystal structure of IL-10 revealed the dimeric form of naturally occurring IL-10 [8, 9]. It was shown that dimeric IL-10 binds in a 1:2 complex to IL-10R1, leading to a shared binding site through which IL-10R2 is bound, leading to a 1:2:2 complex [6, 10, 11]. Striking however, is that IL-22, another Class II family member, only sharing 22% sequence identity with IL-10, is formed with similar six peptide chains but exists in the monomeric form [7, 16]. Comparing IL-22 to IL-10 explains the possibility of a functional active IL-10 monomer, binding to the IL-10R1 in a 1:1 complex [12]. Comparison of the structure of IL-22 with that of IL-10, suggests that peptide chains E and F of IL-10, under certain conditions,

are able to fold back within the hydrophobic core of the monomeric form, thereby forming a fully functional monomeric IL-10 unit. An explanation for monomerization of wild-type IL-10 might be the involvement of glycosylated Asn116. This potential glycosylation site is positioned in the DE loop, which is known to be critical for dimerization [8, 12]. Under normal conditions, however, IL-10 is hardly glycosylated [1], whereas we know that IL4-10 FP is differentially glycosylated and that the specific potential glycosylation site Asn116 is likely to be glycosylated (*in silico* prediction via the NetNGlyc tool (www.cbs.dtu.dk/services/NetNGlyc/)). It can thus be speculated that differential glycosylation of Asn116 determines the ability of IL4-10 FP to dimerize. However, another possible explanation is that the presence of IL-4 at the N-terminal site of IL-10 hinders dimerization of IL4-10 FP.

In conclusion, this study for the first time shows that the monomeric sequence of IL-10 has similar functional activity as compared to dimeric IL-10. Our results also underline the fact that dimerization of IL-10 is more likely to be the result of interactions between amino acids in the sequence, than that the dimerization is needed for binding to the receptor and, subsequently, proper functional activity.

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8 General Discussion

This thesis describes the development, characterization and therapeutic potential of a fusion protein of the regulatory cytokines IL-4 and IL-10: IL4-10 fusion protein (IL4-10 FP). Regulatory cytokines have great potential for the treatment of inflammatory diseases. However, due to limitations such as a short half-life and unwanted pro-inflammatory activities, the success of regulatory cytokines as stand-alone therapeutics in clinical trials have been limited [1, 2]. By developing IL4-10 FP we aimed to overcome some limitations of the regulatory cytokines as stand-alone treatments. This thesis describes studies on the characterization of this fusion protein, and on its performance in preclinical disease models.

8.1 | Main findings presented in this thesis

8.1.1 | Development of a functionally active IL4-10 FP

IL4-10 fusion protein is expressed mainly as a 70 kDa glycosylated dimeric protein **(Chapters 4, 5, 7)**. IL4-10 FP is functional active in various *in vitro* assays and dose-dependently inhibits production of pro-inflammatory cytokines such as TNF in the whole blood assay **(Chapter 2)**. Importantly, we show that both cytokine moieties of the IL4-10 fusion protein are functionally active as blocking either of its receptors results only in a partial loss of the inhibitory activity IL4-10 FP **(Chapters 2 and 4)**.

Although the majority of IL4-10 FP is expressed as dimeric protein, batches of IL4-10 FP contain monomeric protein as well. It is thought that IL-10, normally present as a non-covalently linked homo-dimer, can only signal efficiently in its dimeric form. We tested whether a monomeric form with the normal human IL-10 sequence is bioactive and found that dimeric and monomeric IL4-10 FP have similar functional activity **(Chapter 7)**.

8.1.2 | IL4-10 FP has unique immunoregulatory activity

Next to inhibition of TNF, other pro-inflammatory cytokines like IL-6, IL-8 and IL-1 are also potently inhibited by IL4-10 FP in LPS-stimulated whole blood cultures. In contrast, levels of IL-1RA and sTNFR are hardly affected by IL4-10 FP, resulting in an altered balance between pro-inflammatory cytokines and their naturally occurring inhibitors **(Chapter 2)**. In addition, IL4-10 FP reduced IFNγ and IL-17 levels and stimulated IL-5 production in SEB-stimulated PBMC cultures, whereas T-cell proliferation and the number of regulatory T-cells remained unchanged. Thus upon superantigen stimulation, IL4-10 FP skews Th1/Th17 activity towards

Th2 activity **(Chapter 2)**. Another remarkable finding is the untouched expression of FcγRI, IIa, III in the presence of IL4-10 FP, which is in contrast to the significant upregulation of these receptors by IL-10, a known, major unwanted pro-inflammatory activity of IL-10. IL4-10 FP significantly shifted the FcγRIIa/FcγRIIb ratio in favour of the inhibitory FcγRIIb. Also the expression of FcεR is largely unaffected by IL4-10 FP, whereas this receptor is markedly upregulated by IL-4, associated with the risk of allergic-type responses. These findings indeed support the hypothesis that by combining both cytokines in a fusion protein, IL-4 and IL-10 ameliorate each other's unwanted pro-inflammatory effects **(Chapter 2)**.

8.1.3 | The potential of IL4-10 FP in inflammatory diseases

In supernatants of *ex vivo* cultures of diseased synovial biopsies and cartilage explants of both RA and OA patients, a strong inhibition of pro-inflammatory activity was observed upon addition of IL4-10 FP **(Chapter 2, Chapter 3)**. In addition, the fusion protein had a chondroprotective effect since it improved proteoglycan turnover in human cartilage cultures of OA patients **(Chapter 3)**. The ability of IL4-10 FP to attenuate disease activity was confirmed *in vivo* in rodent and canine models for RA, OA, and inflammatory pain. Systemic administration of IL4-10 FP suppressed experimental, proteoglycan-induced arthritis in mice, without inducing B-cell hyperactivity **(Chapter 2)**. Next, intra-articular injection of IL4-10 FP decreased the pain of dogs with experimentally induced OA, reflected by increased joint loading of the affected legs **(Chapter 3)**. IL4-10 FP dose-dependently inhibited hyperalgesia in several chronic inflammatory pain models. Remarkably, the analgesic effect of IL4-10 FP was larger than the effect of the combination of IL-4 plus IL-10. Upon three subsequent injections of IL4-10 FP, inflammatory pain was completely resolved and did not return after the last injection **(Chapter 4)**. Thus, IL4-10 FP is more potent than the combination of both individual cytokines to inhibit inflammatory pain, and is even able to permanently resolve pain. All together these data support the potential of IL4-10 FP as disease-modifying drug in several inflammatory and degenerative diseases, and in chronic pain.

8.1.4 | Glyco-engineering of IL4-10 FP for various therapeutic applications

Since administration routes and dose frequency of a therapeutic protein may depend on the disease treated as well as on some properties of the protein that determine its bioavailability, we investigated several glyco-engineered forms of IL4-10 FP *in vitro* as well as *in vivo*. We

developed sialic acid-engineered IL4-10 FP in stable CHO cell lines. We confirmed that the functional activity of IL4-10 FP *in vitro* is independent of the extent of sialylation ("capping") of the protein. However, a remarkably fast initial clearance of low-sialylated IL4-10 FP from the circulation was found in rats upon systemic administration. This feature is advantageous when IL4-10 FP is administered in a local compartment since a fast systemic clearance minimizes the systemic exposure upon leakage from the local compartment. This initial fast clearance was mediated by the asialoglycoprotein receptor in the liver, as blockade of this receptor by asialofetuin prevented the initial clearance of low-sialylated IL4-10 FP. In agreement herewith, high-sialylated IL4-10 FP displayed a slower initial clearance from the circulation upon intravenous injection, and this glycoform might be the preferred option for systemic treatment **(Chapter 5)**. To even further improve the bioavailability of IL4-10 FP for systemic applications, we applied additional glycoengineering as a strategy to increase its molecular size and extend its half-life. Based on a comparison of human IL-4 and IL-10 sequences with those of various animal species, we designed mutants of IL4-10 FP with up to three additional glycosylation sites. Although *in vitro* the functional activity of mutants with two or more additional glycosylation sites was impaired compared to IL4-10 FP consisting of wild-type IL-4 and IL-10 sequences, the plasma half-life of clearance of the IL4-10 FP variant with three additional glycosylation sites was prolonged upon intravenous administration, as compared to non-mutated IL4-10 FP **(Chapter 6)**.

8.2 | Discussion & Future Perspectives

8.2.1 | The unique mode of action (MoA) of IL4-10 FP

Our data support that both moieties of IL4-10 FP are functionally active **(Chapter 2, Chapter 4, Chapter 7)**. The contribution of each moiety, leading to signaling, differs per cell type and disease setting. For example, the potency of IL4-10 FP to inhibit LPS-induced TNF production in whole blood, is somewhat less compared to that of recombinant IL-10 **(Chapter 2)**. In **Chapter 7** we discuss that whole blood may contain cells, other than monocytes, with IL-4 receptors that bind IL4-10 FP. Such cells, presumably T-cells [3, 4], thus effectively decrease the amount of IL4-10 FP available to inhibit LPS-triggered production of TNF by monocytes. It might also be that, due to the presence of a linker sequence and the IL-4 moiety, binding/signaling of IL4-10 FP through IL-10R1 is affected. However, based on other results

achieved by IL4-10 FP, we have no evidence for the latter explanation. In **Chapter 4** we show that the analgesic effect of IL4-10 FP in an inflammatory pain model in mice, is superior compared to the effect of IL-4, IL-10 and, remarkably, also to the effect of the combination of IL-4 and IL-10. Moreover we have obtained *in vitro* evidence confirming the superior effect of the fusion protein over the combination of IL-4 and IL-10. TNF mediated sensitization of capsaicin-induced calcium responses in sensory neurons *in vitro* was inhibited by IL4-10 FP whilst the combination of IL-4 and IL-10 did not inhibit neuronal sensitization in this model to the same extent. Unravelling the superior effect of IL4-10 FP on chronic pain revealed that IL4-10 FP uniquely promotes clustering of IL-4R and IL-10R on sensory neurons, leading to unique intracellular signaling (Prado et al., manuscript submitted). Prado used a proximity ligation assay on sensory neurons to demonstrate clustering of IL-4R and IL-10R by IL4-10 fusion protein. Such clustering was not observed with the combination of cytokines or vehicle treatment. Further experiments showed that IL4-10 fusion protein, by clustering IL-4R and IL-10R, drives unique downstream signaling of neurons involving amongst others JAK1 activation. Whether non-natural cytokine receptor clustering completely explains the superior effect of IL4-10 FP in pain models needs to be established. Nevertheless, this unique mode of action underlines the potential of IL4-10 FP for the treatment of inflammatory diseases.

8.2.2 | Therapeutic Efficacy of IL4-10 FP

Next to a unique cellular signaling profile, the longer half-life of IL4-10 FP as compared to wild-type IL-4 and IL-10, is an advantage of IL4-10 FP as therapeutic protein. The extended half-life can, in certain situations, compensate the somewhat impaired *in vitro* activity that was discussed in the previous paragraph. In **Chapter 5** we report a fourfold increase in half-life for IL4-10 FP, as compared to wild-type IL-4 or IL-10. We also show, as has been reported before, that IL-4 is more rapidly cleared from the circulation than IL-10 [5, 6], explained by the fact that IL-10 is a dimer, has a larger molecular size than IL-4, and is therefore less rapidly cleared via the kidneys [7]. As mentioned earlier, although there is quite some evidence that the combination of IL-4 and IL-10 is beneficial to attenuate inflammatory disorders, a combination therapy is costly and would not solve the limited *in vivo* bioactivity of both cytokines. An additional limitation would be the difference in half-life of both cytokines, since this will lead to a molar ratio that varies in time after administration. We speculate that a variable molar ratio may hinder the additive and counteracting effects of the combination of IL-4 and IL-10.

In case of IL4-10 fusion protein, the molar ratio of both cytokines is predictable and constant since the cytokines are linked to each other, though at this moment we cannot exclude completely that some of the fusion protein can be cleaved *in vivo*. We have no data to support that the half-life of IL4-10 FP, though extended, is already optimal for therapeutic application. So it might still be that, as for IL-4 and IL-10 stand-alone therapy, multiple injections are required for optimal efficacy.

On the other hand, efficacy does not necessarily require permanent presence of IL4-10 FP. In preliminary experiments with IL4-10 FP in cultures with M0 macrophages we observed that IL4-10 FP is able to skew these macrophages into an anti-inflammatory M2-like phenotype. This effect is based on the phenomenon of macrophage plasticity, due to which macrophages, depending on cytokines and other mediators present, change their phenotype [8]. The duration of the described effect is determined by the presence and activity of M2 macrophages, rather than the presence of IL4-10 FP. Ultimately, skewing cells into an anti-inflammatory phenotype by IL4-10 FP to induce complete resolution of inflammation, may require a single or a few IL4-10 FP injections. Such a long-term effect through polarization of cells may be involved in the full resolution of inflammatory pain we observed in two different mouse models of persistent inflammatory pain upon three intrathecal injections of IL4-10 FP (**Chapter 4**). Importantly, this effect of IL4-10 FP was not explained by its anti-inflammatory effects on the peripheral inflammation in the paws of these mice, since local production of inflammatory cytokines in the paw was not affected by IL4-10 FP intrathecal injection.

8.2.3 | Formulation of IL4-10 FP as therapeutic protein

In **Chapter 7**, we describe a dimeric and monomeric form of IL4-10 fusion protein, and show that both forms have comparable activity. For therapeutic purpose, a preparation consisting of a heterogenic mixture of dimers and monomers is not desirable. GMP manufacturing for further development of a well-characterized drug substance preparation, may require a choice between the dimeric or the monomeric form. As a first step to a possible selection, further characterization of the dimeric and monomeric form of IL4-10 FP is required. From what we know now, the dimeric form is the preferred form in terms of systemic exposure, since due to its larger size it is expected to have a longer half-life. Moreover, probably, the IL-10 moiety of the dimeric fusion protein may be less immunogenic since wild-type IL-10 almost exclusively occurs as a domain-swapped dimer. However, future studies should reveal what the exact

functional activity of dimeric and monomeric IL4-10 FP is, and whether or not these forms have unique properties.

In **Chapter 6** we show, based on the HP-SEC pattern, that hyperglycosylation increases dimerization of IL4-10 FP as compared to wild-type IL4-10 FP. We did not explore whether engineering of IL4-10 FP to further improve both glycosylation and sialylation, yields a product that mainly consists of dimers. Obviously, such a preparation could be a good starting point for the development of a formulation used for systemic administration. For local administration, the monomeric, low-sialylated (but glycosylated) form of IL4-10 FP may be an attractive option, since systemic exposure upon leakage of this glycoform from a local compartment, is expected to be low due to rapid removal of low-sialylated IL4-10 FP from the circulation by both ASGPR-mediated and kidney-mediated clearance. Such a treatment option might be relevant for e.g. effective treatment of OA by IL4-10 FP, as demonstrated in **Chapter 3**.

Recombinant IL-4 and IL-10 as stand-alone therapy, have been evaluated in clinical studies [1, 2, 9, 10, 11]. These studies did not raise concerns about potential immunogenicity of either cytokine. Therefore, the use of wild-type human IL-4 and IL-10 sequences for IL4-10 FP in itself seems to be safe in humans regarding immunogenicity. However, introduction of the linker sequence may result in the formation of T cell epitopes partially involving the linker sequence and wild-type IL-4 or IL-10 sequences. In an early stage of the project, we have therefore investigated if the linker sequence indeed introduces new T cell epitopes in the IL4-10 FP sequence. An *in silico* analysis by Antitope Ltd (Cambridge, UK; now Abzena), however, revealed that no new T cell epitopes are introduced in the sequence of IL4-10 FP by incorporation of the linker sequence (unpublished data). Therefore, we do not expect specific concerns regarding immunogenicity of IL4-10 FP in humans.

Other concerns include the pro-inflammatory activities of IL-10 and the ability of IL-4 to induce a strong Th2 response and increase the risk of development of allergic reactions. **Chapter 2** shows that side-effects of both IL-4 and IL-10 are mutually attenuated by each other in IL4-10 FP. Moreover, it should be noted that during clinical trials with IL-4 or IL-10, no drug-related allergic responses were observed, even not upon high doses of IL-4 [9, 10, 11].

8.2.4 | Broader perspective for IL4-10 FP

In this thesis we describe the potential of IL4-10 FP for the treatment of inflammatory diseases like rheumatoid arthritis, and inflammatory pain. We hypothesize that patients with other inflammatory diseases like inflammatory bowel disease, for which treatment with IL-10 has been investigated [12, 13], would also benefit from the treatment with IL4-10 FP. Clinical studies with IL-10 revealed some, but very limited, efficacy. Possible explanations include that, as for RA [14], pro-inflammatory activities of IL-10 hinder optimal efficacy [15]. In IBD one may postulate that a therapeutic agent should be present for a longer period of time, or that immune cells would need to be re-programmed in order to effectively attenuate inflammation in the gut. Based on the evidence presented in this thesis, IL4-10 FP would be an attractive candidate to evaluate in pre-clinical models of IBD.

In **Chapter 3** we describe experiments that support competence of IL4-10 FP as a disease-modifying drug for osteoarthritis (OA). Previous trials with conventional and biologic disease-modifying drugs for rheumatoid arthritis (RA) like MTX and anti-TNF show that these drugs are not effective in OA [16]. Although these results at first glance argue against an important role of inflammation in OA, it might also be that effective drugs for OA should target three pathophysiologic mechanisms. Such a drug ideally should prevent, or at least reverse, cartilage and subchondral bone damage, attenuate synovial inflammation, and combat OA related pain (**Figure 1**). According to the data presented in **Chapter 3**, we believe that IL4-10 FP targets all three mechanisms and therefore qualifies for a disease-modifying osteoarthritis drug (DMOAD). This was confirmed in an exporatory study, in which canine IL4-10 FP was administered in the OA canine groove model [17]. Future studies should further establish to what extent IL4-10 FP can meet the promise to act as DMOAD.

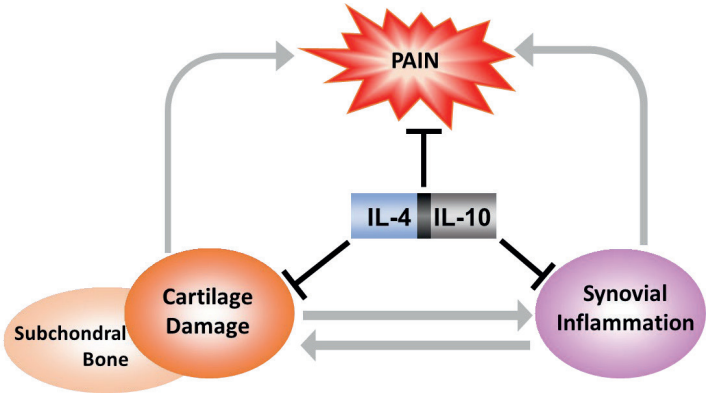

Figure 1. Disease-modifying osteoarthritic drug (DMOAD) potential of IL4-10 FP. treatment of OA requires a triangle approach, using so called disease-modifying osteoarthritis drugs (DMOAD's). IL4-10 FP is a potential DMOAD, affecting cartilage and subchondral bone damage, synovial inflammation, and OA related pain. *Adapted from an image by Lafeber, F.P.J.G.*

Another disease in which treatment with IL4-10 FP could play a role is sepsis. Sepsis is a life-threatening disease caused by a dysregulated host response to infection, which leads to organ dysfunction and in severe cases is followed by death [18]. Sepsis is notoriously difficult to treat, since the disease may develop very rapidly, and the type of immune response differs per patient. Therefore precision immunotherapy together with close monitoring at the sites of infection have been suggested [19, 20]. Notably, sepsis can either result in hyperinflammation ("cytokine storm"), particularly in the early stage, and in immunoparalysis later on, in which case the body cannot generate a pro-inflammatory response at all. Pro- and anti-inflammatory cytokines play a role in hyperinflammation and immune paralysis of sepsis, respectively [21]. Administration of pro-inflammatory cytokines like TNF and IL-1, induces septic shock in animals, and septic shock-like symptoms in humans [22], while administration of anti-inflammatory cytokines, such as IL-10, mitigates severe experimental sepsis in animals. Indeed, animals with endotoxemia that were administered with IL-10 had less TNF release and a better overall survival [23]. Many other pre-clinical studies in several experimental models support that administration of IL-10 attenuates sepsis, but until now recombinant IL-10 has not been tested in humans with sepsis [24]. Probably, negative results of extensive and expensive clinical studies with other anti-cytokine agents like anti-TNF and IL-1RA in sepsis, in spite of impressive positive results in preclinical models, have discouraged extensive exploration of recombinant IL-10 in human sepsis. Notably, patients with a severe Sars-CoV-2 infection develop a syndrome which is very similar to sepsis. These symptoms are triggered by a massive production of inflammatory cytokines, the so-called cytokine storm [25], which can be lethal when resulting in acute respiratory distress syndrome and multi-organ dysfunction. The urgent medical need for drugs that attenuate this syndrome in life-threating COVID-19 may renew interest in the potential of IL-10 and IL-4 to combat the cytokine storm.

We explored the potential of IL4-10 FP in a pilot study in mice with experimental endotoxemia. Preliminary data show a moderate effect of IL4-10 FP on the release of pro-inflammatory cytokines TNF and IL-6 (**Figure 2**), and on the overall course of the inflammation, as reflected

by a slightly reduced body temperature response. Thus, IL4-10 FP may have potential to treat sepsis, however future studies wil have to determine whether the fusion protein outperforms IL-10 or even the combination of IL-4 and IL-10.

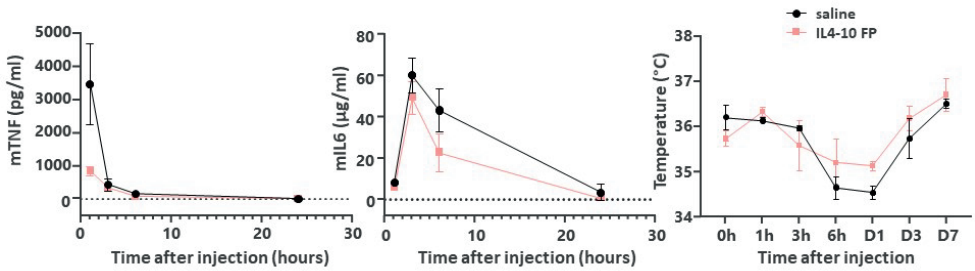

Figure 2. The potential of IL4-10 FP to attenuate endotoxemia upon systemic administration. Systemic mild endotoxemia was induced in male C57BL/6 Mice by an IV injection of 850 µg/kg LPS (Escherichia coli, strain O111:B4 from Sigma). 1 minute after LPS injection, mice were injected IV with 10 µg/kg IL4-10 FP (n=4) preparation or with sterile saline (n=3). Blood (50 µl) was collected at timepoints 1, 3, 6 and 24 hours after LPS injection. Temperature was monitored on these time points as well, as well as on Day 3 and Day 7 after injection of LPS. All blood samples were collected in EDTA tubes and mouse TNF and IL-6 levels were measured with ELISA kits (DuoSet, R&D), according to manufacturer's instructions.

8.2.5 | Potential for other cytokine fusion proteins

The potential of IL4-10 FP in various disease models, supports the idea to combine other cytokines into a fusion protein. Especially the superior efficacy of IL4-10 FP in the pain models compared to that of the combination of IL-4 and IL-10, which most likely results from non-natural cross linking of involved cytokine receptors, makes it attractive to evaluate the potential of other cytokines in this regard, and to explore how unique signaling by other cytokine fusion proteins can be used in human disease.

The concept that the functional effect of a cytokine fusion protein can be larger than that of the sum of the activities of each individual cytokine has been observed before by other research groups, studying respectively the activity of GIFT fusokines and synthekines [26, 27]. GIFT fusokines are developed to combine the activities of two different pro-inflammatory cytokines into one protein as a novel cancer immunotherapy [28]. As a prototype, a GIFT fusokine was made of IL-2 and GM-CSF (GIFT-2) with the aim to improve NK cell stimulation and enhance IFN-γ production, an effect that was significantly improved by GIFT-2 as compared to the

combination of GM-CSF or IL-2 [26]. The beneficial effects of fusokines compared to combination therapy of cytokines could only be observed with cells expressing receptors for both cytokines and resulted in receptor clustering upon binding of GIFT fusokines. The unique interaction of the receptors resulted in the transduction of an enhanced signal compared to normal signaling of these receptors by wild-type cytokines [29]. In another platform, synthekines, fusion proteins of two different cytokines, were developed to study receptor dimerization following chimeric protein signaling [27]. The idea is to generate cytokine receptor dimers that do not naturally occur, in order to discover novel signal-transduction programs, after which those are explored for clinical application. To that end, synthetic cytokines were engineered consisting of a dominant and a negative version (DN) of two different cytokines that were fused together using a short linker sequence comparable to the sequence we used in IL4-10 FP. Two synthekines were generated, namely IFN-α/IL-4 (SY1) dimerizing the IFN-αR with the IL-4R and IL-2/IL-4 (SY2) dimerizing the IL-2R with the IL-4R. Indeed, the signaling and functional response induced by the synthekines differed from the natural signaling by the respective combinations of IL-2, IL-4 and IFN-α. Synthekines not only induce signaling that resembles that of the parent cytokines but also induce novel signaling programs. Although aberrant signaling is mechanistically not yet fully understood, it is suggested that the unnatural heterodimer receptor pairing results in novel gene expression [27]. The results of IL4-10 FP, which we have termed synerkine, described in this thesis thus resemble those of synthekines regarding the unique activity these cytokine fusion proteins may have on cells. Moreover, this thesis describes a potential therapeutic application of unique signaling of cells through non-natural cross-linking of cytokine receptors, i.e. the treatment of chronic inflammatory pain.

8.2.6 | In conclusion: a platform for the development of novel fusion proteins

In conclusion, the feasibility to make a biochemically stable and functional fusion protein of regulatory cytokines is presented in this thesis. Furthermore, tools to manipulate half-life and systemic exposure of such a fusion protein are provided. We postulate that the potential of IL4-10 FP in various pre-clinical models is modulated by receptor crosslinking of IL-4R and IL-10R. Thus, this thesis presents a proof of concept that unique functional effects can be created by combining regulatory cytokines in a fusion protein. The challenge remains to explore which cytokine combinations can induce unique signaling through non-natural cross-linking of cytokine receptors, and how cell biological effects of this unique cell signaling can be used for

clinical applications. The results of IL4-10 FP described in this thesis support the application of IL4-10 FP in RA, OA, chronic pain, and other inflammatory conditions.

8.3 | References

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Appendices

English Summary
Nederlandse samenvatting
Dankwoord
Curriculum Vitae
List of Publications

ENGLISH SUMMARY

Inflammation is an important response of the human body to harmful stimuli. The course of inflammation is orchestrated amongst others by regulatory proteins called cytokines. In case of an inappropriate response, or an imbalance between inflammatory and regulatory cytokines, inflammation can become chronic. In a variety of human diseases, chronic inflammation contributes to the development and continuation of the disease. Rheumatoid arthritis (RA) and osteoarthritis (OA) are well-known examples of chronic inflammatory diseases. Recent studies point to involvement of inflammation in many more conditions, traditionally not considered to be an immune or inflammatory condition. The latter include various forms of chronic pain. **Chapter 1** describes that, in general, treatment of chronic inflammatory diseases is challenging, reflecting the complexity of the human immune system. Regulatory cytokines seem attractive candidates for therapeutic drugs to treat chronic inflammatory diseases as they are natural compounds and inhibit the formation and release of multiple inflammatory mediators. However, results of clinical studies with regulatory cytokines have been disappointing. Reasons for clinical failure are discussed in chapter 1, as well as a possible approach to improve therapy with regulatory cytokines, which is the use of fusion proteins of these cytokines. In this thesis, the development and characterization of IL4-10 fusion protein is described, in which the activity of regulatory cytokines IL-4 and IL-10 is combined in a single, unique molecule.

The IL4-10 fusion protein was created by linking IL-4 covalently to IL-10, using a linker sequence that allows maximal flexibility between both cytokine moieties. IL4-10 FP was expressed in HEK293 cells as a 70 kDa glycosylated dimeric protein **(Chapter 4)**.

As a starting point, we studied the functional activity of IL4-10 FP in the context of RA, as described in **Chapter 2**. IL4-10 FP potently inhibits production of pro-inflammatory cytokines such as TNF, IL-1, IL-6, and IL-8, while levels of IL-1RA and sTNFR are hardly affected by IL4-10 FP. In addition, IL4-10 FP reduced Th1 levels and stimulated Th2 cytokine production, whereas T-cell proliferation and the number of regulatory T-cells remained unchanged. These results suggest that IL4-10 FP is able to shift a pro-inflammatory phenotype of cells towards a more immunoregulatory phenotype. Furthermore, we confirmed that both the IL-4 and the IL-10

moiety contribute to the functional activity of IL4-10 FP. In RA, upregulation of activating FcγRs potentially enhances immune complex-mediated inflammation, a side effect seen in IL-10 treated RA patients. In the presence of IL4-10 FP, however, the expression of FcγRI, IIa and III is unaffected. IL4-10 FP significantly shifted the FcγRIIa/FcγRIIb ratio in favor of the inhibitory FcγRIIb. Also the expression of FcεR is largely unaffected by IL4-10 FP, whereas this receptor is markedly upregulated by stand-alone IL-4, enhancing a risk of allergic-type responses. These findings support the hypothesis that by combining both cytokines in a fusion protein, IL-4 and IL-10 ameliorate each other's unwanted pro-inflammatory effects. Furthermore, the ability of IL4-10 FP to reduce IL-6 and IL-8 production in *ex vivo* cultures with arthritic tissue and the ability to attenuate experimental arthritis in mice, suggests a therapeutic potential of IL4-10 FP in RA.

In **Chapter 3** we studied the potential of IL4-10 FP as a disease modifying osteoarthritis drug (DMOAD). OA is a rheumatic disease in which inflammation, cartilage degeneration and pain contribute to disease severity, and an effective treatment of OA may need to target these three pathophysiological phenomena. We studied the effectiveness of IL4-10 FP on each of the phenomena. In *ex vivo* cultures with human synovium and cartilage of OA patients, the fusion protein reduced levels of pro-inflammatory cytokines like IL-6 and IL-8 and showed an improved proteoglycan turnover. This OA modifying effect was furthermore supported by reduction in catabolic and pain-related mediators, like MMP3, VEGF and NGF. Finally, intra-articular injection of IL4-10 FP decreased the pain of dogs with experimentally induced OA, reflected by increased joint loading of the affected legs.

The potential of IL4-10 FP to attenuate chronic inflammatory pain was evaluated in **Chapter 4**. Intrathecal injection of IL4-10 FP dose-dependently inhibited hyperalgesia in several chronic inflammatory pain models in mice. Remarkably, the analgesic effect of IL4-10 FP was larger than the effect of the combination of IL-4 and IL-10. Upon three subsequent injections of IL4-10 FP, inflammatory pain – induced by carrageenan or complete Freund's adjuvant – was completely and permanently resolved. Thus, IL4-10 FP is more potent than a combination therapy of both individual cytokines to inhibit inflammatory pain, and is even able to permanently resolve pain in this experimental mouse model.

The results of IL4-10 FP described in chapters 2, 3 and 4 are promising, yet, we considered that optimization of the therapeutic use of IL4-10 FP may require further biochemical modification of the fusion protein. Strategies to achieve this, are described in the subsequent chapters.

In **Chapter 5** we studied the effect of differential sialylation of the glycans of IL4-10 fusion protein on *in vitro* and *in vivo* bioactivity and pharmacokinetic parameters. To that end, we generated CHO cell lines, stably expressing a low and a high sialylated form of IL4-10 FP. We concluded that sialylation of the fusion protein does not influence it's *in vitro* activity. Also its *in vivo* efficacy upon local injection, as evaluated in the inflammatory pain model in mice, was unaffected. However, sialylation markedly influences the clearance of IL4-10 FP from the circulation. The low sialylated variant was rapidly cleared from the circulation via the asialoglycoprotein receptor in the liver, resulting in low recovery after systemic injection in rats. In contrast, the high sialylated variant had a better recovery. Therefore, engineering the sialylation of IL4-10 FP may be used as a strategy to optimize IL4-10 FP for local or systemic application.

Next, glycoengineering as strategy to increase the plasma half-life of our IL4-10 FP was studied in **Chapter 6**. Additional N-linked glycosylation sites were introduced in the human IL4-10 FP sequence, based on homology with sequences of several animal species. Although *in vitro* the functional activity of IL4-10 FP mutants with two or more additional glycosylation sites was somewhat impaired compared to IL4-10 FP consisting of wild-type sequences, the plasma half-life of the IL4-10 FP variant with three additional glycosylation sites was prolonged upon intravenous administration, as compared to non-mutated IL4-10 FP.

Functional wild-type IL-10 is a non-covalently linked dimer under physiological conditions. Batches of human IL4-10 FP indeed contain non-covalently linked dimers, but also monomeric IL4-10 FP. In **chapter 7** we describe whether the functional activity of the IL-10 moiety within IL4-10 FP is dependent on the non-covalent dimerization of the fusion protein, by isolating and testing both monomeric and dimeric IL4-10 FP. We observed an equal dose-dependent inhibition of LPS-induced TNF by monomeric and dimeric IL4-10 FP in whole blood cultures. Moreover, the addition of IL-4 receptor blocking antibodies showed a similar activity of

monomeric and dimeric IL4-10 FP. These results suggest that dimerization of the wild-type IL-10 sequence is not mandatory for its functional activity.

In **Chapter 8**, the main findings described in this thesis are discussed, as well as the potential of IL4-10 FP in the treatment of other inflammatory disease, like inflammatory bowel disease (IBD) and sepsis. Furthermore, we suggest that the unique activity of IL4-10 FP might be mediated by receptor cross-linking, leading to alternative signaling of cells with IL4R and IL10R. This insight leaves the opportunity to combine other regulatory cytokines into fusion proteins with unique properties.

In conclusion, in this thesis the potential of the novel IL4-10 fusion protein for the treatment of inflammatory diseases is presented. The novelty lies in the combination of IL-4 and IL-10 in one molecule, shifting a pro-inflammatory phenotype towards a more immunoregulatory phenotype, mutually inhibiting each other's pro-inflammatory effects, and – in some conditions – leading to unique signaling of cells by non-natural crosslinking of IL-4R and IL-10R. Finally, this thesis provides a platform for the development of biochemically stable and functional fusion proteins of cytokines, including strategies to manipulate half-life and systemic exposure of such fusion proteins.

NEDERLANDSE SAMENVATTING

Een ontstekingsreactie is een proces in het menselijk lichaam als reactie op schadelijke prikkels. Het verloop van een ontsteking wordt onder meer gestuurd door "ontstekingsremmende eiwitten" die cytokines worden genoemd. Er zijn grofweg twee categorieën cytokines: ontstekingsbevorderende cytokines en ontstekingsremmende cytokines. Tijdens een ontsteking zijn beide groepen cytokines van belang om ervoor te zorgen dat het lichaam de schadelijke prikkel efficiënt opruimt. Het kan echter gebeuren dat de activiteit van beide groepen cytokines niet goed op elkaar is afgestemd. Er kan daardoor een verstoorde balans ontstaan, waardoor een goed bedoelde ontstekingsreactie chronisch kan worden en zijn oorspronkelijke doel voorbijschiet. Bij verschillende ziektebeelden draagt chronische ontsteking bij aan de ontwikkeling en continuering van de ziekte. Reumatoïde artritis (RA) en (osteo)artrose (OA) zijn bekende voorbeelden van chronische ontstekingsziekten. Recente onderzoeken wijzen op betrokkenheid van ontsteking bij veel meer aandoeningen, die traditioneel niet worden beschouwd als ontstekingsziekten. Tot deze aandoeningen behoren ook verschillende vormen van chronische pijn. **Hoofdstuk 1** beschrijft dat succesvolle behandeling van chronische ontstekingsziekten over het algemeen ingewikkeld is, vanwege de complexiteit van het menselijke afweersysteem. De toepassing van ontstekingsremmende cytokines als geneesmiddel voor de behandeling van chronische ontstekingsziekten lijkt aantrekkelijk, omdat cytokines lichaamseigen zijn én omdat ontstekingsremmende cytokines meerdere ontstekingsmediatoren tegelijk kunnen remmen. Op deze manier zou de cytokinebalans in het lichaam weer hersteld kunnen worden. De resultaten van klinische studies met ontstekingsremmende cytokines zijn tot nu toe echter onvoldoende succesvol gebleken. Redenen voor de teleurstellende resultaten van deze klinische studies worden besproken in hoofdstuk 1, evenals een mogelijke aanpak om de therapie met ontstekingsremmende cytokines te verbeteren, namelijk het gebruik van combinaties van deze ontstekingsremmende cytokines in zogenaamde fusie-eiwitten. In dit proefschrift wordt de ontwikkeling en karakterisering van zo'n combinatie, het IL4-10 fusie-eiwit, beschreven, waarbij de activiteit van de ontstekingsremmende cytokines IL-4 en IL-10 wordt gecombineerd in één uniek eiwit.

Het IL4-10 fusie-eiwit is gemaakt door IL-4 covalent (een vaste verbinding) te koppelen aan IL-10, met behulp van een linkersequentie, bestaande uit een aantal extra aminozuren (de bouwstenen van een eiwit), die maximale flexibiliteit tussen beide cytokines mogelijk maakt. Het IL4-10 fusie-eiwit werd vervolgens in specifieke cellen (HEK293-cellen) tot expressie gebracht (geproduceerd). Het geproduceerde IL4-10 fusie-eiwit bestond uit een geglycosyleerd dimeereiwit van 70 kDa, dit wil zeggen dat het suikerketens (glycanen) bevat zoals ieder natuurlijk eiwit en dat het uit twee identieke delen bestaat die aan elkaar verbonden zijn (dimeer) (**Hoofdstuk 4**).

Als eerste uitgangspunt bestudeerden we de functionele activiteit van IL4-10 fusie-eiwit in de context van reumatoïde artritis, zoals beschreven in **Hoofdstuk 2**. In een, in het laboratorium nagebootste, ontstekingsreactie zoals deze wordt gezien bij reuma liet IL4-10 fusie-eiwit sterke vermindering zien in de productie van ontstekingsbevorderende cytokines zoals TNF, IL-1, IL-6 en IL-8, terwijl IL-1RA en sTNFR, de natuurlijk voorkomende remmers van IL1 en TNF, nauwelijks werden beïnvloed door IL4-10 fusie-eiwit. Daarnaast verminderde het fusie-eiwit de cytokineproductie van specifiek stimulerende ontstekingscellen (Th1-cellen), terwijl de cytokineproductie van specifiek remmende ontstekingscellen (Th2-cellen) werd verhoogd. Deze resultaten suggereren dat IL4-10 fusie-eiwit in staat is een ontstekingsbevorderend celtype (Th1) te verschuiven naar een meer ontstekingsremmend celtype (Th2). In deze experimenten is bovendien bevestigd dat zowel het IL-4- als de IL-10 cytokine bijdragen aan de functionele activiteit van IL4-10 fusie-eiwit. Bij het verloop van reumatoïde artritis kan een verhoogd aantal activerende Fc-gamma receptoren de ziekte verergeren. Bij reumapatiënten die behandeld werden met IL-10 werd dit als bijwerking waargenomen, naast de gewenste ontstekingsremmende activiteit. Echter, in experimenten met het IL4-10 fusie-eiwit, waarbij de activiteit van IL-10 gecombineerd is met die van IL-4, werd de hoeveelheid van deze Fc-gamma receptoren niet beïnvloed. Daarentegen liet IL4-10 fusie-eiwit juist een verbeterde balans tussen activerende en remmende Fc-gamma receptoren zien. Aan de andere kant is van IL-4 bekend dat dit cytokine een specifieke receptor stimuleert (de Fc-epsilon receptor), waardoor bij een therapie met IL-4 het risico op de ontwikkeling van allergische reacties aanwezig is. Echter: door de aanwezigheid van het IL-10 cytokine naast IL-4 in het fusie-eiwit werd ook de expressie van deze specifieke receptor door het IL4-10 fusie-eiwit nauwelijks beïnvloed. Deze bevindingen ondersteunen de hypothese dat door het combineren van beide

cytokines in een fusie-eiwit, IL-4 en IL-10 elkaars ongewenste effecten tegengaan. Daarnaast laten we in dit hoofdstuk het directe effect van IL4-10 fusie-eiwit op reuma zien. Enerzijds door ontstekingsweefsel uit het gewricht van RA-patiënten samen met IL4-10 fusie eiwit in het laboratorium in kweek te brengen. In deze kweken werd een sterke vermindering in productie van ontstekingsbevorderende cytokines zoals IL-6 en IL-8 gezien. Anderzijds werd in een artritis muismodel aangetoond dat IL4-10 fusie-eiwit de ontsteking remt. Deze resultaten onderstrepen de potentie die het IL4-10 fusie-eiwit heeft voor de behandeling van ontstekingsziekten zoals reumatoïde artritis.

In **Hoofdstuk 3** hebben we de mogelijkheid van het IL4-10 fusie-eiwit als therapeutisch eiwit voor het behandelen van artrose bestudeerd. Artrose is een reumatische ziekte waarbij ontsteking bijdraagt aan de degeneratie van kraakbeen en aan de pijn en daarmee aan de ernst van de ziekte. Voor een effectieve behandeling van artrose lijkt het gelijktijdig onderdrukken van deze drie punten (ontsteking, kraakbeenschade en pijn) de beste aanpak te zijn. De effectiviteit van IL4-10 fusie-eiwit is dan ook op elk van deze drie punten bestudeerd. In laboratoriumkweken met weefsel van artrosepatiënten verminderde het fusie-eiwit de productie van ontstekingsbevorderende cytokines zoals IL-6 en IL-8 en daarnaast verbeterde het de kwaliteit van het kraakbeen. Deze effecten werden bovendien ondersteund door vermindering van weefsel destructieve en pijn gerelateerde eiwitten, zoals MMP3, VEGF en NGF. Ten slotte verminderde een intra-articulaire injectie van IL4-10 fusie-eiwit de pijn van honden met artrose, weerspiegeld door verhoogde gewrichtsbelasting van de aangetaste poten. Concluderend vermindert IL4-10 fusie-eiwit het verloop van artrose op alle drie genoemde punten.

Vervolgens is in **Hoofdstuk 4** de potentie van het IL4-10 fusie-eiwit om chronische ontstekingspijn te verzwakken geëvalueerd. Een injectie van IL4-10 fusie-eiwit in de ruimte naast het ruggenmerg (intrathecale toediening) liet een dosisafhankelijke afname van hyperalgesie (verhoogde gevoeligheid voor een pijnsimulatie) zien, in verschillende pijnmodellen bij muizen. Opvallend was dat het remmende effect op de pijn door IL4-10 fusie-eiwit groter was dan het effect door de combinatie van de individuele IL-4 en IL-10 cytokines. Bovendien werd bij drie opeenvolgende injecties met IL4-10 fusie-eiwit een volledige en permanente verlichting van de pijn gezien. IL4-10 fusie-eiwit is dus beter in staat om

ontstekingspijn te remmen dan de combinatietherapie van beide individuele cytokines, en is zelfs in staat om permanente pijnverlichting te geven in dit experimentele muismodel. Deze resultaten onderstrepen de uniciteit van het fusie-eiwit.

De resultaten van IL4-10 fusie-eiwit beschreven in de hoofdstukken 2, 3 en 4 zijn veelbelovend, maar het therapeutisch gebruik van IL4-10 fusie-eiwit vereist verdere biochemische aanpassingen om het effect te optimaliseren. Om dit te bereiken hebben we diverse technieken toegepast, welke worden beschreven in de volgende hoofdstukken.

In **Hoofdstuk 5** is het effect bestudeerd van bepaalde veranderingen (sialylering) van de suikerketens (glycanen) van het IL4-10 fusie-eiwit op de functionele activiteit in laboratoriumkweken en in diermodellen. In dierproeven is daarbij ook gekeken naar de farmacokinetische parameters, de parameters die in het lichaam van invloed zijn op de beschikbaarheid van het fusie-eiwit. In specifieke cellen (CHO-cellijnen) is een hoog- en een laag-gesialyleerde vorm van IL4-10 fusie-eiwit tot expressie gebracht. Beide gesialyleerde vormen van het fusie-eiwit lieten eenzelfde activiteit in laboratoriumkweken zien. Ook de activiteit op ontstekingspijn in muizen werd niet beïnvloed door de mate van sialylering. Sialylering heeft echter een invloed op de duur van de aanwezigheid van IL4-10 fusie-eiwit; de zogenaamde klaring uit de circulatie (verwijdering uit het lichaam). De laag-gesialyleerde variant werd snel uit de circulatie verwijderd (via de specifieke asialoglycoproteïne-receptor in de lever), na systemische injectie bij ratten. Daarentegen bleef de hoog gesialyleerde variant veel langer in de circulatie aanwezig. Dit laatste effect maakt de hoog-gesialyleerde variant van het IL4-10 fusie-eiwit geschikt voor systemische toepassing. De laag-gesialyleerde variant is juist geschikt voor lokale toepassing (bijvoorbeeld een gewricht bij de behandeling van reuma of artrose), zodat deze snel door het lichaam geklaard kan worden wanneer er lekkage naar de bloedbaan is vanuit het lokale compartiment.

In **Hoofdstuk 6** hebben we optimalisatie van de glycosylering toegepast als strategie om de duur van het IL4-10 fusie-eiwit in de circulatie (de halfwaardetijd) te verlengen. In de samenstelling van het IL4-10 fusie-eiwit zoals we die in de mens zien (de humane sequentie) werden een aantal aminozuren vervangen om additionele N-gebonden glycanen (suikerketens) aan IL4-10 fusie-eiwit toe te voegen. De plaatsing van deze aminozuren werd

gebaseerd op vergelijkbare aminozuurvolgordes in de IL-4 en IL-10 sequentie van verschillende diersoorten. Dit om, ondanks aangepaste aminozuren, binding aan de receptor en dus de functionele activiteit te kunnen waarborgen. In laboratoriumkweken zagen we echter dat de functionele activiteit van IL4-10 varianten (mutanten) met twee of meer extra glycanen enigszins verminderd was ten opzichte van het niet-aangepaste IL4-10 fusie-eiwit. De duur van de aanwezigheid in de circulatie (gemeten aan de halfwaarde tijd) van de IL4-10 fusie-eiwitvariant met drie extra glycanen was daarentegen ongeveer twee keer beter in vergelijking met het niet-aangepaste IL4-10 fusie-eiwit.

Van nature komt IL-10 voor als een dimeer; dit wil zeggen dat twee identieke eiwitten samen een molecuul vormen. Preparaten van het IL4-10 fusie-eiwit bestaan deels uit dimeren, maar bevatten daarnaast ook monomeer IL4-10 fusie-eiwit. Wij hebben onderzocht of de functionele activiteit van IL-10 binnen IL4-10 fusie-eiwit afhankelijk is van de dimerisatie van het fusie-eiwit en dit is beschreven in **Hoofdstuk 7**. Hiertoe is monomeer en dimeer IL4-10 FP van elkaar gescheiden en is de functionele activiteit van de twee preparaten bekeken. We zagen in het laboratorium een gelijke dosisafhankelijke remming van het ontstekingsbevorderende TNF-cytokine, in een nagebootste ontstekingsactiviteit, door zowel monomeer als dimeer IL4-10 FP. De vergelijkbare functionele activiteit bleef intact na het blokkeren van de functie van IL-4 in het fusie-eiwit door toevoeging van een IL-4-receptor blokkerend antilichaam. Dit suggereert dat de IL-10 activiteit in zowel de monomeer als dimeer variant van het IL4-10 FP gelijk is. Deze resultaten laten zien dat dimerisatie van de wildtype IL-10 sequentie niet persé noodzakelijk is voor de functionele activiteit.

In **Hoofdstuk 8** worden de belangrijkste bevindingen besproken die in dit proefschrift worden beschreven, evenals de potentie van IL4-10 FP bij de behandeling van andere ontstekingsziekten, zoals inflammatoire darmaandoeningen (IBD) en sepsis. In dit hoofdstuk wordt ook besproken dat het IL4-10 fusie-eiwit in staat is om de IL-4 en IL-10 receptor met elkaar te “verbinden” (crosslinking), waardoor een unieke, alternatieve signalering van cellen optreedt. Dit fenomeen is in één van onze meest recente onderzoeken vastgesteld en is mogelijk onderliggend aan de unieke effecten van het IL4-10 fusie-eiwit in de ontstekingspijnmodellen. Dit inzicht biedt de mogelijkheid om ook andere regulatoire cytokines te combineren tot fusie-eiwitten met unieke eigenschappen.

Concluderend wordt in dit proefschrift de potentie van het nieuwe IL4-10 fusie-eiwit voor de behandeling van (chronische) ontstekingsziekten gepresenteerd. De uniciteit van IL4-10 fusie-eiwit ligt in de combinatie van IL-4 en IL-10 in één molecuul. IL-4 en IL-10 versterken elkaar, waardoor een ontstekingsbevorderende conditie wordt verschoven naar een ontstekingsremmende conditie. Daarnaast remmen IL-4 en IL-10 elkaars ongunstige effecten en - onder bepaalde omstandigheden - leidt de combinatie in het fusie-eiwit tot een unieke signalering van cellen door de verbinding van de IL-4 en de IL-10 receptoren op cellen, hetgeen van nature niet voorkomt. Ten slotte biedt dit proefschrift een platform voor de ontwikkeling van biochemisch stabiele en functionele fusie-eiwitten, inclusief technologieën om de halfwaardetijd en klaring van dergelijke fusie-eiwitten te verbeteren.

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&

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Sunshine Room Ladies! It was so nice to spend the last year of my PhD in your company, Eveline, Vania, Annelisa, Sangeeta and Ryana! This room truly is the most sunny room of CTI-Applied :) I hope we can have a goodbye dinner sometime soon… Looking forward to catch up with you!

En nu, na tien jaar UMC, weer terug bij Genmab en dat is leuk! Dank BioA, voor de passende functie, de fijne sfeer en gezelligheid én voor het meeleven rondom de afronding van mijn proefschrift. Ik hoop dat we met elkaar mogen blijven bijdragen aan hoopgevende medicatie voor patiënten.

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CURRICULUM VITAE

Cristine Steen-Louws was born on the 25th of January, 1985, in Vlissingen, the Netherlands. She attended the first years of primary school at the Graaf Jan van Nassauschool in Vlissingen, after which she continued and completed her primary school in Iboko, Nigeria, where she and her family lived for 5 years. In 1997 she started secondary school via the IVIO Wereldschool and continued in the second year at Calvijn College in Middelburg, the Netherlands. Cristine passed her MAVO exam in 2001, where after she continued her education at the Laboratory Education (MLO) of the ROC Zeeland, at that time located in Goes. She did an internship at the Clinical Biochemistry and Haematology Laboratories of the Oosterschelde Ziekenhuis in Goes and the Princess Royal University Hospital in Orpington, nearby London, England. In 2005 she started her Bachelor study Life Sciences (HLO) at Avans Hogeschool, Breda. Her interest to contribute to new therapeutics started here, and she specialized herself in Biotechnology/Biochemistry. She finalized her Bachelor study, which she passed with honors, with an internship at the Molecular Pharmacology department of Organon (MSD), Oss. Here, Cristine studied heterodimerization of G-protein coupled receptors (GPCR's) with the novel Bioluminescence Resonance Energy Transfer (BRET) technology. In 2008 she moved to Utrecht to start her first job as a research associate within the Assay Development & Qualification department of Genmab. During 2 years of employment she was involved in (pre-) clinical assay development. Thereafter she started as a research technician in the research group of Erik Hack, at the UMC Utrecht. In 2015 she got the opportunity to start a PhD on the project that she was involved in from the start: the development of a novel fusion protein for the treatment of inflammatory diseases: IL4-10 fusion protein. The results of her PhD-project are described in this thesis and are published in several scientific papers. She is co-inventor on the main patent application of the fusion protein portfolio of originally the UMC Utrecht Holding B.V., more recently licensed to Synerkine Pharma B.V. Currently, Cristine is employed as a Scientist within the Bioanalytical Science department at Genmab, where she coordinates and supervises the bioanalysis of pre-clinical studies.

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