Improving health from the inside

Use of engineered intestinal microorganisms as in situ cytokine delivery system

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Abbreviations: A, absorbance; BRP, bacteriocin-release protein; EBV, Epstein-Barr virus; EnN, E. coli Nissle 1917; hIL-10, human interleukin-10; hIL-10, human interleukin-10; HlyA, hemolysin A; HCMV, human cytomegalovirus; IBD, inflammatory bowel disease; IL-10, interleukin-10; mAB, monoclonal antibody; mIL-10, murine interleukin-10; M2, modar m Euras; OmpT, outer membrane protease T; SD, Shime-Dalgarno; STAT3, signal transducer and activator of transcription 3; Tyr, tyrosine

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Introduction

The anti-inflammatory cytokine interleukin-10 and its viral homologs were chosen as model proteins for the development of drug delivery systems based on probiotic carriers like E. coli Nissle 1917, E. coli G3/10, and Saccharomyces boulardii. Exterior cytokine secretion was achieved by a modified E. coli hemolysin transporter. Release of interleukin-10 transported to the periplasm via the OmpF signal peptide was enabled by a T4 phage lysis system under control of the araC-PBAD activator-promoter. The yield of interleukin-10 delivered by the phage lysis system was too low for functional analysis whereas the fusion protein secreted by the hemolysin transporter proved to be biologically inactive. Moreover, partial processing of the fusion protein by the E. coli membrane protease OmpT had no effect on the protein’s functionality. Using the α-mating factor signal sequence, the yeast S. boulardii proved to be suitable for secretory expression of biologically active viral interleukin-10.

In recent years, bacteria engineered for autodisplay or secretion of heterologous proteins have moved into focus as live carrier systems for the in situ delivery of vaccine candidates or therapeutic molecules. In inflammatory bowel disease (IBD), in situ synthesis of immunomodulating cytokines by genetically modified microorganisms represents a new therapeutic approach. A promising candidate for use in therapeutic interventions is the cytokine interleukin-10 (IL-10) due to its key role in downregulating inflammatory cascades. Secretion of biologically active IL-10 was first accomplished by genetically modified Lactococcus lactis, a nonpathogenic, noninvasive Gram-positive bacterium, which is mainly used to produce fermented foods. Localized IL-10 synthesis in the intestine by a transgenic L. lactis strain prevented experimental enterocolitis in mice. and showed a decrease in disease activity in a phase I clinical trial. In Gram-negative bacteria, the design and construction of in vivo delivery systems for biologically active molecules is more complex since both the inner and outer cell membrane are involved in the translocation process. Although Gram-negative bacteria possess a broad variety of transport systems which can be manipulated for heterologous protein export, secretory expression of IL-10 remains challenging for two reasons. First, the secondary structure of IL-10 is composed of ca. 60% α-helices which is maintained by two disulfide bonds. Reduction or modification of these disulfide bonds results not only in a structural instability of the protein lowering the α-helical content of its structure to approximately 50% but also in a loss of biological activity of the cytokine. Thus, correct disulfide bond formation is a critical factor during export out of the bacterial carrier. Second, IL-10 exists under physiological conditions predominantly as noncovalently linked homodimer, and...
in dimeric state strictly correlates with the biological activity indicating that the dimer is the active species in signaling. For recovery of protein functionality, it is imperative that the selected bacterial transporter be tailored to the traits of IL-10 in a way that enables dimerization of the recombinant monomers either in the periplasm or in the extracellular space. Since recombinant bacteria secrete its dimeric state strictly correlates with the extracellular space where dimer formation is observed. Therefore, secretory expression of functionally active IL-10 in Gram-negative bacteria has only been achieved in a Shigella flexneri strain using a modified Shigella type III secretion apparatus. Since S. flexneri is a potential pathogen with invasive properties and L. lactis does not colonize the gut mucosa, it must be engineered to produce a more suitable carrier system ensuring safe and efficient intestinal IL-10 delivery. Furthermore, since dimer stability is also temperature-dependent, in vivo conditions (low protein concentration, 37°C) most likely promote the formation of significant amounts of inactive IL-10 monomers. For future applications in a clinical setting, highly efficient carrier systems with good colonization traits and the ability to produce IL-10 analogs with higher molecule stability are needed.

Secretory Expression of Murine IL-10 via a Modified E. coli Hemolysin A Transporter

Recently, we have established a bacterial transporter construct which allows export of human IL-10 (hIL-10) to the periplasmic compartment in a biologically active form. In the culture supernatant, only a small amount of the E. coli produced hIL-10 was detected. Moreover, recombinant hIL-10 from the extracellular fraction did not show any activation of signal transducer and activator of transcription 3 (STAT3), suggesting that the predominant species in the culture supernatant is the monomeric form. In order to achieve higher yields of recombinant IL-10 in the extracellular space, we evaluated the E. coli hemolysin A (HlyA) translocase for secretory expression of murine IL-10 (mIL-10). The E. coli HlyA transporter is encoded by the hlyCABD operon, which is mainly associated with unpathogenic strains. In the ARC (ATP-binding cassette)-dependent type 1 secretion machineries of Gram-negative bacteria, secretion of the hemolytic toxin HlyA requires the C-terminal signal sequence HlyA, the two specific translocator proteins HlyB and HlyD, as well as the pore-forming outer membrane protein ToC. The E. coli HlyA secretion apparatus has been proven to be a powerful tool for secretory delivery of heterologous proteins in different hosts. It is essentially indiscriminate in that it may accommodate alleles of various sizes and composition. For example, export of functionally active human interleukin-6 into the growth medium was demonstrated in both E. coli and S. typhimurium strains by utilizing the E. coli hemolysin signal sequence and apparatus. For heterologous protein secretion via the E. coli hemolysin system, the pUC19-derived expression vector pMH80 (Fig. S1A) was constructed comprising the complete hly operon with its intrinsic promoter region of E. coli strain N96. However, the operon was made devoid of the toxin sequence of the hlyA gene. Subsequently, the mature form of the mIL-10 gene without its eukaryotic signal sequence was inserted in-frame via a NsiI restriction site into a residual part of the hlyA gene which encodes the C-terminal 62-aa secretory recognition signal of hemolysin A. The resulting plasmid was designated pMH84 (Fig. S1B). After transformation of prokaryotic E. coli strain Nisle N96777 (EcN) with plasmid pMH84, secretion of the mIL-10-HlyA fusion protein was verified by ELISA and immuno blot analysis of cell-free supernatants from EcN pMH84 overnight cultures. Immunoblot analysis revealed two mIL-10-specific protein bands, one major band with a molar mass (M) of ca. 25 kDa most probably representing the full-length mIL-10-HlyA fusion protein (calculated M \text{f} = 25.2 \text{ kDa}, 222 aa) and a minor band with an M of ca. 20 kDa, suggesting that this protein is a proteolytic degradation product of mIL-10-HlyA (data not shown). The mIL-10-HlyA protein concentrations in culture supernatants of pMH84 transformed EcN measured by a commercial ELISA were in the range of 46–52 ng/ml. Next, we assessed the biological activity of the secreted mIL-10-HlyA proteins by tracking the intracellular IL-10 signaling pathway in a previously described cell-based immunoblot assay.11 However, the secreted mIL-10-HlyA proteins were not able to activate STAT3 by phosphorylating its Tyr705 residue (data not shown). Thus, dimerization of the mIL-10-HlyA fusion protein which is a prerequisite for recovery of IL-10 functionality11 does not appear to occur significantly in the culture supernatant. Since hybrid proteins adapted to Hly-specific secretion always carry the HlyA transport signal at the C-terminus, we hypothesized that the unprocessed secretory peptide markedly affects either the correct folding of the IL-10 monomers or proper assembly of the IL-10 dimeric form.

Processing of the HlyA, Sequence in mIL-10-HlyA, Fusion Proteins

To abrogate potential steric or electrostatic constraints introduced by the bulk of extra residues fused to the C-terminus, it was investigated as to whether cleavage of the C-terminal HlyA signal sequence by an intrinsic E. coli protease was feasible and added to the ability of the recombinant mIL-10 protein to restore its biological activity. The E. coli outer membrane protease T (OmpT) was shown to be responsible for the degradation of various recombinant proteins during purification from cell extracts.16–18 Furthermore, due to its narrow substrate specificity, OmpT is a useful processing enzyme of recombinant fusion proteins.17–19 OmpT primarily recognizes a sequence with two adjacent dibasic residues, e.g., RK, KR, RR, and KK, and cleaves between these amino acids.19 In order to test whether OmpT might be capable of removing the HlyA signal peptide from the mIL-10 fusion protein when a suitable cleavage site is inserted between the alanine and HlyA, a mIL-10 fusion protein was designed containing a polylinker sequence by constructing the expression vectors pMH84L, pMH84LmmpF3-5,
and pH84L_SD_ompT (Fig. 1A; plasmid maps see Fig. S1C–E). All plasmids encode a fusion protein with two OmpT recognition sites (KIRI and KIIIRII) within the linker sequence which are flanked by two spacer regions consisting of several α-helix breaking amino acids (P, G) for preferred OmpT recognition (Fig. 1A and B). Plasmids pH84L_ompT3.5 and pH84L_SD_ompT additionally harbor one copy of the ompT gene amplified from E. coli which was cloned downstream from the hly operon via a Nco restriction site. In plasmid pH84L_SD_ompT, an extra Shine-Dalgarno (SD) sequence was introduced upstream of the ompT start codon for enhanced gene expression. The expression vectors were introduced into EcN, and the amount of recombinant mIL-10 proteins in cell-free supernatants from overnight cultures was determined by ELISA. All three vector constructs delivered similar mIL-10 protein concentrations to the supernatant of overnight cultures which were in the range of 40–60 ng/ml and therefore rather low (Fig. 2A). To assess whether removal of the HlyA sequence from the mIL-10 fusion protein was successful, concentrated supernatants were analyzed by SDS-PAGE and Immunoblotting using a gradient gel for improved band resolution. As shown in Figure 2B, the mIL-10-HlyA fusion proteins (at ca. 26 kDa) are the major secreted proteins in most cases (D-marked arrow). In addition, two smaller bands running at ca. 20 kDa (B-marked arrow) and 24 kDa (C-marked arrow), respectively, are observed in all three transformants. The lower band (Fig. 2B, B-marked arrow) has a size which is slightly larger than the commercial mIL-10 control, suggesting cleavage at the second OmpT site (K170/171) within the linker sequence (M19.6 kDa, 170 aa). The upper band might be the result of proteolytic degradation within the HlyA sequence (Fig. 2B, C-marked arrow) although HlyA does not contain a dibasic residue motif. However, OmpT is also known to cleave monobasic amino acid sites such as R-A,40 the only possible dipeptide motif within mIL-10-HlyA which might fit the substrate specificity of OmpT. Cleavage between residues R211 and A212 within HlyA would yield a product with a calculated M of 23.8 kDa (211 aa, Fig. 1B). A fourth specific protease (Fig. 2B, A-marked arrow) is weakly secreted by those mutants which harbor an additional ompT copy on the plasmid, e.g., pH84L_ompT3.5 and pH84L_SD_ompT. It has the same electrophoretic mobility as the commercial mIL-10 control, suggesting cleavage at the first OmpT site (K161/162, Fig. 1B) within the linker sequence. The expected size of the processed mIL-10 is 18.9 kDa (161 aa, Fig. 1B) compared with 18.8 kDa for the full-length mature mIL-10 protein (160 aa). When the ompT gene is present in a single chromosomal copy (vector pH84L), a relevant portion of the secreted mIL-10-HlyA fusion protein is processed. Interestingly, compared with the mIL-10-HlyA fusion protein at ca. 26 kDa (Fig. 2B, lane 45, D-marked arrow), the processing efficiency could not be increased in the presence of a higher ompT gene dose provided by plasmids pH84L_ompT3.5 (Fig. 2B, lane 55, D-marked arrow) and pH84L_SD_ompT (Fig. 2B, lane 65, D-marked arrow). Since the additional ompT gene in these vector constructs is located far downstream of the hly promoter region, the level of transcription might be insufficient for OmpT overexpression despite a SD sequence upstream of the ompT start codon as in construct pH84L_SD_ompT. To overcome incomplete processing, it may be favorable to provide ompT with an appropriate promoter. The processing capacity seems to be also strongly strain-dependent, as can be seen in Figure 2B by comparison of EcN with E. coli MD54240. While the HlyA sequence contains no additional OmpT sites apart from an atypical dibasic OmpT motif (Fig. 2A, B-marked arrow), the linker D denotes the unprocessed fusion protein aa, amino acid, FP, fusion protein; M, molar mass; S, Shine-Dalgarno sequence.

![Figure 1](image-url)

**Figure 1.** Scheme showing the design of the modified hemolysin transporter and the possible OmpT cleavage products of the mIL-10-HlyA fusion protein. (A) The modified E. coli J96 hemolysin operon is depicted as harbored by the expression vectors pH84L, pH84L_ompT3.5, and pH84L_SD_ompT, respectively. The linker DNA sequence is given with both OmpT recognition sites underlined. Arrows indicate possible cleavage at the first (K161) and second (K170) dibasic OmpT motif. (B) The full-length mIL-10-HlyA fusion protein is shown with arrows indicating the preferred OmpT processing sites within the linker and HlyA moieties. Alphabetic characters (A–C) indicate the amino acids involved in the cleavage process and the calculated M of the truncated mIL-10-HlyA fusion proteins. The letter D denotes the unprocessed fusion protein. aa, amino acid. 

![Figure 2](image-url)

**Figure 2.** HlyA is produced in recombinant E. coli BL21 (DE3) (New England Biolabs). Finally, the STAT3 assay was used to test whether incomplete processing of the fusion protein would be sufficient for recombinant mIL-10 to regain its biological functionality.
Another, yet lytic mechanism which can be exploited for the release of recombinant periplasmic proteins is the controlled expression of phage native lytic proteins. Moreover, lysis based protein delivery systems may confer biological containment of genetically modified bacteria. Such organisms raise legitimate concerns about their use in medicine due to unintentional disposal into the environment and possible transfer of cloned virulence attributes. Consequently, we assessed a lysis conveying vector construct for enhanced hIL-10 release from the periplasm: the plasmid pGA9 (Fig. S1F) contains a BioBrick phage lysis device (part code BBa_K112808) present in the MIT Registry of Standard Biological Parts. The araC PBAD activator-promoter was installed with the T4 phage device to control lysis of the bacteria. The particular genetic parts of pGA9 were synthesized and cloned into a high copy number vector backbone with ColE1-type origin of replication by Geneart, Regensburg, Germany. After co-transformation of E. coli strain BL21 (DE3) with hIL-10 expression plasmid pAZ1 and lysis vector pGA9, the hIL-10 concentration in the absence of arabinose as inductor was determined from overnight cultures in both, the periplasm and the growth medium. Surprisingly, in contrast to the reference E. coli BL21 (DE3) pAZ1, the hIL-10 concentration of the co-transformant substantially dropped to ca. 5 ng/ml in the periplasmic fraction and ca. 50 pg/ml in the culture supernatant, respectively. Furthermore, in contrast to E. coli BL21 (DE3) transformed with pGA9 alone, the co-transformant was prone to spontaneous lysis in the absence of the inductor, indicating a less tightly restricted araC PBAD activator-promoter. Next, we investigated the influence of different inductor concentrations on the viability and growth rate of the cells. Lysis was assayed by measuring the absorbance at 600 nm (A600), and the percent reduction of A600 was used as an indirect measurement of the amount of lysed cells. Prior to induction, cells were grown to the stationary phase since hIL-10 production by E. coli cells was marginal during the exponential growth phase (data not shown). As outlined in Figure 3A, a maximum cell lysis equivalent to a ca. 65

However, concentrated supernatants from overnight cultures of all transformants were not able to activate STAT3 (data not shown), indicating that a fair amount of active dimers has not been generated due to misfolding, false assembly or low concentration of processed monomers.

Heterologous Protein Release by Induced Cell Lysis

E. coli-derived hIL-10 was shown to be delivered to the periplasm in a biologically active form. Since extracellular hIL-10 concentrations generated by the pAZ1 expression vector were rather low, we considered enhancing hIL-10 release to the growth medium by either induced lysis of the carrier cells or by increased permeability of the carrier’s outer membrane. For example, co-expression of kil, out and ady genes or the gene of the bacteriocin-release protein (BRP) leads to the formation of permeable zones in the outer cell membrane allowing periplasmic proteins to pass into the culture medium.
percent reduction of A₆₀₀ can be achieved at inductor concentrations from 0.8 mM arabinose upwards. In parallel, the bacterial count decreased from ca. 10⁶ CFU/mL to less than 10² CFU/mL (data not shown), indicating a significant loss of cell viability. Lastly, we studied the kinetics of hIL-10 release to the supernatant at different arabinose concentrations. Ca. 60 min after addition of 0.25, 1, and 4 mM arabinose, a distinct increase of hIL-10 in the growth medium to a peak concentration of 2.7–6.5 ng/mL was measured, suggesting efficient lysis of the carrier (Fig. 3B). However, a timely follow-up of 6 h revealed a steady decline of the hIL-10 concentration in the supernatant. This may indicate a beginning proteolytic degradation of hIL-10, most probably due to activated E. coli proteases in the growth medium. A functional analysis of the released recombinant hIL-10 was not performed due to low yield, but with other transformants delivering similar hIL-10 concentrations to the supernatant, e.g., E. coli BL21 (DE3) pAZ1, no in vitro biological activity was experienced.

**Probiotic S. boulardii as Delivery Chassis for IL-10 Homologs**

The yeast strain *S. boulardii* has proven its importance as a live pharmaceutical for the prevention and treatment of various gastrointestinal disorders. Recently, we engineered the *S. boulardii* strain present in the probiotic drug Perenterol for secretion of biologically active mIL-10 and evaluated its anti-inflammatory capacity in a colitis mouse model by using nuclear magnetic resonance imaging. In this study, no significant difference in the decrease of disease activity could be demonstrated between the genetically modified and the wild-type strain. Since the IL-10 monomer-dimer equilibrium is affected by temperature, protein concentration, and pH, we presumed that in vivo conditions favor dissociation of the active dimer species. Furthermore, interaction with luminal gut components such as mucins might add to the instability of the IL-10 dimer. The maximum cytokine output procured by *S. boulardii* carrying mIL-30 expression vector pSC1m² at high cell densities (A₆₀₀ 25–30) was in the range of 140–190 ng/mL (data not shown), suggesting in vivo concentrations to be much lower due to a highly competitive habitat. Thus, proteins combining IL-10-like functions with enhanced biological potency and half-life are highly desirable therapeutics. Naturally occurring IL-10 homologs are encoded in the genomes of herpes viruses such as Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV). They share many biological activities of cellular IL-10 but as a consequence of selection during virus-host coevolution they also display unique traits such as increased molecule stability and lack of immunostimulatory functions. These attributes may render viral IL-10 more effective as immunosuppressive agent than its eukaryotic counterpart. For secretory expression of viral IL-10, vectors pSC1c (HCMV IL-10) and pSC1e (EBV IL-10) were constructed as described previously. After lithium acetate transformation, *S. boulardii* cells were grown at 30°C for 48 h.

**Figure 3.** Evaluation of the T4 phage lysis construct encoded by plasmid pGα9 for hIL-10 release. (A) The extent of E. coli BL21 (DE3) (New England Biolabs) cell lysis as a function of different inductor concentrations ranging from 0 to 64 mM arabinose is shown. (B) The kinetics of bacterial cell lysis and hIL-10 release into the supernatant is indicated for E. coli BL21 (DE3) pCλ217 pGα9 induced at 0.25 (red lines), 1 (green lines) and 4 (blue lines) mM arabinose during stationary phase. A₆₀₀ values are represented by solid lines and hIL-10 concentrations in the supernatant as dotted lines. As reference for hIL-10 release into the growth medium E. coli BL21 (DE3) pAZ1 was used (black lines), as negative control (yellow lines) E. coli BL21 (DE3) pGα9 pUC19. A, absorbance; c, concentration.
to an A\textsubscript{600} of approx. 25–30, pelleted, and viral IL-10 levels were quantified in cell-free culture supernatants by using either a commercial (EBV IL-10) or an in-house (HCMV IL-10). The expression efficiency of both viral proteins significantly differed, with EBV IL-10 reaching a mean of ca. 920 ng/mL and HCMV IL-10 of ca. 100 ng/mL, respectively (Fig. 4A). Unexpectedly, HCMV IL-10 expression in S. boulardii was even lower than in different E. coli strains using the modified HlyA transporter for secretory production of HCMV IL-10 (Fig. 4A). Immunoblot analysis of culture supernatant from pSCLe transformed S. boulardii revealed a product of ca. 30 kDa in size (Fig. 4B), resembling the unprocessed EBV IL-10 protein (calculated M\textsubscript{r} 26.5 kDa (234 aa) for the mature EBV IL-10 including the N-terminal α-mating factor signal sequence). Compared with the viral IL-10-αH\textsubscript{a} fusion proteins of E. coli (data not shown), both yeast-derived recombinant viral IL-10 proteins demonstrated in vitro biological activity in a STAT3 cell assay using either the mouse macrophage cell line J774.1 (EBV IL-10, Fig. 5A) or the human Daudi’s Burkitt lymphoma cell line (HCMV IL-10, Fig. 5B).

Conclusions

The use of microorganisms with the ability of in situ drug delivery has tremendous potential for improving the treatment of various diseases. Probiotics represent an ideal chassis for genetic engineering since most of them have had an unbeaten record of safe application in humans for several decades. However, high-level expression of functional proteins, especially those from eukaryotic sources, is often difficult and strongly dependent on the expression platform. We genetically modified different probiotics such as EcN, E. coli Symbioflor G3/10, a component of the probiotic drug Symbioflor 2, and S. boulardii for secretory expression of IL-10 proteins which represent promising candidates for in situ bacteriotherapy of IBD. Due to their unique structure—the active species is a noncovalently linked dimer—IL-10 proteins impose particularly high demands on the foreign host’s transport machinery. Thus, adapting a selected transporter to the needs of the cytokine is a real challenge. In E. coli, the periplasm seems to provide a favorable milieu for dimer assembly while demonstration of the active species in the growth medium failed due to either low protein content shifting the equilibrium toward the monomeric form or, in case of the modified HlyA transporter, incomplete processing of the C-terminal signal peptide, thus impeding proper IL-10 folding. Moreover, in vivo conditions may negatively influence dimer stability emphasizing the necessity of more potent analogs with longer half-life for a successful therapeutic intervention. IL-10-like proteins encoded by herpes viruses are naturally occurring homologs of cellular IL-10 with unique traits which make them promising therapeutic candidates. Consequently, we engineered the probiotic yeast strain S. boulardii for secretory expression of biologically active viral IL-10 homologs. In vivo colitis models will provide future evidence whether or not in situ delivery of viral IL-10 by genetically modified probiotic yeasts has the potential as innovative alternative for the treatment of IBD.

Disclosure of Potential Conflicts of Interest

No potential Conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here: http://www.landesbioscience.com/journals/bioengineered/article/22646/
Figure 5. Activation of STAT1 phosphorylation by L. boulardii secreted recombinant viral IL-10. STAT1 phosphorylation (STAT1-pY701) was assayed by immunoblot analysis of protein extracts from either macrophage cell J774.1 treated with yeast-derived recombinant EBV IL-10 (A) or (B) from human DAUD’s Burkitt lymphoma cells treated with yeast-derived recombinant HCMV IL-10 (B). Total STAT1’s antibodies were used as loading control to ensure equal amounts of protein in all lanes. The supernatant of L. boulardii pre-Kana1 and commercial viral IL-10 was used as controls. (C) superimposed; (D) STAT3, total STAT3.

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