Bioengineered 4:3, 172–179; May/June 2013; © 2013 Landes Bioscience

Improving health from the inside

Use of engineered intestinal microorganisms as in situ cytokine delivery system

Christoph Pöhlmann,[1,2,*] Mandy Thomas,[2] Sarah Förster,[2] Manuela Brandt,[2] Maike Hartmann,[3] André Bleich[4] and Florian Gunzer[2,*]

[1]Department of Laboratory Medicine; Robert-Bosch Hospital; Stuttgart, Germany; [2]Institute of Medical Microbiology and Hygiene; Faculty of Medicine Carl Gustav Carus; TU Dresden; Dresden, Germany; [3]Institute for Cellular Chemistry; Hannover Medical School; Hannover, Germany; [4]Institute for Laboratory Animal Science; Hannover Medical School; Hannover, Germany

Keywords: *Escherichia coli*, *Saccharomyces boulardii*, interleukin-10, probiotics, hemolysin A, transport system, bacterial cell lysis, biological containment

Abbreviations: A, absorbance; BRP, bacteriocin-release protein; EBV, Epstein-Barr virus; EcN, *E. coli* Nissle 1917; 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; M_r, molar mass; OmpT, outer membrane protease T; SD, Shine-Dalgarno; STAT3, signal transducer and activator of transcription 3; Tyr, tyrosine

Submitted: 07/24/12

Revised: 10/21/12

Accepted: 10/23/12

http://dx.doi.org/10.4161/bioe.22646

*Correspondence to: Christoph Pöhlmann and Florian Gunzer; Email: Christoph.Poehlmann@rbk.de and florian.gunzer@tu-dresden.de

Addendum to: Pöhlmann C, Brandt M, Mottok DS, Zschüttig A, Campbell JW, Blattner FR, et al. Periplasmic delivery of biologically active human interleukin-10 in *Escherichia coli* via a sec-dependent signal peptide. J Mol Microbiol Biotechnol 2012; 22:1-9; PMID:22353729; http://dx.doi.org/10.1159/000336043

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.

Introduction

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.[1,2] 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.[3] Secretion of biologically active IL-10 was first accomplished by genetically modified *Lactococcus lactis*,[4] 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[5] and showed a decrease in disease activity in a phase I clinical trial.[6] 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[7] 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-helix which is maintained by two disulfide pairs.[8] Reduction or malformation 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.[8] 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

its dimeric state strictly correlates with the biological activity indicating that the dimer is the active species in signaling.[9] 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 subunit dissociation occurs at low protein concentrations (< 50 μg/ml),[9] the bacterial periplasm seems to be a more appropriate milieu for dimerization than the extracellular space where dilution effects favor dimer dissociation. So far, successful 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.[10] Since *S. flexneri* is a potential pathogen with invasive properties and *L. lactis* does not colonize the gut mucosa, it must be searched for a more suitable carrier system ensuring safe and efficient intestinal IL-10 delivery. Furthermore, since dimer stability is also temperature-dependent,[9] 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 of high recombinant protein output as well as natural or engineered 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.[11] 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.[11] 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 *hly*CABD operon,[12] which is mainly associated with uropathogenic strains,[13] and belongs to the ABC (ATP-binding cassette)-dependent type I secretion machineries of Gram-negative bacteria. Secretion of the hemolytic toxin HlyA requires the C-terminal signal sequence $HlyA_s$, the two specific translocator proteins HlyB and HlyD, as well as the pore-forming outer membrane protein TolC.[14] The *E. coli* HlyA secretion apparatus has been proven to be a powerful tool for secretorial delivery of heterologous proteins in different hosts.[15] It is essentially indiscriminate in that it may accommodate allocrites of various sizes and composition.[16] 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 secretion signal and apparatus.[17] 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 J96.[18] However, the operon was made devoid of the toxin sequence of the *hly*A gene. Subsequently, the mature form of the mIL-10 gene without its eukaryotic signal sequence was inserted in-frame via a *Nsi*I restriction site into a residual part of the *hly*A gene which encodes the C-terminal 62-aa secretorial recognition signal of hemolysin A. The resulting plasmid was designated pMH84 (**Fig. S1B**). After transformation of probiotic *E. coli* strain Nissle 1917[19] (EcN) with plasmid pMH84, secretion of the mIL-10-$HlyA_s$ fusion protein was verified by ELISA and immunoblot 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_r) of ca. 25 kDa most probably representing the full-length mIL-10-$HlyA_s$ fusion protein (calculated M_r 25.2 kDa, 222 aa) and a minor band with an M_r of ca. 20 kDa, suggesting that this protein is a proteolytic degradation product of mIL-10-$HlyA_s$ (data not shown). The mIL-10-$HlyA_s$ 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-$HlyA_s$ proteins by tracking the intracellular IL-10 signaling pathway in a previously described cell-based immunoblot assay.[11] However, the secreted mIL-10-$HlyA_s$ fusion proteins were not able to activate STAT3 by phosphorylating its Tyr_{705} residue (data not shown). Thus, dimerization of the mIL-10-$HlyA_s$ fusion protein which is a prerequisite for recovery of IL-10 functionality[20] does not appear to occur significantly in the culture supernatant. Since hybrid proteins adapted to Hly-specific secretion always carry the $HlyA_s$ transport signal at the C-terminus, we hypothesized that the unprocessed secretorial peptide markably affects either the correct folding of the IL-10 monomers or proper assembly of the IL-10 dimeric form.

Processing of the $HlyA_s$ Sequence in mIL-10-$HlyA_s$ Fusion Proteins

To abrogate potential steric or electrostatic constraints introduced by the bulk of extra 62 aa 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.[21-24] Furthermore, due to its narrow substrate specifity, OmpT is a useful processing enzyme of recombinant fusion proteins.[25-27] OmpT primarily recognizes a sequence with two adjacent dibasic residues, e.g., RK, KR, RR, and KK, and cleaves between these amino acids.[28] In order to test whether OmpT might be capable of removing the $HlyA_s$ signal peptide from the mIL-10 fusion protein when a suitable cleavage site is inserted between the allocrite and $HlyA_s$, a mIL-10 fusion protein was designed containing a polylinker sequence by constructing the expression vectors pMH84L, pMH84L*ompT*3_5,

and pMH84L_SD_*ompT* (**Fig. 1A**; plasmid maps see **Fig. S1C–E**). All plasmids encode a fusion protein with two OmpT recognition sites (K^IR^I and $K^{II}R^{II}$) 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 pMH84L*ompT*3_5 and pMH84L_SD_*ompT* additionally harbor one copy of the *ompT* gene amplified from EcN which was cloned downstream from the *hly* operon via a *Nco*I restriction site. In plasmid pMH84L_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 $HlyA_s$ 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-$HlyA_s$ 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 (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 [K^{II}(170)-R^{II}(171), **Figure 1B**] within the linker sequence (M_r 19.6 kDa, 170 aa). The upper band might be the result of proteolytic degradation within the $HlyA_s$ sequence (**Fig. 2B**, C-marked arrow) although $HlyA_s$ does not contain a dibasic residue motif. However, OmpT is also known to cleave monobasic amino acid sites such as R-A,[29] the only possible dipeptide motif within mIL-10-$HlyA_s$ which might fit the substrate specifity of OmpT. Cleavage between residues R211 and A212 within $HlyA_s$ would yield a product with a calculated M_r of 23.8 kDa

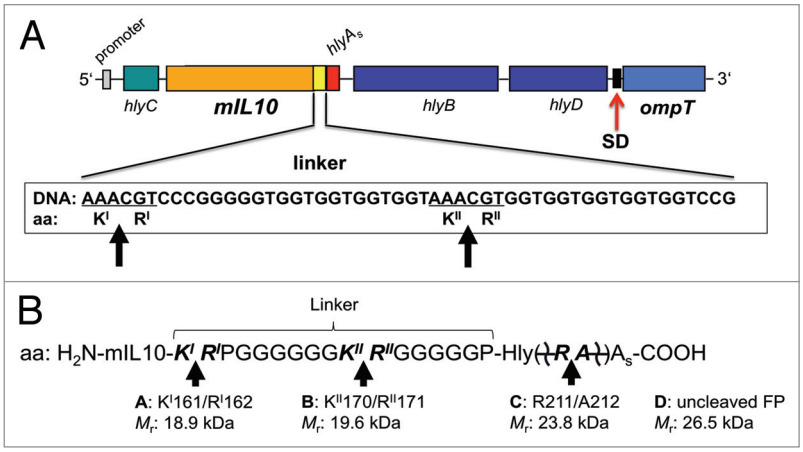

Figure 1. Scheme showing the design of the modified hemolysin transporter and the possible OmpT cleavage products of the mIL-10-$HlyA_s$ fusion protein. (**A**) The modified *E. coli* J96 hemolysin operon is depicted as harbored by the expression vectors pMH84L, pMH84L*ompT*3_5, and pMH84L_SD_*ompT*, respectively. The linker DNA sequence is given with both OmpT recognition sites underlined. Arrows indicate possible cleavage at the first (K^IR^I) and second ($K^{II}R^{II}$) dibasic OmpT motif. (**B**) The full-length mIL-10-$HlyA_s$ fusion protein is shown with arrows indicating the preferred OmpT processing sites within the linker and $HlyA_s$ moieties. Alphabetic characters (A-C) indicate the amino acids involved in the cleavage process and the calculated M_r of the truncated mIL-10-HlyAs fusion proteins. The letter D denotes the unprocessed fusion protein. aa, amino acid; FP, fusion protein; M_r, molar mass; SD, Shine-Dalgarno sequence.

(211 aa, **Fig. 1B**). A fourth specific protein (**Fig. 2B**, A-marked arrow) is weakly secreted by those mutants which harbor an additional *ompT* copy on the plasmid, e.g., pMH84L*ompT*3_5 and pMH84L_SD_*ompT*. It has the same electrophoretic mobility as the commercial mIL-10 control, suggesting cleavage at the first OmpT site [K^I(161)-R^I(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 pMH84L), a relevant portion of the secreted mIL-10-$HlyA_s$ fusion protein is processed. Interestingly, compared with the mIL-10-$HlyA_s$ fusion protein at ca. 26 kDa (**Fig. 2B**, lane 4S, D-marked arrow), the processing efficiency could not be increased in the presence of a higher *ompT* gene dose provided by plasmids pMH84L*ompT*3_5 (**Fig. 2B**, lane 5S, D-marked arrow) and pMH84L_SD_*ompT* (**Fig. 2B**, lane 6S, 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 pMH84L_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* MDS42.[30] While the $HlyA_s$ sequence contains no additional OmpT site apart from an atypical motif at position 211/212, there is only one putative OmpT site present in the mature mIL-10 moiety, namely RR at position 106/107. **Figure 2B** suggests that within the mIL-10-$HlyA_s$ fusion protein the $K^{II}R^{II}$ site within the linker (position 170/171) and a yet undefined cleavage site, possibly the atypical OmpT motif at position 211/212 within the $HlyA_s$ sequence, are preferentially recognized by OmpT. This preferential cleavage pattern remained unchanged in the presence of an increased *ompT* gene dose. Of note, proteolysis by a yet unknown protease was not ruled out by introducing the corresponding plasmids in an *ompT*-deficient *E. coli* strain, e.g., *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.

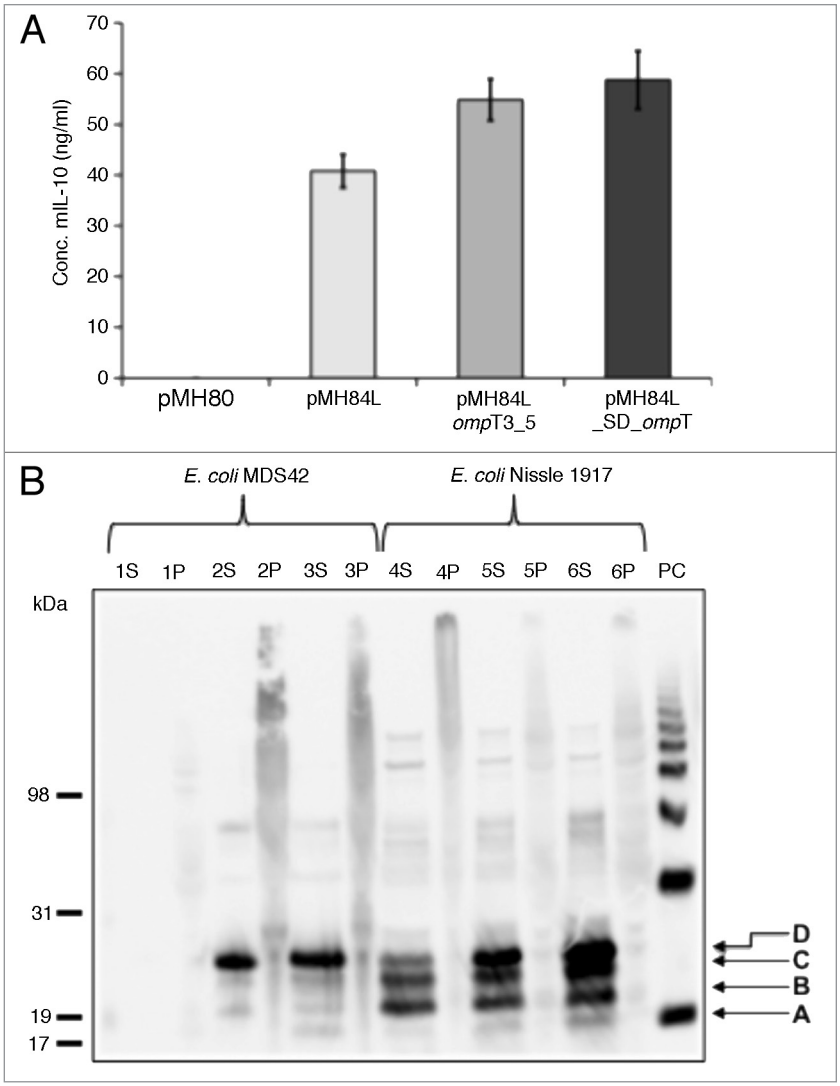

Figure 2. Secretory expression of mIL-10 via the modified hemolysin transporters. (**A**) Recombinant mIL-10 concentrations in culture supernatants from *E. coli* Nissle 1917[19] transformed with either pMH84L, pMH84L*omp*T3_5, and pMH84L_SD_*omp*T are compared (negative control pMH80). (**B**) Immunoblot analysis of periplasm (P) and supernatant (S) from *E. coli* strains MDS42[30] and Nissle 1917, respectively, transformed with either pMH84L (lanes 1 and 4), pMH84L*omp*T3_5 (lanes 2 and 5), and pMH84L_SD_*omp*T (lanes 3 and 6) is shown. Arrows designated A–D indicate the different mIL-10-HlyA$_s$ cleavage products (A–C) as well as the unprocessed fusion protein (D). Conc., concentration; PC, positive control (commercial mIL-10, R&D Systems); P, periplasm; S, supernatant.

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.[11] Since extracellular hIL-10 concentrations generated by the pAZ1 expression vector[11] 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*,[31] *out*[32] genes or the gene of the bacteriocin-release protein (BRP)[33] leads to the formation of permeable zones in the outer cell membrane allowing periplasmic proteins to pass into the culture medium. Another, yet lytic mechanism which can be exploited for the release of recombinant periplasmic proteins is the controlled expression of phage native lytic proteins.[34] 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.[35] 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 (A_{600}), and the percent reduction of A_{600} 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

percent reduction of A_{600} can be achieved at inductor concentrations from 0.8 mM arabinose upwards. In parallel, the bacterial count decreased from ca. 10^6 CFU/mL to less than 10^2 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.[36] 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.[37] 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,[9] 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-10 expression vector pSC1m[37] at high cell densities (A_{600} 25–30) was in the range of 140–190 ng/mL

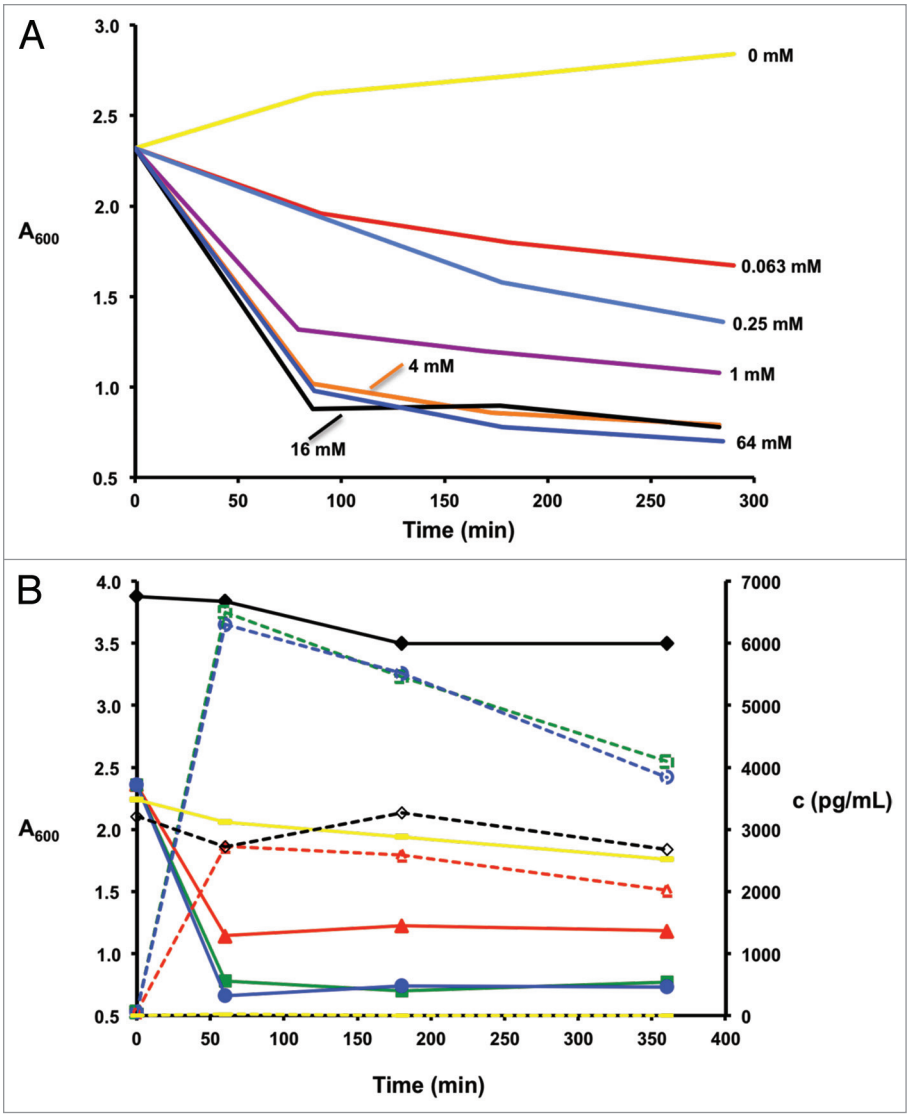

Figure 3. Evaluation of the T4 phage lysis construct encoded by plasmid pGA9 for hIL-10 release. (**A**) The extent of *E. coli* BL21 (DE3) (New England Biolabs) cell lysis as a function of different inductor concentrations ranging from 0 to 64 mM arabinose is shown. (**B**) The kinetics of bacterial cell lysis and hIL-10 release into the supernatant is indicated for *E. coli* BL21 (DE3) pAZ1[11] pGA9 induced at 0.25 (red lines), 1 (green lines) and 4 (blue lines) mM arabinose during stationary phase. A_{600} 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) pGA9 pUC19. A, absorbance; c, concentration.

(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.[38,39] 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.[37] After lithium acetate transformation, *S. boulardii* cells were grown at 30°C for 48 h

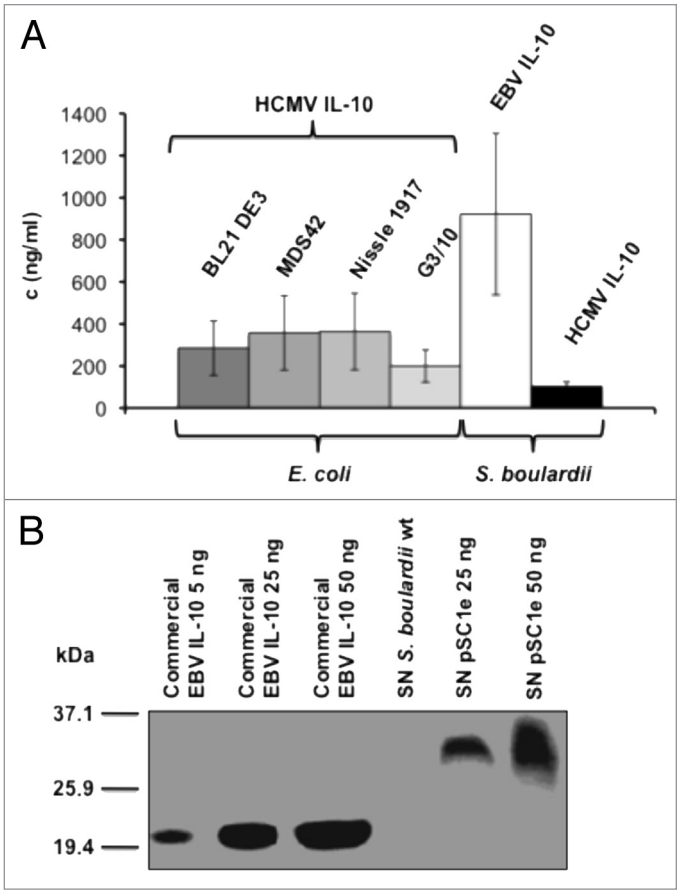

Figure 4. Secretory expression of viral IL-10 homologs in both pro- and eukaryotic carrier hosts. (**A**) Codon-optimized synthetic viral IL-10 genes were introduced in either *E. coli* hemolysin transporter plasmid pMH80 or yeast expression vector p426-Kana, as described previously.[37] Viral IL-10 concentrations in supernatants from 24 h bacterial and 48 h yeast cultures are compared. (**B**) Immunoblot analysis of supernatant (SN) from *S. boulardii*[36] transformed with EBV IL-10 expression vector pSC1e is demonstrated. The calculated M_r of the unprocessed EBV IL-10 protein carrying the α-mating factor signal sequence is 26.5 kDa (234 aa) compared with 17.1 kDa (145 aa) for the mature EBV IL-10. aa, amino acid; c, concentration; M_r, molar mass; SN, supernatant; wt, wild-type.

to an A_{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 ELISA (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 pSC1e transformed *S. boulardii* revealed a product of ca. 30 kDa in size (**Fig. 4B**), resembling the unprocessed EBV IL-10 protein [calculated M_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-$HlyA_s$ 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* Symbio G3/10,[40] 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/bioe/article/22646/

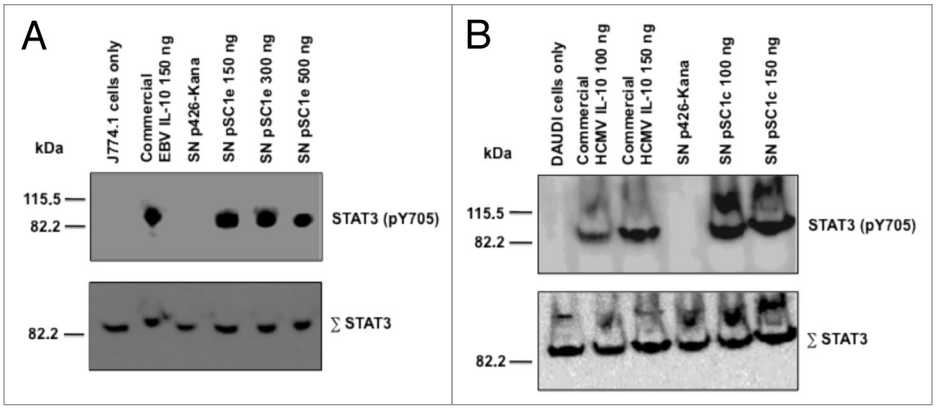

Figure 5. Activation of STAT3 phosphorylation by *S. boulardii* secreted recombinant viral IL-10. STAT3 phosphorylation (STAT3-pY705) was assessed by immunoblot analysis of protein extracts from either macrophage cells J774.1 treated with yeast-derived recombinant EBV IL-10 (**A**) or from human DAUDI's Burkitt lymphoma cells treated with yeast-derived recombinant HCMV IL-10 (**B**). Total STAT3 antibodies were used as loading control to ensure equal amounts of protein in all lanes. The supernatant of *S. boulardii* p426-Kana[37] and commercial viral IL-10 served as controls. SN, supernatant; Σ STAT3, total STAT3.

References

1. Chamekh M. Immunomodulation using genetically engineered bacteria for type III-mediated delivery of heterologous antigens and cytokines: potential application in vaccine and therapeutical developments. Immunopharmacol Immunotoxicol 2010; 32:1-4; PMID:19778176; http://dx.doi.org/10.3109/08923970902817890.
2. Medina E, Guzmán CA. Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. Vaccine 2001; 19:1573-80; PMID:11166877; http://dx.doi.org/10.1016/S0264-410X(00)00354-6.
3. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. Immunol Rev 2008; 226:205-18; PMID:19161426; http://dx.doi.org/10.1111/j.1600-065X.2008.00706.x.
4. Schotte L, Steidler L, Vandekerckhove J, Remaut E. Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. Enzyme Microb Technol 2000; 27:761-5; PMID:11118583; http://dx.doi.org/10.1016/S0141-0229(00)00297-0.
5. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, et al. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 2000; 289:1352-5; PMID:10958782; http://dx.doi.org/10.1126/science.289.5483.1352.
6. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, et al. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol 2006; 4:754-9; PMID:16716759; http://dx.doi.org/10.1016/j.cgh.2006.03.028.
7. Durand E, Verger D, Rêgo AT, Chandran V, Meng G, Fronzes R, et al. Structural biology of bacterial secretion systems in gram-negative pathogens--potential for new drug targets. Infect Disord Drug Targets 2009; 9:518-47; PMID:19594425; http://dx.doi.org/10.2174/187152609789105722.
8. Windsor WT, Syto R, Tsarbopoulos A, Zhang R, Durkin J, Baldwin S, et al. Disulfide bond assignments and secondary structure analysis of human and murine interleukin 10. Biochemistry 1993; 32:8807-15; PMID:8364028; http://dx.doi.org/10.1021/bi00085a011.
9. Syto R, Murgolo NJ, Braswell EH, Mui P, Huang E, Windsor WT. Structural and biological stability of the human interleukin 10 homodimer. Biochemistry 1998; 37:16943-51; PMID:9836587; http://dx.doi.org/10.1021/bi981555y.
10. Chamekh M, Phalipon A, Quertainmont R, Salmon I, Sansonetti P, Allaoui A. Delivery of biologically active anti-inflammatory cytokines IL-10 and IL-1ra in vivo by the *Shigella* type III secretion apparatus. J Immunol 2008; 180:4292-8; PMID:18322242.
11. Pöhlmann C, Brandt M, Mottok DS, Zschüttig A, Campbell JW, Blattner FR, et al. Periplasmic delivery of biologically active human interleukin-10 in *Escherichia coli* via a sec-dependent signal peptide. J Mol Microbiol Biotechnol 2012; 22:1-9; PMID:22353729; http://dx.doi.org/10.1159/000336043.
12. Felmlee T, Pellett S, Welch RA. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J Bacteriol 1985; 163:94-105; PMID:3891743.
13. Welch RA, Dellinger EP, Minshew B, Falkow S. Haemolysin contributes to virulence of extra-intestinal *E. coli* infections. Nature 1981; 294:665-7; PMID:7031483; http://dx.doi.org/10.1038/294665a0.
14. Holland IB, Schmitt L, Young J. Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review). Mol Membr Biol 2005; 22:29-39; PMID:16092522; http://dx.doi.org/10.1080/09687860500042013.
15. Gentschev I, Mollenkopf H, Sokolovic Z, Hess J, Kaufmann SH, Goebel W. Development of antigen-delivery systems, based on the *Escherichia coli* hemolysin secretion pathway. Gene 1996; 179:133-40; PMID:8955639; http://dx.doi.org/10.1016/S0378-1119(96)00424-6.
16. Holland IB, Kenny B, Steipe B, Plückthun A. Secretion of heterologous proteins in *Escherichia coli*. Methods Enzymol 1990; 182:132-43; PMID:2179663; http://dx.doi.org/10.1016/0076-6879(90)82013-R.
17. Hahn HP, Hess C, Gabelsberger J, Domdey H, von Specht BU. A *Salmonella typhimurium* strain genetically engineered to secrete effectively a bioactive human interleukin (hIL)-6 via the *Escherichia coli* hemolysin secretion apparatus. FEMS Immunol Med Microbiol 1998; 20:111-9; PMID:9544778; http://dx.doi.org/10.1111/j.1574-695X.1998.tb01117.x.
18. Welch RA, Hull R, Falkow S. Molecular cloning and physical characterization of a chromosomal hemolysin from *Escherichia coli*. Infect Immun 1983; 42:178-86; PMID:6311743.
19. Nissle A. Über die Grundlagen einer neuen ursächlichen Bekämpfung der pathologischen Darmflora. Dtsch Med Wochenschr 1916; 42:1181-4; http://dx.doi.org/10.1055/s-0028-1135392.
20. Zdanov A, Schalk-Hihi C, Gustchina A, Tsang M, Weatherbee J, Wlodawer A. Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. Structure 1995; 3:591-601; PMID:8590020; http://dx.doi.org/10.1016/S0969-2126(01)00193-9.
21. Sugimura K, Higashi N. A novel outer-membrane-associated protease in *Escherichia coli*. J Bacteriol 1988; 170:3650-4; PMID:3136144.
22. Grodberg J, Dunn JJ. ompT encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. J Bacteriol 1988; 170:1245-53; PMID:3277950.
23. Sedgwick B. *In vitro* proteolytic cleavage of the *Escherichia coli* Ada protein by the *ompT* gene product. J Bacteriol 1989; 171:2249-51; PMID:2649494.
24. Yam CH, Siu WY, Kaganovich D, Ruderman JV, Poon RY. Cleavage of cyclin A at R70/R71 by the bacterial protease OmpT. Proc Natl Acad Sci U S A 2001; 98:497-501; PMID:11136238; http://dx.doi.org/10.1073/pnas.98.2.497.
25. Hanke C, Hess J, Schumacher G, Goebel W. Processing by OmpT of fusion proteins carrying the HlyA transport signal during secretion by the *Escherichia coli* hemolysin transport system. Mol Gen Genet 1992; 233:42-8; PMID:1603076; http://dx.doi.org/10.1007/BF00587559.
26. Klauser T, Pohlner J, Meyer TF. Selective extracellular release of cholera toxin B subunit by *Escherichia coli*: dissection of *Neisseria* Iga beta-mediated outer membrane transport. EMBO J 1992; 11:2327-35; PMID:1600950.
27. Yabuta M, Ochi N, Ohsuye K. Hyperproduction of a recombinant fusion protein of *Staphylococcus aureus* V8 protease in *Escherichia coli* and its processing by OmpT protease to release an active V8 protease derivative. Appl Microbiol Biotechnol 1995; 44:118-25; PMID:8579825; http://dx.doi.org/10.1007/BF00164490.
28. Sugimura K, Nishihara T. Purification, characterization, and primary structure of *Escherichia coli* protease VII with specificity for paired basic residues: identity of protease VII and OmpT. J Bacteriol 1988; 170:5625-32; PMID:3056908.
29. Lassen SF, Mortensen KK, Sperling-Petersen HU. OmpT proteolysis of *E. coli* initiation factor IF2. Elimination of a cleavage site by site-directed mutagenesis. Biochem Int 1992; 27:601-11; PMID:1417895.

30. Pósfai G, Plunkett G 3rd, Fehér T, Frisch D, Keil GM, Umenhoffer K, et al. Emergent properties of reduced-genome *Escherichia coli.* Science 2006; 312:1044-6; PMID:16645050; http://dx.doi.org/10.1126/science.1126439.
31. Robbens J, Raeymaekers A, Steidler L, Fiers W, Remaut E. Production of soluble and active recombinant murine interleukin-2 in *Escherichia coli*: high level expression, Kil-induced release, and purification. Protein Expr Purif 1995; 6:481-6; PMID:8527934; http://dx.doi.org/10.1006/prep.1995.1064.
32. Zhou S, Yomano LP, Saleh AZ, Davis FC, Aldrich HC, Ingram LO. Enhancement of expression and apparent secretion of *Erwinia chrysanthemi* endoglucanase (encoded by *celZ*) in *Escherichia coli* B. Appl Environ Microbiol 1999; 65:2439-45; PMID:10347024.
33. Fu Z, Hamid SB, Razak CN, Basri M, Salleh AB, Rahman RN. Secretory expression in *Escherichia coli* and single-step purification of a heat-stable alkaline protease. Protein Expr Purif 2003; 28:63-8; PMID:12651108; http://dx.doi.org/10.1016/S1046-5928(02)00637-X.
34. Morita M, Asami K, Tanji Y, Unno H. Programmed *Escherichia coli* cell lysis by expression of cloned T4 phage lysis genes. Biotechnol Prog 2001; 17:573-6; PMID:11386882; http://dx.doi.org/10.1021/bp010018t.
35. MIT. Registry of Standard Biological Parts. http://partsregistry.org.
36. Kelesidis T, Pothoulakis C. Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. Therap Adv Gastroenterol 2012; 5:111-25; PMID:22423260; http://dx.doi.org/10.1177/1756283X11428502.
37. Michael S, Keubler LM, Smoczek A, Meier M, Gunzer F, Pöhlmann C, et al. Quantitative phenotyping of inflammatory bowel disease in the IL-10-deficient mouse by use of noninvasive magnetic resonance imaging. Inflamm Bowel Dis 2012; PMID:22570250; http://dx.doi.org/10.1002/ibd.23006.
38. Jones BC, Logsdon NJ, Josephson K, Cook J, Barry PA, Walter MR. Crystal structure of human cytomegalovirus IL-10 bound to soluble human IL-10R1. Proc Natl Acad Sci U S A 2002; 99:9404-9; PMID:12093920; http://dx.doi.org/10.1073/pnas.152147499.
39. Ding Y, Qin L, Kotenko SV, Pestka S, Bromberg JS. A single amino acid determines the immunostimulatory activity of interleukin 10. J Exp Med 2000; 191:213-24; PMID:10637267; http://dx.doi.org/10.1084/jem.191.2.213.
40. Zschüttig A, Zimmermann K, Blom J, Goesmann A, Pöhlmann C, Gunzer F. Identification and characterization of microcin S, a new antibacterial peptide produced by probiotic *Escherichia coli* G3/10. PLoS One 2012; 7:e33351; PMID:22479389; http://dx.doi.org/10.1371/journal.pone.0033351.