The Early Steps in the Development of a Novel Allergy Vaccine

Production and Purification of an Interleukin-18-Thioredoxin Fusion Protein

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Abstract

The prevalence of allergies is increasing not only in humans, but also in other animals, especially dogs. Unfortunately, the number of available treatments is still limited. The treatments that do exist are not always effective, especially not for humans and dogs with severe asthma or atopic dermatitis with very high IgE levels. We have therefore started the development of a new treatment strategy for patients with these problems by attacking the pro-inflammatory interleukin 18, an important regulator of T\(_{\text{H}1}\) and T\(_{\text{H}2}\) responses. cDNA of dog and mouse interleukin-18 were ligated into a bacterial expression vector containing an \textit{E.coli} thioredoxin (Trx) gene. The combined IL-18-Trx was transferred to the \textit{E.coli} plasmid pET21a(+), and then transferred into the \textit{E.coli} Rosetta strain. One of the goals of this project was to see how much protein we could produce. We also wanted to know how much protein could be dissolved as well as to see what the final exchange would be. The results were very satisfying, giving a high purity of the proteins in high concentrations. Following an additional purification step, the proteins should be ready to be injected as a vaccine against allergies into mice, dogs, and, hopefully, eventually humans.
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Introduction

An estimated 30% of the population in the industrialized countries of our world suffers from allergic disorders, such as asthma, conjunctivitis, rhinitis and atopic dermatitis. These disorders have already become a significant socio-economic problem, costing billions of US dollars.

The common denominator for these diseases is the production of allergen-specific IgE against often relatively harmless environmental antigens. The IgE molecules bind the FcεR1 receptors on effector cells, resulting in an immediate release of anaphylactogenic mediators, see Figure 1. The symptoms of common allergies are normally not life-threatening. Despite this, itching, broncho-constriction, and nausea negatively affect many peoples’ every day lives. In addition, the common, mild symptoms, can unfortunately develop into severe respiratory and cardiovascular symptoms.

The treatments available on the market today for allergic disorders and asthma include antihistamines, corticosteroids, and anti-IgE antibody therapy. These all require long-or even lifelong-administration. The only available “cure” is the allergen-specific immunotherapy (SIT). This method is sometimes effective for less complex allergic disorders, such as rhinitis or insect venom allergy, but has a limited efficacy in patients with more complex disorders such as atopic dermatitis. Because of this, and also because that SIT is not perfect with side-effects and limited duration, there is an urgent need for the development of new curative strategies against IgE-mediated disorders (Crameri and Rhyner, 2006). We have therefore started the development of an allergy vaccine that targets the cytokine interleukin-18 (IL-18). IL-18 is an early cytokine of the inflammatory cascade, which has a high potential to become a potential therapeutic target molecule for the treatment of allergic disorders (Sugimoto et al 2004).

IL-18 is a member of the IL-1 cytokine family (Nakanishi et al 2002). This interleukin, which was identified 10 years ago, is synthesized into a 24 kD precursor protein, which is then cleaved by caspase-1, an IL-1β converting enzyme.
IL-18 mRNA is expressed in a number of cells even under normal conditions (Mojtahedi et al. 2005), both immune, like T-cells, natural killer cells (NK), mast cells, and basophils, and non-immune, like keratinocytes, epithelial cells, and astrocytes, see figure 2 (Nakanishi et al. 2002).

After antigen stimulation via molecules on antigen presenting cells, called major histocompatibility complexes (MHC class II), naïve CD4+ Th cells differentiate into two kinds of effector helper T-cells: Th1 and Th2, having distinct cytokine production profiles and immunological effects, see figure 3 (Tsutsui et al. 2002). The two groups of T-helper cells are recognized by their cytokine production profiles (El-Mezayen and Matsumoto, 2004).

Our target, IL-18, a pro-inflammatory and multifunctional cytokine, is an important regulator of the Th1/Th2 balance by modulating the cytokine response of the helper T-cells (figure 4). When activated macrophages have produced IL-18, IL-18 synergizes with IL-2 and IL-12 to induce the synthesis of interferon-γ (IFN-γ). IFN-γ then promotes Th1 cytokine response, see figure 5 (El-Mezayen and Matsumoto, 2004).

By itself, IL-18 can induce IL-4, which favors Th2 development and IgE production that stimulate allergic inflammation, as seen in figure 6 (Nakanishi et al. 2002). This indicates a promotion of Th2 cell response by IL-18, especially the production of IL-13 (El-Mezayen and Matsumoto, 2004).

Most researchers in this field agree that the Th2 cytokines IL-4, 5, 9, and 13 together with the granulocyte macrophage colony-stimulating factor are responsible for inducing bronchial asthma (Sugimoto et al. 2004). IL-18 has an important role in host defence against infections (Dinarello and Fantuzzi 2003). For example, it serves a key role controlling infections caused by Salmonella, Candida, and Mycobacterium.
among others. High levels of IL-18 have been detected in these patients (Nakanishi et al 2002). This is partly because of its IFN-γ-inducing property and partly because of the induction of endothelial adhesion molecules, which both facilitates the clearing of invading microbes (Dinarello and Fantuzzi 2003). IFN-γ stimulates B-cells and macrophages to produce oxygen radicals and immunoglobulins to fight infectious microbes. Other positive effects of IL-18 includes viral clearance, by activation of CD8+ T cells, and stimulation of the cytotoxic activity of CD8+ T cells and NK cells (Nakanishi et al 2002).

An interesting theory for the increased prevalence of allergies (Mojtahedi et al 2005) and asthma (in which the immune response is dominated by T_{H2} cells) (Lee et al 2006) is the hygiene hypothesis. This statement is based on the observations that exposure of microbial agents in early life leads to protections against disorders such as allergies and asthma later in life, probably because it provides an important impact on the T_{H1}/T_{H2} balance. Infections during early life either modulate the immune system development or downregulate the immune response to autoantigens and allergens, or maybe both. These effects are, at least partly, exerted through modulation of IL-18. One hypothesis is that reduced exposure to microbial agents in the early years of life leads to a dysfunctional production of IL-18, affecting the host response to microbes, allergens, and even autoantigens for the rest of the host life. Microbial infections early in life have been proposed to direct the developing immune system towards a T_{H1} type. Therefore, a reduction in microbial exposure results in an excessive activity of T_{H2} cells, allowing an increase of allergic diseases.

Through evolution, it seems that microorganisms have selected IL-18 as an old friend, mediating the beneficial effects of microbes on our immune system (Mojtahedi et al 2005). However, an overproduction of interleukins 12 and 18 induces severe inflammatory disorders, leading to the conclusion hat IL-18 is a potent pro-inflammatory cytokine with pathophysiological roles in many inflammatory conditions, not only within the immune system, but also in the endocrine, nervous, and vascular systems. IL-18 may also be associated with diabetes, multiple sclerosis, rheumatoid arthritis, Chron’s disease, sepsis, and of course allergic disorders (Nakanishi et al 2002). A characteristic of the pathophysiologies of many allergies, like atopic dermatitis, is a dysfunction in the balance of T_{H1} and T_{H2} immune responses as well as in the control of these mechanisms, often because of a dysfunctional regulation of IL-18 production. This is shown through high IL-18 levels in the sera of affected patients, see figure 7 (Novak et al 2005). The levels increase with pathogenesis (Mojtahedi et al 2005), and inflammation in atopic dermatitis is even initiated by an overrelease of IL-18 (Konishi et al 2002).

Due to its potential involvement in many important human and animal diseases, IL-18 is an interesting target for the development of novel treatment strategies. We have here looked into the possibility of modulating excessive IL-18 levels by vaccination. In the development of a vaccine against a self-protein several important issues have to be addressed. One particularly difficult question is the tolerance. So to make a vaccine, a foreign protein is needed to stimulate an immune response against a self-protein by breaking the T-cell tolerance.
The making of a vaccine
The basic procedure for making a therapeutic vaccine against a self-protein is to connect a self-protein (or part of a self-protein) to a, for the body, foreign protein to recruit non-tolerized T-cell epitopes to the self-protein. T-cells that then recognize these epitopes make the otherwise unproliferative autoreactive B-cell go into a proliferative phase which results in a potent antibody reaction against the self-protein. The normal tolerance against the self-protein is then broken since the immune system starts to produce antibodies that bind the self-protein. Macrophages can then more easily eliminate the self-protein, which in our case is IL-18, from the circulation. It is important that the foreign protein and the self-protein is as close in size as possible for optimal balance between self-epitopes stimulating B-cells and non-tolerized T-cell epitopes stimulating the T-cells. For my project I used thioredoxin (Trx) from \textit{E.coli} as a foreign protein (figure 8). The thioredoxin was connected with IL-18 by ligating the coding regions to form a fusion protein construct.

Production of the IL-18-thioredoxin fusion protein in bacteria.
Many proteins produced in bacteria aggregate and form so called inclusion bodies. These inclusion bodies can however often be solubilized and refolded. Aggregation and accumulation into inclusion bodies is caused by a high level of expression of recombinant proteins (Lilie et al 1998). Unwanted interactions between hydrophobic residues in the core of proteins are believed to be the reasons. The solution to be sought after in the production of recombinant proteins from \textit{E. coli} is to convert the inactive, insoluble proteins into soluble and correctly folded products more efficiently. To recover the correctly folded proteins from these amorphous aggregates two major steps are performed. The first step, solubilizing the inclusion body material, has to be done with strong denaturants (chaotropic agents) such as 6M GdmCl (guanidinium hydrochloride) or 8M urea. When using high concentrations of chaotropic reagents in solubilizing inclusion bodies, the result will be loss of secondary structures, leading to a random coil of the proteins which exposes the hydrophobic surfaces. This loss of the secondary structures, together with the interactions between refolding proteins, are considered to be the main reasons for the poor recovery of bioactive proteins from inclusion bodies (Singh et al 2005). In other words, using a too high of a concentration of a chaotropic agent will lead to the solubilization of the inclusion bodies instead of the desired dissociation of the inclusion body associated proteins (Lilie et al 1998). Restoring, i.e. refolding the protein into its native shape from the unfolded, soluble state and removal of the denaturating agent is the second step, using dialysis for example (Vincentelli et al. 2004). Dialysis against PBS has proved efficient. The proteins have regained a bioactivity close to that of the native material (Hattori et al). The problem with membrane-based dialysis is the risk of proteins binding to the membranes. To maintain the cystein residues in a reduced state, and so prevent the formation of non-native intra-and interdisulfide bonds, a reducing agent(Clark 2001), like 10 mM β-mercaptoethanol, is added to the denaturant solution (Vincentelli et al. 2004).

Despite the troubles that need to be gone through to take care of the inclusion bodies, these aggregates are also advantageous in a number of ways. Partly because their isolation from cell homogenate is an easy and effective way of purifying the specific protein, but also because the expression of the specific protein is very high. The degradation of the proteins is low, there is a resistance in the inclusion bodies against proteolytic attack by
cellular proteases, and finally, the homogeneity of the specific proteins is high, reducing the number of purification steps. The loss in the recovery steps is compensated by a very high level of expression of the specific protein in \textit{E.coli} (Singh et al 2005).

Cells containing inclusion bodies are most commonly disrupted by high-pressure homogenization followed by low-speed centrifugation to remove the soluble proteins from the particulate containing the inclusion bodies. Centrifugation results in a higher protein purity compared to membrane filtration for example, probably because it takes advantage of the density differences between cell debris and inclusion bodies. To remove membrane proteins and other contaminants, a number of washing steps are performed (Clark 2001).

\section*{Materials and Methods}

\textit{Construction of expression vectors and transfection of these vectors into bacterial hosts for protein production}

Bacteria cultures with dog IL-18 His-TAG.Clone4/pET21a(+)Trx and mouse IL-18 His-TAG.Clone6/pET21aTrx in the \textit{Rosetta} strain of \textit{E.coli} had been prepared and stored in -70°C. The preparation of the two cultures was done by the same procedure. I will therefore describe only the preparation of the dog-IL-18-Trx culture.

Total IL-18 RNA was prepared from a dog skin sample. From this total RNA, mRNA was purified using PolyAtract mRNA Isolation System I (SDS Promega\textregistered) mixing biotin Oligos (dT) (primers) and Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) with the RNA, magnetizing and eluting the purified mRNA with nuclease free water. Following this, the purified mRNA went through Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The RT process was used to make a copyDNA (cDNA) strands from the mRNA, using Oligo (dT) as a primer. The cDNA-strands were then amplified through PCR, generating numerous doublestranded cDNAs by using dog-IL-18 primer 1064 (Invitrogen\textregistered), see figure 9. The ends of the cDNA were then cleaved by the restriction enzymes XhoI and BamHI (figure 9).

| 1064-D-IL18-5' | 5'-PRIMER BamHI- site GG*GGATCCTACTTTGGCAAGCTTGAACCTAAA** |
| 1114-D-IL18-3' | 3'-PRIMER XhoI -site GGG*CTCGAGCCTAGCTTTTGAACAGTGAA** |

*For the restrictions enzymes to be able to cleave at their sites, a few base pairs have to be added. **The sequence is ended with a polyA tail so the primers will know where to stop.

\textit{Figure 9. Primers used for the amplification of canine IL-18 (for insertion into pThioHis)}

The PCR products were run on an agarose gel whereafter the fragments 5'-BamHI-IL18-XhoI-3' were extracted by E.Z.N.A Gel Extraction Kit (200; D2500-02 Omega Bio-Tek\textregistered). By ligation with a ligase (T4 DNA Ligase) buffer, the fragments were inserted into a pThioHis vector (figure I, Appendix A), containing a modified thioredoxin gene allowing purification of fusion proteins on metal-chelating resins (Invitrogen\textregistered). Before ligation, the vector had been cleaved with the same restriction enzymes as IL-18 (figure 9).

After IL-18 had been inserted into a pThioHis vector, the plasmid was transformed with competence treatment, leading to the bacteria (\textit{E.coli}) picking up the DNA (pThioHis-IL-18). This is performed on an ampicillin plate, letting only the proper plasmids (with ampicillin resistant genes) form colonies. To test if the wanted gene fragment exists within the plasmid,
a mini-preparation kit, E.Z.N.A Plasmid Miniprep Kit 1 (200; D6943-02 Omega Bio-Tek®), is performed, using the same restriction enzymes as for the ligation step.

PCR reaction was used to excise IL-18-Thioredoxin and inserting it into a pET21a(+) vector (Novagen®) (figure II, Appendix A). The only difference between the first and second round of plasmid insertion was the restriction enzymes for the cDNA cleavage and mini-preparation kit, adjusted to the final plasmid pET21a(+) (figure 10).

| 1106-D-IL18-Trx-5' 5'-PRIMER NdeI-site | CGACATATGAGCGATAAAATTATTCACCTGATG |
| 1114-D-IL18-Trx-3' 3'-PRIMER BamHI-site | AGCGGATCCGCTACCAGAACCAGAACCACCAGCCAG |

*Figure 10. Primers used for the amplification of canine IL-18-Trx (for insertion into pET21a(+))*

Having done the second mini-preparation kit to verify the IL-18-Trx gene in the pET21a(+) plasmid, small amounts of the cultures were frozen, serving as the origin to my overnight cultures.

**Analysis of protein expression by SDS-PAGE gels**

Protein containing samples were analysed on 12.5% polyacrylamide gels. These gels were composed of 2.5 ml acrylamide, 1.5 ml lower buffer and 2 ml water for the stacking gel and 1.75 ml of water, 0.75 ml of upper buffer and 0.5 ml of acrylamide for the running gel. All samples were mixed with different fractions of blue-colored SampleBuffer to ensure that all proteins were unfolded. Before application on the gel, the samples were boiled for 5 minutes as a complement to the samplebuffer. To be able to locate our proteins on the gels, a rainbow marker (Sigma®) was used on each gel, see figure 11.
Figure 12. Estimations of protein amounts by using albumin markers of different concentrations

How to calculate the concentration of each sample:
1. Compare the intensity of the sample to the Albumin markers A x µg/5µl
2. Since the amount of samples as well as of markers always was 10 µl, the amounts are as follows:
   X=8= 8 µg/5µl = 16 µg/10µl
   X=4= 4 µg/5µl = 8 µg/10µl
   X=2= 2 µg/5µl = 4 µg/10µl
   X=1= 1 µg/5µl = 2 µg/10µl
   X=0.5= 0.5 µg/5µl = 1 µg/10µl
   X=0.25= 0.25 µg/5µl = 0.5 µg/10µl
3. Depending on the dilution of the sample, divide the amount of protein (µg) by the volume of sample applied to the gel.
   Ex: the sample contains 1:1 samplebuffer:sample. This means 5 µl sample + 5 µl samplebuffer in a 10 µl sample. Therefore, the amount of protein is divided by 5 to get the protein concentration in the particular sample.
4. Multiply the protein concentration of the sample with the total amount of sample volume (before dilution with samplebuffer) in ml, and the total protein amount is received in mg

Albumin markers (figure 12) were used to estimate protein amounts.

Fermentation
The fermentation of dog IL-18 His-TAG.Cl4/pET21a(+)Trx in the E.coli Rosetta strain was carried out in an Infors HT, 5 l Minifors fermentor. Preparations were begun by calibrating the pH electrode in buffers 7 and 4, rinsing in between. After calibration, the pH and pO$_2$ electrodes were mounted on the fermentor and reagent bottles filled with H$_2$O. When the tubes had been filled up with water, ensuring proper sterilisation of the tubing, 1 l 4 x LB (Luria Broth) and 700 ml H$_2$O (including an extra 100 ml to compensate for evaporation loss during autoclavation) was added into the fermentor vessel.

Following sterilization in an autoclave, sterile solutions of 200 ml glucose solution (200 g/mL), 200 ml MgSO$_4$ solution (5 g/l), 2 ml ampicillin stock (50 mg/ml), and 2 ml trace element solution was connected to the fermentor vessel. This gives a fermentation medium containing 2 x LB, 20 g/l glucose, 0.5 g/l MgSO$_4$, 50 mg/L ampicillin and 1 ml/l trace element solution*. The four bottles of water were replaced with the reagent bottles with antifoam, acid (1M H$_3$PO$_4$), base (25%(W/W) NH$_3$) and feeding solution**. The fermentor was then started, and the tubes filled with each of the four solutions. pH was adjusted to 7 and the water cooling system, temperature regulator, antifoam regulator, and airflow were turned on. This was left overnight for the pO$_2$ electrode to polarize. Lastly, a 200 ml overnight culture with dog IL-18 His-TAG.Cl4/pET21a(+)Trx Rosetta 91LJXVI, in LB containing 50 mg/l ampicillin and 20 g/l glucose was started.

*Trace element solution contains 2.8 g FeSO$_4$ x 7 H$_2$O, 2 g MnCl$_2$ x 4 H$_2$O, 2.37 g CoCl$_2$ x 6 H$_2$O, 1.5 g CaCl$_2$ x 2 H$_2$O, 0.2 g CuCl$_2$ x 2 H$_2$O, and 0.3 g ZnSO$_4$ x 7 H$_2$O in 111 M HCl
**Feeding solution contains 4 x LB, 40 g/l glucose, 2 g/l MgSO$_4$, and trace element solution 2 ml/l.

The following day pH was adjusted slightly. The medium was then saturated with air, and the pO$_2$ electrode calibrated. When the computer with the Iris program had been started, a new fermentation could be started. Inoculation with dog IL-18 His-TAG.Cl4/pET21a(+)TrxRosetta 91LJXVI from the overnight culture was done, and when the OD reached 4, feeding was begun at speed 10 (see figure 16).

At OD 8-10, induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) took place and the feeder speed was changed to 4. IPTG (figure 13) mimics alloctase, a lactose
metabolite, and induces the activity of an enzyme called β-galactosidase. This enzyme usually promotes lactose utilization by inhibiting the lac repressor in a vector. The lac operon has in our case been replaced by our recombinant protein, so when β-galactosidase is activated, our protein will be expressed. The sulphur prevents degradation of IPTG because of the chemical bonds it creates. These bonds are non-hydrolyzable by the cell.

The final vessel concentration of IPTG was 3 mM, and the induction took place for 3 hours. After 3 hours, the bacteria were harvested.

Preparation of inclusion bodies
The dog IL-18 His-TAG.Clone4/pET21a(+)Trx and mouse IL-18 His-TAG.Clone6/pET21aTrx were cultured over night on a shaker in 37°C in LB, 0.1% ampicillin, and 1% glucose. The next day the cultures where added to 9/10 of its volume of LB, 0.1% ampicillin, and 1% glucose and allowed to grow on a 37°C shaker until an OD₆₀₀ of 0.5 was reached.

At this point, one mouse and one dog IL-18-Trx culture was cooled on ice for a few minutes, the volume of 1:100 100 mM IPTG was added, and then put on a room temperature shaker (200 rpm). At the same time, one mouse and one dog IL-18-Trx culture was put on the 37°C shaker for three hours after the adding of 1:100 volume of 100 mM IPTG.

After 3 h and 20 h respectively, the four different cultures were centrifuged at 6000 rpm for 10 min at 4°C. Following centrifugation, the pellet was washed with as much PBS + 1% triton as possible, and subsequently centrifuged at 6000 rpm for 10 minutes at 4°C. This washing step was repeated three times. The pellet was then resuspended in a volume of 1:50 PBS (phosphate buffer saline) + 0.1% Tween to the original culture volume. This PBS-solution was sonicated (Soniprep 150®) 5 times for 1 minute s at an amplitude of 14-20 microns and then centrifuged for 10 min at 6000 rpm and 4°C (RC 3B Sorvall®). This sonication procedure was repeated 5 times.

Solubilizing the inclusion bodies
The cell pellets were dissolved in PBS, centrifuged 10 min at 14000 rpm (EBA 12R Hettich zentrifugen), and then dissolved in different concentrations of urea. The urea solutions were heated at 37°C and vortexed twice before the insoluble particles were spun down at 14000 rpm for 10 min (EBA 12R Hettich zentrifugen).

Refolding of IL-18-Thioredoxin
To the supernatants from the urea solutions 10 mM β-mercaptoethanol (Sigma®) was added. These solutions were then dialyzed against PBS for 2 hrs and then overnight. The day after the solution was spun down at 14000 rpm for 10 min at 4°C (RC 3C Plus Sorvall®). For smaller samples (5-10ml), Spectra/Por® 0.32 ml/cm was used for dialysis, and for larger samples (50 ml and above) Sigma® 150 ml/ft was used.

Purification of our proteins
To the dialysis supernatants Ni-NTA agarose (400 µl for smaller samples, 20ml for larger samples) was added. After rotaring for 1 h, all components were added on a syringe with a Sartonius glass filter serving as a column. The column with the pearls in it was washed 4 times with as much PBS + Tween 0.1% + 20 mM Imidazol as the syringe could hold.

Subsequently, 500 mM Imidazol were used to elute the proteins from the column. Since not all dog or mouse proteins would elute from the pearls, we added another elution step with an
acetate buffer. This buffer consisted of 100 mM pH 5.5 (consisting of 90 µl acetic acid and 1.5 g natriumacetate according to www.egr.msu.edu/sch-group/tools/acetate/acetate.html).

Results

Cloning of IL-18-thioredoxin

Before I began the production and purification steps of IL-18-thioredoxin, bacteria cultures with dog IL-18 and mouse IL-18 had been prepared. The results can be seen in figure 14.

![Dog IL-18, mouse IL-18 and thioredoxin DNA and protein sequences](image)

**Figure 14.** Dog IL-18, mouse IL-18 and thioredoxin DNA and protein sequences
Preparation of inclusion bodies

We tested if it was more effective to, after the addition of IPTG, to put the cultures on a shaker for 3h in 37°C or over night in room temperature (RT). After 3 h and 20 h respectively, the four different cultures were centrifuged, washed and sonicated. After running all the final pellet samples on a 12.5% protein gel, it was obvious that most of our protein was in the pellet of the RT cultures (figure 15).

Fermentation

In order to be able to produce larger amounts of the fusion protein and to standardize the production for future GMP productions, we decided to test and optimize production in a fermentor. Despite the fact that RT pellets contatined more protein than pellets from 37°C pellet we decided to do the fermentation at 37°C. The amount of bacteria obtained from a fermentor can be very high. We could easily culture up to OD:s around 30, see figure 16. Production at 37°C involves shorter production times compared to RT fermentations which normally takes several days, which is why this was more effective and beneficial than to use RT for fermentation.

Solubilizing the inclusion bodies

Dog IL-18 needed 8 M urea to dissolve, while mouse IL-18 needed only 4 M urea (figure 17). These conditions were used for all further experiments. In figure 18 it is obvious that the majority of the inclusion bodies were dissolved.
Figure 17. Solubilizing IL-18-Trx. To dissolve as many proteins as possible in the lowest concentration possible, 8 M urea was needed for dog IL-18-Trx (D8) while only 4 M urea was needed to dissolve mouse IL-18-Trx (M4). In the other lanes the protein amounts are very small, which means that most proteins are still in the inclusion bodies. Numbers 8, 6, 4, and 2 represent the urea concentrations (M), D = dog IL-18-Trx, M = mouse IL-18-Trx, Ma = rainbow marker

Refolding of IL-18-Thioredoxin
Dialysis was used to refold our proteins and to remove the urea. As seen in figure 18, not much of the proteins aggregated during dialysis.

Figure 18. Inclusion bodies of dog-IL-18-Trx dissolved in 8M urea (Us = urea supernatant and Up = urea pellet after centrifugation), and then dialysed (urea supernatant dialysed, centrifuged and resulting in Ds = dialysis supernatant and Dp = dialysis pellet). As seen, there are almost no proteins undissolved (Up). Ma = rainbow marker, A8 – A0.25 = albumin markers (see figure 12)

Purification of our proteins
The fusion proteins, which all contained histidine tags, were purified on Ni chelating columns. After application and washings of Ni-NTA agarose with bound proteins on the syringe, 500 mM imidazol were used to elute the proteins from the column. Since not all dog or mouse proteins would elute from the pearls (see figure 19), we added another elution step with an acetate buffer. The fractions were eluted into tubes with TRIS pH 7.9 so that the proteins would not be denatured due to low pH (figure 20). The reason for low yield was apparently not that the protein stayed at the column but instead that a large portion (70-80%) of the proteins did not bind to the Ni-pearls at all, as can be seen in figure 21. Therefore we skipped the natriumacetate step in the succeeding purifications.
Figure 19. A sample from the Ni-NTA in the column (c) showed that much of the protein did not elute. Numbers 5, 4, 3, 2, and 1 represent the eluted fractions of dog (D) and mouse (M) IL-18-Trx. Nisup = unbound proteins in solution, Ma = rainbow marker.

Figure 20. Trying to elute dog-IL-18 from the Ni pearls using acetate buffer 100 mM pH 5.5. Numbers 1-6 represent the eluted fractions, Ma = rainbow marker, $A_8 - A_{0.5} = $ albumin markers (see figure 12).

Figure 21. Unbound proteins in solution (Ni\textsubscript{sup}). Ni\textsubscript{sup} contains an estimated amount of 2000 mg of unbound proteins, which is about 70-80% of the starting amount. Ma = rainbow marker, $A_8 - A_{0.5} = $ albumin markers (see figure 12).

We therefore added the Ni-NTA agarose to the solution that had passed through the solution the first time, and let it bind for another hour, whereafter the washing and elution procedure was performed a second time. By this procedure, we extracted close to as much protein as in the first round. Unfortunately, the unbound protein percentage was still 70-80%. At the same time as trying the “two-round-elution,” we also tried to use lower concentrations of Imidazol. As seen in figure 22, the amount eluted was as much with 100 mM imidazol as with imidazol 500 mM. The first 6 fractions are with 100 mM, the succeeding 6 are with 200 mM and the
final 6 are with 500 mM. The amounts in the fractions follow the same patterns as when using 500 mM imidazol the whole time.

Figure 22. First (a) and second (b) rounds of elution steps with imidazol for dog-IL-18-Trx using 0.1 M, 0.2 M, and 0.5 M fractions of imidazol. (c) shows first round of elution with only 0.5 M imidazol, and (d) the second round with only 0.5 M of imidazol. If one compares figure (a) and (c), it can be seen that the pattern of eluated amounts looks the same. The same is true with figure (b) and (d). The conclusion to be drawn is that the eluation that we used in (a) and (b) is as effective as using only 0.5 M imidazol but with lower imidazol concentration. We strived towards the lowest imidazol concentration possible. Numbers 1-18 (a) and 1-11 (c) = fractions eluated in the first round. Numbers 1-18 (b) and 1-11 (d) = fractions eluated in the second round. Ma = rainbow marker, A\(_8\) - A\(_{0.25}\) = albumin markers (see figure 12) All samples contained 1:4 samplebuffer:sample

The fractions with high protein concentration were pooled, and then dialysed. The result was highly satisfying, showing very clean samples, leaving no aggregated proteins, and yielding protein concentrations of approximately 3 mg/ml in a volume of 170 ml (figure 23).

Figure 23. Dialysed fractions of dog-IL-18-Thioredoxin. Samples diluted 1:1 and 1:49 sample:samplebuffer to ensure proper estimations. Ma = rainbow marker, A\(_8\) - A\(_{0.25}\) = albumin markers (see figure 12)
**Calculations of protein yield from the final fermentation**

In the last fermentation of dog IL-18-Trx, we started with the original pellet P (after harvesting), which contained 2600 mg, according to our estimation. Only about 50 mg of the inclusion bodies did not dissolve in the 8M urea, which is only 2% (50/2600). During dialysis of the urea solution, only 5 mg aggregated, 0.2% (5/2550).

Before starting the Ni-NTA agarose purification step, we therefore had 2545 mg (2545/2600), being 98% of the total protein amount in the pellet P. Large amounts of these solubilized proteins did not bind to the Ni-pearls. However, the proteins not bound to the Ni can not be seen as a loss. About 15% of the total amount of proteins was eluted with washes, or still bound to the pearls. The biggest loss, 30-50%, however happened in the last dialysis step. The reason for this may be that the proteins bound to the dialysis tube membrane. If we had diluted the samples before dialysis, the concentrations in the tubes would not have been so high, leading to fewer proteins bound to the membrane.

Compared to our original pellet of 2600 mg, our final yield of 475 mg was just below 20%. However, since we only put a certain amount into dialysis, and since a lot of proteins are still in solution, able to be further purified, this result is not fully correct. Taking into account the total amount we were able to elute in additional purification of unbound material the total yield is instead closer to 50%.

Calculations show that our estimations of protein amounts were quite accurate through the whole process, which makes these numbers reliable.

**Discussion**

The goal of this project was to evaluate the capacity of the process as well as to see how clean the final proteins would be (optimization of the process). Looking at the final gels, our samples are very clean; therefore the purification can be seen as successful. The 98% recovery in the solubilization step was very good, and to be able to produce 2-3 g of non-purified proteins every time is more than one should expect. However, the proteins will need one additional purification step to be able to use in animal studies. The purity that we achieved in our process is, despite the need for another round, as clean as it will get. The final concentration of the protein was also were significantly higher than is really needed for a vaccine, so these results look very promising for a future vaccine production.

Concerning targeting Interleukin-18 with a vaccine, my personal opinion is that it has the potency to be very successful. However, to avoid side-effects I think it will only be safe to give it to individuals with very severe allergic and inflammatory disorders. After all, IL-18 is very important for our immune system and the elimination of microbial invaders, which is why it is dangerous to lower the levels too much. In individuals with very high levels of IL-18 however, I do not think that this function of IL-18 will not be significantly affected, as long as therapeutic windows have been well studied before administrations to patients.
References


Dinarello, A.C., Fantuzzi, G. Interleukin-18 and host defence against infection. Journal of Infectious Diseases 187 (2003) 370-384

El-Mezayen, R.E.H., Matsumoto, T. In vitro responsiveness to IL-18 in combination with IL-12 or IL-2 by PBMC from patients with bronchial asthma and atopic dermatitis. Clinical Immunology 111 (2004) 1: 61-68


Nakanishi, K., Yoshimoto, T., Tsutsui, H., Okamura, H. Interleukin-18 regulates both Th1 and Th2 responses. Annual Reviews Immunology 19 (2001) 423-474


Sugimoto, T., Ishikawa, Y., Yoshimoto, T., Hayashi, N., Fujimoto, J., Nakanishi, K. Interleukin 18 acts on memory T helper cells type I to induce airway inflammation and


Appendix A

Figure I. pThioHis vector from Invitrogen®

Figure II. pET-21a(+) vector from Novagen