VI Summary and Conclusions
TNF-α is an important cytokine implicated in a variety of cellular processes. Its multiple role in normal immune responses and other stimulatory/inhibitory functions can be underlined by the fact that TNFα receptors have been found to be expressed in almost all the cell types examined. A variety of extracellular signals induce the expression of TNF-α and its expression is regulated at the levels of transcription, pre-mRNA splicing, sequestering of the mRNA in cytoplasm, translation and post-translational control. Extracellular regulation of the soluble form of TNF-α, is supposed to be achieved by shedding of TNF-α receptors, which bind to the cytokine and either make it unavailable or increase its half life. The level of regulation involved in the expression and maintenance of this cytokine in the serum indicate that excess of TNF-α might be deleterious and that biological system have learnt to exploit the benificial aspects of this molecule by putting it under very tight regulation. Indeed, overproduction of TNFα has been a major cause in leading to several human pathological states with both acute and chronic effects. TNF-α is known to be the major cytokine involved in progression and maintenance of rheumatoid arthritis, myesthenia gravis, Crohn's disease and is the cause of septic shock leading to death, upon bacterial infections, in hospital wards treating post-surgery patients.

It is necessary to find a safe means to control the expression of TNF-α to be able to provide relief to patients afflicted with diseases involving excess TNF-α production. Various means of control of expression of this cytokine have been tried and tested. Unfortunately, none of these methods could provide the expected level of control and a few have their inherent problems of safety. We have embarked on an ambitious goal of achieving complete control of TNF-α by using ribozymes designed to specifically cleave and inactivate TNF-α mRNA. The cleaved fragments are unsuitable for translation and thus it is expected that ribozymes would abolish gene expression.

**Aim of the study:**

- Cloning of cDNA for human TNF-α.
- Design and cloning of multitarget ribozymes against TNF-α mRNA.
• Evaluation of the cleavage efficiency of designed ribozymes, *in vitro*.
• Evaluation of various factors affecting ribozyme-mediated catalysis *in vitro*.
• *In vitro* evaluation of ribozyme-protein interactions using purified cytosolic proteins.
• Detection of RNA-protein interactions using human cytoplasmic extracts.
• Design and construction of vectors for expression of ribozymes in human cell lines.
• Detection of ribozyme cleavage activity *in vivo*.
• Evaluation of various strategies for *in vivo* ribozyme expression.
• Ribozyme-mediated control of biological function of TNF-α.
• To explore strategies for over expression of human TNF-α in *E.coli*.

**Summary of results:**
In the present study, two ribozymes (Rz₃₁₆ and Rz₇₆₂) were designed against human TNF-α RNA to achieve greater control of the transcript *in vivo*. We have cloned cDNA for human TNF-α, which allowed us to test the designed ribozymes *in vitro*, along various parameters, prior to the delivery of ribozymes *in vivo*. *In vitro* studies evaluated the efficacy of the designed ribozymes to cleave longer transcripts under varied conditions. Ribozyme-substrate interaction at varied temperatures, substrate length and pH conditions were studied. Also, the specificity of ribozyme cleavage reaction was probed using a combination of primer extension and sequencing. This was done to ensure that the designed ribozyme cleaved exactly at the expected nucleotide on longer substrates. This evaluation is important to demonstrate that the ribozyme has the ability to recognize and correctly cleave at the designated target site in presence of excess of unrelated polynucleotide sequences and possibly prohibitive substrate conformations, thus estimating its biosafety. *In vitro* evaluation of the ribozymes were carried out to study its interactions with some common cytosolic proteins. These proteins were selected based on their ability to interact with the chemical components of the ribonucleotides. The ribozyme Rz₇₆₂, exhibited protein-mediated dimerization while Rz₃₁₆ did not form any novel conformations under similar conditions. The
interaction of \( R_{762} \) with four different cytosolic proteins was studied by employing techniques like, UV cross linking, RNase mapping, gel retardation assays. Also, binding of the ribozymes to cytosolic proteins was detected, employing gel retardation assays, using purified cytoplasmic extracts of human THPI cells (monocyte-macrophage).

Three different strategies were employed to evaluate the *in vivo* efficacy of the designed ribozymes to control TNF-\( \alpha \) gene expression. The ribozymes were expressed as polyadenylated messenger RNA (for directing the ribozymes for cytoplasmic localization), as intron-embedded (for achieving nuclear localization of the ribozymes) and \( T_7 \) based expression (for studying the effect of addition of exogenous ribozyme-stabilizing sequences). The first two modes of expression were achieved by transfecting the respective ribozyme constructs into human TNF\( \alpha \) producing cell line, THPI. The synthesis of TNF-\( \alpha \), in THP1 cells, was induced by using bacterial lipopolysaccharide, a highly potent inducer of TNF-\( \alpha \). The third mode of expression was done in a subclone of human embryonic kidney cell line (U293), which contained a stably integrated T7 RNA polymerase gene under the control of human cytomegalovirus promoter (CMV). This allows *in vivo* expression of plasmids containing T7 promoter in a mammalian cell line. Since U293 cells do not express TNF-\( \alpha \), these cell line was co-transfected with the full-length TNF-\( \alpha \) cDNA along with the ribozyme constructs.

Ribozyme-mediated cleavage of TNF-\( \alpha \) was detected, *in vivo*, using a combination of highly sensitive techniques like northern blotting, RNase protection assay, cell viability assay and flow cytometry.

Although excess TNF-\( \alpha \) production is deleterious, we experimented with prokaryotic systems to explore the possibility of conveniently expressing human TNF-\( \alpha \) cDNA in bacteria. Our choice of expressing human TNF-\( \alpha \) with a N-terminal 6x Histidine tag proved suitable for large scale isolation of the expressed protein using Ni-NTA matrix. The Histidine tag binds with very high affinity to the column and purification of TNF-\( \alpha \) can be easily achieved.

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Conclusions

- A 1.44kb cDNA for human tumor necrosis factor alpha (TNF-α) was amplified by RT-PCR and cloned in suitable in vitro and in vivo expression vectors (a modified pBluescript vector KS(−), pStuI, pQE30 and; pCI-neo).

- Two separate ribozymes, directed at two different sites on TNF-α mRNA, were designed based on secondary structure considerations (predicted by FOLD RNA, GCG, Wisconsin).

- The ribozyme coding sequences were obtained as partial oligonucleotides with complementarity at their 3’ ends. The oligonucleotides were annealed and extended to full-length by end filling and cloned in pStuI.

- Ribozyme-mediated cleavage of TNF-α substrate, in vitro, indicates that the both the designed ribozymes efficiently cleaved a 951nt in vitro synthesized TNFα substrate, at 32°C and 37°C.

- Increasing the temperature of incubation conditions led to reduction of cleavage efficiency on a 951nt substrate, but showed improved cleavage when a 308 substrate was used. This indicated that temperature related changes in the substrate structure could influence cleavage efficiency of the ribozyme.

- The two ribozymes when used independently, exhibited high level of activity, but when used in combination, Rz316 inhibited the cleavage of substrate by Rz762. This indicates that ribozyme binding at the target site induces structural changes in the substrate which could be prohibitive for binding or formation of an optimal hammerhead structure of the other ribozyme.

- Evaluation of the specificity of ribozyme cleavage reaction by using a combination of primer extension and sequencing indicated that the ribozyme cleaves exactly at the predicted nucleotide even in presence of unrelated substrate sequences.

- In vivo expression of the ribozymes were done in mammalian system. Ribozymes were cloned in suitable expression vectors (pCI-neo;
pVR1012-neo) allowing them to be expressed as polyadenylated RNA, intron-embedded RNA and T7 terminator-containing RNA.

- Expression of ribozymes, in vivo, in mammalian cells lead to decreased levels of TNF-α, as assessed by northern blotting.
- RNase protection assay was able to detect in vivo ribozyme cleavage product.
- A combination of cell viability assay and flow cytometry indicated that all the three strategies of expression of ribozymes were competent in protecting the TNF-α sensitive cell line (WEHI) from TNF-induced apoptosis.
- TNF-α was expressed in E.coli as a N-terminal histidine tagged protein. Small-scale purification (from 200ml cultures) of TNF-α showed that the expression system could be used for convenient large scale isolation of this important cytokine.