

# Repression of allo-cell transplant rejection through CIITA ribonuclease P<sup>+</sup> hepatocyte

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## Abstract

**AIM:** Allo-cell transplant rejection and autoimmune responses were associated with the presence of class II major histocompatibility complex (MHC II) molecules on cells. This paper studied the effect of Ribonuclease P (RNase P) against CIITA, which was a major regulator of MHCII molecules, on repressing the expression of MHCII molecules on hepatocyte.

**METHODS:** M1-RNA is the catalytic RNA subunit of RNase P from *Escherichia coli*. It were constructed that M1-RNA with guide sequences (GS) recognizing the 452, 3408 site of CIITA by PCR from *pTK117* plasmid, then were cloned into the *EcoRI/BglII* or *EcoRI/SalI* site of vector *psNAV* (*psNAV*-M1-452-GS, *psNAV*-M1-3408-GS) respectively. The target mould plate (3176-3560) of CIITA was obtained from Raji cell by RT-PCR, and then inserted into the *XhoI/EcoRI* of *pGEM-7zf(+)* plasmid (*pGEM*-3176). These recombinant plasmids were screened out by sequence analysis. *psNAV*-M1-452-GS, *psNAV*-M1-3408-GS and its target RNA *pGEM*-3176 were transcribed and then mixed up and incubated *in vitro*. It showed that M1-3408-GS could exclusively cleave target RNA that formed a base pair with the GS. Stable transfectants of hepatocyte cell line with *psNAV*-M1-3408-GS were tested for expression of class II MHC through FCM, for mRNA abundance of MHCII, II and CIITA by RT-PCR, for the level of IL-2 mRNA on T cell by mixed lymphocyte reaction.

**RESULTS:** When induced with recombinant human interferon-gamma (*IFN-γ*), the expression of HLA-DR, -DP, -DQ on *psNAV*-M1-3408-GS<sup>+</sup> hepatocyte was reduced 83.27 %, 88.93 %, 58.82 % respectively, the mRNA contents of CIITA, HLA-DR, -DP, -DQ and II decreased significantly. While T cell expressed less IL-2 mRNA in the case of *psNAV*-M1-3408-GS<sup>+</sup> hepatocyte.

**CONCLUSION:** The Ribonuclease P against CIITA-M1-

3408-GS could effectively induce antigen-specific tolerance through cleaving CIITA. These results provided insight into the future application of M1-3408-GS as a new nucleic acid drug against allo-transplantation rejection and autoimmune diseases.

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## INTRODUCTION

The donor shortage has become the major restriction on liver transplantation with the increasing demands for it<sup>[1]</sup>. Now people are trying to produce artificial liver with hepatocytes and various biological materials<sup>[2,3]</sup>, but are faced with the challenge of rejection in allo-hepatocyte transplantation<sup>[4]</sup>. Allo-transplant rejection, was associated with the presence of class II major histocompatibility complex (MHCII) on the tissues and organs<sup>[5,6]</sup>. MHCII played a critical role in the induction of immune responses by presenting fragments of alloantigenic peptides to CD4<sup>+</sup> T lymphocytes, then resulting in the activation of CD8<sup>+</sup> T lymphocytes. So it was more important for compatibility of MHCII in allo-transplantation. Moreover, the abnormal expression of MHCII molecules was associated with autoimmune disease too<sup>[7-9]</sup>. There are codominance and multiple allele for MHCII molecules which lead to their complicated polymorphism, so it is difficult to repress every MHCII molecules expression directly. MHC class II transactivator (CIITA) was the major rate-limiting factor for both constitutive and inducible MHCII expression., and with rare exceptions, its expression parallels to that of MHCII transcripts<sup>[10-12]</sup>. There was no rejection in allo-skin graft<sup>[5]</sup> or prolonged survival time in allo-cardiac graft<sup>[13]</sup> of CIITA(-/-) according to the latest investigation.

Ribozymes included hammerhead-, hairpin-, Ribonuclease P (RNase P), *et al.* M1-RNA was the catalytic RNA subunit of RNase P from *Escherichia coli*. RNase P was a ribonucleoprotein complex that catalyzed the hydrolysis reaction by removing a 5' leader sequence from tRNA precursors<sup>[14]</sup>. Hammerhead ribozymes required presence of specific nucleotide sequences in the target RNA to be cut<sup>[15,16]</sup>, and these requirements could not always be fulfilled. M1-RNA could be used as a tool to cleave any specific mRNA sequence simply by the 3' terminal addition to the ribozyme sequence of a so-called guide sequence (GS) complementary to the target mRNA, that formed a base-pair with it and left a 5' -ACCAC-3' unpaired stretch needed for the M1-GS RNA to recognize and cleave this artificially created substrate (Figure 1B). Thus, M1-GS RNA, apart from some requirements to improve its cleavage efficiency, could be specifically directed to cut any mRNA sequences. This study represents, to our knowledge, the first gene therapy approach that makes use of the catalytic activity of M1-RNA for allo-cell transplant rejection in hepatic tissue engineering and the autoimmune diseases.

Hepatocytes were transfected with 0.4  $\mu\text{g}$  psNAV-M1-3408-GS by nanometer vector. According to Effectene (QIAGEN) kit's instructions, seeded  $2.5 \times 10^5$  cells/well the day before transfection. The cell number seeded should produce 40-80 % confluence on the day of transfection. psNAV-M1-3408-GS were diluted in 100  $\mu\text{l}$  EC buffer, mixed with Enhancer 3.2  $\mu\text{l}$ , incubating 2-4 min at RT, then adding Effectene 10  $\mu\text{l}$ , at RT 7-8 min, mixed with 600  $\mu\text{l}$  medium containing serum and antibiotics, and immediately transferred the total volume to the above cells in the 6-well plate.

### The expression of MHCII antigens on hepatocyte by FCM

Hepatocytes were collected and washed with 1.5 g/L MPBS buffer (10 g/L BSA and 1 g/L NaN<sub>3</sub>) once at the density of  $1 \times 10^6$ /ml, adding IgG2a, HLA-DR, DP, DQ 10  $\mu$ l respectively, incubating at 4 °C for 30 min, detecting the expression of MHCII molecules by Flow cytometry (COULTER, EPICSXL).

### RNA analysis

RT-PCR was done according to the instructions of TRIZOL<sup>R</sup> and TITANIUM<sup>R</sup> one-step RT-PCR kit. In a total 50  $\mu$ l volume, 50 °C 1 h, 94 °C 5 min, 94 °C 30 s, 65 °C 30 s, 68 °C 1 min, 30 cycles, 72 °C extending 7 min. Primers sequences (synthesized by Shanghai Bioengineering Company) referred to Table 1.

**Table 1** The primers of CIITA, MHCII and RNaseP

Primer	Sequence	Length
CIITA mould	L 5'-CCGCTCGAGAGCTGAAGTCTTGGA-3'	384bp
	R 5'-GCGGAATTCGAACATGCCTGTCCAGAGC-3'	
CIITA	L 5'-CCG CTC GAG GCT GCC TGG CTG GGA TT -3'	410bp
	R 5'-GCG GAA TTC CGA TCA CTT CAT CTG GTC CTAT-3'	
M1-452-GS	L 5'-GCGGAATTCTAATACGACTCACTATAG-3'	446bp
	R 5'-GAAGATCTGTGTTCTTCCAGGACTGCCAAGCTTGC-3'	
M1-3408-GS	L 5'-GCGGAATTCTAATACGACTCACTATAG-3'	454bp
	R 5'-ACGCGTCGACGTGGTGCAGCTCGCTGATTACGCCAAGC-3'	
HLA-DR	5'-AAT GGC CAT AAG TGG AGT CC-3'	335bp
	5'-GGA GGT ACA TTG GTG ATC GG-3'	
HLA-DP	5'-CAG AGC TGT GAT CTT GAG AG-3'	197bp
	5'-AGA TGC CAG ACG GTC TCC TT-3'	
HLA-DQ	5'-CTC TGA CCA CCG TGA TGA GC-3'	153bp
	5'-CTC TCC AGG TCC ACG TAG AA-3'	
Ii	5'-CCA GAT GCA CAG GAG GAG AA-3'	714bp
	5'-CCT CTG CTG CTC TCA CAT GG-3'	
Neo gene	L 5'-ACA ATC GGC TGC TCT GAT -3'	349bp
	R 5'-CTC GCT CGA TGC GAT GTT -3'	
$\beta$ -actin	L 5'-ATC ATG TTT GAG ACC TTC AA -3'	310bp
	R 5'-CAT CTC TTG CTC GAA GTC CA -3'	

### Mixed lymphocyte reaction (MLR)

It were incubated at 37 °C, 5 % CO<sub>2</sub> (keeping away light) after adding mitocin-C (25  $\mu$ g/ml, Sigma) into IFN- $\gamma$  induced psNAV-M1-3408-GS<sup>+</sup> hepatocyte ( $1 \times 10^7$ /ml), and washed with RPMI1640 twice, plated at a density of  $1 \times 10^6$ /well as stimulating cells. Then added peripheral blood mono-nucleated cells (PBMNC,  $1 \times 10^6$ ) from healthy donors into above stimulating cells. The level of IL-2 mRNA from PBMNC after 48 h incubation was Detected through RT-PCR<sup>[17]</sup>.

## RESULTS

### The expression of MHCII molecules on hepatocyte

**Hepatocyte without IFN- $\gamma$  induction** The expression of HLA-DR, DP, DQ on hepatocyte was low, (0.14 $\pm$ 0.04) %, (26.76 $\pm$ 5.26) %, (2.12 $\pm$ 0.56) % respectively.

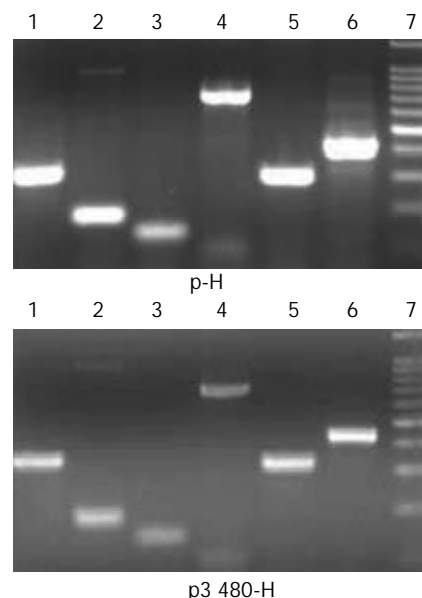
**Hepatocyte after IFN- $\gamma$  induction** The expression of HLA-DR, DP, DQ on hepatocyte with IFN- $\gamma$  (40 ng/ml) induction for 3 d increased significantly, (18.68 $\pm$ 2.94) %, (41.78 $\pm$ 4.90) %, (4.78 $\pm$ 1.26) % respectively.

### Rnase P down-modulating MHCII expression on hepatocyte

The expression of MHCII on psNAV-M1-3408-GS<sup>+</sup> hepatocyte after the induction of IFN- $\gamma$  (40 ng/ml) for 3 d was repressed. Compared with void-vector<sup>+</sup> hepatocyte, the expression of HLA-DR, DP, and DQ on psNAV-M1-3408-GS<sup>+</sup> hepatocyte was inhibited 83.27 %, 88.93 %, and 58.82 % respectively.

### The expression of MHCII, Ii and CIITA mRNA through RT-PCR detection

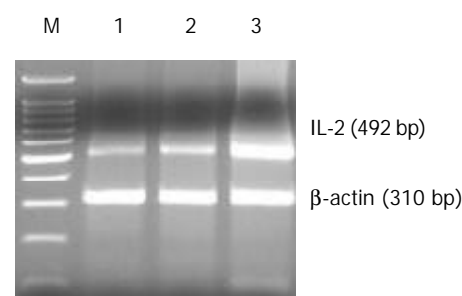
The induction of MHCII, Ii and CIITA mRNA on psNAV-M1-3408-GS<sup>+</sup> hepatocyte was detected through RT-PCR (Figure 2). Compared with void vector, the amount of MHCII and Ii mRNA of psNAV-M1-3408-GS<sup>+</sup> hepatocyte was significantly down-modulated, the CIITA mRNA was down-modulated 71 %.



**Figure 2** Comparison of CIITA, MHCII mRNA abundance of psNAV-M1-3408-GS<sup>+</sup> hepatocyte with void vector<sup>+</sup> following IFN- $\gamma$  induction. The total RNA of psNAV-M1-3408-GS<sup>+</sup> (p3408-H) or void vector<sup>+</sup> (p-H) hepatocyte after IFN- $\gamma$  (30 ng/ml, 3 d) induction was both extracted, then RT-PCR. 1: HLA-DR (335bp); 2: HLA-DP (197bp); 3: HLA-DQ (153bp); 4: Ii (714bp); 5:  $\beta$ -actin (310bp); 6: CIITA (410bp); 7: 100bp DNA Ladder.

### Hepatocyte inducing mixed lymphocyte reaction

The excretion of IL-2 from PBMNC stimulated by psNAV-M1-3408-GS<sup>+</sup> hepatocyte after the induction of IFN- $\gamma$  for 3 d referred to Figure 3. Void-vector<sup>+</sup> hepatocyte could induce PBMNC high amount of IL-2 mRNA, but negative control and psNAV-M1-3408-GS<sup>+</sup> hepatocyte could hardly induce the excretion of IL-2 mRNA from PBMNC.



**Figure 3** The secretion of IL-2 mRNA from PBMNC through RT-PCR. M: 100bp DNA ladder; 1: Negative control; 2: The IL-2 from PBMNC stimulated by psNAV-M1-3408-GS<sup>+</sup> hepatocyte after IFN- $\gamma$  induction; 3: The IL-2 from PBMNC stimulated by void vector control after IFN- $\gamma$  induction.

## DISCUSSION

In allo-cell transplantation or autoimmune diseases, some cytokines such as IFN- $\gamma$ , induced some low/non MHCII antigen expressing cells to express these molecules highly<sup>[18,19]</sup>. We selected IFN- $\gamma$  to induce hepatocyte for 3 days, the expression

of HLA-DR, DP, DQ antigens increased exactly, while the expression of HLA-DP was most apparent.

CIITA regulated the transcription of MHCII gene by interacting with the trans-acting factors such as RFX, X2BP and NFY. The expression of CIITA paralleled to that of MHCII molecules and appeared only in the MHCII-positive cells<sup>[10,11]</sup>. In the hepatocyte detected by us, the expression of CIITA was consistent with that of MHCII molecules: without IFN- $\gamma$  induction, all hepatocytes didn't express MHCII molecules and CIITA gene; following IFN- $\gamma$  induction, these cells expressed MHCII molecules and CIITA gene simultaneously; in the case of anti-CIITA psNAV-M1-3408-GS positive hepatocytes, the induced MHCII expression on their surface was nearly completely lost, and their CIITA mRNA detected by RT-PCR was also defect, perhaps the latter was the direct reason that MHCII expression didn't react to IFN- $\gamma$  induction. This view was coincidence with that of Luder *et al*<sup>[18,20]</sup>: TOXO plasma gondi parasite lowered the MHCII expression by inhibiting its induced CIITA expression. Moreover, HMG-CoA reductase inhibitors, cyclosporine and phosphatidylethanolamine-linked hyaluronic acid (HYPE) could completely repress MHCII expression of human microvascular endothelial cells by reducing its induced CIITA mRNA contents *ex vitro*<sup>[19,21-23]</sup>.

Gene blocking techniques were mainly made up of anti-sense oligonucleotide, anti-sense RNA, ribozyme and RNA interference (RNAi), and so on. Anti-sense oligonucleotide referred to a small fragment of single-strand deoxyribonucleic acid (14-23 bases) synthesized artificially, which could hybridize with target DNA or mRNA. However, there were still some problems of stability and efficiency of entering cell *in vivo* about it. Anti-sense RNA, ribozyme and RNAi all took action on target mRNA, namely anti-sense complementation, cutting and interference respectively. The novel RNAi technique was the double-strand RNA connected by anti-sense RNA and sense RNA in essence, and was more efficient than single anti-sense RNA<sup>[24,25]</sup>. The mechanism of which was still not clear and might be related to activating ribonuclease to degrade target mRNA. But when it is larger than 30bp, the action of it was not specific<sup>[24]</sup>. Compared with above-mentioned gene blocking techniques, ribozyme not only sealed mRNA, but also cut mRNA with specificity. Moreover, ribozyme could be used repeatedly, so it had higher efficiency. Both hammerhead ribozyme and hairpin ribozyme require GUC sequence to identify in target sequence. However, RnaseP was not limited to this and could aim at any site in the target sequence, so it had wider selective range<sup>[14]</sup>. There was no report on RnaseP yet at home. According to human RNA sequences published in the NCBI Gene Bank, our experiment selected 452, 3408 site in the CIITA gene as target sites of M1-RNA after eliminating the possibility of their homology. PST and LRR regions initiated by 452, 3408 site were very essential to the transcription activation of CIITA. Moreover, the secondary structure around them was relatively simple and accessible. The GS of M1-3408-GS and M1-452-GS were programmed as 11 and 12 nucleotides respectively, to fit for the combination with their own substrate, the disconnection of cutting products with the ribozyme, and the specificity of the ribozyme. The 5'-terminal of M1-RNA had a TAATA box (T7 promoter), and M1-RNA was cloned into the psNAV vector (without T7 promoter), then the transcription was gone on owing to T7 promoter in the ribozyme itself. This could avoid supplementary sequence of the psNAV vector and objectively reflect the cutting activity of the Rnase P. The result of our experiment revealed the expected cutting stripes in the electrophoresis of cutting products of M1-3408-GS and CIITA mould plate *ex vitro*.

The reason why our experiment used nanometer-vector to mediate the transfection of M1-RNA into hepatocyte was that

nanometer had the advantages of both virus vector and non-virus vector<sup>[26,27]</sup>. For instance, adenovirus vector<sup>[28,29]</sup> or retroviral vectors<sup>[30]</sup> could cause too strong immunological reaction of body to fit for the study of inhibiting the immunological rejection in our experiment; Especially, nanometer-vector could mediate exogenous gene to integrate into the chromosome DNA of host cell so that the long-term and stable expression of transgene could be obtained. In our experiment using the novel nanometer vector Effectene to transfect human hepatocyte, the rate was about 11 %, and it could rise to 60-80 % after the screening with G418 for 1 wk. Nanometer vector, however, has just begun to be used in the field of gene therapy. So far, internationally, there has no report on it used in the gene therapy of clinical or pre-clinical study.

Moreover, hepatocytes induced by IFN- $\gamma$  could stimulate the secretion of IL-2 mRNA from exogenous T cell, while psNAV-M1-3408-GS<sup>+</sup> hepatocyte after IFN- $\gamma$  induction lost this ability. Therefore, M1-3408-GS inhibited CIITA mRNA and thus the family of MHCII molecules regulated by CIITA, then down-regulated the ability of stimulating mixed lymphocyte reaction. In conclusion, our research will have important theoretical and practical meaning on the study of transplantation immune in the whole hepatocyte tissue engineering and the therapy of autoimmune diseases.

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