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# Genome-wide analysis of TIAR RNA ligands in mouse macrophages before and after LPS stimulation

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ABSTRACT

TIA-1 related protein (TIAR) is a RNA-binding protein involved in several steps of gene expression such as RNA splicing Aznarez et al. (2008) [1] and translation Piecyk et al. (2000) [2]. TIAR contains three RNA recognition motifs (RRMs) allowing its interaction with specific sequences localized in the untranslated regions (UTRs) of several mRNAs. In myeloid cells, TIAR has been shown to bind and regulate the translation and stability of various mRNA-encoding proteins important for the inflammatory response, such as TNFα Piecyk et al. (2000), Gueydan et al. (1999) [2,3], Cox-2 Cok et al. (2003) [4] or IL-8 Suswam et al. (2005) [5]. Here, we generated two macrophage-like RAW 264.7 cell lines expressing either a tagged full-length TIAR protein or a RRM2-truncated mutant unable to bind RNA with high affinity Dember et al. (1996), Kim et al. (2013) . By a combination of RNA-IP and microarray analysis (RIP-chip), we identified mRNAs specifically bound by the full-length protein both in basal conditions and in response to LPS (GSE77577).



| Specifications [standardized info for the reader] | |
|---|---|
| Organism/cell line/tissue | Murine RAW 264.7 cells |
| Sex | None |
| Sequencer or array type | MEEBO Mouse array |
| Data format | Raw and analyzed |
| Experimental factors | Microarray hybridization was performed on TIAR-FLAG-associated mRNAs or TIARΔRRM2-FLAG-associated mRNAs in unstimulated and LPS-stimulated RAW264.7 cells. |
| Experimental features | Enrichment of TIAR-FLAG-associated mRNAs and TIARΔRRM2-FLAG-associated mRNAS were normalized to total transcriptome of TIAR-FLAG and TIARΔRRM2-FLAG expressing-RAW264.7 cells respectively. After amplification and labelling, sample pairs were hybridized onto Mouse Exonic Evidence Based Oligonucleotide (MEEBO) arrays containing on average 38,784 mouse 70mer oligonucleotide probes (Stanford University, US). Hybridizations were replicated with dye swap. |
| Consent | Level of consent allowed for reuse if applicable |
| Sample source location | [1]Laboratoire de Biologie Moléculaire du Gène, Faculté des Sciences, Université Libre de Bruxelles (ULB) |

## 1. Direct link to deposited data [provide URL below]

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77577

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## 2. Experimental design, materials and methods

### 2.1. Generation of RAW264.7 cells expressing tagged full-length or RRM2-truncated TIAR proteins

Full-length murine TIARb [8] (Genbank accession number U55861) or a truncated version lacking RRM2 were FLAG-tagged at the 3′ end and cloned in a modified version of the lentiviral plasmid *pHR'trip CMV-eGFP* where the CMV promoter was replaced by the chicken β-actin promoter (kind gift of Fabrice Bureau, Université de Liège). Lentiviral particles were produced as previously described [9]. Subsequently, $4 \times 10^6$ RAW cells were seeded in 96-well plates and infected with the lentiviral particles for 48 h. Cells were washed with PBS and the colony expanded. Subsequently, single cell clones were isolated by limit dilution and amplified. Expression of TIAR-FLAG and TIARΔRRM2-FLAG was verified by Western blot using FLAG M2 mouse monoclonal antibody (Sigma) as described elsewhere [10]. To avoid binding artifacts due to overexpression of TIAR-FLAG proteins, we selected clones expressing TIAR-FLAG or TIARΔRRM2-FLAG at levels similar to the endogenous TIAR protein (Fig. 1).

The expression of TIAR-FLAG and TIARΔRRM2-FLAG was measured by Western blot as described in materials and methods.

### 2.2. Cell culture and LPS treatment

RAW 264.7 cell clones were cultured in Glutamax Dubelcco Modified Eagle Medium (Gibco-BRL®) complemented with 5% fetal bovine

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serum, 1 mM Na pyruvate and 1% penicillin–streptomycin. $2 \times 10^6$ cells were seeded and were either left untreated or treated for 2 h with 100 ng/ml LPS from *Escherichia coli* (0127:B8; Sigma®).

### 2.3. Immunoprecipitation of RNA-protein complexes

We used the RIP-chip method [11] to identify mRNAs bound by TIAR. Briefly, RAW 264.7 cells expressing TIAR-FLAG or TIARΔRRM2-FLAG were lysed in polysome lysis buffer containing 10 mM HEPES pH 7.0, 100 mM KCl, 25 mM EDTA, 2 mM DTT, 5 mM $MgCl_2$, 0.5% NP40 and 2 μl RNAse OUT™ (Invitrogen®). TIAR-FLAG or TIARΔRRM2-FLAG was then immunoprecipitated overnight with anti-FLAG M2 agarose beads (Sigma®) according to the manufacturer's instructions and then eluted in a buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 2 μl de RNase OUT™ (Invitrogen®) and the FLAG peptide (Sigma®) at a final concentration of 250 μg/ml. mRNAs were then isolated by Trizol (Invitrogen®) extraction according to the manufacturer's protocol.

### 2.4. Microarray, quality control and data processing

Total or immunoprecipitated RNA was purified on RNeasy kit columns (Qiagen). Double-stranded cDNA was synthesized from 1 μg of total RNA, followed by production of antisense RNA containing the modified nucleotide 5-(3-aminoallyl)-uracil triphosphateP using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, Texas). After labelling with Cy3 or Cy5 (GE Healthcare Bio-Sciences, NJ), samples were hybridized on the Mouse Exonic Evidence Based Oligonucleotide array (Stanford Functional Genomics Facility, CA). The oligonucleotide set consists of 38,784 70-mer probes that were designed using a transcriptome-based annotation of exonic structure for genomic loci. Hybridizations were replicated with dye swap.

Slides were scanned using a Molecular Devices 4000B laser scanner and expression levels were quantified using GenePix Pro 6.1 image analysis software (Axon Instruments, CA). Image acquisitions were performed with automatic photomultiplier gain adjustment. Artefact-associated spots were eliminated by both visual and software-guided flags, as well as spots with a signal/background fluorescence ratio less than 2. The fluorescence values were imported into Acuity 4.0 software package (Molecular Devices, Union City, CA). A non-linear locally weighted scatter plot normalization method applied to each individual block (print-tip option) was carried out. The resulting data files were used for further data analysis. In order to identify differentially expressed genes, normalized log2 ratio obtained from the individual hybridization experiments was selected by a threshold of absolute log2 value > 1.

Enrichment of TIAR-FLAG or TIARΔRRM2-FLAG-associated mRNAs was evaluated by comparison to the total mRNA input used for immunoprecipitation.

The importance of RRM2 for high-affinity binding of TIAR to RNA ligands has been documented in two independent studies [6,7]. Therefore, to identify high-affinity RNA ligands of TIAR, we discarded from the analysis all the mRNAs that co-precipitated both with TIAR-FLAG and the TIARΔRRM2-FLAG mutants.

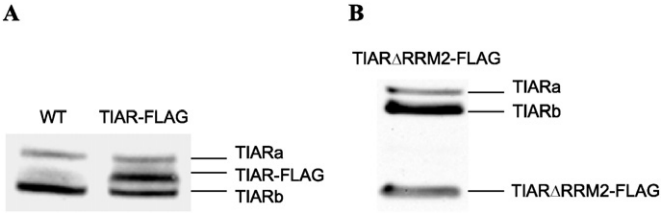


**Fig. 1.** Stable expression of TIAR-FLAG and TIARΔRRM2-FFLAG in RAW 264.7 macrophages.

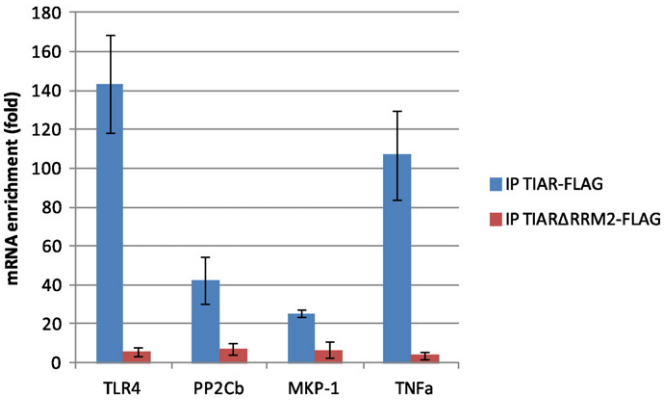


**Fig. 2.** TIAR binds to mRNAs encoding TLR4, PP2Cb, MKP-1 and TNFα in response to LPS.

### 2.5. Consolidation of microarray data by qPCR

To validate the microarray data, we analyzed by RT-qPCR on independent RIP samples prepared from LPS-stimulated cells the binding of two already known targets of TIAR, TNFα [3] and MKP-1 [12] and two new targets, TLR4 and PP2Cb. As shown in Fig. 2, this assay confirmed that all four tested mRNAs co-precipitate with TIAR-FLAG with high efficiency as compared to TIARΔRRM2-FLAG.

## 3. Results

### 3.1. Comparative analysis of TIAR RNA ligands in unstimulated and LPS-stimulated RAW 264.7 macrophages

Our data revealed that 351 transcripts co-precipitated with TIAR in untreated cells and 779 transcripts were found in the RIP-chip performed with LPS-stimulated cells. Of these, 343 transcripts were precipitated in both conditions, 436 exclusively in LPS-treated cells and only 8 exclusively in untreated cells (Fig. 3), showing that the repertoire of mRNAs bound by TIAR increases importantly in response to LPS.

Analysis of gene ontology terms or KEGG pathway annotations using DAVID bioinformatics resources [13] shows that in both conditions, TIAR binds to mRNAs important for the inflammatory response, cell proliferation, cell death or metabolism. Terms such as “protein catabolic process”, “cell cycle”, “regulation of apoptosis” or “chemokine signaling pathway” are significantly enriched both in unstimulated and LPS-stimulated samples. Strikingly, the term “positive regulation of I-kappaB kinase/NF-kappaB cascade” was exclusively enriched among the mRNAs bound in response to LPS (Table 1).

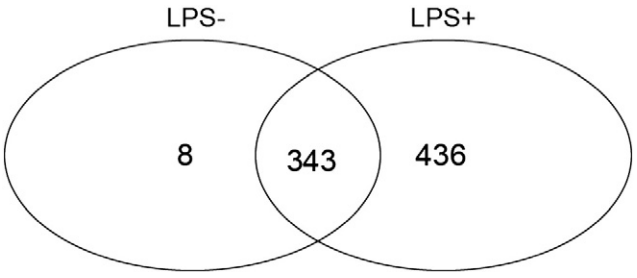


**Fig. 3.** Number of transcripts bound by TIAR in untreated cells (LPS−), LPS-stimulated cells (LPS+) or in both conditions.