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**Truncation of c-Mip (Tc-mip) , a new proximal  
signaling protein, induces c-maf-dependent Th2  
pathway and cytoskeleton reorganization**

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## Summary

Several arguments suggest that Minimal Change Nephrotic Syndrome (MCNS) results from yet unknown systemic disorder of T cell function. By screening a cDNA library from T cell relapse, we identified a new Pleckstrin homology (PH) domain-containing protein encoded by a gene located on chromosome 16q24. Its expression was restricted to fetal liver, kidney and PBMC in which two transcripts were identified. The first species (c-mip) was expressed in normal PBMC but weakly detected in PBMC from MCNS patients. The second form, lacking a part of the N-terminal PH domain (Tc-mip, standing for Truncated c-maf inducing protein), was specifically recruited in CD4<sup>+</sup> T cells of patients with MCNS. Overexpression of Tc-mip in T cells induced c-maf, transactivated the IL-4 gene and downregulated IFN expression, characteristic of a Th2 commitment. Moreover, the overexpression of Tc-mip induced T cell clustering and a cellular redistribution of the cytoskeleton-associated L-plastin, by a PI3 kinase independent pathway. Tc-mip represents therefore the first identified protein, which links proximal signaling to c-maf induction.

## Introduction

Minimal Change Nephrotic Syndrome (MCNS) is the most frequent glomerular disease in children, characterized by heavy proteinuria with relapse and remission courses (1). Although no immune cell infiltration, or immune complex deposit could be identified in the kidney, many arguments suggest that MCNS results from a systemic disorder of T cell function (2). Indeed, relapses often occur in the context of immune challenge initiated by infectious, allergic stimuli or vaccine and they are usually sensitive to drugs known to inhibit the immune system (i.e./glucocorticoids, ciclosporine and ciclophosphamide). Nonetheless, the link between immune disorders and this glomerular disease remains unresolved.

Recently, we have shown that T lymphocytes from MCNS were rather driven toward a Th2 phenotype. Supporting this view, we found that T cells display a downregulation of the IL-12 receptor  $\beta$ 2 subunit (IL-12R  $\beta$ 2), whereas the second component of the IL-12R, the  $\beta$ 1 chain, was normally expressed (3). The IL-12R  $\beta$ 2 is selectively expressed by Th1 cells and plays a key role in the transduction of IL-12 signaling through the Jak/Stat pathway. The downregulation of the IL-12R  $\beta$ 2 is compatible with a lack of IL-12 production during relapse (4). The commitment of MCNS T cells along the Th2 pathway involves the

recruitment of c-maf of which the functional expression appears closely related to MCNS activity (Valanciute A., submitted). It has been shown that c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation (5), (6). As a matter of fact, patients with MCNS are often unable to mount an effective Th1 response that might account for observed defects in delayed-type hypersensitivity response and recall response to antigens (2, 7).

In order to understand in depth the molecular mechanisms involved in this T cell dysfunction, we recently identified by subtractive cloning and differential screening, transcripts upregulated during the active phase of the disease (3). Among them, we isolated a truncated form of a new protein, involved in c-maf signaling pathway, that we named Tc-mip (for truncated c-maf inducing protein). The natural transcript, c-mip, corresponds to the product of the Kiaa1694 gene, previously identified in the human brain (8). Tc-mip exhibited a deletion within the N-terminal pleckstrin homology domain (PH) of c-mip and its transcript is selectively induced in active phase of the disease whereas it is hardly detected in T cells of normal subjects. We show here that overexpression of Tc-mip in Jurkat T cells, strongly induces the c-maf protein and increases the IL-4 promoter-mediated transcription, whereas it concomitantly represses the INF expression. Moreover, overexpression of Tc-mip in Jurkat T cells, induces T cell clustering and

cellular redistribution of L-plastin, a cytoskeleton-associated protein, by a PI3 kinase independent pathway.

These results suggest that Tc-mip plays a critical role in Th2 signaling pathway and represents the first proximal signaling protein which links TCR-mediated signal to the activation of c-maf Th2 specific factor.

## Material and Methods

**Patients.** The cohort of patients analyzed in this study has been described previously (9). In children, the criteria of the International Study of Kidney Diseases were used for diagnosis and management of MCNS (10). In adults, the diagnosis of MCNS or Membranous Nephropathy (MN) was confirmed by renal biopsy before inclusion. All patients with relapse (children and adults) had proteinuria over 3g/24h, and low serum albumin levels (below 3 g/dl), at the time of blood sampling, which was performed before the beginning of steroid treatment.

Remission samples were collected during periods of inactive disease, defined by a proteinuria below 0.2g/24h. Controls consisted of normal children studied while undergoing routine analysis, normal adult volunteers, as well as patients with MN.

Informed consent was obtained from the parents and whenever possible from the pediatric patients, as well as from adult patients and normal volunteers.

**Antibodies and reagents.** Immunoselection of T cell subsets and monocytes was performed using a cocktail of hapten-conjugated antibodies (Miltenyi Biotech. GmbH, Auburn, CA, USA).

A polyclonal antibody common to c-mip and Tc-mip was produced against a peptide of 15 aa, corresponding to amino acids 194-209 of the Tc-mip (Eurogentec, Belgium). Antibodies against c-maf (sc-7866) and phospho Akt (ser-473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and New England Biolabs (Beverly, Ma, USA), respectively. Monoclonal antibody of L-Plastin (Anti-LPLA4.1) was a gift of Drs Eric J. Brown and Hua Shen (University of California, San Francisco, CA) and was used at 1µg/ml. Wortmanin was purchased from Sigma (Paris, France).

**Purification of PBMC and T cell subsets.** Peripheral blood mononuclear cells (PBMC) and T cell subsets were purified as previously described (9). The purity of the preparations was 88-96%, as assessed by flow cytometric analysis, using FITC-conjugated CD2, CD4<sup>+</sup>, CD19, and CD8 antibodies. Activation was performed with 5µg/ml PMA (phorbol ester myristate) and 5µg/ml calcium ionophore (ionomycin), for four hours. Th1 and Th2 polarized T cells were a gift of Dr Pellegrini (Institut Pasteur, Paris). They were isolated from blood umbilical cord and cultured under Th1 (in the presence of IL-12 and anti-IL-4 antibody) or Th2 (in the presence of IL-4 and anti-IL-12 antibody) skewing conditions.

**Immunocytochemistry.** Cells were cytopun at 10<sup>5</sup> cells/slide, fixed and permeabilized by using methanol at -20°C, then processed for



immunoreactivity. Cells were incubated in the blocking solution (10% normal sheep serum (NSS), 1% Bovine Serum Albumin (BSA) for 40 min, washed twice with phosphate-buffered saline (PBS), then incubated with Tc-mip (1/200) or L-plastin (1  $\mu$ g/ml) antibody (in 5% NSS, 1% BSA, 0.1% Tween 20) for 2 hours at room temperature. Slides were washed three times with PBS, then incubated with anti-rabbit Cy3-labelled antibody (1/1000 in blocking solution) for 30 min. Slides were mounted in a Vectashield DAPI (Vector laboratories, Burlingame, California), and analyzed on an Axioplan Zeiss microscope equipped for epifluorescence. The percentage of positive cells was determined on an average of 200 cells.

**Reverse transcription-PCR (RT-PCR).** Total RNAs were treated by DNase I and purified using Rneasy kit (Qiagen SA), following the supplier's protocol. The 5' and 3' primers of Tc-mip and c-mip were selected in the first respective exon. The sequence of the primers, and PCR parameters are indicated in Table 1.

Semiquantitative RT-PCR was performed as previously reported (9). Each cycle consists of denaturation at 94°C for 30 sec, annealing at the indicated temperature for 30 sec and extension at 68°C for 2 min. Amplified products were detected on Southern blots with [<sup>32</sup>P] labeled specific internal oligonucleotide probes and quantified by using a phosphoimager (Storm 840, Molecular dynamics SA), coupled to the

ImageQuant v1.11 analysis software. PCR reactions were normalized for GAPDH expression.

**Northern blot.** Human multiple-tissue Northern blots (Clontech, CA) were hybridized with a 560 bp cDNA probe common to c-mip and Tc-mip. Hybridization and blot processing were performed as previously described (3).

**DNA sequencing.** Preparation and sequencing of double stranded plasmid DNA template and sequencing were performed as previously reported (3). Nucleic acid and protein database searches were done using resources of the National Center Biotechnology Information.

**Western blots and electromobility shift assays (EMSA)**

Preparation and quantification of protein extracts, as well as SDS-PAGE immunoblotting, and EMSA experiments, were performed as previously described (9). The double-stranded oligonucleotide probes (100 ng), (Genset, France) consisting of the wild (5'-GGAATTGCTGACTCAGCATTACT-3') and the mutant (5'-GGAATTGCTGACTCATTGTTACT-3') MAREs containing the c-Maf recognition sequence (underlined).

**Cloning of the 5'end of c-mip by rapid amplification of cDNA ends (RACE).** Additional sequence upstream of the 5'end of the c-mip mRNA was explored by using the 5'/3'RACE kit (Clontech) and an universal antisense primer included in the kit and a 28 mer antisense

oligonucleotide located at the position 145-172 of the c-mip-exon 1 (5'-GCTGAGAAAGGTCCGCGGGTGCCGGATG-3'). The PCR product was blunt ended and cloned into Topo Zero blunt end vector (Invitrogen), according to manufacturer's protocol. Preparation and sequencing of double stranded plasmid DNA template and sequencing were performed as previously described (3).

**Plasmids construction.** The cDNA corresponding to the coding sequences of Tc-mip and c-mip were obtained by RT-PCR using PBMC RNA from a patient with MCNS relapse and a normal subject, respectively. The specific primers are reported in table 1. The amplified products were subcloned into pcDNA3.1/V5-His TOPO cloning vector (Invitrogen, CA). The IL-4 promoter chloramphenicol acetyltransferase (IL4-CAT) plasmid (fragment corresponding to -65 base pairs upstream of the IL-4 transcription initiation start site) was a gift of Dr V Casolaro (Johns Hopkins University School of Medicine).

**Transient transfections.** Jurkat T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI 1640 supplemented with 10% FCS. Transient transfections were performed by electroporation of  $30 \times 10^6$  cells with 30  $\mu$ g of each plasmid (Tc-mip, c-mip or empty vector), or in combination with 10  $\mu$ g of CAT-IL4-reporter construct, using a Bio Rad Gene Pulser set at 250V and 960  $\mu$ F. The transfected cells were allowed to recover

overnight, then were divided into identical aliquots in a 24 well plates. One aliquot was left unstimulated whereas the other was stimulated with OKT3 ascite fluid (1:5000) for 6 hours. In some experiments, wortmanin (100 nM) was added to the culture medium 6 hours following transfection and left overnight. CAT assays were performed according to the manufacturer's protocol (Promega, Madison, USA). CAT activities, normalized by protein content, were determined using a liquid scintillation counting assay (Packard, Meriden CT), and were shown as fold induction relative to control, between stimulated and unstimulated conditions.

## Results

### 1-Identification and characterization of Tc-mip

A Tc-mip cDNA clone (4.1kb, GenBank accession number: AY172689) was isolated by differential screening of a full length cDNA library, generated from T cell-enriched PBMC of MCNS relapse, using subtracted probes from relapse versus remission (unpublished data). The Tc-mip mRNA corresponds to an alternative species of c-mip (4.2 kb), isolated from human brain<sup>8</sup>. Both Tc-mip and c-mip are transcribed from a single gene comprising 21 exons spanning 268 Kb in length and located on chromosome 16q24 (Fig 1). A Tc-mip 5' RACE did not reveal upstream 5' sequence in c-mip and Tc-mip (data not shown), suggesting that both mRNA are transcribed from distinct promoter regions.

The Tc-mip and c-mip cDNAs contain an open reading frame of 2040 and 2220 nucleotides, encoding 679 (75kDa) and 739 (85kDa) amino acid proteins respectively. Both initiation codons (ATG) are flanked by the Kozak consensus sequence. The c-mip protein contains a pleckstrin homology (PH) domain encoded by exons 1, 2, 3 and 4. The PH domain starts at position 38 of exon 1 that encodes 66 amino acids. The Tc-mip transcript does not contain the first exon of c-mip but starts with a new

145 nucleotide-exon, encoding 6 amino acids and which is located 50 Kb downstream of c-mip-exon 1 on the genome (Fig 1A). Thus, Tc-mip exhibit a N-terminal truncation of 29 aa of the PH domain. This truncation delete the sheet 1, the sheet 2 as well as the loop 1 2 of the PH domain including but the rest of the protein remains unchanged (Fig 1B). Beside the PH domain, Tc-mip and c-mip proteins contain a leucine-rich repeat (LRR) domain encoded by the exon 19. The remainder of the protein does not exhibit significant homology with others known domains but contains several patterns, including protein kinase C (PKC), casein kinase II and tyrosine, phosphorylation sites. The truncation of Tc-mip removes the putative Thr 61, Ser 62 and Lys 63 PKC phosphorylation sites as compared to c-mip.

We hybridized multiple-tissue Northern blots with a cDNA probe common to Tc-mip and c-mip (Fig 2A). We only detected a signal at 4.25 kb corresponding to c-mip mRNA. The transcript was highly expressed in PBMC, kidney and fetal liver and to a lesser extent in adult brain and liver. We were not able to make a northern blot of Tc-mip transcript in PBMC of MCNS patients was not done because of the scarcity of the biological material.

## **2-Expression of Tc-mip in resting and activated T cells**

Since Northern-blot analyses revealed a basal expression of c-mip in PBMC, we analyzed by semi-quantitative RT-PCR, the relative expression

of Tc-mip and c-mip mRNA in PBMC subsets purified by immunomagnetic selection. In resting cells, we found that the c-mip transcript was expressed in monocytes, CD8<sup>+</sup>, CD4<sup>+</sup> T cells and B lymphocytes, whereas the Tc-mip transcript was only detected in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig 2B). In order to determine whether these transcripts are induced in response to activating conditions, cells were exposed to phorbol ester myristate (PMA) and calcium ionophore (ionomycin), for four hours. The expression of c-mip and Tc-mip mRNAs was markedly downregulated in activated cells (Fig 2B). Then we analysed the expression their respective proteins by immunoblotting protein extracts with a polyclonal antibody recognizing the amino acid sequence 194-209 common to Tc-mip and c-mip proteins. In contrast with the basal mRNA expression, we were unable to detect Tc-mip by immunoblotting, whereas c-mip was very faintly or not detected, either in resting or in activated cells (data not shown), suggesting post transcriptional regulation mechanisms.

Our recent investigations argue for an early commitment of MCNS T cells along a Th2 phenotype(3) . The identification of a new gene upregulated in this disease, markedly expressed in CD<sup>+</sup>T cells led us to investigate its expression in polarized CD4<sup>+</sup> T cells along Th1 or Th2 pathway. We found that Tc-mip transcript is highly expressed in Th2

relative to Th1 cells (Fig 2C). This result constitutes an additional element strengthening the Th2 bias in MCNS.

### **3-Specific induction of Tc-mip in MCNS.**

We analyzed by semi-quantitative RT-PCR the expression level of c-mip and Tc-mip transcripts during the relapse and the remission phases in nine patients who experienced a first episode of MCNS. The expression of Tc-mip mRNA was increased during the relapse in the nine patients tested, as compared to remission, whereas the eight normal subjects exhibited very low levels of this transcript (Fig 3A). In contrast, the c-mip mRNA was expressed to the same low extent in relapse and in remission phase, and highly expressed in normal subjects in accordance with the previous experiments (Fig 2B). The upregulation of Tc-mip mRNA appeared specific to MCNS since four patients with membranous nephropathy, and a similar range of proteinuria, exhibited a Tc-mip and c-mip mRNA levels comparable to normal subjects.

We further studied whether the induction of Tc-mip was restricted to a cell subset during the active phase of the disease. We purified by immunomagnetic selection, the CD4<sup>+</sup> and non-CD4<sup>+</sup> T cells species from PBMC of patients with relapse. The highest level of Tc-mip mRNA was observed in the CD4<sup>+</sup> T cell subset (Fig 3B).

Since we could not detect Tc-mip protein in normal PBMC cells, we determined whether it was induced during MCNS disease. We



immunoblotted total protein extracts from PBMC of six patients in relapse. We detected a major band of 75kD specific of Tc-mip in patients with MCNS relapse whereas the signal was undetectable in normal subjects (fig 3C). In contrast, the expected 85kD band related to c-mip was weakly detectable in MCNS patients as well as in normal subjects.

Cellular distribution of Tc-mip was analyzed in PBMC from patients with MCNS. In relapse, we detected Tc-mip in nuclear and cytoplasmic compartments, whereas its expression was restricted to the cytoplasm in remission (Fig 3D). In those samples, 20% of the PBMCs were Tc-mip positive whereas no cellular staining was visualized in PBMC from normal controls in accordance with the western blotting results (data not shown).

#### **4-Tc-mip induces c-maf nuclear expression in Jurkat cells**

Recently, we have shown that c-maf was strongly induced in MCNS and shuttled between nuclear and cytoplasmic compartments during the relapse and the remission phases, respectively (Valanciute A, submitted). Since Tc-mip and c-maf are mainly expressed in CD4<sup>+</sup>T cells, we examined whether Tc-mip could influence c-maf expression. We transfected Jurkat cells, which do not express either endogenous c-mip or Tc-mip, with c-mip or Tc-mip expression vectors. Transfection with Tc-mip induced c-maf expression at the mRNA and protein level

(Fig 4A), whereas c-mip promoted a lower but significant induction of c-maf relative to controls. Importantly, the expression pattern of the c-maf protein was radically different: in cells overexpressing Tc-mip, c-maf was primarily detected in nuclei (Fig 4B, upper panel), whereas its expression was much lower and mostly restricted to periphery of the cell in c-mip transfected cells (Fig 4B, middle panel). The induction of c-maf and its nuclear localization in transfected cells did not indicate that c-maf is transcriptionally active. To address this question, we analyzed by EMSA experiments, c-maf-dependent DNA binding activity in nuclear extracts of Tc-mip-transfected Jurkat cells. We only detected a slow migrating complex binding to MARE probe in Tc-mip transfected Jurkat cells whereas no complex was visible in c-mip transfected cells. This complex was specific since it was not formed in the presence of mutant MARE oligonucleotide (Fig 4C). These results suggest that Tc-mip induced a functional c-maf in Jurkat T cells.

Confocal fluorescence microscope analysis revealed that the c-mip protein was restricted to peripheral area of transfected cells, whereas Tc-mip was diffusely expressed, particularly in nuclear compartment (Fig 4D). Thus, Tc-mip and c-maf display a similar distribution in nuclei and cytoplasmic compartments but we were not able to co-immunoprecipitate both proteins. Altogether, these results suggest that truncation of the PH domain affects the cellular distribution of the Tc-

mip protein, which might regulate the induction and the cellular compartmentalization of c-maf.

### **5-Overexpression of Tc-mip results in differential effects on IL-4 and INF $\gamma$ production**

Since c-maf is a transactivator of the IL-4 gene (5,11), the identification of Tc-mip as an upstream inducer of c-maf lead us to determine whether Tc-mip could be able to activate the IL4 gene. To that end, we analyzed the effects of a transient transfection of Tc-mip on IL-4 promoter activity in Jurkat cells. In unstimulated-transfected Jurkat cells, no significant variation of the IL-4-dependent CAT activity was detected after transfection with Tc-mip (Fig 5A). When transfected Jurkat T cells were stimulated with anti-CD3 antibody, Tc-mip and c-mip induced a four fold and two fold increase of Il-4 promoter activity relative to control, respectively (Fig 5B).

Since Tc-mip induces the expression of c-maf, we supposed that these cells acquired a Th2 phenotype. In order to test this hypothesis we analyzed in parallel IFN and IL4 mRNA expression, two reliable markers of Th1 and Th2 profile respectively. In unstimulated transfected cells, Tc-mip, and c-mip did not altered significantly the the IL-4 and IFN mRNA expressions, relative to controls (Fig 5B). Upon stimulation with anti-CD3 antibody, IL-4 mRNA expression was considerably increased, whereas IFN mRNA expression was sharply

reduced in Tc-mip-overexpressing cells (Fig 5B). By contrast, c-mip did not reduced IFN mRNA, whereas the IL-4 mRNA was slightly increased relative to Tc-mip-transfected cells. This result suggests that signals emanating from TCR engagement provided additional requirement for c-maf-mediated Th2 pathway in which Tc-mip but not c-mip might play a potent role.

#### **6-Tc-mip-overexpressed T cells display striking changes in cellular redistribution of cytoskeleton-associated L-plastin.**

Our precedent results support the role of TCR activation in c-maf signaling pathway, as it has been previously reported (12), but upstream proteins involved in c-maf induction remain unidentified. Since Tc-mip behaves as a proximal inducer of c-maf expression, we wished to determine whether Tc-mip could mimic TCR signaling. To test this hypothesis, we analysed the distribution of L-plastin, a cytoskeleton protein that is upregulated in active MCNS disease (3). We transfected Jurkat T cells with Tc-mip and c-mip expression vectors and 16 hours later, cells were recovered from medium alone, fixed and incubated with L-plastin monoclonal antibody. T cells transfected with c-mip or empty vector exhibited an homogeneously distribution of L-Plastin in the cytoplasm area, whereas Tc-mip induced a cell clustering with distribution changes of L-plastin that was relocalized in cytoplasmic zones facing the cell contact (Fig 6A). We conclude that overexpression

of Tc-mip promotes the cytoskeleton rearrangement partially mimicking TCR stimulation. To assess whether this effect involved the PI3-kinase pathway, we treated Tc-mip-transfected cells with wortmanin, a PI3-k inhibitor. We found that cell clustering as well as the relocalization of L-plastin were not affected by wortmanin treatment (Fig 6B), suggesting that Tc-mip-induced cytoskeleton reorganization was independent of PI3 kinase enzymatic activity.

## Discussion

We isolated from T lymphocyte of patients with MCNS a new protein, which induce c-maf transcription factor. This protein, Tc-mip corresponds to a truncated form of the protein c-mip, both proteins being produced by a single gene through two alternative transcripts. The main difference between these proteins is a deletion in Tc-mip of the N-terminal part of a PH domain which is fully functional in c-mip. These two proteins display striking differences in cellular expression and distribution, as well as in functional characteristics.

We showed that Tc-mip is specifically expressed in CD4<sup>+</sup> T cells in patients with MCNS and plays a driving role in c-maf-mediated Th2 signaling pathway. Such findings extend our previous data regarding the specific recruitment of c-maf in MCNS (Valanciute A., submitted). Conversely, c-mip is mainly detectable in PBMC of normal subjects and exerts slight effects on c-maf expression that is restricted to cytoplasmic compartment.

Under unstimulated conditions, c-mip and Tc-mip proteins were weakly or not detected despite the basal expression of their respective transcripts, as reported for other genes expressed in immune cells (13). Under stimulation conditions under which an increase in (Ca<sup>2+</sup>)<sub>i</sub> occurs (e.g. PMA plus ionomycin), we observed a dramatic fall in Tc-mip and c-

mip mRNA levels. It is likely that these agents activate PKC, which exerts a negative regulatory role on Tc-mip/c-mip signaling pathway as recently demonstrated for the PI3k/AKT pathway (14). On the other hand, under Th skewing conditions, Tc-mip is preferentially recruited in Th2 but not in Th1 cells. In addition c-maf is induced in Tc-mip-transfected T cells and there is an increase of IL-4 promoter activity. The IL-4 induction in Tc-mip-, and to a lesser extent, in c-mip-overexpressing T cells, was observed only after TCR engagement following stimulation by the anti-CD3 antibody. These data suggest that additional signals, stemming from TCR, are required for the IL-4 induction by c-maf, as previously reported (12).

Intracellular molecules, known as cytosolic adapter proteins (CAPs) and lacking intrinsic enzymatic or transcriptional activity, contain modular domains which serve to recruit proteins to couple proximal events initiated by TCR ligation with more distal signaling pathways. Tc-mip/c-mip might fall into this CAP family as the first protein identified so far and linking TCR proximal signaling to c-maf transcription factor.

By comparison, NFATc-mediated Th2 signaling uses apparently distinct pathway, involving the activation of lck and the phosphactivation of the PH domain-containing protein Itk/Emt, a T cell associated Tec kinase (15). As matter of fact Th2 development fails in lck-deficient T cells (16). Nuclear localization of NFATc is sharply reduced in itk-deficient T

cells upon stimulation by anti-TCR/CD28 (17). However, activation of PKC with calcium ionophore restores NFATc-mediated IL-4 expression. Importantly, Itk does not exhibit any influence on Th1 cytokines (18). These data fundamentally differentiate Itk/Emt from Tc-mip by at least two aspects: i) Tc-mip bypasses the requirement for calcium signaling; ii) Tc-mip induces c-maf and represses the IFN expression. Nevertheless, whether the downregulation of IFN is a direct effect of Tc-mip or a consequence of the induction of c-maf remains to be clarified.

PH domain-containing proteins are members of a large family involved in cellular signaling (19), (20) (21). The consensus sequence of the PH domains is weak but they share similar core sandwich structure (22). The PH domains comprise approximately 100 amino acids, likely to recruit molecules to the membrane by specific interactions with phosphoinositide lipids (22). These interactions might contribute to the attachment of the plasma membrane to the cytoskeleton through lipid-protein and protein-protein interactions leading to a regulation of the cytoskeleton organization (23). We showed here that Tc-mip partially mimics TCR signaling and induced T cell clustering and refolding of the cytoskeleton protein L-plastin, independently of PI3 kinase pathway.

The truncation of the PH domain of Tc-mip is associated with a nuclear location of the protein. This suggests that the PH domain of c-mip acts



cooperatively with membrane phosphoinositides to cluster c-mip at and near the plasma membrane. The nuclear translocation of Tc-mip might result from the alteration of the sheet structure present in the phosphoinositide-binding module of the PH domain of c-mip, as previously shown for other PH domain-containing proteins (23) (24) (25).

The inhibitory role of the PH domain on cellular signaling has been documented for other proteins. The PH domain of the guanine nucleotide exchange factors (GEFs) Vav-1, acts as a negative, intramolecular regulator of Dbl homology (DH) domain function. Mutations in the PH domain alters the phosphoinositide binding of Vav1 and induces an increase in GEF activity (26). In addition, a Vav variant, lacking the PH domain, induces JNK activation leading to cytoskeletal reorganization (27). As other examples, deletion of the PH domain of the protein kinase D (PKD) or point mutation in PH domain from Bruton's tyrosine kinase (BTK), increased kinase activities (28, 29).

Alterations of the PH domain have also been associated with several human disease: i) a point mutation in the PH domain of Btk is associated with X-linked agammaglobulinaemia (30); ii) alterations in the DH-PH domain of proteins such as Ost, Lfc, Dbs or the FGD-1 gene

are implicated in human oncogenesis and developmental disorders (31) (32) (33) (34).

Inasmuch that c-mip constitutes the normal protein isoform in T lymphocytes of normal subjects, the occurrence of Tc-mip at high level in MCNS patients allows important regulatory changes in the putative signaling pathway. Truncation of the PH domain might suppress the anchorage of Tc-mip to membrane-phosphoinositides, precluding proximal interactions of Tc-mip with negatively regulating molecules. Nevertheless, Tc-mip conserves other domains allowing its interactions with downstream molecules. Following TCR stimulation, Tc-mip upregulates the activation cascade that ultimately induces c-maf and affect its cytokine repertoire.

Growing evidence suggests that glomerular diseases with nephrotic syndrome are associated with alterations of cytoskeleton scaffold in podocyte cells (35) (36). Since Tc-mip/c-mip is also preferentially expressed in kidney, it has to be demonstrated whether Tc-mip alters the cytoskeleton organization in Minimal Change Nephrotic Syndrome.

In conclusion, we identified in this study a new protein, Tc-mip, which is specifically recruited during MCNS. We showed that Tc-mip links TCR-mediated signal to the activation of c-maf Th2 specific factor. The presence of a truncated PH domain allows a nuclear translocation of the protein which promote a high c-maf expression level in MCNS patients.

It also gives additional evidence regarding the inhibitory function of the PH domain on protein activity and its critical role on protein localization. Finally, it gives strong support to the potential involvement of unknown alternative species of transcripts in human diseases.

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mRNA	oligonucleotides	Accession number	expected size (bp)	Tm annealing (°C)	PCR cycles
GAPDH	S: 5'-ACCACAGTCCATGCCATCAC-3' AS: 5'-TCCACCACCCTGTTGCTGTA-3' I: 5'-CTCAAGGGCATCCTGGGCTACACTGAGCAC-3'	NM004048	374	58	25
c-maf	S: 5'-TGCACTTCGACGACCGCTTCTC-3' AS: 5'-CGCTGCTCGAGCCGTTTTCTC-3'	AF055376	326	62	32
Tc-mip	S: 5'-CCTGCGAGGAGGGAAGTTACAGATCTC-3' AS: 5'-CAGGTAGCTATTGGCAGCCTGCAGTAAG-3' I: 5'-ATGGGACAGGCTGCTGAGCCAACT-3'	AY172689	209	62	32
c-mip	S: 5'-GAAGGCACGAAGATGGGCGCCGTGCCCT-3' AS: 5'-GCTGAGAAAGGTCCGCGGGTGCCGGATG-3'	ABO51481	130	75	32
IL-4	S: 5'-TTCTCCTGATAAACTAATTGCCTCACATTGTC3' AS: 5'-GGTGATATCGCACTTGTGTCCGTGG-3'	XM004053	143	60	34
IFN	S: 5'-GGTTCTCTTGGCTGTTACTGC-3' AS: 5'-GTCATCTCGTTTCTTTTGTGCT-3'	XM006883	294	60	34
Tc-mip (CS)	S: 5'-ATGGGACAGGCTGCTGAGCCAA-3' AS: 5'-CTTCCTGCCTTGAGCTGGGAGC-3'	AY172689	2150	63	32
c-mip (CS)	S: 5'-ATGGGCGCCGTGCCCT-3' AS: 5'-CTTCCTGCCTTGAGCTGGGAGC-3'	ABO51481	2270	56	32

Table 1. Sets of primers used in semiquantitative RT-PCR and for PCR amplification of coding sequences of c-mip and Tc-mip. The oligonucleotides sense (S) and antisense (AS) were selected from the

sequences with the indicated Accession numbers. The size of each amplified product, annealing temperature of each oligonucleotides , and the number of PCR cycles are indicated (I: internal oligonucleotide probe) CS: Coding sequence



## Legends

### **Figure 1 Comparative structure of c-mip and Tc-mip.**

Partial c-mip and Tc-mip genes structure are reported are indicated on the top and bottom of panel A. Both transcripts differ the first exon: c-mip exon 1 encodes for 66 amino acid (aa) of which the 29 aa C-terminal (position 38-66) contribute to PH domain (109 aa); Tc-mip exon-1 encodes for 6 aa belonging to PH domain (87aa). Protein structure was analyzed by Smart software (Simple Modular Architecture Research Tool), which detected the PH domain (encoded by exons 1, 2, 3 and 4 and the Leucine-rich repeats (LRR), encoded by the exon 19). B. Prediction of sheet structures of the PH domain using the Garnier Peptide structure tool (<http://biotools.umassd.edu/cgi-bin/biobin/garnier>). Amino acid sequence corresponding to the PH domain is indicated in bold characters.

### **Figure 2: Northern blot analysis of c-mip and Tc-mip expression and differential expression of c-mip and Tc-mip mRNA in PBMC subsets. Preferential induction of Tc-mip in Th2.**

A. Multiple tissue-Northern blots (Clontech) were hybridized with a 560 bp cDNA probe common to Tc-Mip and c-Mip and exposed on a

phospho-imager Storm for 24h. PBMC: peripheral blood mononuclear cells. LN: Lymph node; BM: Bone marrow; B: Brain; H: Heart; SK: Skeletal muscle; C: Colon; T: Thymus; S: Spleen; K: Kidney; L: Liver; I: Intestine; P: Placenta; Lu: Lung. B. Resting (-) and PMA/iono-activated (+) cells were isolated and analyzed for c-mip and Tc-mip mRNA expression as described under "Material and Methods". The expression of GAPDH was monitored in parallel. PBMC: peripheral blood mononuclear cells. BL: B lymphocyte. C. Naive T cells were purified from blood umbilical cord, then polarized under Th1 or Th2 skewing conditions and analyzed for c-mip/Tc-mip and IL-4 mRNA expression by semiquantitative RT-PCR as described under "Material and Methods." The expression of GAPDH was monitored in parallel.

**Figure 3: Specific induction of Tc-mip in MCNS.** A. RT-PCR analyses of Tc-mip mRNA expression. Nine patients with MCNS were studied in relapse and in remission as well as eight normal subjects and four nephrotic patients with membranous nephropathy. The expression of GAPDH was monitored in parallel, in order to control the initial amount of mRNA. The lower panel shows quantification of the PCR bands in panel A determined by using the Image Quant V 1.11 analysis software, after normalization against the corresponding GAPDH bands; B. induction of Tc-mip was higher in the CD4<sup>+</sup> T cell subset. Total RNA

from CD4<sup>+</sup>, non-CD4<sup>+</sup> (nCD4) subsets was purified from PBMC of patients with MCNS relapse, as described under "Material and Methods" and analyzed for Tc-mip expression by RT-PCR.

C. Western blot analyses using anti-Tc-mip/c-mip antibody. Total protein extracts (50 µg) of PBMC from six patients in relapse and six normal subjects were tested for Tc-mip/c-mip expression; The blots were stripped and reprobed with anti-actin antibodies; D. immunostaining of PBMC of a pediatric patient with MCNS (4-year-old, first relapse and remission). PBMC were purified, methanol fixed and incubated with anti-Tc-mip/c-mip antibody, as described under "Material and Methods" (X40, magnification).

**Figure 4: Tc-Mip induces c-maf nuclear expression.** A. RT-PCR and Western blot analyses for c-maf expression in Jurkat cells transfected with either Tc-mip, c-mip or empty vector, as described under Materials and Methods. B. Immunofluorescence detection of c-maf in transfected Jurkat cells. The data are representative of six separate experiments. GAPDH and Actin were assayed as control. C. c-maf DNA binding activity. EMSA analyses of nuclear extracts (15 µg) from Tc-mip- (Lane 1-3) and c-mip- (Lane 4) transfected Jurkat cells. The specificity of the band shift (black arrow) was demonstrated by the loss of this band, in the presence of the mutant MARE oligonucleotide (mt



MARE, line 5). D. Confocal imaging of Tc-mip and c-mip after transient transfection in Jurkat cells with respective expression vectors. This result is representative of several cells in four independent experiments.

**Figure 5: Tc-Mip induces the transactivation of the IL4 gene and inhibits the  $\text{INF}\gamma$  expression in stimulated Jurkat T cells.**

A. Jurkat T cells were transiently co-transfected with the IL-4/CAT plasmide and either Tc-mip, c-mip or empty vector. Cells were then incubated with medium alone or with anti-OkT3 monoclonal antibody as described under “Material and Methods”. Data are expressed as fold induction of intracellular CAT and represent the mean results of 4 independent experiments. B. RT-PCR of total RNA extracted using IL4 and INF specific primers (table 1). The expression of GAPDH was monitored in parallel.

**Figure 6 Overexpression Tc-mip in T cells induces a spatial and PI3 kinase-independent redistribution of cytoskeleton-associated L-plastin.** A Immunofluorescence detection of L-plastin in unstimulated Jurkat cells transfected with either Tc-mip, c-mip or empty vector. The data are representative of three independent experiments. B Pharmacologic inhibitors of PI3-K did not inhibit redistribution of cytoskeleton-associated L-plastin. Jurkat T cells

transfected with T-cmip and wortmanin (100 nM) was added to medium culture, six hours following transfection and left overnight. This result was reproduced in three independent experiments.

Fig 1

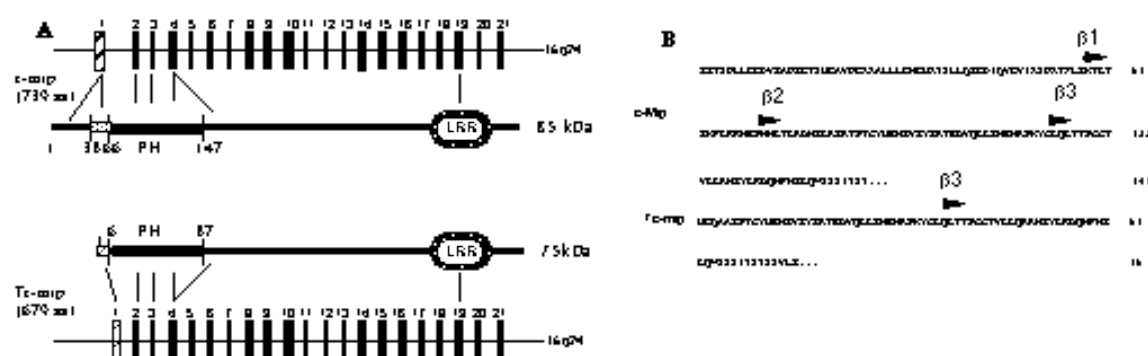


Fig2

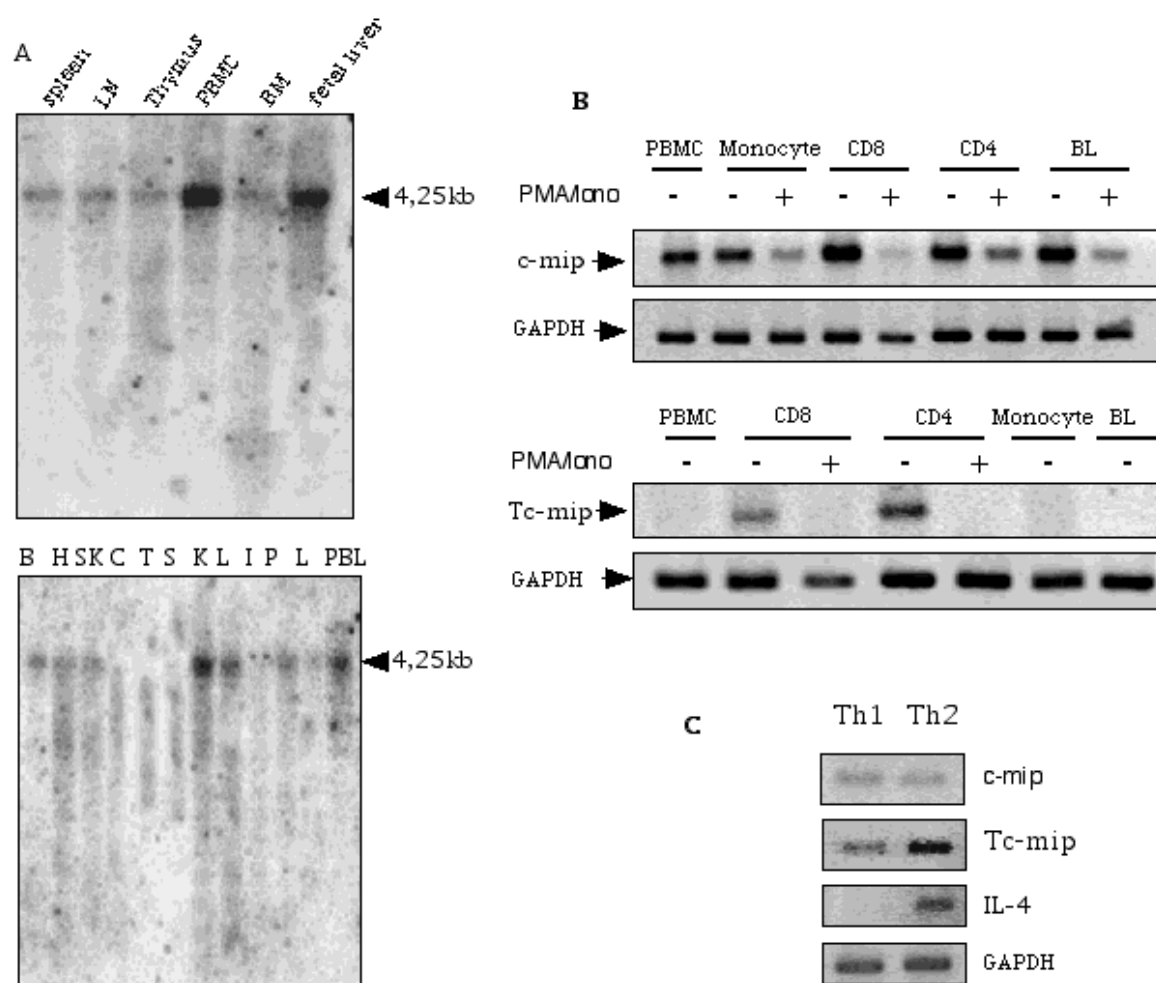


Fig 3

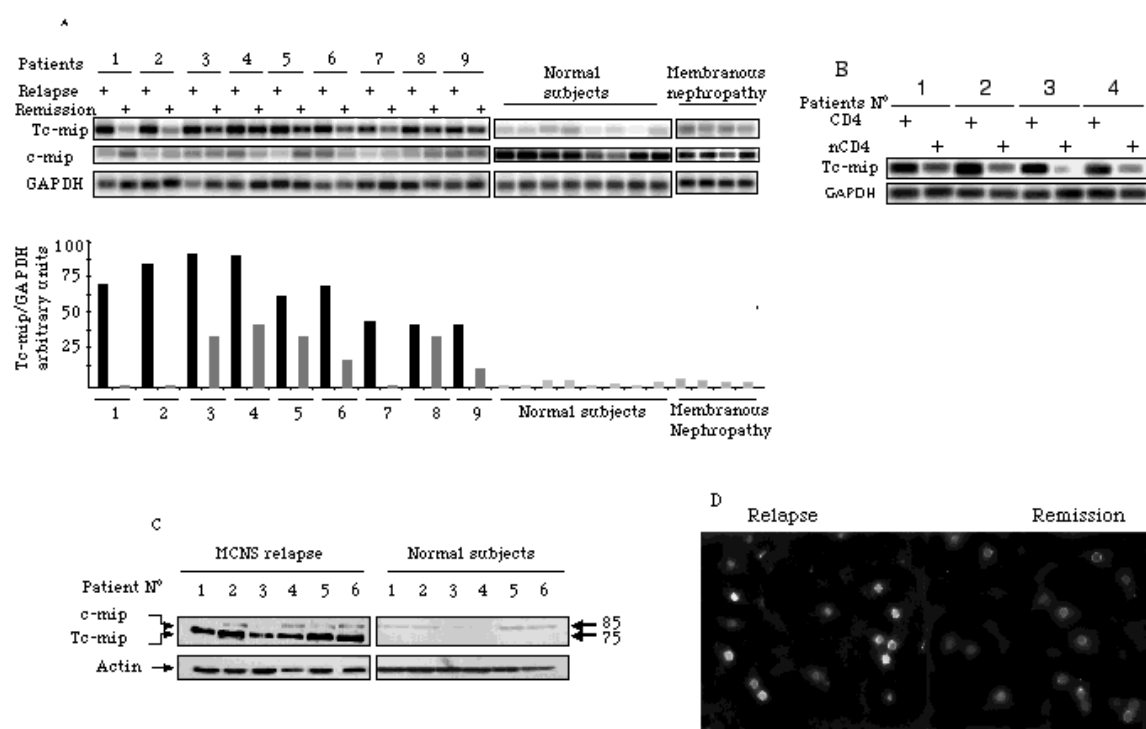


Fig4

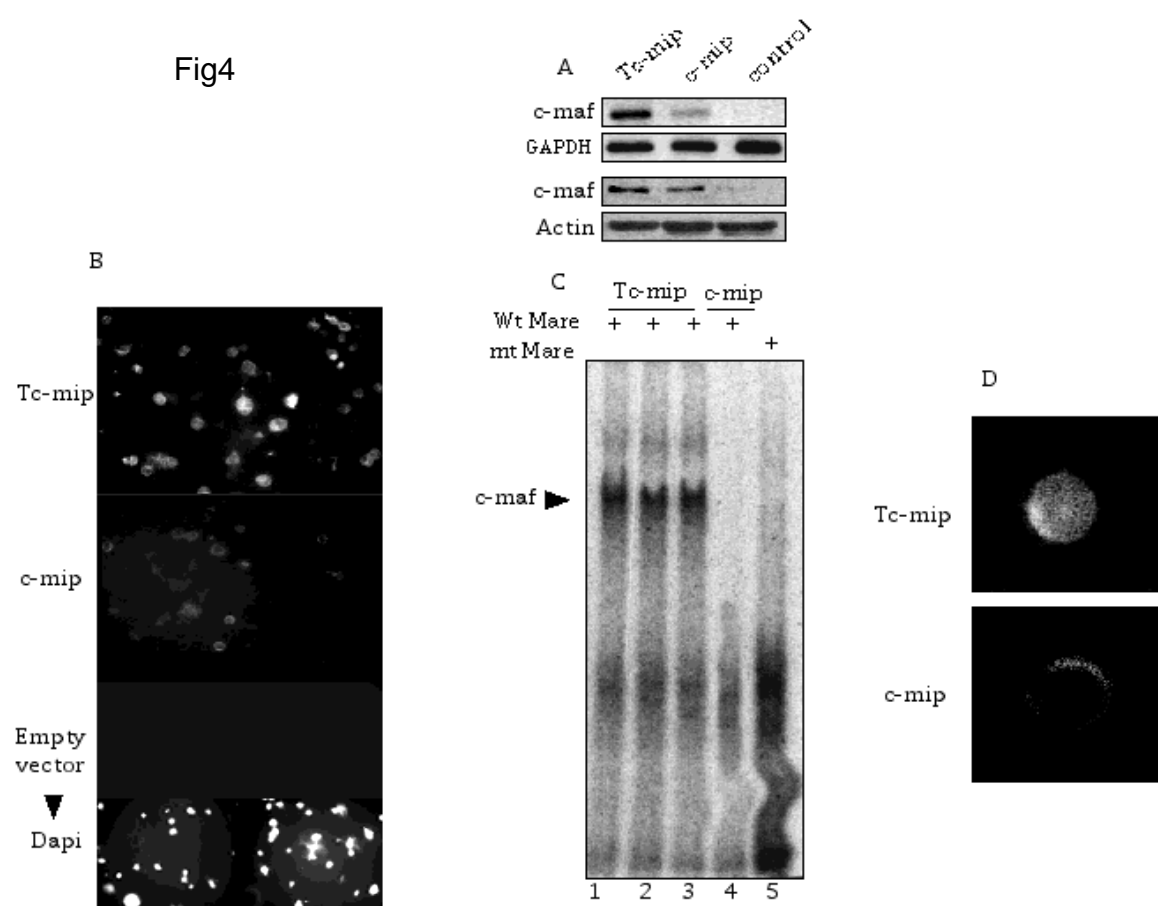


Fig 5

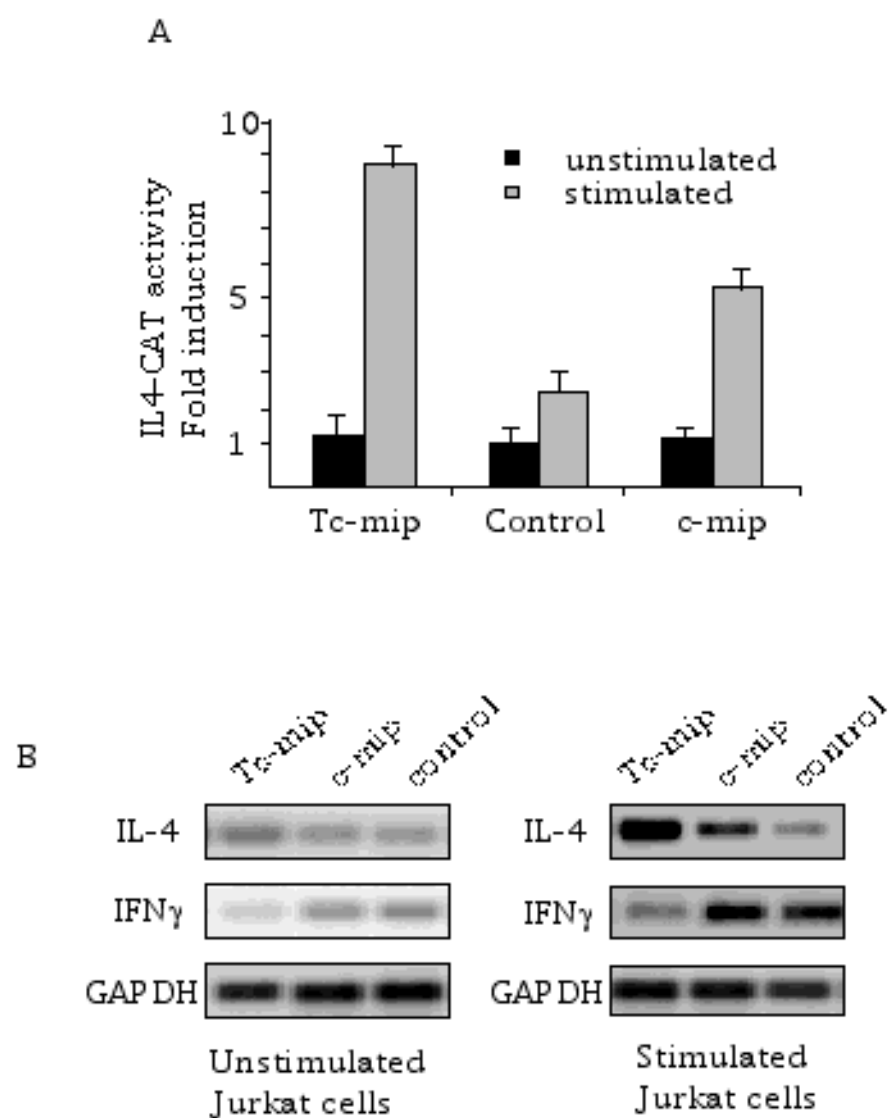


Fig 6

