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Original article

# The protective effect of 1-methyltryptophan isomers in renal ischemia-reperfusion injury is not exclusively dependent on indolamine 2,3-dioxygenase inhibition

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ABSTRACT

*Background and Purpose:* Indolamine 2,3-dioxygenase (IDO), an enzyme that catalyses the metabolism of tryptophan, may play a detrimental role in ischemia-reperfusion injury (IRI). IDO can be inhibited by 1-methyl-tryptophan, which exists in a D (D-MT) or L (L-MT) isomer. These forms show different pharmacological effects besides IDO inhibition. Therefore, we sought to investigate whether these isomers can play a protective role in renal IRI, either IDO-dependent or independent.

*Experimental Approach:* We studied the effect of both isomers in a rat renal IRI model with a focus on IDO-dependent and independent effects.

*Key Results:* Both MT isomers reduced creatinine and BUN levels, with D-MT having a faster onset of action but shorter duration and L-MT a slower onset but longer duration (24 h and 48 h vs 48 h and 96 h reperfusion time). Interestingly, this effect was not exclusively dependent on IDO inhibition, but rather from decreased TLR4 signalling, mimicking changes in renal function. Additionally, L-MT increased the overall survival of rats. Moreover, both MT isomers interfered with TGF-β signalling and epithelial-mesenchymal transition. In order to study the effect of isomers in all mechanisms involved in IRI, a series of *in vitro* experiments was performed. The isomers affected signalling pathways in NK cells and tubular epithelial cells, as well as in dendritic cells and T cells.

*Conclusion and Implications:* This study shows that both MT isomers have a renoprotective effect after ischemia-reperfusion injury, mostly independent of IDO inhibition, involving mutually different mechanisms. We bring novel findings in the pharmacological properties and mechanism of action of MT isomers, which could become a novel therapeutic target of renal IRI.

*Abbreviations:* 3−OH-kyn, 3- hydroxykynurenine; Ccl5/RANTES, Chemokine (C-C motif) ligand 5/ Regulated on activation, normal T cell-expressed and secreted; C-DCs, control DCs; C-TECs, control TECs; DCs, dendritic cells; D-IRI-DCs, IRI-induced DCs treated with D-MT; D-IRI-TECs, IRI-induced TECs treated with D-MT; D-MT, 1-methyl-D-tryptophan; EMT, epithelial-mesenchymal transition; FoxP3, Forkhead box P3; GATA3, GATA-binding protein 3; HEK, human embryonic kidney; IDO, indolamine 2,3-dioxygenase; IDOt, indolamine 2,3-dioxygenase transduced; IFN-γ, interferon gamma; IL, interleukin; IRI, ischemia-reperfusion injury; IRI-DCs, IRI-induced DCs; IRI-TECs, IRI-induced TECs; KMO, 3-hydroxykynurenine; kyn, kynurenine; L-IRI-DCs, IRI-induced DCs treated with L-MT; L-IRI-TECs, IRI-induced TECs treated with L-MT; L-MT, 1-methyl-L-tryptophan; MT, methyltryptophan; NK, natural killer; NKG2D, natural killer group 2D; NLRP3, NOD-like receptor, pyrin domain containing-3; RORγT, Retinoid-related orphan receptor gamma T; T-bet, T-box transcription factor T-bet; TECs, tubular epithelial cells; TGF-β, transforming growth factor β; TLR4, toll-like receptor 4; TPH1, Tryptophan hydroxylase 1; trp, tryptophan; TTS, Tryptophanyl-tRNA synthetase; αSM, α smooth muscle actin.

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## 1. Introduction

Currently, there is no specific treatment available for renal ischemia-reperfusion injury (IRI) [1]. Indolamine 2,3-dioxygenase (IDO) may be an interesting therapeutic target in renal IRI [2].

IDO catalyses the conversion of tryptophan (trp) along the kynurenine pathway, generating metabolites collectively referred to as kynurenines (kyn). IDO has immunosuppressive properties and promotes tolerance towards transplanted organs [3]. Moreover, IDO gene therapy attenuates both acute rejection and chronic transplant dysfunction after renal transplantation in rats [4,5]. In contrast, increased IDO expression augmented injury to tubular epithelial cells (TECs) and renal parenchymal cells in both *in vitro* and *in vivo* models of IRI [2], suggesting that IDO has both negative and positive effects in renal transplantation. Therefore, fine-tuning IDO activity using specific inhibitors may be required to maximize the therapeutic potential of IDO in renal transplantation.

Two isomers of 1-methyltryptophan (IDO inhibitor; MT) exist: 1-methyl-D-tryptophan (D-MT) and 1-methyl-L-tryptophan (L-MT). Contradictory results have been reported regarding the inhibitory potential of MT isomers; L-MT was proven to be a more potent IDO inhibitor than D-MT *in vitro*, but D-MT was shown to have higher antitumour activity [6,7]. Furthermore, the pharmacokinetic properties of these isomers vary [8]. Taken together, these findings demonstrate that MT isomers possess different effects.

A hallmark of IRI is tubular epithelial cell damage, followed by the development of tubulointerstitial fibrosis with subsequent loss of renal function [9]. Transforming growth factor β (TGF-β), as a mediator of fibrosis, promotes extracellular matrix production and proliferation of myofibroblasts and fibroblasts. During IRI, TGF-β contributes to renal fibrosis through epithelial-mesenchymal transition (EMT), when polarized renal epithelial cells assume a mesenchymal phenotype [10]. TECs lose epithelial markers, such as E-Cadherin, and increase the production of mesenchymal proteins, such as α smooth muscle actin (αSMA) [11] and N-Cadherin [12]. It has recently been shown that D-MT may influence the fibrotic processes described above [13]; however, the role of MT isomers in renal fibrosis following IRI is currently unknown.

Toll-like receptor 4 (TLR4) signalling plays a role in the damage of renal cells in IRI [14]. TLR4 also influences fibrosis/wound repair [15] [16], the expression of natural killer (NK) cell ligands in renal TECs [17] and EMT of renal TECs through a NOD-like receptor, pyrin domain containing-3 (NLRP3) pathway [18]. By its activation TLR4 mediates renal IRI and is expressed by TECs in the kidney [14], and that activation of its signalling contributes to expression of the natural killer group 2D (NKG2D) ligands by renal TECs [17]. NKG2D ligands then induce NK cells, leading to TECs apoptosis [19]. D-MT has been shown to interfere with TLR4 signalling [20]. Whether it influences NKG2D and/or NKG2D ligand expression, or what the role of L-MT is in TLR4 signalling in renal IRI, is not yet clear.

Dendritic cells (DCs) are also involved in the pathogenesis of renal IRI [21]. The interaction between DCs and T cells during and after ischemia leads to a Th1/Th2 switch and has relevant consequences in renal IRI, as the Th1 favoured by IRI are perceived as pathogenic, whereas Th2 as protective. DCs influenced by the TLR4 signalling pathway mediate renal damage in IRI. Racemic DL-MT can interfere with DCs TLR4 signalling, leading to secretion of Th2 cytokines rather than Th1. In this way MT isomers may influence DCs present in the ischemic kidney and attenuate renal damage.

Considering the different effects and properties of the two 1-MT isomers, we here aim to investigate the effect of the D-MT andMT isomers in a rat IRI model. In addition, we studied IDO activity and possible IDO-dependent or independent protective mechanisms of the MT isomers, as well as the role of MT isomers in prefibrosis, TLR4 signalling and the signalling of DCs and the phenotype of their effector T cells.

## 2. Results

### 2.1. L-MT is a stronger inhibitor of IDO activity than D-MT in vitro

First, due to the conflicting findings published prior to our research, we assessed the ability of both D-MT and L-MT to inhibit IDO activity [6–8,22,23]. We therefore studied the effect of D-MT and L-MT on HEK293 cells transfected with an adenoviral vector carrying the human IDO1 gene. We measured the levels of tryptophan (trp, Fig. 1A) and its metabolite – kynurenine (kyn, Fig 1B) – and we estimated IDO activity (kyn/trp ratio, Fig. 1C). IDO activity was elevated in IDO transduced (IDOt) cells compared to controls. Both D-MT and L-MT decreased IDO activity compared to IDOt cells, although L-MT had a stronger inhibitory effect on IDO activity than D-MT.

### 2.2. Renoprotective effect of L-MT and D-MT differs – D-MT acts faster and for a shorter time, while the effect of L-MT is delayed, lasts longer and increases survival in the IRI rat model

Considering the various properties of MT isomers and the difference in their IDO inhibitory potential, D-MT/L-MT may have different effects on renal IRI. In our IRI model, rats underwent 30 min of bilateral renal ischemia followed by 24, 48, or 96 h reperfusion.

After 24 h reperfusion, plasma blood urea nitrogen (BUN) and creatinine (CR) were increased in the IRI group compared to SHAM. Treatment with L-MT had no effect (Fig. 2A, B, respectively); however, treatment with D-MT decreased BUN and showed a trend to decreased plasma CR ($p = 0.08$; Fig. 2A, B, respectively) already at 24 h.

After 48 h reperfusion, BUN and CR were increased in the IRI group in comparison with SHAM (Fig. 2A, B, respectively). Treatment with either D-MT or L-MT decreased both BUN and CR levels (Fig. 2A, B, respectively).

After 96 h reperfusion, plasma levels of BUN and CR were increased in the IRI group compared to SHAM rats (Fig. 2A, B, respectively). D-MT treatment no longer influenced the BUN and CR plasma levels, but treatment with L-MT still decreased the levels of both BUN and CR compared to the IRI rats (Fig. 2A, B, respectively).

The overall survival of rats in our IRI model with various reperfusion time points was assessed by Kaplan-Meier analysis using the Breslow test. The survival of rats in the IRI group compared to SHAM was decreased, and D-MT did not affect survival. However, L-MT treatment did lead to a significant increase in the survival rate (Fig. 2C).

### 2.3. Protective effect of L-MT and D-MT is not exclusively dependent on IDO inhibition, while IDO expression in the kidney is only transiently altered

Increased IDO expression following IRI was proposed as a possible mechanism leading to renal injury [2], therefore we determined IDO expression in the kidney in our rat IRI model. After 24 h reperfusion increasing trend in IDO protein expression was found in the IRI group ($p = 0.08$), however no differences in IDO expression were found in any other group throughout the whole experiment (Fig. 3A).

To get insight into the mechanisms behind the renoprotective effect of D-MT/L-MT in our IRI model, we assessed the plasma levels of trp, kyn and 3−OH-kyn as well as the kyn/trp ratio and KMO (3−OH-kyn/kyn) activity. After 24 h reperfusion, the kyn/trp ratio was increased in the IRI group (Fig. 3B), whereas KMO activity remained unchanged (Fig. 3C). D-MT treatment did not influence either the increased kyn/trp ratio (Fig. 3B) or KMO activity (Fig. 3C), despite its beneficial effect on renal function. Further, treatment with L-MT did not affect the kyn/trp ratio and KMO activity as well (Fig. 3B and 3C, respectively). In 48 h reperfusion, the kyn/trp ratio was increased in the IRI group (Fig. 3B). Even though at this time point both MT isomers revealed a beneficial effect on renal function, only the D-MT treated group showed a decreased kyn/trp ratio compared to the IRI group (Fig. 3B). On the

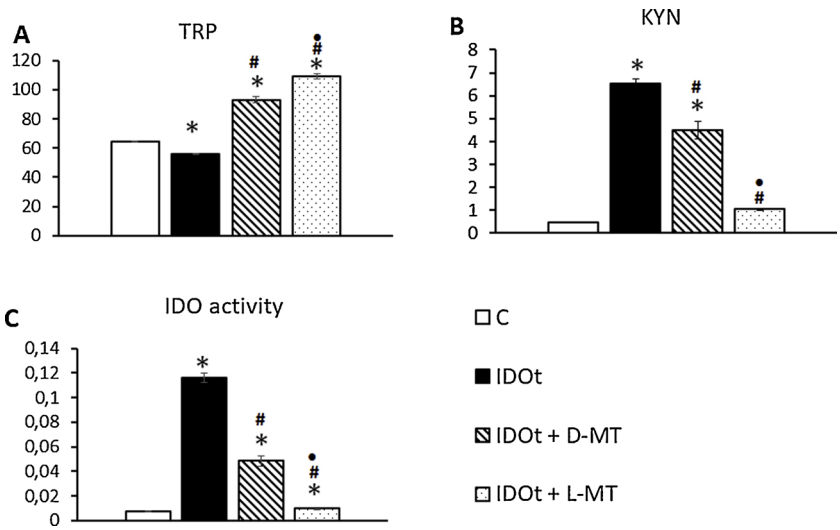


**Fig. 1.** Human IDO1 gene transduced HEK293 cells treated by D-MT/L-MT. The concentration of trp (A) and kyn (B) in the cell culture medium; the estimated activity of IDO (C). *p < 005 vs control, #p < 005 vs IDOt, •p < 0,05 vs IDOt + D-MT; C - control cells; IDOt - cells transfected with RGD-AdTIDO; IDOt + D-MT - cells transfected with RGD-AdTIDO treated with D-MT; IDOt + L-MT - cells transfected with RGD-AdTIDO treated with L-MT; trp - tryptophan (μmol/L); kyn - kynurenine (μmol/L); kyn/trp - estimated IDO activity. Data are presented as mean ± SEM.

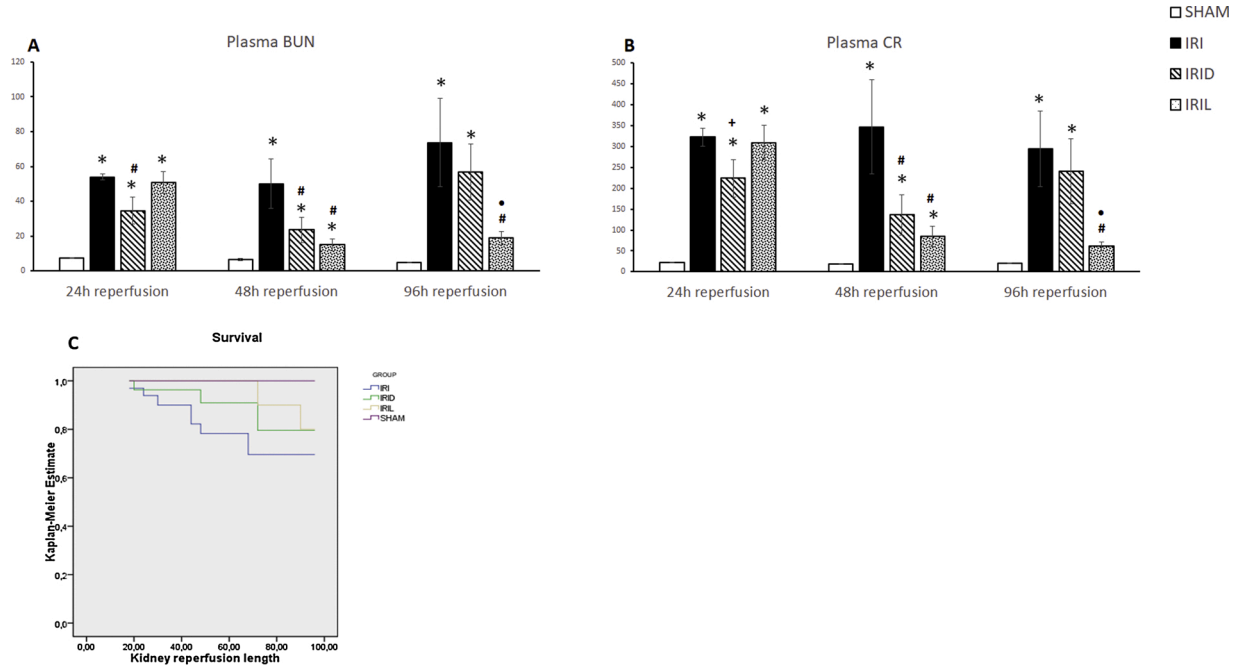


**Fig. 2.** Renal function and overall survival of IRI-induced rats treated with D-MT/L-MT. Plasma concentration after 24, 48, and 96 h reperfusion of BUN (A), CR (B). Overall survival of rats in renal IRI model with various reperfusion time points (C) - IRI vs SHAM p = 0044; IRI vs IRIL p = 0,048. *p < 0,05 vs SHAM, #p < 0,05 vs IRI, •p < 0,05 vs IRID; + vs IRI p = 0,08; ° vs. SHAM p = 0,08; SHAM - sham-operated rats; IRI - rats that underwent IRI; IRID - rats that underwent IRI treated with D-MT; IRIL - rats that underwent IRI treated with L-MT. Data are presented as mean ± SEM.

other hand, L-MT treatment decreased KMO activity (Fig. 3C), which was increased as a result of IRI. Although L-MT did not affect the increased kyn/trp ratio in 48 h reperfusion, L-MT decreased the elevated kyn/trp ratio later in 96 h reperfusion in the IRI group (Fig. 3B), which is in concordance with its beneficial effect on renal function (Fig. 3B). Moreover, L-MT treatment decreased KMO activity (Fig. 3C). Treatment with D-MT did not affect the kyn/trp ratio. Trp, kyn and 3−OH-kyn values are depicted in Table 1.

### *2.4. L-MT and D-MT attenuate mediators and markers of renal prefibrosis following IRI in rat as well as they affect TGF-β signalling in NIH/3T3 fibroblasts*

Since MT was suggested to influence fibrosis in human bladder cancer cells [13], we investigated the effect of MT isomers on mediators and markers of renal prefibrosis in our IRI rat model. TGF-β mRNA expression was increased in the IRI group at all time points (Fig. 4A). In accordance with the effect of the renal function, TGF-β mRNA expression was decreased after 24 h reperfusion in the group treated with the D isomer, after 48 h reperfusion in groups treated with both isomers, and after 96 h reperfusion in the group treated with L-MT (Fig. 4A).

At 24 h reperfusion, we found increased αSMA expression in the IRI group (Fig. 4B). Treatment with D-MT decreased αSMA expression (Fig. 4B), while treatment with L-MT had no effect. At 48 h reperfusion, αSMA expression was increased in the IRI group and was not affected by MT treatment (Fig. 4B). At 96 h reperfusion, we found increased αSMA expression in the IRI group (Fig. 4B) and it was decreased by treatment with L-MT (Fig. 4B).

Further, we focused on proteins of TGF-β signalling. At 24 h reperfusion, pP38 expression and MEK and ERK phosphorylation were

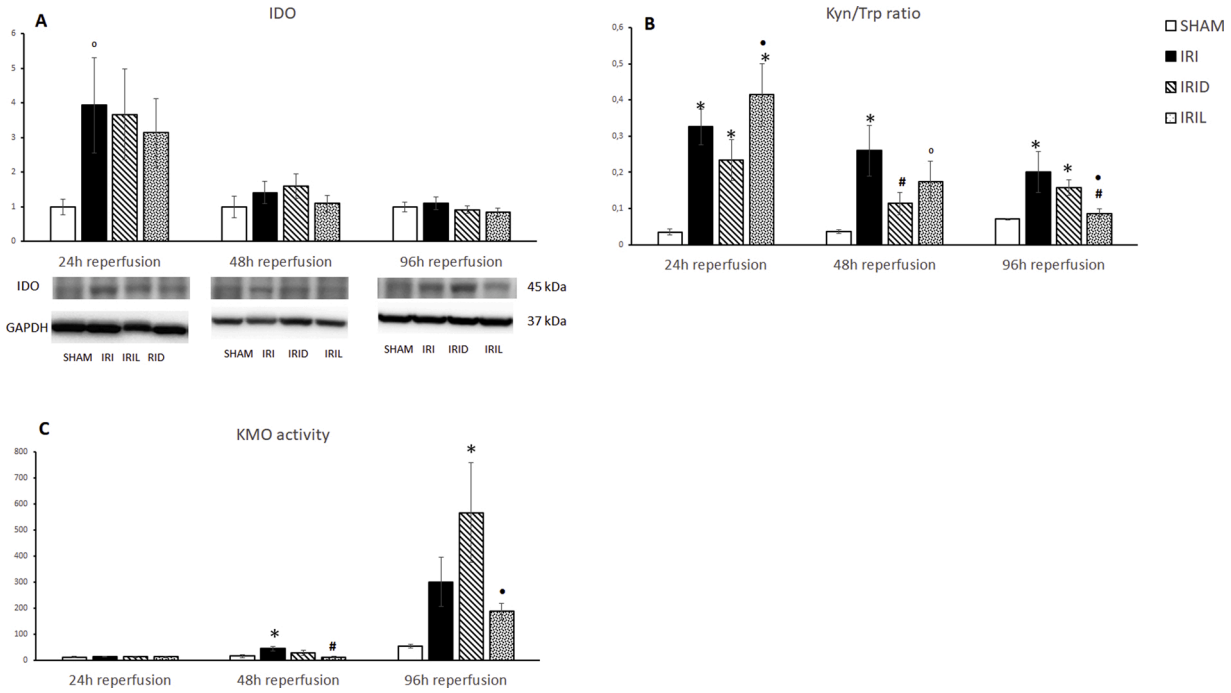


**Fig. 3.** Indolamine 2,3-dioxygenase (IDO) expression, kyn/trp ratio, and KMO activity of IRI-induced rats treated with D-MT/L-MT. IDO protein expression in 24, 48, and 96 h of reperfusion, including representative western blots (A). Estimated IDO activity (kyn/trp ratio) in 24, 48 and 96 h of reperfusion (B). Estimated KMO activity in 24, 48, and 96 h hours of reperfusion (C). *p < 0,05 vs SHAM, #p < 0,05 vs IRI, •p < 0,05 vs IRID; + vs IRI p = 0,08; ° vs. SHAM p = 0,08; SHAM - sham-operated rats; IRI - rats that underwent IRI; IRID - rats that underwent IRI treated with D-MT; IRIL - rats that underwent IRI treated with L-MT. Data are presented as mean ± SEM. IDO - indolamine 2,3-dioxygenase (kynurenine/tryptophan); KMO - kynurenine 3-monooxygenase (3-hydroxykynurenine/ kynurenine). Data are presented as mean ± SEM.

**Table 1**
Plasma concentration of tryptophan (trp), kynurenine (kyn) and 3-hydroxykynurenine (3−OH-kyn) in rat IRI model.

| | Group | Kidney reperfusion length | | |
|---|---|---|---|---|
| | | 24 h | 48 h | 96 h |
| TRP (μmol/l) | SHAM | 89.27 ± 9.90 | 88.80 ± 2.81 | 82.18 ± 8.51 |
| | IRI | 27.63 ± 3.38* | 43.88 ± 8.02* | 48.11 ± 12.67* |
| | IRID | 38.02 ± 10.59 * | 55.74 ± 8.89* | 68.27 ± 8.98 |
| | IRIL | 27.43 ± 5.01* | 53.40 ± 6.48* | 107.51 ± 6.40*, #,• |
| KYN (μmol/l) | SHAM | 4.265 ± 1.077 | 3.255 ± 0.494 | 5.820 ± 0.622 |
| | IRI | 7.825 ± 0.979° | 5.715 ± 0.831 * | 9.932 ± 1.349* |
| | IRID | 6.995 ± 1.053 | 4.837 ± 1.058 | 10.052 ± 1.021* |
| | IRIL | 9.707 ± 1.598 * | 8.486 ± 1.599*,• | 9.088 ± 1.371* |
| 3-OH-KYN (nmol/l) | SHAM | 53.01 ± 25.40 | 32.67 ± 5.47 | 289.78 ± 36.42 |
| | IRI | 126.76 ± 11.56* | 238.44 ± 45.80* | 2944.89 ± 984.71 |
| | IRID | 83.48 ± 15.93 | 154.89 ± 46.46 | 5250.15 ± 1596.18* |
| | IRIL | 118.54 ± 31.75° | 105.55 ± 39.39# | 1655.58 ± 351.72• |

SHAM – sham-operated rats; IRI – rats that underwent IRI; IRID – rats that underwent IRI treated with D-MT; IRIL – rats that underwent IRI treated with L-MT. Data are presented as mean ± SEM.
* vs SHAM p < 0.05.
# vs IRI p < 0.05.
• vs IRID p < 0.05.
° vs SHAM p = 0.07.

increased in the IRI group, all of them being decreased by treatment with D-MT (Supp. 1A-D). At 48 h reperfusion, pP38 expression as well as ERK and JNK phosphorylation were increased in the IRI group and treatment had no effect (Supp. 1A-D). At 96 h reperfusion, we found increased pP38 expression, as well as MEK, ERK, and JNK phosphorylation, in the IRI group and all of them were decreased by treatment with L-MT (Supp. 1A-D). These results are in line with αSMA expression described above, suggesting that both isomers similarly affect the TGF-β pathway as well as renal prefibrosis in IRI.

To study possible interference of MT isomers with TGF-β signalling, NIH/3T3 fibroblasts were stimulated with recombinant TGF-β1 for 24 h, 48 h and 96 h and treated with either D-MT or L-MT correspondingly with rat IRI model. We assessed αSMA, IDO, and pP38 expression as well as MEK, ERK, and JNK phosphorylation.

TGF-β stimulation for 24 h increased αSMA and pP38 expression. Treatment with both MT isomers decreased αSMA, while only the D isomer decreased pP38 expression (Fig. 4C and D, respectively). After 48 h and 96 h of TGF-β stimulation, αSMA and pP38 were also increased and decreased by both MT isomers (Fig. 4C and D, respectively).

After 24 h of TGF-β stimulation, IDO expression was increased and subsequently decreased by the D isomer, whereas the L isomer had no effect. There was no change in IDO expression after 48 h of TGF-β stimulation, while after 96 h the expression of IDO was increased. However, neither of the MT isomers had an effect (Fig. 4E). MEK and JNK phosphorylation was not changed after 24 h of TGF-β stimulation; however, L-MT treatment resulted in increased phosphorylation of both MEK and JNK (Fig. 4F and G, respectively). After 48 h, JNK phosphorylation was increased and the L isomer led to its decrease (Fig. 4G), while MEK phosphorylation remained unchanged (Fig. 4F). After 96 h of TGF-β stimulation, MEK and JNK were increased and decreased by both the L and D isomers (Fig. 4F and G, respectively). ERK phosphorylation was increased after 24 h of TGF-β stimulation; the D isomer had no effect and L-MT resulted in its increase. After 48 h of TGF-β stimulation, ERK phosphorylation remained unchanged but was increased after 96 h. Both D-MT and L-MT decreased ERK phosphorylation (Fig. 4H).

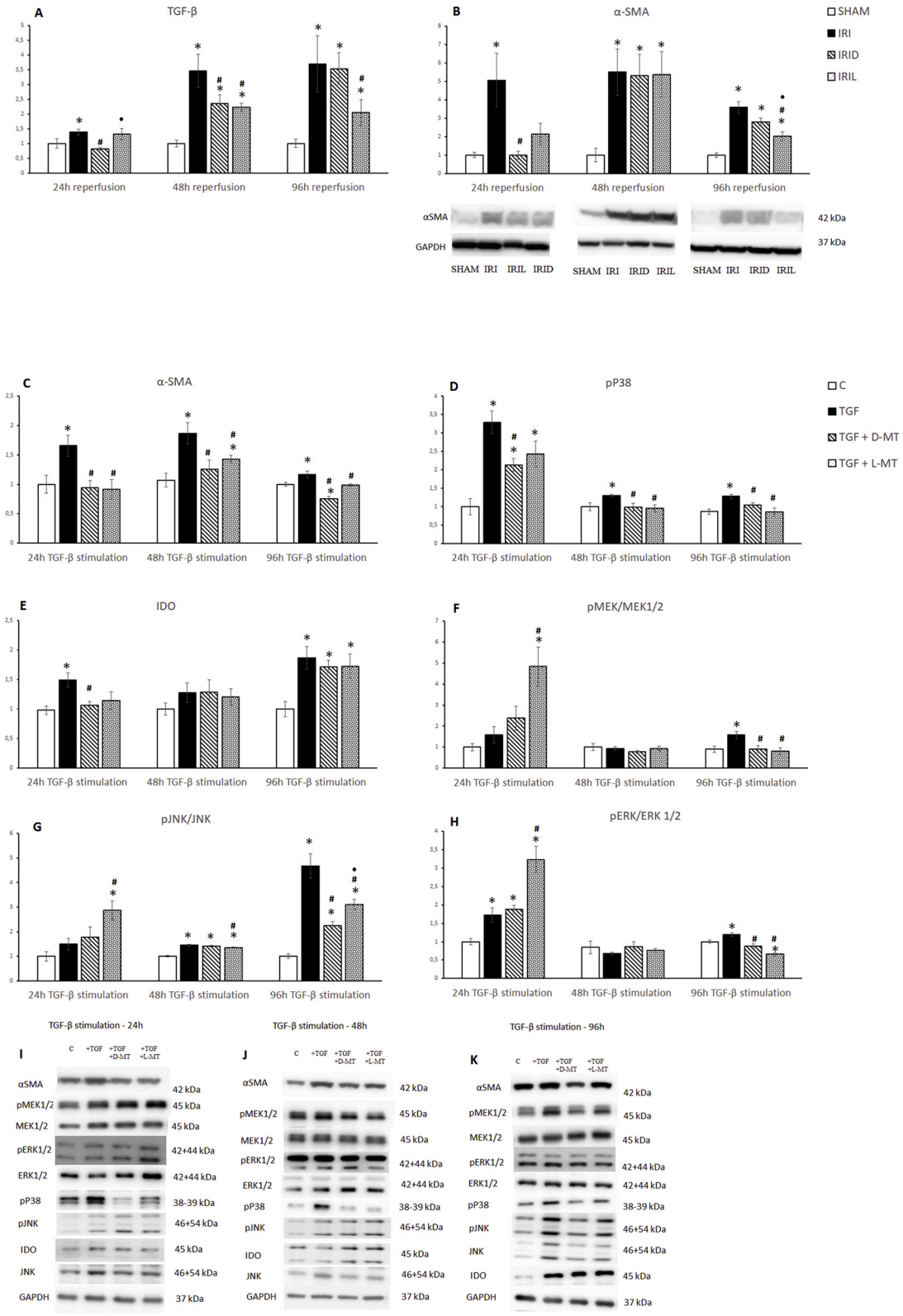


**Fig. 4.** Prefibrosis in the kidneys of IRI-induced rats treated with D-MT/L-MT and NIH/3T3 fibroblasts stimulated with recombinant TGF-β1 treated with D-MT/L-MT. IRI-induced rats: TGF-β mRNA expression in 24, 48, and 96 h of reperfusion (A). α-SMA protein expression in 24, 48, and 96 h of reperfusion, including representative western blots (B). Fibroblasts stimulated with TGF-β1 for 24 h, 48 h or 96 h: Protein expression of α-SMA (C), pP38 (D), IDO (E), pMEK/MEK 1/2 (F), pJNK/JNK (G), pERK/ERK 1/2 (H). Representative western blots 24 h (I), 48 h (J), and 96 h (K). *$p < 0,05$ vs C/SHAM, #$p < 0,05$ vs TGF/IRI, •$p < 0,05$ vs TGF + D-MT/IRID; C - control cells; TGF - cells stimulated by recombinant TGF-β1; TGF + D-MT - cells stimulated by recombinant TGF-β1 treated with D-MT; TGF + L-MT - cells stimulated by recombinant TGF-β1 treated with L-MT; SHAM - sham-operated rats; IRI - rats that underwent IRI; IRID - rats that underwent IRI treated with D-MT; IRIL - rats that underwent IRI treated with L-MT. Data are presented as mean ± SEM.

### 2.5. L-MT and D-MT inhibit epithelial-mesenchymal transition of tubular epithelial cells in an in vitro IRI model

We showed that both MT isomers can attenuate renal prefibrosis as a consequence of IRI and interfere with TGF-β signalling. We thus hypothesized that MT isomers could influence epithelial-mesenchymal transition (EMT) in renal tubular epithelial cells (TECs), attenuating renal prefibrosis, since EMT contributes to this process. TECs lose epithelial markers (like E-Cadherin) and increase TGF-β1-mediated production of mesenchymal proteins such as α-SMA [11] and N-Cadherin [12]. Hence, we induced IRI in isolated TECs correspondingly with the rat IRI model and treated them with MT isomers. We determined the mRNA expression of TGF-β (Fig. 5A) and transcriptional factors of EMT [24] – Snai1, Snai2/Slug, Twist1 and Zeb1 (Supp. 2C-F) – as well as the protein expression of αSMA, pP38, MEK, ERK, JNK, E-Cadherin, N-Cadherin and IDO (Fig. 5B-E, Supp. 3A-D).

In IRI-induced TECs (IRI-TECs) with 24 h reperfusion αSMA expression was elevated compared to control TECs (C-TECs) (Fig. 5B), while the expression of E-Cadherin was decreased (Fig. 5C). TGF-β, N-Cadherin and IDO expression were not increased in IRI-TECs compared to C-TECs (Fig. 5A, D, and E, respectively). TECs treated with D-MT (D-IRI-TECs) showed decreased αSMA and N-Cadherin (Fig. 5B and 5D, respectively) compared to IRI-TECs. Further, treatment with the L isomer (L-IRI-TECs) decreased N-Cadherin expression (Fig. 5D).

After 48 h reperfusion, the expression of TGF-β, αSMA and N-Cadherin was increased and the expression of E-Cadherin decreased in IRI-TECs (Fig. 5A, B, D and C, respectively) in comparison with C-TECs, while the expression of IDO (Fig. 5E) remained unchanged. Treatment with both MT isomers decreased TGF-β, αSMA and N-Cadherin. Moreover, both isomers increased E-Cadherin (Fig. 5A, B, D and C, respectively).

In IRI-TECs with 96 h reperfusion a substantial increase in TGF-β, αSMA and N-Cadherin and a decrease in E-Cadherin (Fig. 5A, B, 5D and C, respectively) were found compared to C-TECs. However, IDO expression (Fig. 5E) was unchanged in IRI-TECs. Both treatments decreased TGF-β, αSMA and N-Cadherin and increased E-Cadherin expression (Fig. 5A, B, 5D and C, respectively) in comparison with IRI-TECs. Additionally, the L isomer decreased IDO expression compared

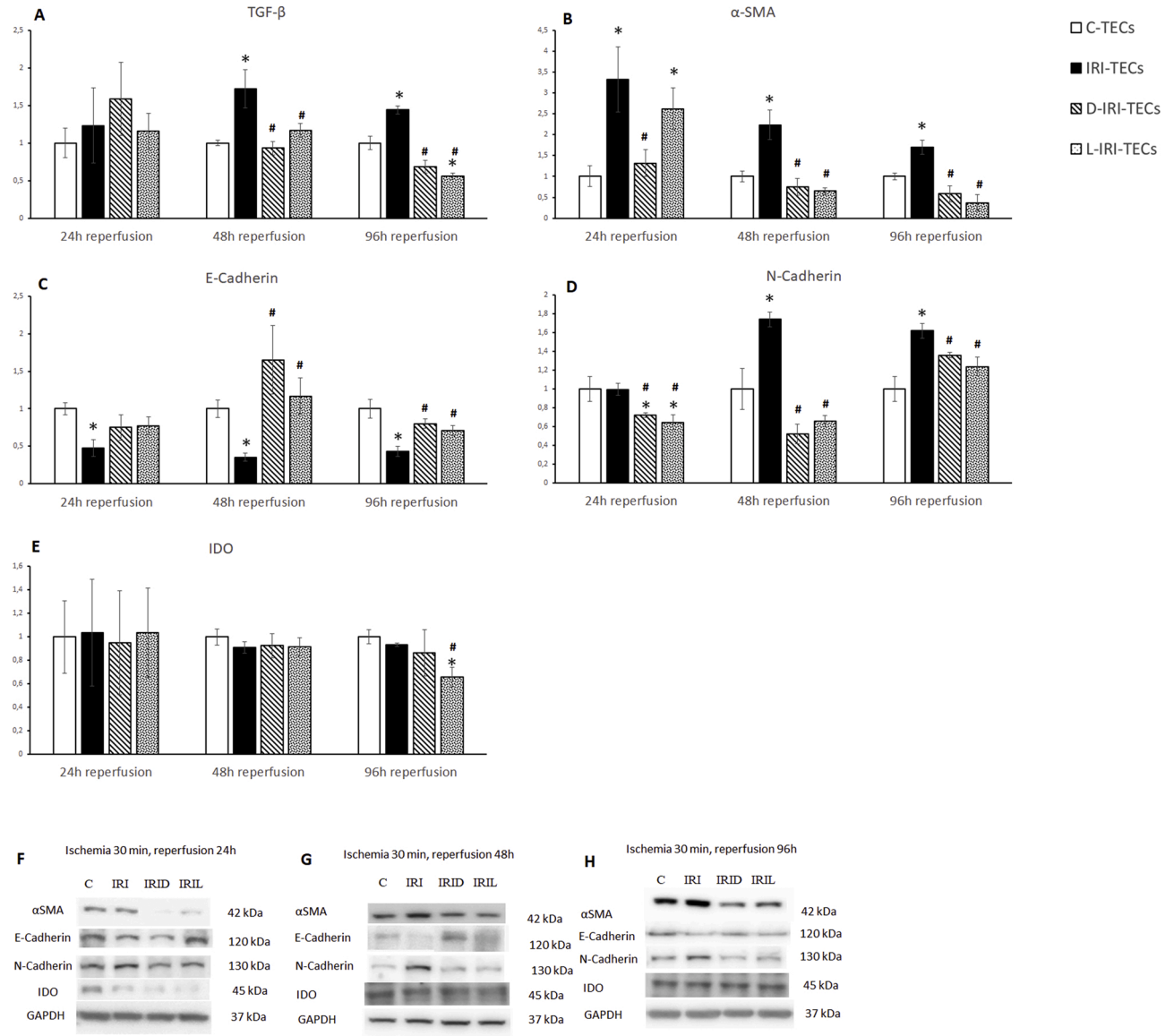


**Fig. 5.** Epithelial to mesenchymal transition of IRI-induced and D-MT/L-MT treated tubular epithelial cells (TECs). mRNA expression in 24, 48, and 96 h of reperfusion of TGF-β (A). Protein expression after 24, 48, and 96 h reperfusion of α-SMA (B), E-Cadherin (C), N-Cadherin (D) and IDO (E). Representative western blots 24 h (F), 48 h (G), and 96 h (H). *p < 0,05 vs C-TECs, #p < 0,05 vs IRI-TECs; •p < 0,05 vs D-IRI-TECs; C-TECs - control cells (TECs); IRI-TECs - IRI-induced TECs; D-IRI-TECs - IRI-induced TECs treated with D-MT; L-IRI-TECs - IRI-induced TECs treated with L-MT. Data are presented as mean ± SEM.

to both C-TECs and IRI-TECs (Fig. 5E). The relative expression of TGF-signalling proteins (pP38 expression; MEK, ERK and JNK phosphorylation) is depicted in the Supplement (Supp. 3A-D) as well as the mRNA expression of E-Cadherin, N-Cadherin, Snai1, Snai2/Slug, Twist1 and Zeb1 (Supp. 2A-F).

### 2.6. L-MT and D-MT downregulate TLR4 signalling induced by IRI both in vivo and in vitro

The observed effect of MT isomers on TECs EMT and on renal damage in the rat model points to the possible involvement of toll-like receptor 4 (TLR4) signalling, which has a considerable impact on IRI-related damage to the kidney. It plays a role in fibrosis [15,16], the expression of NK cell ligands in renal TECs [17], and EMT of renal TECs through the NLRP3 pathway [18]. Hence, we investigated TLR4 signalling both *in vivo* and *in vitro*. In the rat and TEC IRI model, we assessed the mRNA expression of TLR4 and NLRP3, which links TLR4 activation to EMT and TGF-β-mediated cascade [25] and TLR4 downstream molecules – IL-1β, IL-6, IL-17 – as well as the protein expression of proinflammatory cytokine Ccl5/RANTES.

In the 24 h reperfusion *in vivo* model, we found increased NLRP3, IL-1β and IL-6 mRNA expression in the IRI group (Fig. 6B, C and D, respectively), while TLR4, IL-17 and Ccl5 expression were not changed (Fig. 6A, 6E and 6 F, respectively). D-MT treatment decreased TLR4, NLRP3, IL-1β, IL-6 and Ccl5 (Fig. 6A, B, C, D and F, respectively), while L-MT treatment did not affect the expression of all the assessed molecules (Fig. 6A-F). This effect occurs in parallel with improved renal function in this group. In 48 h reperfusion, the expression of NLRP3, IL-1β, IL-6, IL-17 and Ccl5 was increased in the IRI group (Fig. 6B, C, D, E and F, respectively). Treatment with D-MT decreased the expression of IL-6, whereas L-MT had no effect (Fig. 6D). However, the expression of TLR4 was decreased by both isomers compared to the IRI group, in accordance with improved renal function (Fig. 6A). In 96 h reperfusion, we found a significant increase in the expression of all the investigated molecules (TLR4, NLRP3, IL-1β, IL-6, IL17, Ccl5) in the IRI group, which was decreased by treatment with L isomer, again in parallel with improved renal function. Treatment with D-MT had no such effect (Fig. 6A-F, respectively).

Likewise, we assessed the expression of TLR4, NLRP3 and Ccl5 in our TEC *in vitro* IRI model. After 24 h reperfusion, there was no change in the expression of any assessed molecule (Fig. 6G, H and I, respectively). However, in 48 h and 96 h reperfusion the expression of TLR4, NLRP3 and Ccl5 were increased in IRI-TECs compared to C-TECs (Fig. 6G, H and I, respectively), while both isomers decreased the expression of the above-mentioned molecules (Fig. 6G, GH and I, respectively).

### 2.7. L-MT and D-MT decreased NKG2D and NKG2D ligands expression both in vivo and in vitro

During IRI, the activation of TLR4 leads to the expression of NKG2D ligands by TECs, resulting in NK cell-mediated attack upon tubules [19]. Therefore, we investigated the effect of MT isomers on the expression of NKG2D and its ligands in the rat IRI model. We detected the mRNA expression of NKG2D receptor expressed by NK cells as well as its ligands, RRLT and Raet1d, expressed by TECs.

We found that in the rat IRI model with 24 h reperfusion, an increase in Raet1d and RRLT expression was found in the IRI group (Fig. 7A and 7B, respectively) compared to SHAM, while NKG2D expression was unaffected (Fig. 7C). Treatment with both MT isomers led to a significant decrease in Raet1D and RRLT (Fig. 7A and B, respectively). Furthermore, D-MT decreased NKG2D expression (Fig. 7C). In 48 hr reperfusion, NKG2D, Raet1d and RRLT expression were increased in the IRI group in comparison with SHAM, and treatment with D-MT decreased all the above-mentioned mRNA (Fig. 7C, A and B, respectively), while treatment with L isomer decreased the expression of NKG2D and Raet1d (Fig. 7C and A, respectively). In 96 h reperfusion, we found a trend towards an increase in NKG2D expression and increased expression of Raet1d and RRLT in the IRI group compared to SHAM (Fig. 7C, A and B, respectively). L-MT decreased Raet1d expression (Fig. 7A), while on the other hand, D-MT treatment decreased RRLT expression (Fig. 7B).

To confirm our findings suggesting that MT isomers can influence this pathway, we co-cultured freshly isolated TEC induced by IRI and treated it with either D-MT or L-MT with NK cells. First, we confirmed the phenotype of the isolated NK cells by FACS, which revealed that 67.37 % of the NK cells were NKG2D positive (Fig. 7D).

We found that NKG2D and TLR4 expression in NK cells co-cultured for 48 h and 96 h with IRI-TEC was increased compared to C-TEC co-cultured NK cells (Fig. 7E and F, respectively). NKG2D and TLR4 in NK cells were decreased by co-cultivation with both D-IRI-TECs and L-

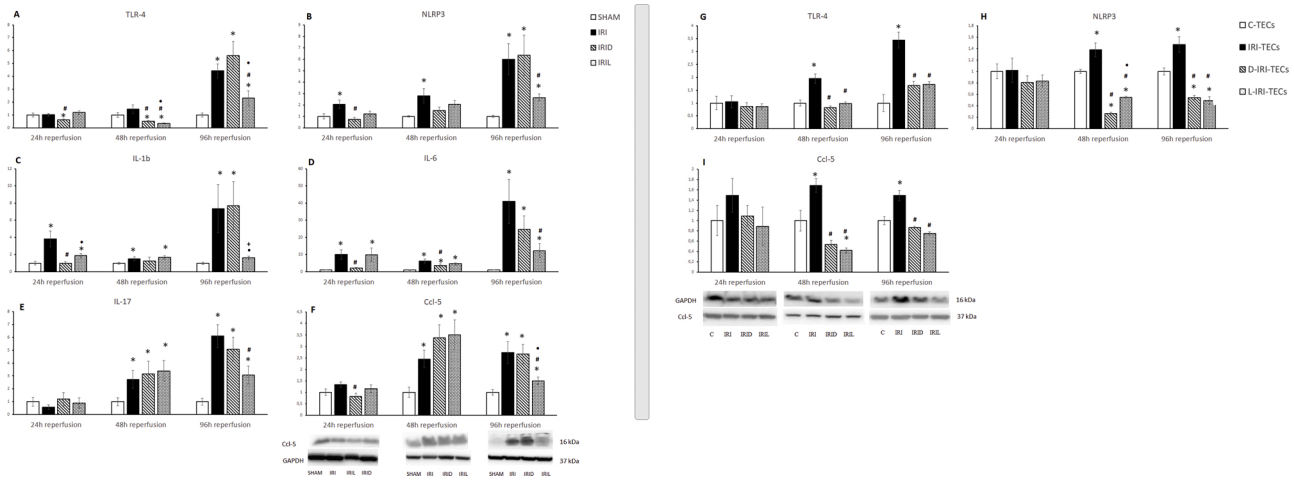


**Fig. 6.** TLR-4 signalling in kidneys of IRI-induced rats and tubular epithelial cells (TECs) treated with D-MT or L-MT. IRI induced rats: mRNA expression after 24, 48 and 96 h reperfusion of TLR-4 (A), NLRP3 (B), IL-1b (C), IL-6 (D), IL-17 (E). Ccl-5 protein expression in 24, 48, and 96 h of reperfusion, including representative western blots (F). IRI-induced TECs: mRNA expression in 24, 48, and 96 h of reperfusion of TLR-4 (G) and NLRP3 (H). Protein expression after 24, 48, and 96 h reperfusion of Ccl-5, including representative western blots (I). *$p < 0,05$ vs SHAM/C-TECs, #$p < 0,05$ vs IRI/IRI-TECs, •$p < 0,05$ vs IRID/D-IRI-TECs; + vs. IRI $p = 0,07$; SHAM - sham-operated rats; IRI - rats that underwent IRI; IRID - rats that underwent IRI treated with D-MT; IRIL - rats that underwent IRI treated with L-MT; C-TECs - control cells (TECs); IRI-TECs - IRI-induced TECs; D-IRI-TECs - IRI-induced TECs treated with D-MT; L-IRI-TECs - IRI-induced TECs treated with L-MT. Data are presented as mean ± SEM.

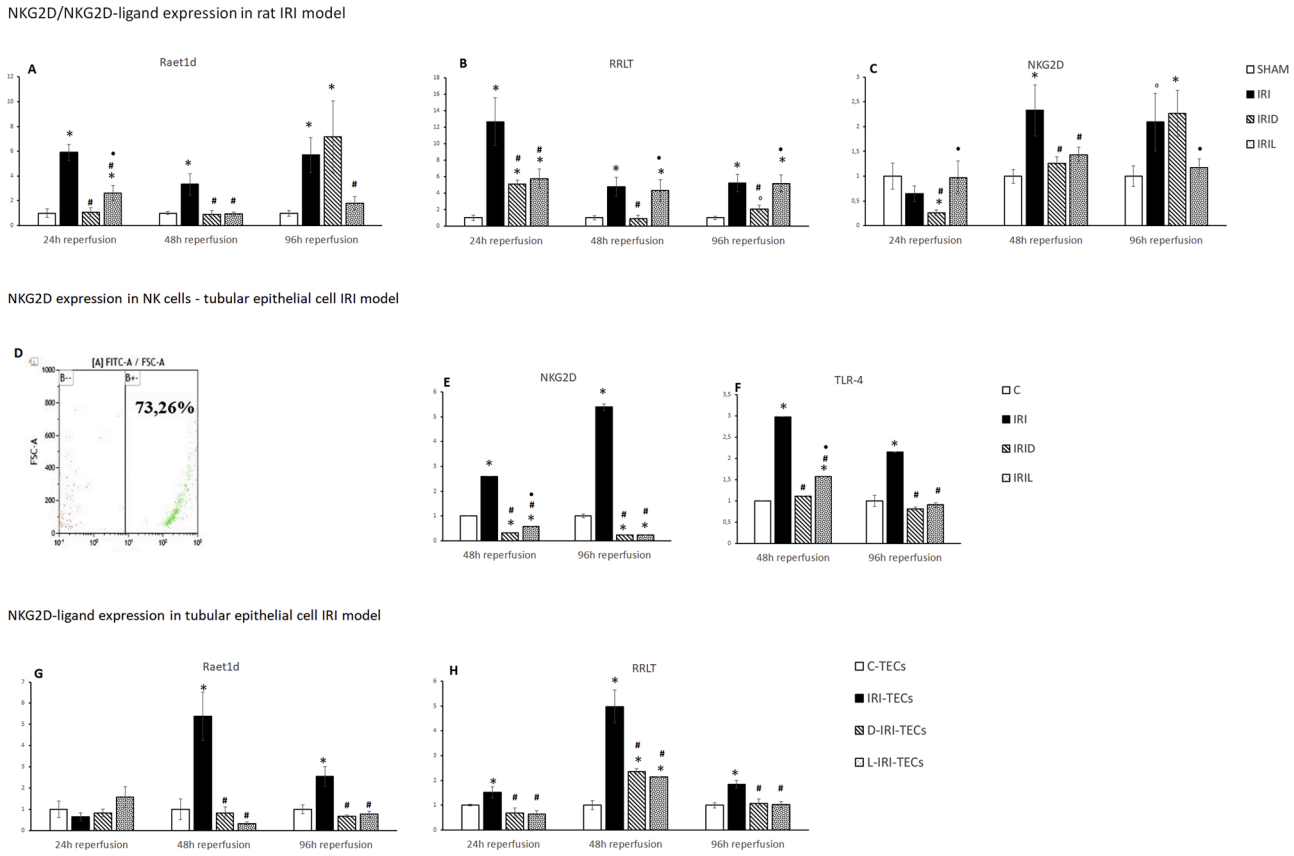


**Fig. 7.** NKG2D/NKG2D-ligand expression *in vivo* and *in vitro*. IRI-induced rats treated with D-MT or L-MT: mRNA expression after 24, 48, and 96 h reperfusion of Raet1d (A), RRLT (B), and NKG2D (C). NK cells co-cultured with IRI-induced TECs treated with D-MT or L-MT: NKG2D expression in NK cells (D). NKG2D (E) and TLR-4 (F) mRNA expression in NK cells co-cultured with TECs after 48 and 96 h of reperfusion. Raet1d (G) and RRLT (H) mRNA expression in IRI-induced TECs after 24, 48, and 96 h of reperfusion. *$p < 0,05$ vs C/C-TECs/SHAM, #$p < 0,05$ vs IRI/IRI-TECs, •$p < 0,05$ vs IRID, ° vs. SHAM $p = 0,07$; C - NK cells co-cultured with C-TECs; IRI - NK cells co-cultured with IRI-TECs/ rats that underwent IRI; IRID - NK cells co-cultured with D-IRI-TECs/ rats that underwent IRI treated with D-MT; IRIL - NK cells co-cultured with L-IRI-TECs/ rats that underwent IRI treated with L-MT; C-TECs - control cells (TECs); IRI-TECs - IRI-induced TECs; D-IRI-TECs - IRI-induced TECs treated with D-MT; L-IRI-TECs - IRI-induced TECs treated with L-MT; SHAM - sham-operated rats. Data are presented as mean ± SEM.

IRI-TECs (Fig. 7E and F, respectively). Additionally, we assessed the expression of NKG2D ligands Raet1d and RRLT in TECs co-cultured with NK cells. We found a significant increase in Raet1d in both 48 h and 96 h reperfusion IRI-TECs compared with C-TECs (Fig. 7G). In 24 h reperfusion, we did not find such a change in Raet1d expression in IRI-TECs or as a result of D-MT or L-MT treatment (Fig. 7G). However, we did find a significant increase in RRLT expression in IRI-TECs in both 48 h and 96 h reperfusion (Fig. 7H). Treatment with both isomers decreased both NKG2D ligands in 48 h and 96 h reperfusion and the expression of RRLT in 24 h reperfusion (Fig. 7G and 7H, respectively) in IRI-TECs co-cultured with NK cells.

### *2.8. L-MT and D-MT affect TLR4 and MAPK signalling in ischemic dendritic cells differently and possibly attenuate renal IRI via affecting T cell proliferation and differentiation and their tryptophan hydroxylase 1 expression*

Dendritic cells (DCs) influenced by the TLR4 signalling pathway play a role in the pathogenesis of renal IRI. We thus investigated the effect of both MT isomers on DCs and the effector T cells in the IRI *in vitro* model. Fresh isolated DCs were cultured until mature, treated with either L-MT or D-MT, underwent ischemia, and were co-cultured with T cells in a mixed leukocyte reaction (MLR). We assessed the mRNA and protein expression of IDO, the mRNA expression of tryptophanyl-tRNA synthetase (TTS; upregulated by IDO activity), the expression of TGF-β, interferon-gamma (IFN-γ), TLR4, NLRP3 and the protein expression of Ccl-5. Lastly, we assessed the protein expression of pP38 and phosphorylation of MEK and ERK due to previously observed changes in MAPKs phosphorylation associated with the effect of D-MT on DC TLR4 signalling, independent of IDO activity.

We found that in IRI-induced DCs (IRI-DCs) the mRNA expression of IDO was decreased (Fig. 8A) compared to control DCs (C-DCs). D-MT (D-IRI-DCs) had no effect on IDO expression; however, L-MT (L-IRI-DCs) increased IDO expression compared to all groups (Fig. 8A). On the other hand, IDO protein level was decreased in the L-IRI-DCs group compared to both C-DCs and IRI-DCs (Fig. 8B).

The expression of TTS was increased in IRI-DCs (Fig. 8C) and not changed in D-IRI-DCs, while in L-IRI-DCs it was decreased compared to both IRI-DCs and D-IRI-DCs (Fig. 8C). TGF-β was increased in IRI-DCs (Fig. 8D), and treatment with the L isomer led to its decrease (Fig. 8D). The expression of TLR4 signalling molecules TLR4, NLRP3 and Ccl5 was increased in IRI-DCs, and both MT isomers led to its decrease (Fig. 8E, 8 F and 8 G, respectively). This was accompanied by a significant decrease in MEK, and ERK phosphorylation increased in IRI-DCs (Fig. 8H and 8I, respectively). Also, pP38 expression was increased in IRI-DCs (Fig. 8J) and decreased in L-IRI-DCs (Fig. 8J); however, it was increased in D-IRI-DCs even compared to untreated IRI-DC (Fig. 8J).

Different T cell subsets (influenced by DCs) were shown to have various roles in IRI (Th1 – harmful, Th2 – possibly protective, Th17 – possibly harmful, Treg – protective). Therefore, we assessed the mRNA expression of characteristic cytokines and transcription factors for these subsets of T cells stimulated by IRI-DC treated with either D-MT or L-MT. We found an increase in the expression of IFN-γ, T-box transcription factor (T-bet), GATA-binding protein 3 (GATA3), IL-4, IL-17, Retinoid-

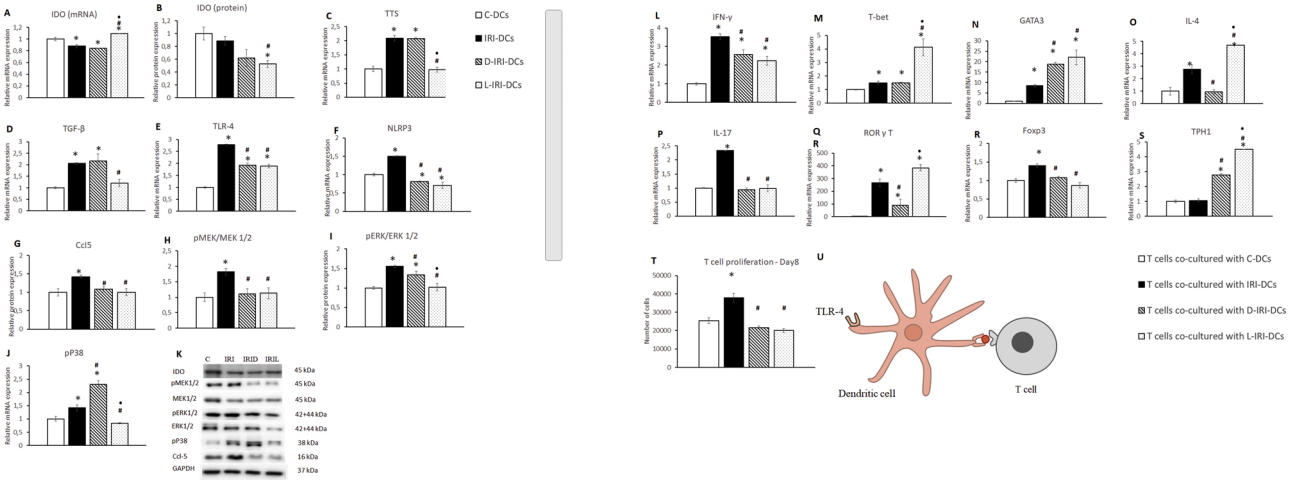


**Fig. 8.** Effect of D-MT/L-MT treatment on ischemic dendritic cells: mRNA expression of IDO (A), TTS (C), TGF-β (D), TLR-4 (E), NLRP3 (F). Protein expression of IDO (B), Ccl5 (G), pMEK/MEK1/2 (H), pERK/ERK 1/2 (I), pP38 (J). Representative western blots (K). Effect of ischemic dendritic cells treated with D-MT/L-MT on T cells. mRNA expression of IFNγ (L), T-bet (M), GATA3 (N), IL-4 (O), IL-17 (P), RORγT (Q), Foxp3 (R), TPH1 (S); T cell proliferation on day 8 in mixed leukocyte reaction (T); Schematic image – the interaction of DC with T cell (U). *p < 0,05 vs C-DCs/ T cells co-cultured with C-DCs, #p < 0,05 vs IRI-DCs/T cells co-cultured with IRI-DCs; • p < 0,05 vs. D-IRI-DCs/ T cells co-cultured with D-IRI-DCs; C-DCs - control cells; IRI-DCs – IRI-induced DCs; D-IRI-DCs – IRI-induced DCs treated with D-MT; L-IRI-DCs – IRI-induced DCs treated with L-MT; TTS - Tryptophanyl-tRNA synthetase. Data are presented as mean ± SEM.

related orphan receptor gamma T (RORγT) and (Forkhead box P3) FoxP3 in T cells co-cultured with IRI-DCs (Fig. 8L, 8 M, 8 N, 8O, 8 P, 8Q and 8R, respectively). In T cells co-cultured with D-IRI-DCs the expression of IFN-γ, IL-4, IL-17, RORγT and FoxP3 was decreased, indicating a possible decrease in the presence of all Th phenotypes (Fig. 8L, 8O, 8 P, 8Q and 8R, respectively). On the other hand, D-IRI-DCs increased GATA3 expression in co-cultured T cells (Fig. 8N), which could indicate the presence of protective Th2 T cells. L-IRI-DC decreased the expression of IFN-γ and IL-17 in co-cultured T cells (Fig. 8L and 8 P, respectively), which is in parallel with its beneficial effect on renal IRI. Moreover, T cells co-cultured with L-IRI-DCs exhibited increased IL-4 and GATA3 expression (Fig. 8O and 8 N, respectively), indicating differentiation towards the Th2 protective phenotype. We also found an increase in T-bet expression (Fig. 8M) and a decrease in FoxP3 expression (Fig. 8R) in T cells co-cultured with L-IRI-DCs compared to T cells co-cultured with IRI-DC.

Furthermore, we assessed the expression of tryptophan hydroxylase 1 (TPH1). We did not find any change in TPH1 expression in T cells co-cultured with IRI-DCs compared to T cells co-cultured with C-DCs (Fig. 8S). Nonetheless, we found a significant increase in TPH1 in T cells co-cultured with D-IRI-DCs compared to C-DCs and an even higher increase in T cells co-cultured with L-IRI-DCs (Fig. 8S). Additionally, we assessed the proliferation of IRI-DCs co-cultured T cells and found that IRI-DC leads to increased T cell proliferation in comparison with C-DCs (Fig. 8T) and both D-IRI-DCs and L-IRI-DCs markedly decreased T cell proliferation compared to IRI-DC-stimulated T cells (Fig. 8T).

The effects of the 1-MT isomers are depicted in an additional diagram (Fig. 9) and summarized in Table 4.

## 3. Discussion

In this study, we show for the first time that both 1-methyl-tryptophan isomers – D-MT and the rather rarely investigated L-MT – have a protective effect in renal IRI. Moreover, we show that the effect of both isomers is not exclusively dependent on IDO activity and/or its expression; it varies with reperfusion length and differs on a molecular level.

First, we confirmed the ability of L-MT/D-MT to inhibit IDO activity *in vitro*. Although both MT isomers inhibited IDO activity, L-MT was more potent than the D isoform. Considering IDO inhibition as a suggested protective mechanism of MT in renal IRI, this observation supports L-MT as a more suitable choice than D-MT in IRI treatment. Our finding is in agreement with those of Peterson et al., who documented a stronger ability of the L isomer to inhibit recombinant IDO1 in both a cell-free environment as well as in interferon (IFN)-γ-treated HeLa cells [22,26]. Furthermore, it was shown that the L isomer, but not the D isomer, reversed the arrest of T cell proliferation in human cancer cells *in vitro,* suggesting the stronger IDO inhibitory potential of L isomer [27].

Further, we investigated the effect of MT isomers in a rat IRI model. At 24 h reperfusion, D-MT improved renal function and decreased markers of prefibrosis, while L-MT had no effect. However, the beneficial effect of D-MT was not accompanied by changes in IDO protein expression or its activity. At 48 h reperfusion both isomers improved renal function; however, only D-MT treatment was associated with decreased IDO activity. IDO expression was unchanged. On the other hand, the effect of the L isomer was accompanied by decreased KMO activity. At 96 h reperfusion the D isomer had no effect on renal function or prefibrosis, and it no longer changed IDO activity or expression, while L-MT did improve renal function as well as markers of prefibrosis. This was associated with a decrease of both IDO and KMO activity, suggesting at least partly an enzyme-depend protective effect of L-MT. In conclusion, our data show that the effect of D-MT and L-MT on renal IRI is independent of IDO protein expression and not exclusively dependent on IDO activity. Since IDO inhibition was earlier proposed as a mechanism of MTs effect, our results point to another mechanism of action than was originally suggested. A possible explanation of the delayed effect of the L isomer may be a different organ distribution of MT isomers after the application of treatment. It was shown that after i.v. administration, the L isomer bypasses the kidneys in the first 20 min, with the highest distribution observed in the pancreas, whereas the D isomer is distributed directly to the kidney [8]. Differences in organ distribution could explain the clear differences in MT isomers' effect in the *in vivo* model and the more subtle or no differences in our *in vitro* models. Another explanation of the different *in vivo* MT effects may be its possible dependency on the affected cell/tissue type. It was shown that the L isomer abrogates IDO activity in dendritic cells stimulated with lipopolysaccharide, [6], whereas the D isomer interferes with TLR4 signalling independently of IDO [20].

Additionally, we have shown that L-MT decreased KMO activity in accordance with renal function improvement and increased survival of the rats. It has been reported that mice lacking KMO activity had preserved renal function, reduced renal tubular cell injury, and a fewer number of infiltrating neutrophils [28]. Moreover, 3−OH-kyn (as a

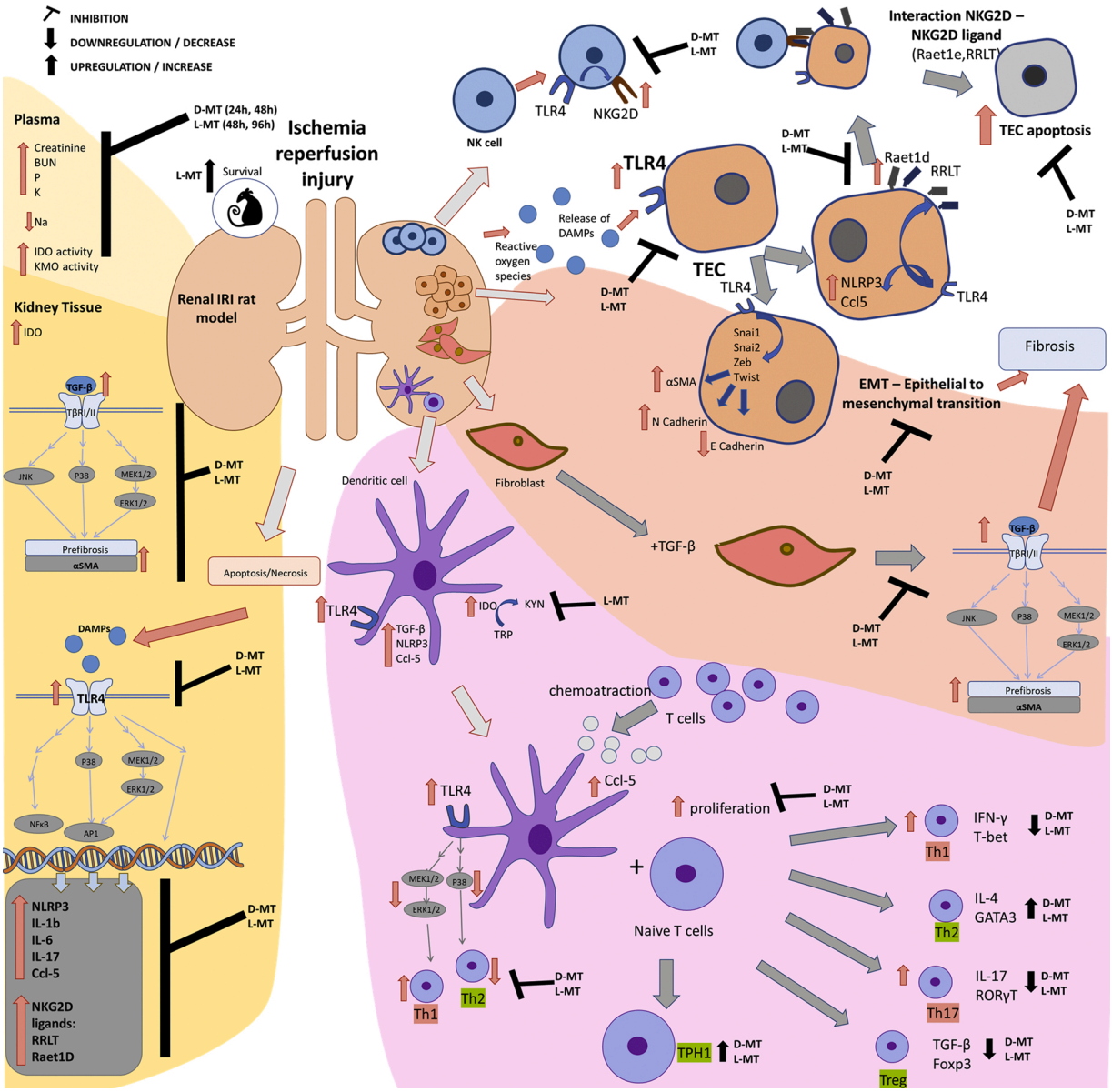


**Fig. 9.** Scheme depicting effects od 1-MT isomers.

result of KMO activity) mediates cytotoxicity and induces cell apoptosis [29], which suggests a possible link between decreased KMO activity and improved renal function in L-MT-treated rats.

Our data show for the first time the antifibrotic properties of MT isomers, as documented by the decreased expression of TGF-β, as well as the proteins involved in the TGF-β-mediated pathway, and decreased αSMA, both *in vivo* (paralleling improved renal function) and *in vitro*. Data on the effect of MT (particularly D-MT) on prefibrosis are scarce and in contradiction with our results. Previously it was reported that D-MT (1 mM) acts as a profibrotic agent via enhancing epithelial to mesenchymal transition (EMT) of MDCK (Madin-Darby Canine Kidney) cells simultaneously stimulated by TGF-β1 1 ng/mL [13] and also in T24 human bladder cancer cells stimulated with TGF-β1 5 ng/mL [30]. In our TECs model, we used different types of cells without any additional TGF-β1 stimulation, which could account for the difference.

Another possible explanation of the renoprotective MT effect in our study is its impact on EMT in TECs. EMT causes cell cycle arrest and leads to decreased expression of transporters in TECs [10]. We have shown in our *in vitro* TECs IRI model that MT isomers influenced paracrine TGF-β as well as the transcriptional factors of EMT, epithelial markers and mesenchymal proteins, indicating attenuation of EMT. Moreover, this effect was analogous to the beneficial effect of MT on renal function and prefibrosis *in vivo*. Limited data are available regarding this issue and furthermore are contradictory to our data – D-MT induced EMT in a model of unilateral ureteral obstruction through IDO inhibition [13]. Contrarily, the effect of D-MT in T24 cells stimulated by TGF-β1 [30] was IDO independent.

Interference with TLR4 may represent another IDO-independent protective mechanism of MT, particularly D-MT [20,30–32]. TLR4 signalling plays a considerable role in mediating kidney cell damage [14], fibrosis [15], the responses of fibroblast to TGF-β [16], EMT of renal TECs [18] and upregulation of NK cell ligands, expressed by renal TECs [17]. We found that both MT isomers decreased the expression of TLR4 signalling molecules in parallel with the improvement of renal function *in vivo*, as well as in our TECs IRI model and in NK cells co-cultured with IRI-stimulated TECs. Hence, we show for the first time the association between the renoprotective effect of both MT isomers and TLR4 signalling. This is in accordance with Writhgen et al., who reported TLR4 interference of MT in a model of septic shock in LPS-challenged pigs [32]. TLR4 engagement can lead to an increase in Ccl5 expression [33]

concordantly with our research and it can result in increased infiltration of lymphocytes. We did not observe such an increase; however, it can occur later than after 96 h of reperfusion. During IRI, TLR4 engagement leads to NKG2D ligands expression in renal TECs [17], and its upregulation induces NK cells to attack tubules [34], resulting in tubular apoptosis. However, information concerning rat ligands of the NKG2D receptor on NK cells is scarce [35]. To the best of our knowledge, we are the first to show that NKG2D and NKG2D ligands RRLT and Raet1d are increased in rat postischemic kidneys and IRI-stimulated TECs and that both MT isomers decreased them, in parallel with improved renal function. Additionally, we found that IRI-stimulated TECs treated with MT decreased NKG2D expressed by co-cultured NK cells. It has been previously shown that NKG2D expression can be influenced by an IDO metabolite – kynurenine [36]. However, in our study, this effect seems to be IDO independent. Hence, we can conclude that influencing the expression of NKG2D ligands in TECs and NKG2D in NK cells represents another mechanism by which both MT isomers may participate in their renoprotective effect in IRI.

Mohib et al. suggested that D-MT improves renal IRI via IDO inhibition, as IDO can enhance the injury of TECs through Fas/FasL [2]. However, we found that changes in Fas and FasL expression were not always in parallel with improved renal function or IDO activity. Moreover, Fas/FasL-associated changes were related mainly to our *in vitro* experiments (Supp. 5). We can therefore assume that although changes in FasL expression indicated MT interposed cell death *in vitro*, it does not provide sufficient explanation in our *in vivo* experiment.

Lastly, we investigated the influence of MT isomers on IRI-induced DCs and their effector function on T cells. DCs infiltrate the renal interstitium during IRI, inducing pro-inflammatory mediators [21,37]. It has also been shown that DCs play a role in the TLR4 signalling pathway *in vitro* [38] and in myocardial [39], intestinal [40] and hepatic [41] IRI, and that D-MT interferes with this signalling independent of IDO activity [20].

We found a phenotype steered towards Th2 in T cells induced by DCs treated with both isomers. Th2 shows rather a protective effect in renal IRI [42]. This finding is consistent with Agaugue et al., when D-MT-treated DCs steered the T cell phenotype towards Th2 [20]. Stimulation of TLR4 is crucial in the differentiation of T cells towards Th1, as TLR4 engagement induces p38 phosphorylation and suppresses ERK activation. p38 is a critical inducer of IL-12(p70), which is pivotal for Th1 proliferation. Therefore, we hypothesized that decreased TLR4 expression in DCs treated with MT isomers could lead to this phenotype shift, as suppression of the activation of TLR4 promotes Th2 differentiation [43]. Additionally, we observed a decreased proliferation of T cells stimulated with IRI-induced DCs and treated with both MT isomers. In DCs, both isomers decreased the expression of TLR4 signalling molecules, which was accompanied by decreased MEK and ERK phosphorylation. Moreover, the effect of the L isomer was paralleled by decreased pP38 expression. Thus, we have shown that both MT isomers affect IRI-DCs with some differences and alter their TLR4 signalling pathway as well as MAPK phosphorylation, which could have a renoprotective effect. Furthermore, D-MT did not change IDO expression either on the mRNA or on the protein level in DCs; however, the L isomer increased IDO mRNA expression. In contrast, the protein expression of IDO was decreased by L-MT, as was the expression of TTS. This suggests that the L isomer inhibited IDO activity and led to a compensatory increased mRNA. The difference is in accordance with the previously described observation that the L isomer, not the D isomer, can abrogate IDO activity in human DCs [6]. We thus showed a different mechanism of action for each isomer.

Previous evidence suggests that D-MT could upregulate TPH1 expression (enzyme catalysing trp conversion to serotonin) and thus the synthesis of melatonin [44], which has protective properties in renal IRI [45]. We found an increase in TPH1 in T cells that were co-cultured with D-IRI-DCs and an even higher increase in T cells co-cultured with L-IRI-DCs. Our data show that influencing DCs and their effector T cells can represent another possible mechanism of the renoprotective attributes of MT isomers in IRI.

### 3.1. Conclusion

We have shown that both MT isomers have the potential to improve outcomes after IRI. The D isomer acts faster but for a shorter time, while the L isomer has a delayed effect and lasts longer. Moreover, only L-MT improved the survival of rats. Taken together, this points towards L-MT as a novel therapeutic target in renal IRI. However, in the future treatment of IRI in patients, a DL-MT racemic mixture would be beneficial due to the faster onset of the D isomer's action. We are the first to show the protective mechanism of MT isomers, which may include downregulation of TLR4 and its signalling molecules, a probable decrease in NKG2D-NKG2D ligand-mediated apoptosis in TECs as well as the EMT programme in TECs. We have also shown that some mechanisms of MT isomers varied. L-MT affected DCs and their effector T cells while inhibiting IDO, whereas D-MT acted in a similar manner IDO-independently.

## 4. Materials and methods

### 4.1. Animals

All experimental procedures involving the use of experimental animals were approved by a local Ethics Committee and the State Veterinary and Food Administration of the Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals: Eight Edition (2010), published by the US Committee for the Update of the Guide for the Care and Use of Laboratory Animals of the National Research Council, and to the EU adopted Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for experimental and other scientific purposes.

Wistar rats (male, 10–12 wk old, 200−250 g) and Lewis rats (male, 10–12 wk old, RT1[l], 180−220 g) were used in the experiments (Department of Toxicology and Laboratory Animals Breeding, Dobra Voda, Slovak Republic). Standard housing conditions were maintained (12 h light/dark cycle, well-ventilated room, the temperature of 23 °C, humidity-controlled environment) and rats received water and food *ad libitum*. All rats were acclimatized to the animal house environment at least 5 days before the experiments.

### 4.2. Renal IRI model

For each reperfusion time point (24 h, 48 h, 96 h) Wistar rats were divided into 4 groups: control sham-operated rats (SHAM), rats undergoing ischemia-reperfusion injury (IRI), rats undergoing ischemia-reperfusion injury treated with D-MT (IRID), and rats undergoing renal ischemia-reperfusion injury treated with L-MT (IRIL). Rats that died prematurely from IRI-unrelated causes were excluded from the study. Number of animals that completed the study: 24 h-SHAM n = 7; 24 h-IRI n = 5; 24 h-IRID n = 6; 24 h-IRIL n = 7; 48 h-SHAM n = 8, 48 h-IRI n = 9; 48 h-IRID n = 9; 48 h-IRIL n = 7; 96 h-SHAM n = 6; 96 h-IRI n = 5; 96 h-IRID n = 7; 96 h-IRIL n = 7. Animal count for Kaplan-Meyer analysis: SHAM group (total n = 21, prematurely dead n = 0), IRI group (total n = 26, prematurely dead n = 7), IRID group (total n = 26, prematurely dead n = 4), IRIL group (total n = 23, prematurely dead n = 2). IRI was induced as previously described, with minor modifications [46]. Briefly, rats were anesthetized with 3% isoflurane in $O_2$, a midline abdominal incision was performed and heparin solution (150U/kg) *i.p.* was administered before the surgery. Ischemic injury in rats was induced by bilateral renal pedicle clamping for 30 min at 37 °C using microaneurysm clamps. Body temperature was monitored and controlled by the heating system. After the microaneurysm clamps were released, reperfusion of the kidneys was confirmed visually. The abdomen was closed in two layers using standard 4–0 and 3−0 sutures. Sham-operated

rats received identical surgical procedures, except that microaneurysm clamps were not applied. To maintain fluid balance, all of the rats were supplemented with 1 mL of prewarmed phosphate-buffered saline administered intraperitoneally directly after surgery. Rats received orally applied (by oral gavage) D-MT or L-MT (Sigma-Aldrich) in a dose of 200 mg/kg dissolved in 1% methylcellulose solution or methylcellulose solution alone (SHAM operated animals) 24 h and 1 h before induction of ischemia and every 24 h of following reperfusion (24 h, 48 h or 96 h of reperfusion) until sacrifice. After 24 h, 48 h or 96 h of reperfusion, the rats were anesthetized with isoflurane and an aortic blood sample was collected. Plasma was isolated and stored at −80 °C. The kidney was perfused with saline and removed. Two midcoronal slices were snap-frozen in liquid nitrogen, stored at 80 °C, and further used for mRNA and protein isolation.

### 4.3. Determination of renal function

Kidney function was determined based on serum creatinine, blood urea nitrogen (BUN) and serum Na, P and K levels, which were assessed in commercial laboratories (Alphamedical, Bratislava, SR).

### 4.4. Determination of IDO, hIDO and KMO activity – High-performance liquid chromatography (HPLC) with mass spectrometric detection

To assess the ability of both D-MT and L-MT to inhibit IDO we assessed the levels of tryptophan (trp) and its metabolites, kynurenine (kyn) and 3-hydroxykynurenine (3−OH-kyn). The estimated IDO activity was calculated as the kyn/trp ratio. We also assessed kynurenine 3-monooxygenase (KMO) activity (calculated as 3−OH-kyn/kyn) in the renal IRI rat model, which has been shown to be a critical regulator of renal IRI [28] and could also be influenced by IDO inhibition. High-performance liquid chromatography (HPLC) assay was used: IDO activity was measured in HEK293 cells lysate or in rat plasma by quantifying the metabolization of tryptophan to kynurenines. Concentrations of tryptophan and kynurenines were measured in the cell lysates/rat plasma using a high-throughput online solid-phase extraction-liquid chromatographic-tandem mass spectrometer as described previously [47]. Fifty microliters of lysate/plasma were pre-purified by automated online solid-phase extraction, using strong cation exchange (propylsulphonic acid) cartridges. Chromatographic separation of the analytes and deuterated analogues occurred by C18 reversed phase chromatography. Mass spectrometric detection was performed in the multiple reaction-monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionization. The detection limit was 30 nmol/l for tryptophan and 1 nmol/l for kynurenines.

### 4.5. Transduction of HEK293 cells with an adenoviral vector carrying human IDO1 gene

To assess the ability of both D-MT and L-MT to inhibit IDO due to the contradictory findings published prior to our research, we studied the effect of D-MT/L-MT on HEK293 cells transduced with an adenoviral vector carrying the human IDO1 gene [4]. RGD-modified adenoviruses were used as vectors for gene delivery [48]. A first-generation recombinant adenovirus type 5 having an RGD sequence in the HI loop was kindly provided by Dr David T. Curiel (University of Alabama at Birmingham, Birmingham, Alabama, USA). This adenovirus contains the genes for GFP (T) and firefly luciferase (L), under the control of a cytomegalovirus (CMV) promoter, in the E1 region (RGD-AdTL). To construct the IDO vector, the IDO gene was isolated from human placenta using a polymerase chain reaction (PCR). Next, the cDNA of human IDO gene (hIDO) was cloned into the shuttle-plasmid pAd-Track-CMV [49]. Using homologous recombination in *Escherichia coli* BJ5183, the shuttle was integrated into the RGD adenovirus plasmid pVK503 [50], resulting in an RGD-modified adenovirus genome with GFP and hIDO under the control of a CMV promoter cloned into the E1 region. Viruses were propagated on 293 cells and purified by double CsCl density centrifugation. The amount of VPs was determined spectrophotometrically at 260 nm. The infectivity of the viruses was determined by plaque assay on HEK 293 cells and expressed as plaque-forming units per millilitre of virus stock (pfu/mL). The VP/pfu ratio of the virus stocks was 100:1 for RGD-AdTIDO.

HEK293 cells were cultured in six-well plates (n = 3) in standard conditions (humidified incubator, 37 °C, 5% CO2) in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 4.5 g/L of glucose supplemented with 10 % FBS (Gibco) and 1% penicillin/streptomycin (penstrep, Lonza). Cells were divided into four groups: C, control cells; IDOt, cells transfected with RGD-AdTIDO; IDOt + D—MT, cells transfected with RGD-AdTIDO treated with D-MT; IDOt + L—MT, cells transfected with RGD-AdTIDO treated with L-MT. After 80 % confluency, cells were pre-treated with D-MT/L-MT (750 μmol/l dissolved in 0.1 N NaOH) for 24 h and 1 h before and 24 h after transduction with hIDO (MOI of 50) and harvested 48 h after transduction for further analysis. Briefly, transduced cells were washed three times using phosphate-buffered saline and subsequently lysed in 300 mL of M-PER® Mammalian protein extraction reagent (Pierce Biotechnology). The obtained cell lysates were used in HPLC.

### 4.6. NIH/3T3 fibroblast model

To investigate possible interference of 1-MT isomers with TGF-β signalling, immortalized embryonal mouse fibroblasts NIH/3T3 (ATCC) were stimulated by recombinant TGF-β1 (5 ng/mL, Peprotech) for 24, 48 h and 96 h and treated with D-MT or L-MT correspondingly with the rat IRI model reperfusion length. NIH/3T3 fibroblasts were cultivated in standard conditions in DMEM medium with 4.5 g/L glucose supplemented with 10 % FBS and 1% penstrep, and the cells were passaged with trypsin (Gibco) solution to reach 80 % confluency. Cells were seeded for the experiment in 6-well plates and divided into 4 groups – untreated cells served as control and three groups were stimulated with recombinant TGF-β1 every 24 h until harvest. Two TGF-β1-stimulated groups were treated with either D-MT or L-MT (750 μmol/l dissolved in 0.1 N NaOH) 24 h and 1 h before first TGF-β1 stimulation and then every 24 h until harvest. Cells were harvested for further analysis 24 h, 48 h or 96 h after the first TGF-β1 treatment.

### 4.7. Renal TECs isolation

Renal TECs were isolated from Lewis rats using a standard protocol with $Fe_2O_3$ magnetic separation and selective media as previously described [51], with minor modifications. Rats were anesthetized with 3% isoflurane in $O_2$ and shaved; the aorta was cannulated and the blood sample was extracted. The rats were perfused with a 2 x 20 mL sterile saline solution and subsequently with a 5 x 10 mL $Fe_2O_3$ solution (0.9 *g* Fe2O3/50 mL/rat). Kidneys were removed and transferred to cold DPBS (Gibco). The renal capsule was removed in a sterile bacteriological Petri dish; the kidney cortex was minced with a surgical blade and incubated with 20 mL collagenase IV. for 1 h at 37 °C (40 mg collagenase IV/20 mL DMEM/F12 (Gibco) medium/rat) and resuspended every 10 min. The suspension was transferred to a 50 mL falcon, and 3 mL of DPBS was added to stop the reaction. Then the suspension was filtered through a 100 μm cell strainer into a new 50 mL falcon. The suspension was centrifuged (5 min/800 rpm). The pellet was washed with 20 mL of DMEM/F12 media in a 50 mL falcon and the cell suspension was divided by flipping the magnet device that attracted $Fe_2O_3$-trapped glomeruli. With the magnet held in place, the suspension was transferred into a new falcon (separation was repeated several times until the removal of all glomeruli attached to iron beads) and filled with DMEM/F12 media supplemented with epidermal growth factor (EGF, Peprotech, 10 ng/mL) and hydrocortisone (Hydrocortisone Medochemie, 200 ng/mL) and then divided into 6-well plates. The suspension consisted of tubules that were let to attach to the bottom of the culture flask.

After 5–6 days of culture, tubular epithelial cells grew out of the tubules. Thereafter, the tubules were washed out by PBS and a fresh medium was added. In the next 2–3 days, cells were in 75 % confluency and ready for the experiments. The epithelial nature of these cells was confirmed by their polygonal shape and typical cobblestone appearance. Using this technique no spindle-shaped cells that could resemble renal fibroblasts were observed within the given time. Cells were cultured under standard conditions.

### 4.8. NK cell isolation

NK cells were isolated from Lewis rat spleen as previously described, with minor modifications [52,53]. Briefly, the aorta of anesthetized (3% isoflurane in $O_2$) and shaved rats was cannulated; blood was extracted, and the rat was then perfused with a 20 mL sterile saline solution. Rat spleen was removed and placed into a sterile falcon with a cold saline solution. The spleen was minced with a sterile blade in a sterile bacteriological Petri dish in DPBS. Then 10 mL of Collagenase VI (2 mg/mL in sterile DPBS) was added and the suspension was incubated for 60−90 min at 37 °C. The reaction was stopped with 10 mL DPBS, and the suspension was centrifuged (8 min, 1800 rpm, 20 °C). The supernatant was discarded and the pellet resuspended in 20 mL of RPMI 1640 media (Gibco). The cell suspension was passed through sterile nylon wool columns (0.5 *g* nylon wool/50 mL syringe tube) – 5 mL of cell suspension per one column. The flowthrough of all columns was immediately transferred into one additional column and incubated for 1 h. Columns were washed with 20 mL of media per column without squeezing. The collected cell suspension was centrifuged (8 min, 1800 rpm, 20 °C); the supernatant was removed and cells were resuspended in 2 mL of media and cultured $1 \times 10^6$ cells/well in 24-well plates with recombinant human IL-2 (1000 U/mL, Peprotech) until matured (for 4–8 days) before co-culturing with TECs. The medium was changed and IL-2 was added every second day. The phenotype of the cells was characterized by flow cytometry.

### 4.9. IRI model (hypoxia-reoxygenation) on TECs with subsequent co-culture with NK cells

To test the hypothesis that MT isomers could influence NKG2D-NKG2D ligand-mediated apoptosis of TECs, TECs induced by IRI were co-cultured with NK cells and further analyzed. Renal TECs were cultured in six-well plates (n = 3) in standard conditions in DMEM/F12 medium supplemented as described above with regular medium exchange. After 80 % confluency, the cells were subjected to ischemic (hypoxic) conditions: the medium was removed and TECs were washed with DPBS twice; then 350 µl of sterile mineral oil (Sigma-Aldrich) was applied for 30 min at 37 °C. After ischemia, the mineral oil was washed out with PBS, and a medium with NK cells was added (reoxygenation). Further, TECs were co-cultured with NK cells. Cells were treated with either D-MT or L-MT (750 µmol/l dissolved in 0.1 N NaOH) 24 h and 1 h before induction of ischemia and every 24 h of the following reperfusion for a total period of 24 h, 48 h or 96 h. After 24 h, 48 h or 96 h of reperfusion, NK cells and TECs were collected for further analysis.

### 4.10. Isolation of dendritic cells (DCs) and DCs IRI model

To investigate whether the effect of MT isomers could include changes in postischemic DCs that may result in changes in T cell differentiation and/or proliferation, a model with ischemic DCs treated with either D-MT or L-MT was used. DCs were isolated from Lewis rats using a standard protocol, with minor modifications [54–56]. Femur and tibia were obtained from both legs of the Lewis rat, flushed from both sides with 10 mL of sterile saline, and centrifuged (1800 rpm, 8 min, RT). Erythrocytes were lysed with ice-cold $NH_4Cl$ solution (Sigma Aldrich; 8 min, on ice, 10 mL per tube). DPBS was added to a 50 mL falcon, and the falcon was centrifuged (1800 rpm/8 min/ 20 °C). Cells were resuspended in 1 mL of RPMI 1640 medium supplemented with 10 % FBS (to stimulate full maturation) and penstrep, 20 ng/mL granulocyte-macrophage colony-stimulating factor (GMS-CF, Peprotech) and 20 ng/mL interleukin 4 (IL-4, Peprotech). Cells were cultivated in standard conditions in 24-well plates ($1 \times 10^6$ cells/well) for 9 days. Every second day, 75 % of the medium was replaced with fresh medium containing GM-CSF and IL-4. We divided the cells into 4 groups (n = 3): C-DCs – control cells (DCs); IRI-DCs – IRI-induced DCs; D-IRI-DCs – IRI-induced DCs treated with D-MT; L-IRI-DCs – IRI-induced DCs treated with L-MT. On day 9 some cells received pre-treatment with D-MT/L-MT (750 µmol/l dissolved in 0.1 N NaOH; 24 h and 1 h before ischemia). On day 10 they were then subjected to ischemic conditions as described above, while untreated DCs served as control. The phenotype of cells was characterized by flow cytometry. Thereafter cells were either harvested or used for primary mixed lymphocyte reaction (MLR).

### 4.11. Isolation of T cells and mixed leukocyte reaction (MLR)

T cells were isolated from lymph nodes of Lewis rats using a standard protocol. Right after the rat was sacrificed, lymph nodes from the neck, groin and mesentery area were removed. The lymph nodes were minced with a surgical blade on a sterile bacteriological Petri dish in saline solution. The suspension was passed through a mesh, and the mesh was flushed with the medium; the suspension was then transferred to a 50 mL falcon and centrifuged (8 min, 1800 rpm, 20 °C). The supernatant was discarded and the pellet was resuspended in 4 mL of medium (RPMI 1640 with 20 % FBS and penstrep). Cells were counted and co-cultured with DCs from the IRI DCs model (n = 3) in DC: T cell ratio 1:100 in 24-well plates for 8 days. T cell proliferation was assessed on day 8 using the CyQUANT® Proliferation Assay kit (Invitrogen Life Technologies) and expressed as the intensity of fluorescence. Cells were harvested for further analysis.

### 4.12. Flow cytometry (FC)

FC was performed to confirm the phenotype of the DCs as well as the capability of NK cells to express NKG2D. The following antibodies were used for DC phenotype analysis: anti-CD11c conjugated with Alexa flour 647 (Cell Serotec); anti-MHCII conjugated with phycoerythrin (PE; Serotec); and anti-CD86 conjugated with PE (eBioscience). For analysis of NKG2D expression, anti-NKG2D antibody (ab203353, Abcam) was used with secondary Goat anti Rabbit conjugated with FITC (Abcam). Cells (50,000 cells/antibody) were incubated with antibodies in DPBS supplemented with 2% FBS (30 min, 4 °C), washed with DPBS with 2% FBS, fixed in 2% formaldehyde, and stored until analysis (4 °C, protected from light). The MACSQuant® Analyzer, Miltenyi Biotec, was used, and the obtained data were analyzed with the Kaluza 1.2 cytometry software (Beckman Coulter).

### 4.13. RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (sq-RT-PCR)

Total RNA was isolated using NucleoSpin® RNA XS or NucleoSpin® RNA/protein (Macherey-Nagel) for cells (TECs, NK cells, DCs, T cells) or TRIZOL (TRI- reagent, Sigma Aldrich) for tissue samples (kidneys). cDNA was obtained by reverse transcription (RT) of 500 ng of RNA using the LunaScript® RT SuperMix Kit (New England Biolabs) in Mastercycler personal (Eppendorf®). RT-PCR was performed in QuantStudio™ 3, Real-Time PCR Systems (ThermoFisher) using 5xHot FIREPol EvaGreen qPCR Mix Plus ROX kit (Solis Biodyne). The optimized PCR programme was 95 °C for 15 min, then 40 cycles of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 30 s, followed by a final elongation step at 72 °C for 10 min. The reaction was finished by a dissociation step. Specific primers were designed using Primer-BLAST and acquired from Sigma-Aldrich (Table 2). Results were normalized to GAPDH and analyzed with the standard calibration curve method.

**Table 2**
Primers used in sq-RT-PCR.

| Detected gene | FOR/REV | Sequence | Detected gene | FOR/REV | Sequence |
|---|---|---|---|---|---|
| Cdh1 - E | FOR | TTCAACCCAAGCACGTACCA | NALP3 / NLRP3 | FOR | GACCAGCCAGAGTGGAATGA |
| | REV | TGTACACAGCATTCCACGCT | | REV | ACAAATCGAGATGCGGGAGA |
| Cdh2 - N | FOR | CACCCGGCTTAAGGGTGATT | Raet1d | FOR | TAGAAGAATGCTGGGCGTGT |
| | REV | TGGCAAACTTTCACACGCAG | | REV | AAATAAGGAGTCCAGGCCGC |
| Fas | FOR | TGTCAGCCTGGTGAACGAAA | RORγT | FOR | AACATCTCGGGAGTTGCTGG |
| | REV | AGGCGCACCTTCTGCATTTA | | REV | AGGAGTAGGCCACATTGCAC |
| FasL | FOR | GAACTGGCAGAACTCCGTGA | RRLT | FOR | GCATCCTCTATTCACAGCAGC |
| | REV | TGTGCTGGGGTTGGCTATTT | | REV | CCCTTAAGTCCTGTTTACATC |
| Foxp3 | FOR | CCACACCTCCTCTTCTTCCTT | Snai1 - Snail | FOR | GACGCGTGTGTGGAGTTCA |
| | REV | TGACTAGGGGCACTGTAGGC | | REV | GAGAGAGTCCCAGATGAGGGT |
| GATA3 | FOR | CCACAATATTAACAGACCCCTGAC | Snai2 - Slug | FOR | CACATTAGAACACACACTGGGGA |
| | REV | GAAGTCCTCCAGCGCATCAT | | REV | TGCCCTCAGGTTTGATCTGTC |
| Ido1 | FOR | AGAGGATGCGTGACTTCGTG | Tbx21/T-bet | FOR | AAGGCAGTATGCCAGGGAAC |
| | REV | TTTTTGGGGGTGTCCCAGAC | | REV | TTGGAAGCCCCCTTGTTGTT |
| IFN-γ | FOR | ACTGCCAAGGCACACACTCATT | TGF-β | FOR | ATACGCCTGAGTGGCTGTCT |
| | REV | AGGTGCGATTCGATGACACT | | REV | TGGGACTGATCCCATTGATT |
| IL-1b | FOR | CAGCTTTCGACAGTGAGGAGA | Tlr4 | FOR | GCTGGTTGCAGAAAATGCCA |
| | REV | TGTCGAGATGCTGCTGTGAG | | REV | AGGAAGTACCTCTATGCAGGGAT |
| IL-4 | FOR | TGATGGGTCTCAGCCCCCACCTTGC | TPH1 | FOR | CAGTGGCTTTGAGGTCCTCTTT |
| | REV | CTTTCAGTGTTGTGAGCGTGGACTC | | REV | GTCACTCTCCCCCTTTCTGAG |
| IL-6 | FOR | CATTCTGTCTCGAGCCCACC | Twist1 | FOR | CCGGAGACCTAGATGTCATTGT |
| | REV | GCTGGAAGTCTCTTGCGGAG | | REV | CACGCCCTGATTCTTGTGAAA |
| IL-17 | FOR | ATGTGCCTGATGCTGTTGCTGCTA | Wars/TTS | FOR | GCTACACCGTGGAAAATGCC |
| | REV | TTAGGACGCATGGCGGACAATAGA | | REV | TGGGCTCTGCCCCATATACT |
| Klrk1/NKG2D | FOR | GTCAACTTGAATTGCTTCCTCCA | Zeb1 | FOR | TGGGATGTACGCATGTGACC |
| | REV | ATGATACCATTCTCTCCAGGTCG | | REV | GGGGCCTCTTACCTGTATGC |

### 4.14. SDS-page and western blot

Samples (TECs, NK cells, DCs, T cells) were lysed in RIPA buffer and tissue samples (kidneys) in SDS containing lysis buffer (both containing PMSF/NaF/$Na_3VO_4$/β- mercaptoethanol). The protein concentration of lysates was assessed using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™) and their concentration was adjusted to the same value. Lysates were solubilized in Laemmli sample buffer at 95 °C for 10 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis using Mini Trans-Blot® Cell apparatus (Bio-Rad) at 100 V. After the electrophoresis, proteins were transferred to immobilon-P membranes (0.45 μm) using electrotransfer at 400 mA for 2.5 h. The membranes were incubated in blocking solution (5% bovine serum albumin – BSA/ 5% non-fat dried milk/5% polyvinilpyrolidone) at room temperature for an appropriate amount of blocking time stated in Table 3. For the detection of proteins, the membranes were incubated with primary antibodies (Table 3) at 4 °C overnight. The bound antibodies were detected by secondary antibodies conjugated with horseradish peroxidase (Table 3) and enhanced by chemiluminescent reaction using Immobilon Crescendo Western HRP substrate (Millipore). Chemiluminescence was detected using UVITEC Imaging Systems, Nine Alliance, and the densities of the bands were quantified using myImage Analysis software (Thermo Fischer Scientific).

### 4.15. Statistical analysis

Significance was tested by one-way analysis of variance (ANOVA) followed by an LSD post-hoc test. For assessment of overall survival in the IRI rat model, the Kaplan-Meyer with Beslow test was used, using SPSS Statistics 16.0 software. A P value of $<0.05$ was considered significant, and all declared changes are significant unless stated otherwise (in case of trends, P values are stated). Data are presented as the mean ± standard error of the mean.

**Table 3**
Primary and secondary antibodies used in western blot.

| Primary Antibodies Anti- | Concentration | Product No | Company | Source | Dissolved in |
|---|---|---|---|---|---|
| E-Cadherin | 1:200 | (G-10) sc-8426 | SANTA CRUZ BIOTECHNOLOGY, INC. | mouse | 1% BSA in TBST |
| N-Cadherin | 1:200 | (H-4) sc-271386 | SANTA CRUZ BIOTECHNOLOGY, INC. | mouse | 1% BSA in TBST |
| phospho-MEK1/2 (Ser217/221) | 1:2000 | #9121 | Cell Signalling Technology | rabbit | 5% BSA in TBST |
| phospho-p38α (Thr180/Tyr182) | 1:1000 | #09-272-25UL | EMD Millipore Corporation | rabbit | 1% BSA v TBST |
| Ccl-5 | 1:500 | SAB2107938-100UL | Sigma-Aldrich | rabbit | 1% BSA v TBST |
| SAPK/JNK | 1:1000 | #9252 | Cell Signalling Technology | rabbit | 5% BSA in TBST |
| MEK1/2 (D1A5) | 1:5000 | #8727 | Cell Signalling Technology | rabbit | 5% MILK in TBST |
| IDO1 (Fig. 1) | 1:1000 | * | * | mouse | 5% BSA in TBST |
| IDO1 (Figs. 3,5 and 8) | 1:1000 | ab106134 | Abcam | rabbit | 5% BSA in TBST |
| phospho-SAPK/JNK (Thr183/Tyr185) (81E11) | 1:500 | #4668 | Cell Signalling Technology | rabbit | 5% BSA in TBST |
| phospho-ERK1/2 (pT202/pY204) | 1:1000 | 612358 | BD Transduction Laboratories | mouse | 1% BSA v TBST |
| ERK | 1:10000 | 610124 | BD Transduction Laboratories | mouse | 1% MILK v TBST |
| Human Smooth muscle actin | 1:5000 | M0851 | Dako | mouse | 1% MILK v TBST |
| GAPDH-Peroxidase | 1:200 000 | G9295 | Sigma-Aldrich | mouse | 1% MILK v TBST |
| Secondary antibodies | Concentration | Product No | Company | | |
| Polyclonal Rabbit anti mouse Immunoglobulins/HRP | 1:10000 | P 0261 | Dako | | |
| Polyclonal Rabbit anti goat Immunoglobulins/HRP | 1:1000 | P 0449 | Dako | | |

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## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.111180.

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