

DELETION OF STAT-1 IN PANCREATIC ISLETS PROTECTS AGAINST STREPTOZOTOCIN-INDUCED DIABETES AND EARLY GRAFT FAILURE BUT NOT AGAINST LATE REJECTION


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**Abbreviations**

CsA, cyclosporine A; IL-1ra, interleukin 1 receptor antagonist; MLDS, multiple low-dose streptozotocin-induced diabetes; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PNF, primary islet non-function; STAT-1, signal transducer and activator of transcription-1

## ABSTRACT

**Objective:** Exposure of  $\beta$ -cells to inflammatory cytokines leads to apoptotic cell death through activation of gene networks under control of specific transcription factors, like interferon (IFN)- $\gamma$ -induced signal transducer and activator of transcription (STAT)-1. We previously demonstrated that  $\beta$ -cells lacking STAT-1 are resistant to cytokine-induced cell death *in vitro*. The aim of this study was to investigate the effect of STAT-1 elimination on immune-mediated  $\beta$ -cell destruction *in vivo*.

**Research Design and Methods:** Multiple low-dose streptozotocin (MLDS) was given to C57BL/6 mice after syngeneic STAT-1<sup>-/-</sup> or wild-type islet transplantation. STAT-1<sup>-/-</sup> and wild-type islets were also transplanted in alloxan-diabetic BALB/c and spontaneously diabetic NOD mice. Additionally, mice were treated with IL-1-blockade (IL-1 receptor antagonist, IL-1ra) and low-dose T-cell suppression (cyclosporine A, CsA).

**Results:** When exposed to MLDS in an immune-competent host, STAT-1<sup>-/-</sup> islets were more resistant to destruction than wild-type islets (28% *versus* 100% diabetes incidence,  $p \leq 0.05$ ). STAT-1 deletion also protected allogeneic islet grafts against primary non-function in autoimmune NOD mice (0% *versus* 17% using wild-type islets). However, no difference in survival time was observed. Treating recipients additionally with IL-1ra and CsA prolonged graft survival in chemically-diabetic BALB/c mice, but again no difference was seen between STAT-1<sup>-/-</sup> or C57BL/6 grafts.

**Conclusions:** These data indicate that STAT-1 is a key player in immune-mediated early  $\beta$ -cell dysfunction and death. Considering the many effector mechanisms contributing to  $\beta$ -cell death following islet transplantation, however, multiple combined interventions will be needed for prolongation of  $\beta$ -cell survival in the autoimmune context of type 1 diabetes.

## INTRODUCTION

$\beta$ -cell loss during islet isolation procedures and early after implantation is partly responsible for the present need for multiple donors in islet transplantation (1). Engineering  $\beta$ -cells to become resistant to this early cell loss may have an immediate impact on the outcome of islet transplants.  $\beta$ -cells under immune assault mostly die via apoptosis (2), a complex process mainly driven by local production of pro-inflammatory cytokines including IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ .  $\beta$ -cells are also destroyed by direct action of cytotoxic T-cells, e.g. via Fas- and perforin-mediated mechanisms (2-4).

Insight in the intracellular signaling cascades induced by inflammatory cytokines in pancreatic  $\beta$ -cells is growing, especially through micro-array studies demonstrating that IFN- $\gamma$ , together with IL-1 $\beta$ , alters gene expression of more than 700 transcripts in FACS-purified rat  $\beta$ -cells or in insulin-producing cells (2;5). IL-1 $\beta$  exerts its effects mainly through the NF- $\kappa$ B pathway, while IFN- $\gamma$  acts mostly via activation of the transcription factor STAT-1 (6). Our group has previously shown that absence of STAT-1 in  $\beta$ -cells (from STAT-1<sup>-/-</sup> mice) prevented IL-1 $\beta$  plus IFN- $\gamma$ -induced  $\beta$ -cell death *in vitro*. In addition, STAT-1<sup>-/-</sup> mice are resistant against multiple low-dose streptozotocin-induced diabetes (MLDSD) (7). A major drawback of these findings was that in this *in vivo* system not only  $\beta$ -cells lacked STAT-1, but also the immune system was STAT-1 deficient, making it difficult to determine at which level the STAT-1 disruption was responsible for protection. The present experiments were designed to elucidate whether disruption of the STAT-1 signaling pathway in the  $\beta$ -cells itself improves their resistance against immune destruction *in vivo*. First, we studied a model of MLDSD after syngeneic islet transplantation, reflecting resistance of the STAT-1<sup>-/-</sup> islets against an immune attack in a fully immune-competent host. Secondly, we investigated whether lack of STAT-1 increased the short-term survival of islet transplants in alloxan-diabetic BALB/c mice and spontaneously diabetic NOD mice. In addition, we investigated whether blocking multiple pathways involved in  $\beta$ -cell destruction enhanced long-term survival of allogeneic islet grafts. Therefore, STAT-1<sup>-/-</sup> islets were transplanted in hosts treated with IL-

1 receptor antagonist (IL-1ra) and a subtherapeutic dose of cyclosporine (CsA) (8-10).

## RESEARCH DESIGN AND METHODS

### Animals

STAT-1 knock-out (-/-) mice (C57BL/6 background) were a kind gift of Dr. David Levy (New York University School of Medicine, NY). Eight-week-old wild-type and BALB/c mice were produced from stocks purchased from Harlan Nederland (Horst, The Netherlands). Non-obese diabetic (NOD) mice, inbred in our animal facility (Proefdierencentrum "Leuven", Belgium) since 1989, were used as diabetes-prone animals and diabetes was defined and detected as described (11). STAT-1<sup>-/-</sup> mice were kept under specific pathogen free (SPF) conditions. Experiments were conducted with approval of the Animal Ethics Committee of KULeuven.

### Islet isolation, transplantation and evaluation of graft function

Freshly isolated STAT-1<sup>-/-</sup> or wild-type islets (n=500), were isolated and transplanted under the kidney capsule of recipients as described (11). Alloxan-diabetes was induced and graft function was evaluated as described (11). Graft destruction was defined as return to hyperglycemia (blood glucose >200 mg/dL on two consecutive days after initial normoglycemia). Recipients were killed the day of graft rejection or in a separate experiment for gene analysis in grafts 8 hours post-transplantation.

### Real-time PCR

Islet grafts, retrieved 8 hours post-transplantation, were used for RNA extraction using SV Total RNA Isolation kit (Promega Benelux, Leiden, The Netherlands). cDNA was created and quantitative PCR analysis was performed as described (7). Primer and probe sequences for the determination of mouse cDNAs for house-keeping gene  $\beta$ -actin, IL-1 $\beta$ , IL-15, IFN- $\gamma$ , iNOS, MCP-1, IP-10 and MIP-3 $\alpha$  were as described (7).

### MLDSD after syngeneic islet transplantation

Freshly dissolved streptozotocin (50 mg/kg) (Sigma, St. Louis, MO) was injected for 5 consecutive days intraperitoneally into male wild-type mice that had received an islet

transplantation with 500 STAT-1<sup>-/-</sup> or wild-type islets (after being rendered diabetic by alloxan) and had been normoglycemic for at least 14 days. Graft function was followed for 40 days after the last streptozotocin injection. At that time point, grafts were removed, embedded in paraffin and used for hematoxylin/eosin and insulin staining using a guinea pig anti-insulin antibody (Dakocytomation, Glostrup, Denmark) as described (12). After two additional days, pancreases were removed for histology and insulin content determination (12).

### Treatment regimens

As recommended by Ulrich Feige (Amgen, CA), human recombinant IL-1ra (Kineret®) was delivered by a subcutaneously implanted *in vitro*-primed osmotic pump as described at a dose of 100 mg/kg/day for 15 days starting one day pre-transplantation (10).

A subtherapeutic dose of 7.5 mg/kg CsA (Sandimmune®, Novartis, Switzerland) was administered daily by gavage starting one day pre-transplantation for the duration of normoglycemia.

### Statistical analysis

NCSS 2000 (Kaysville, Utah) software was used for statistical analysis. Data are expressed as mean±SEM. Log-Rank test was performed for graft survival.  $\chi^2$  test was used for the incidence of MLDS. The Student's t-test and ANOVA were used for multiple comparisons, whenever appropriate. Significance was defined at the 0.05 level.

## RESULTS

### Resistance of STAT-1<sup>-/-</sup> islets against MLDS in an immune-competent host

STAT-1<sup>-/-</sup> or wild-type islets were transplanted under the kidney capsule of alloxan-diabetic syngeneic C57BL/6 recipients. Normoglycemia was rapidly reached by STAT-1<sup>-/-</sup> (n=7) and wild-type islets (n=7) ( $3.9 \pm 2.9$  versus  $4.3 \pm 2.8$  days, respectively, p=NS). After 2-3 weeks of normoglycemia, MLDS was induced. At the start of the MLDS treatment, no differences in blood glucose were present between mice transplanted with either STAT-1<sup>-/-</sup> or wild-type islets ( $132 \pm 32$  versus  $121 \pm 20$  mg/dL, respectively, p=NS). However, 10 days after the last injection with streptozotocin there were

clear differences in blood glucose levels ( $159 \pm 32$  versus  $250 \pm 45$  mg/dL in STAT-1<sup>-/-</sup> versus wild-type islet recipients, p<0.05). This difference was maintained until the end of the study, 40 days after the last streptozotocin injection ( $182 \pm 55$  versus  $305 \pm 80$  mg/dL in STAT-1<sup>-/-</sup> versus wild-type islet recipients, p=0.07). After 40 days, 100% of wild-type islet recipients had become diabetic compared to 28% in STAT-1<sup>-/-</sup> islet recipients (p<0.05) (Figure 1). Maintenance of normoglycemia in mice transplanted with STAT-1<sup>-/-</sup> islets was not due to regeneration of recipients' pancreases, demonstrated by low insulin content in pancreases (data not shown). Moreover, clear insulin positivity was seen in removed grafts (Online Appendix Figure 1). In separate mice where no MLDS was induced, STAT-1<sup>-/-</sup> and wild-type islets maintained normoglycemia for >30 days (Figure 1).

### Survival and gene expression of STAT-1<sup>-/-</sup> and wild-type islets transplanted in alloxan-diabetic BALB/c and spontaneously diabetic NOD mice

Absence of STAT-1 in islet cells did not prolong graft survival in chemically-diabetic recipients compared to islets from wild-type mice (Table 1).

Wild-type islet transplantations in spontaneously diabetic NOD mice, where also autoimmune recurrence occurs, failed to normalize glycemia in 2/12 mice, representing 17% of primary islet non-function (PNF). No PNF was observed in transplantations with STAT-1<sup>-/-</sup> islets. However, islets of STAT-1<sup>-/-</sup> and wild-type mice were rejected at the same time (Table 2).

mRNA analysis of STAT-1<sup>-/-</sup> and wild-type islet grafts 8 hours after transplantation revealed analogous levels of inflammatory cytokines in both recipients, illustrating comparable immune responses to the grafts (data not shown). As shown in figure 2 (BALB/c recipients) and online appendix figure 2 (NOD recipients), MCP-1, IL-15, IP-10 and iNOS genes were strongly upregulated in islet allografts of wild-type mice. Induction of IL-15, IP-10 and iNOS mRNA, was markedly suppressed in STAT-1<sup>-/-</sup> grafts (p<0.05).

### Blocking of STAT-1 and IL-1 $\beta$ pathway in combination with low-dose cyclosporine

All chemically-diabetic recipients treated with a combination of IL-1ra and CsA had prolonged graft survival (Table 1). Mean islet graft survival (MST) was however identical for IL-1ra- and CsA-treated mice transplanted with STAT-1<sup>-/-</sup> and wild-type islets (Table 1). The combination of IL-1ra and CsA prevented the PNF observed in wild-type into NOD transplants, but no additional benefit from STAT-1 deletion was observed (Table 2).

## DISCUSSION

Pancreatic islets are markedly susceptible to the cytotoxic effects of inflammatory cytokines (e.g. IFN $\gamma$ , IL1 $\beta$ ) secreted by infiltrating immune cells (2;13). Micro-array studies show that  $\beta$ -cells under attack are not passive bystanders but actively participate in their destruction, up- and down-regulating a complex pattern of genes (2). This altered gene expression profile is most likely responsible for activation of pro-apoptotic genes eventually eliciting a specific destruction of the  $\beta$ -cells (5;14). The transcription factor STAT-1 is a key mediator of biological responses to IFN- $\gamma$ , with IFN- $\gamma$  activating STAT-1 via JAK-mediated tyrosine phosphorylation, allowing dimerization, nuclear translocation and binding of STAT to  $\gamma$ -activated sequences within target promoters (6). As a result, STAT-1 modulates the expression of primary response genes, among them other transcription factors, such as IRF-1, sustaining the transcriptional response and driving expression of secondary response genes (6;15-17).

Here, we used STAT-1<sup>-/-</sup> mice to determine the contribution of this transcription factor to immune-mediated  $\beta$ -cell death *in vivo* (18). We previously showed that both islets and FACS-purified  $\beta$ -cells lacking STAT-1 are completely protected against IFN- $\gamma$  plus IL-1 $\beta$ - or IFN- $\gamma$  plus dsRNA-mediated  $\beta$ -cell death *in vitro* (7;19). Furthermore, STAT-1<sup>-/-</sup> mice are partially resistant to development of diabetes after MLDS treatment (7). It was however impossible to discriminate in this systemic knock-out model between effects of STAT-1 deletion on islet cells and on immune cells. When MLDS is induced in wild-type mice transplanted with STAT-1<sup>-/-</sup> islets, only the effect of the STAT-1 deletion in the islets contributes to differences in diabetes. The higher percentage of diabetic animals at the end of the study and the higher glycemia at all

time points studied after MLDS in mice transplanted with wild-type islets point towards a higher resistance of STAT-1<sup>-/-</sup> islets to the immune consequences of MLDS. We have previously shown that STAT-1<sup>-/-</sup> and wild-type mice are equally sensitive to a single high dose of streptozotocin (7), making it unlikely that the present results are due to specific resistance of STAT-1<sup>-/-</sup> islets against the toxic effects of the drug. In the MLDS-model cytokine and chemokine expression by resident inflammatory cells, endocrine cells and endothelial cells is proposed to be the detrimental trigger leading to islet immune infiltration causing development of hyperglycemia (20). In this regard, we have shown that STAT-1<sup>-/-</sup> islets produce less chemokines compared to C57BL/6 islets when exposed to stress signals (7). Therefore, we hypothesize that our observations in MLDS-model can be explained by differences in chemokine expression and recruitment of immune cells by those chemokines in the graft of STAT-1<sup>-/-</sup> islets compared to wild-type islets. We have previously demonstrated that PNF is mediated by local inflammation (11), and the hypothesis that STAT-1 is a crucial mediator in the cytokine-induced component of  $\beta$ -cell destruction *in vivo* is further strengthened by the observation that STAT-1<sup>-/-</sup> islets were fully protected against PNF after transplantation in spontaneously diabetic NOD mice. Importantly, iNOS expression, which is positively correlated with the prevalence of PNF in islet transplantation (11), was decreased 8 hours post-transplantation in STAT-1<sup>-/-</sup> islets grafted in spontaneously diabetic NOD mice. Taken together, the MLDS and the PNF data indicate that cytokine-mediated cell death is an important component of  $\beta$ -cell demise *in vivo*. Indeed, inflammatory cytokines probably contribute to  $\beta$ -cell death in type 1 diabetes (13). This form of  $\beta$ -cell destruction may be particularly relevant when viral triggers are involved, and transcription factors such as STAT-1 may be crucial messengers in this phenomenon (19).

The absence of prolongation of graft survival by blocking STAT-1 in  $\beta$ -cells indicates that the early beneficial effects of STAT-1 deletion are not sufficient to prevent late and more complex phenomena involved in islet graft destruction. Even additional blocking of IL-1 $\beta$  signaling by IL-1ra and partial blocking of T-cell activation by CsA could not prevent islet graft destruction.

Other mediators such as Fas/Fas ligand and perforin/granzyme probably contribute to late islet graft loss (4;21;22). In fact, recent data involving islet-specific CD8<sup>+</sup> T-cells from T-cell receptor transgenic NOD8.3 mice indicate that both Fas and perforin are implicated in  $\beta$ -cell killing (23).

In conclusion, the present findings point to the very diverse mechanisms that contribute to  $\beta$ -cell destruction *in vivo*. Cytokine-mediated mechanisms clearly contribute to  $\beta$ -cell death, and interfering with cytokine signaling cascades may help to make stronger  $\beta$ -cells. Our data suggest, however, that in order to prevent late  $\beta$ -cell loss, long-term integrated approaches targeting both  $\beta$ -cells and different players in the immune system will be necessary.

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**TABLE 1**Survival of wild-type C57BL/6 or STAT-1<sup>-/-</sup> islets after transplantation in alloxan-diabetic BALB/c mice

Treatment regimen	wild-type C57BL/6 islets in BALB/c				STAT-1 <sup>-/-</sup> islets in Balb/c				
	N° of mice	survival of islets (days)	MST	P vs no treatment	N° of mice	survival of islets (days)	MST	P vs no treatment	P vs wild-type
no treatment	15	6,11,11,11,12,12,13,13 ,13,14,14,14,15,15,17	12.7 ± 2.5		5	11,12,13,13,13	12.4 ± 0.9		NS
IL-1ra	2	10,14	12.0 ± 2.8	NS	2	10,14	12.0 ± 2.8	NS	NS
CsA	7	10,10,10,11,12,12,13	11.1 ± 1.2	NS	ND	ND	ND	ND	ND
IL-1ra+CsA	7	12,14,14,15,15,16,21	15.3 ± 2.8	P < 0.05	6	10,12,14,17,17,23	15.5 ± 4.6	NS	NS

MST: Mean survival time; ND: not done

**TABLE 2**Survival of wild-type C57BL/6 or STAT-1<sup>-/-</sup> islets after transplantation in spontaneously diabetic NOD mice

Treatment regimen	wild-type C57BL/6 islets in NOD				STAT-1 <sup>-/-</sup> islets in NOD				
	N° of mice	survival of islets (days)	MST	P vs no treatment	N° of mice	survival of islets (days)	MST	P vs no treatment	P vs wild-type
no treatment	12	0,0,2,5,5,11,11, 12,13,13,15,17	10.0 ± 4.8		4	[6]*,10,10,13	11.0 ± 1.7		NS
IL-1ra	6	0,5,8,12,12,15	10.4 ± 3.9	NS	2	10,12	11.0 ± 1.4	NS	NS
CsA	7	8,8,8,10,10,11,18	10.4 ± 3.6	NS	3	5, 13, 14	10.7 ± 4.9	NS	NS
IL-1ra+CsA	7	5,10,11,11,12,16,20	12.1 ± 4.7	NS	6	10,11,11,12,12,13	11.5 ± 1.0	NS	NS

MST: mean survival time; \*: died with functioning graft

## FIGURE LEGENDS

**Figure 1: Cumulative diabetes incidence after MLDS treatment performed after syngeneic islet transplantation of STAT-1<sup>-/-</sup> and wild-type control islets.**

MLDS in wild-type mice transplanted with wild-type islets (n=7) (black circles) and in wild-type mice transplanted with STAT-1<sup>-/-</sup> islets (n=7) (white circles) (\*, p<0.05). Follow-up of control mice, not receiving MLDS, showed no rejection of the syngeneic graft [wild-type mice transplanted with wild-type islets: black triangles (n=2); wild-type mice transplanted with STAT-1<sup>-/-</sup> islets: white squares (n=2)].

**Figure 2: Gene expression in STAT-1<sup>-/-</sup> and wild-type islet grafts retrieved from chemically-diabetic BALB/c mice.**

Real-time quantitative PCR analysis of intra-graft IL-15, iNOS, IP-10, MCP-1 and MIP-3 $\alpha$  expression in STAT-1<sup>-/-</sup> islet grafts (hatched bars), wild-type islet grafts (striped bars) and control kidney (white bars) retrieved from alloxan-diabetic BALB/c mice 8 hours post-transplantation. mRNA levels, expressed as ratio between gene of interest and housekeeping gene  $\beta$ -actin, are means  $\pm$  SEM from 2-4 experiments (\*, p<0.05, wild-type *versus* STAT-1<sup>-/-</sup>).

**Online Appendix Figure 1: Histology and immunohistochemistry from grafts at the end of MLDS study.**

Grafts were retrieved 45 days after start of MLDS treatment in wild-type mice transplanted with syngeneic STAT-1<sup>-/-</sup> islets. Panel A (H&E staining) and panel B (immunostaining for insulin in red) show a well preserved graft with positive insulin staining.

**Online Appendix Figure 2: Gene expression in STAT-1<sup>-/-</sup> and wild-type islet grafts retrieved from spontaneously diabetic NOD mice.**

Real-time quantitative PCR analysis of intra-graft IL-15, iNOS, IP-10, MCP-1 and MIP-3 $\alpha$  expression in STAT-1<sup>-/-</sup> islet grafts (hatched bars), wild-type islet grafts (striped bars) and control kidney (white bars) retrieved from spontaneously diabetic NOD mice 8 hours post-transplantation. mRNA levels, expressed as ratio between gene of interest and housekeeping gene  $\beta$ -actin, are means  $\pm$  SEM from 3-5 experiments (\*, p<0.05, wild-type *versus* STAT-1<sup>-/-</sup>).

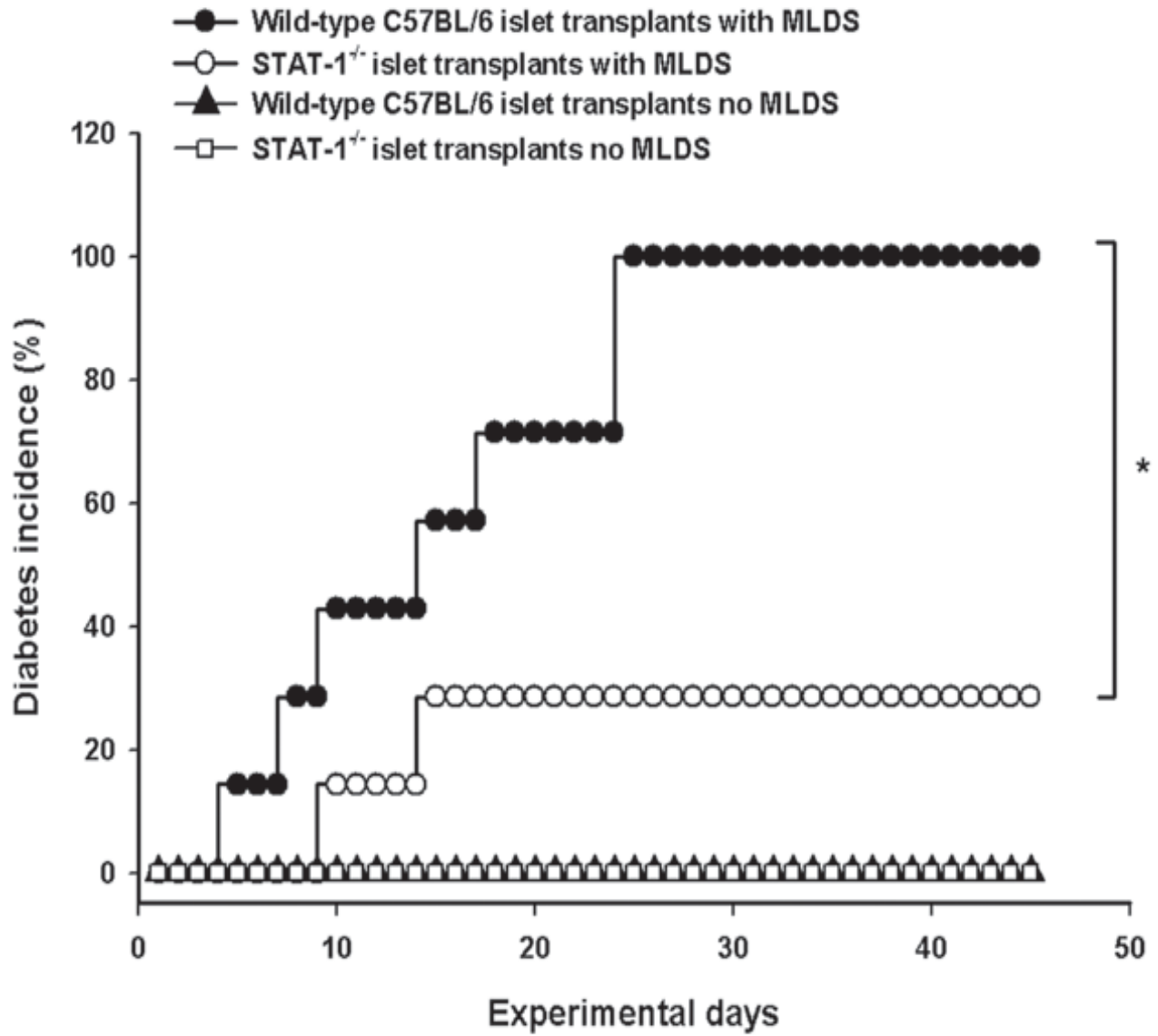


Figure 1

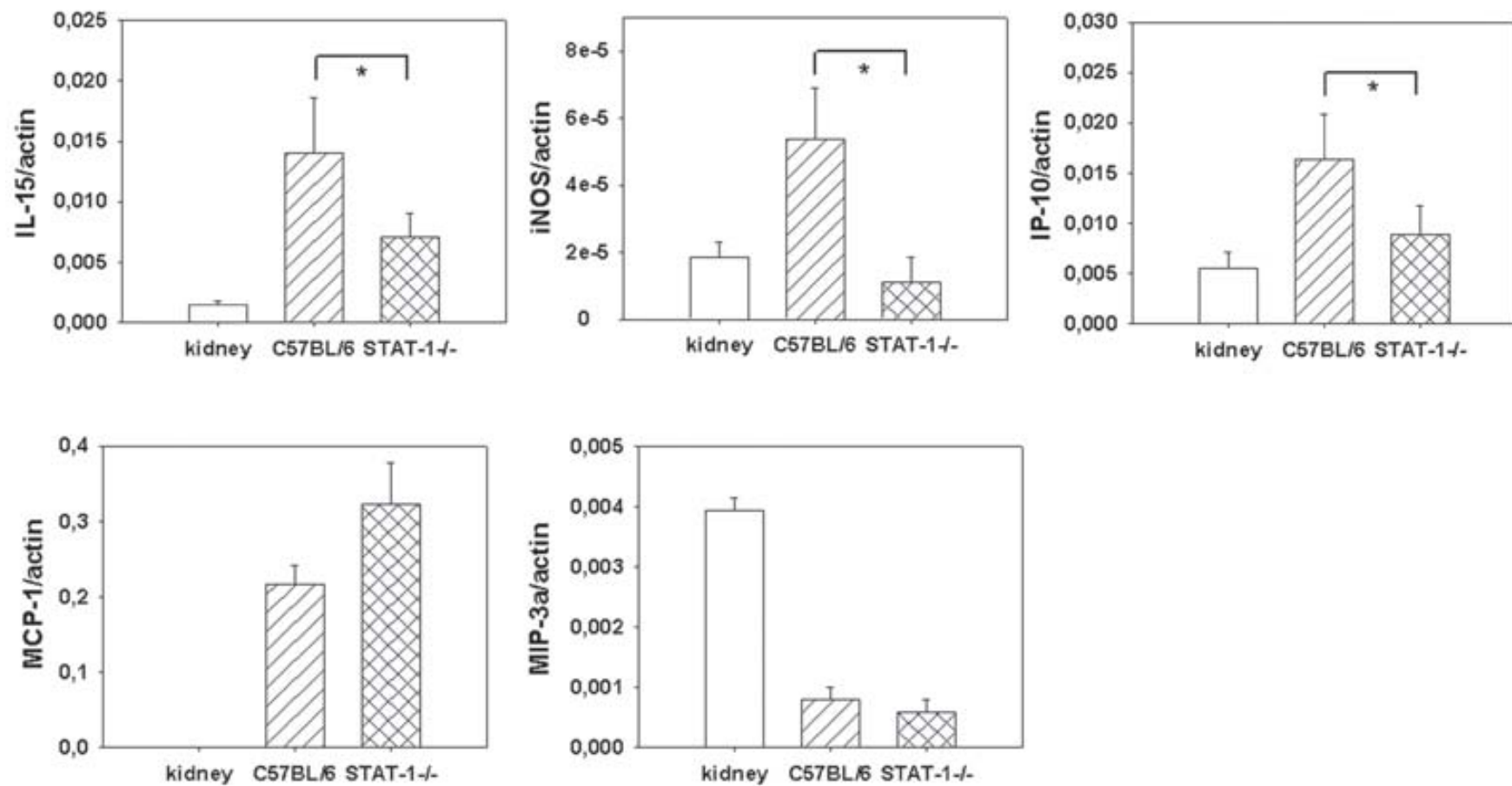


Figure 2