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CD154-CD40 T-cell co-stimulation pathway is a key mechanism in kidney ischemia-reperfusion injury

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Ischemia-reperfusion occurs in a great many clinical settings and contributes to organ failure or dysfunction. CD154-CD40 signaling in leukocyte–endothelial cell interactions or T-cell activation facilitates tissue inflammation and injury. Here we tested a siRNA anti-CD40 in rodent warm and cold ischemia models to check the therapeutic efficacy and anti-inflammatory outcome of *in vivo* gene silencing. In the warm ischemia model different doses were used, resulting in clear renal function improvement and a structural renoprotective effect. Renal ischemia activated the CD40 gene and protein expression, which was inhibited by intravenous siRNA administration. CD40 gene silencing improved renal inflammatory status, as seen by the reduction of CD68 and CD3 T-cell infiltrates, attenuated pro-inflammatory, and enhanced anti-inflammatory mediators. Furthermore, siRNA administration decreased a spleen pro-inflammatory monocyte subset and reduced TNFα secretion by splenic T cells. In the cold ischemia model with syngeneic and allogeneic renal transplantation, the most effective dose induced similar functional and structural renoprotective effects. Our data show the efficacy of our siRNA in modulating both the local and the systemic inflammatory milieu after an ischemic insult. Thus, CD40 silencing could emerge as a novel therapeutic strategy in solid organ transplantation.

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Ischemia-reperfusion injury (IRI) is a common cause of acute kidney injury, contributing significantly to delayed graft function and acute rejection after transplantation.[1–3] The inflammatory response has a critical role in the outcome of IRI[4] and results in endothelial activation, enhanced cell-leukocyte adhesion, and compromised microvascular blood flow.[5] Besides the innate inflammatory immune response, there is growing evidence that T cells also participate in IRI in an antigen-independent manner.[6] Multiple roles of T cells have been described in several studies of ischemia, using T cell–deficient mice with either T cell–specific depleting monoclonal antibodies or knockout animals, combined with adaptive transfer of purified T-cell subsets.[7–9]

CD40 is a co-stimulatory molecule that belongs to the tumor necrosis factor superfamily. The CD40/CD40L dyad participates in T-cell proliferation and in effector functions.[10] Although it was first identified in B cells,[11] it is also expressed in many cell types, including epithelial tubular, endothelial, and immune cells.[12] Several studies have demonstrated the therapeutic effects of blocking CD40–CD40L interaction in ischemia,[13] organ rejection,[14] atherosclerosis[15], and autoimmune disease.[16,17] However, translation of CD40L blockade into non-human primate and human studies revealed thromboembolic complications.[18] Thus, CD40 has become a new emerging target. Although several anti-CD40 monoclonal antibodies (mAbs) have been tested as alternatives to anti-CD40L mAbs,[19] other mechanisms can be used to block the pathway.

The RNA interference phenomenon (RNAi) is an innate gene silencing mechanism that acts at the post-transcriptional level and has emerged as a potent mechanism to specifically knockdown mRNA transcripts.[20] In previous studies, our group developed a small inhibitory RNA (siRNA) against CD40-mRNA that effectively inhibits the translation to CD40 mRNA and protein. This siRNA was used in a model of humoral acute rejection.[17] Thus, local CD40 silencing proved to be a useful strategy to prevent Ab-mediated rejection. Rapid degradation by exo/endonucleases constitutes a serious challenge to the successful intracellular delivery of siRNAs

in vivo and their ultimate biological activity.[21,22] siRNA through cholesterol conjugation has been demonstrated to improve its distribution and cellular uptake.[23] Moreover, chemical modifications introducing phosphorothioate linkages and *O*-methyl greatly prolong half-life in plasma and increase resistance to nucleases. We used a murine-specific siRNA anti-CD40, chemically stabilized and cholesterol conjugated, in a model of autoimmune nephritis and was demonstrated that systemic CD40 blockade induced immune deactivation of the inflamed kidneys.[24]

In the present study, the effect of CD40 blockade with specific siRNAs was analyzed in warm and cold ischemia models in rodents. We hypothesized that blocking the co-stimulatory CD40-CD40L dyad would reduce the inflammatory response developing from IRI. We aimed to evaluate whether the CD40 blockade interfered locally in chemokine-attracting molecules, transduction signals, or cell recruitment. Moreover, we assessed whether systemic siRNA administration affected distant spleen cell populations.

RESULTS

Efficiency of siRNA cholesterol conjugated on renal warm ischemia in rat; dose-finding studies

After confirming the efficacy in mice and the greater efficiency of siRNA conjugated with cholesterol (Supplementary data), we further analyzed different doses to find an optimal dosage for the transplant model. As seen in Figure 1a, the scrambled (SC) and vehicle (Veh) groups showed worse renal function compared with all the treated groups. The higher doses were the most effective. Urea data showed parallel results. Again, the higher doses were the most effective (Figure 1b).

Examination of renal tissue revealed that Veh and SC groups had severe kidney injury, as evaluated by ischemic damage overall scores of 8.3 and 9.0, respectively (Figure 1c). In contrast, this ischemic injury was less severe in all treated groups in a dose-dependent way. Conventional histology showed well-preserved renal architecture in the group treated with 500 μg siRNA (Figure 2).

Assessment of CD40 expression on the renal warm ischemia model

To assess the activation of the CD40 co-stimulatory pathway, kidney RNA expression was measured. CD40 in Veh and SC groups was overexpressed 2.2-fold and 1.8-fold, respectively, compared with healthy kidneys. CD40 gene silencing effectively reduced CD40 expression in all treated groups (Table 1).

CD40 protein expression and location in renal tissue were analyzed by immunostaining. The CD40 overexpression pattern differed in the diverse compartments of the warm ischemic kidneys (tubuli, glomeruli, and vessels). A reduction in CD40 expression was seen in all compartments in the treated groups, with the highest dose being the most effective (Figure 1).

Modulation of renal inflammatory status after warm ischemia by gene silencing

To better characterize the inflammatory cell infiltration observed by conventional histology, CD68 (macrophages) and CD3 (T cells) surface markers were analyzed (Figure 2). CD68 immunostaining revealed a lower degree of macrophage infiltration in siRNA CD40–treated groups, which was significant in those treated with higher siRNA doses. Moreover, CD3 immunostaining showed a significant reduction in CD3+ T cells in all treated groups (Table 1).

Given the innate immune response, gene expression of both pro-inflammatory and anti-inflammatory renal mediators was measured. For Toll-like receptor (TLR)-4, classically related to ischemia damage, there were no differences between groups (not shown). Conversely, TLR2 was reduced in all treated animals. Furthermore, systemic injection of siRNA did not appear to activate the local innate immune response as TLR3 expression was not overexpressed.

The downstream nuclear factor, NF-KB expression, had a similar outcome to TLR2 and CD40. Thus, there was a 2.4-fold overexpression in Veh-treated animals, with a clear reduction in treated kidneys. CD40 silencing induced a significant overexpression of the anti-inflammatory cytokine IL-4 only in animals receiving the highest siRNA dose (Table 1).

We also assessed the expression of chemokines involved in ischemic damage. CXCL9, CXCL10, and CXCL11 were significantly lower after siRNA-CD40 treatment than in the Veh and SC groups. After reperfusion, Veh and SC groups expressed higher levels of CCL2, CCL3, CCL4, and CCL5, which were clearly reduced by CD40 gene silencing (Table 1).

CD40 gene silencing modulates transduction factors triggered by warm ischemia

The previous PROLIGEN study (Supplementary data) defined a gene list relating regeneration versus injury after ischemia insults. There, we validated the renal expression profile of a subset of genes involving defense, immune response, inflammation, and chemotaxis after rat warm ischemic insult.

In present study, we found overexpression of Socs3—a Jak-Stat pathway component—and of several genes related to cell cycle, growth, and cell communication, such as Emp1, Emp3, Fcnb, Reln, and Tnfrsf12a in Veh and SC animals compared with siRNA CD40–treated animals (Figure 3).

In particular, the Ca^{2+}-binding S100 protein family members (S100a6, S100a8, and S100a9), involved in the regulation of neutrophil chemotaxis and inflammation, were overexpressed due to ischemia but were significantly down-regulated by CD40 treatment. Moreover, the expression of galectin family genes (*Lgals1*, *Lgals3*), with immune regulatory functions, was also significantly reduced by siRNA CD40 administration. Neither the S100 nor the galectin gene families had been previously related to CD40 signaling.

We were interested in substantiate changes in the Galectin family, and thus we analyzed protein expression. As shown in Figure 4, galectin 1 and 3 were reduced by CD40 gene silencing in the western blot analysis, with no significant

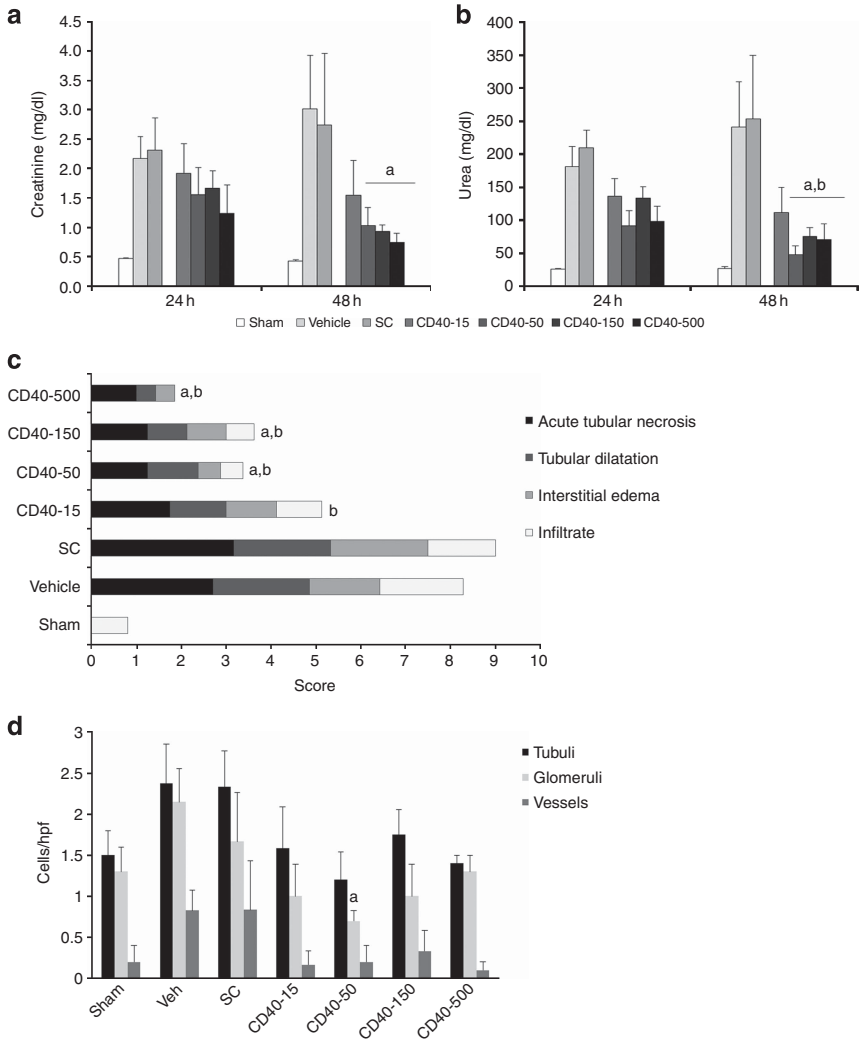

Figure 1 | Renal function and histopathology in warm ischemia studies. Serum creatinine (**a**) and urea (**b**) levels of rats after 40 min of renal warm ischemia. Groups treated with siRNA CD40 showed better renal function compared with Veh (Vehicle) and scrambled (SC) groups. (**c**) Overall score of different histological injury markers. siRNA treatment ameliorates renal histopathology. (**d**) Immune location and quantification of CD40 protein in different kidney compartments. siRNA CD40 reduced its expression in all treated groups in contrast to SC and Veh groups. Data are expressed as a mean ± s.e.m. a, $P < 0.05$ vs. Veh; b, $P < 0.05$ vs. SC.

differences. Interestingly, a reduction in cells expressing galectin was seen, which was clearly significant at higher doses.

Assessment of distant immune-modulatory effects of CD40 silencing after renal warm ischemia

Analysis of the B- and T-cell phenotype in splenocytes population by flow cytometry did not reveal differences in SC or gene silencing–treated rats, 48 h after ischemia (not shown). In treated animals, there were fewer monocytes (ED9) than in Veh and SC groups. Moreover, a positive double-stained $CD43^{high}$/CD161 monocyte population was over-expressed in Veh and SC rats. Interestingly, this cell population clearly decreased in rats after CD40 gene silencing (Figure 5).

To further analyze the systemic inhibitory effect of gene silencing, CD40 expression in spleen B cells (CD45+) was measured. Renal ischemia in SC-treated rats induced CD40 overexpression in non-circulating spleen B cells. Those rats suffering from warm ischemia and treated with siRNA-CD40 had lower CD40 expression, reaching similar values to the healthy group (not shown).

Although no differences in spleen T-cell frequency were observed, intracellular cytokine production was analyzed by flow cytometry. SC-treated splenocytes, obtained 48 h after ischemia, revealed TNFα overproduction in CD3+ cells than splenocytes from healthy rats. In CD3+ T-cell subpopulations, the TNFα secretion levels doubled in the CD4+ cells, whereas the increase in CD8+ cells was lower. Gene silencing caused a decrease in TNFα production in CD3+ T cells, reaching similar levels to that in healthy rats. Apparently, CD4 + T cells were less responsive to gene silencing, as the reduction was modest. Instead, in CD8+ T cells TNFα levels returned to healthy values (Figure 6).

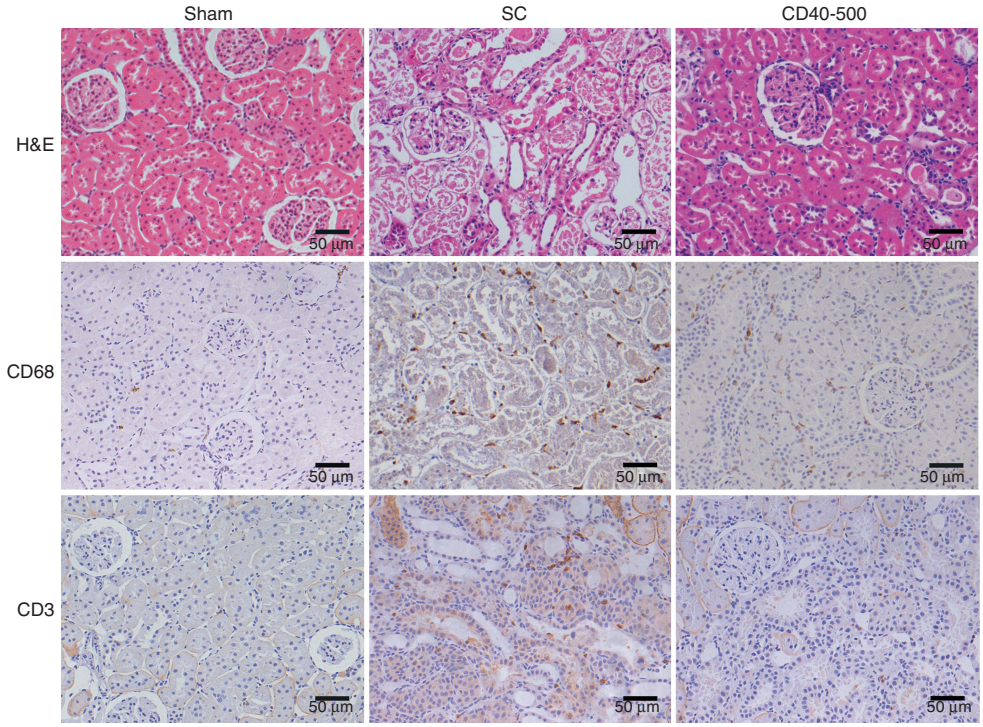

Figure 2 | Representative photomicrographs of hematoxilin & eosin (H&E), CD68 (macrophages), and CD3 (T cell)-positive cells in kidneys, for sham, scrambled (SC), and CD40-500. Original magnification, × 200.

Table 1 | Kidney gene expression and inflammatory cell infiltration characterization

Cells/hpf	Sham	Veh	SC	CD40-15	CD40-50	CD40-150	CD40-500	*P*-value
CD68	10.2 ± 0.8	20.6 ± 4.9	16.3 ± 3.4	12.2 ± 1.66[a]	15.9 ± 2.6	5.2 ± 1.2[a,b]	8.8 ± 1.9[a]	0.010
CD3	0.9 ± 0.2	4.8 ± 0.7	3.3 ± 1.1	1 ± 0.3[a,b]	0.8 ± 0.1[a,b]	0.5 ± 0.2[a,b]	0.6 ± 0.2[a,b]	0.0001
Folds/18s								
CD40	1.2 ± 0.2	2.2 ± 0.6	1.8 ± 0.2	0.8 ± 0.2[a]	0.9 ± 0.3[a]	1.0 ± 0.1[a]	0.9 ± 0.2[a]	0.060
TLR3	0.6 ± 0.1	1.1 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.7 ± 0.8	0.6 ± 0.8	0.8 ± 0.1	0.007
TLR2	0.7 ± 0.5	3.6 ± 1.1	3.7 ± 0.6	2.6 ± 0.7	2.3 ± 0.6	2.6 ± 1.3	1.8 ± 0.3	0.280
NF-Kβ	1.7 ± 0.3	2.4 ± 0.4	2.5 ± 0.4	1.8 ± 0.3	1.1 ± 0.2[a]	1.6 ± 0.3	1.7 ± 0.3	NS
IL-4	2.6 ± 0.9	2.1 ± 0.9	4.0 ± 0.7	3.9 ± 0.9	3.2 ± 0.7	5.0 ± 0.8[a]	5.8 ± 1.2[a]	0.101
CCL2	0.4 ± 0.1	3.5 ± 0.9	3.0 ± 0.4	1.6 ± 0.7[a]	2.6 ± 0.7	2.7 ± 0.4	2.6 ± 0.5	0.064
CCL3	0.6 ± 0.1	1.5 ± 0.3	1.2 ± 0.5	0.5 ± 0.1[a]	0.9 ± 0.3	0.8 ± 0.1	0.9 ± 0.1	NS
CCL4	1.2 ± 0.1	1.9 ± 0.4	1.7 ± 0.4	0.6 ± 0.2[a,b]	1.1 ± 0.3[a]	0.8 ± 0.1[a]	0.8 ± 0.2[a]	0.025
CCL5	0.8 ± 0.1	1.0 ± 0.2	1.3 ± 0.2	0.5 ± 0.1[a,b]	0.4 ± 0.1[a,b]	0.5 ± 0.1[a,b]	0.7 ± 0.1[a]	0.001
CXCL9	0.5 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	0.3 ± 0.1[a,b]	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	NS
CXCL10	1.8 ± 0.2	3.7 ± 0.7	3.2 ± 0.7	1.1 ± 0.4[a,b]	1.2 ± 0.3[a,b]	1.9 ± 0.6[a]	1.7 ± 0.5[a]	0.014
CXCL11	0.8 ± 0.1	2.8 ± 0.6	2.5 ± 0.7	0.9 ± 0.3[a]	1.3 ± 0.3[a]	2.2 ± 0.4	2.1 ± 0.2	0.037

Abbreviations: SC, scrambled; Veh, vehicle. siRNA anti-CD40, especially knocked-down CD40 expression, as well as other pro-inflammatory and chemokine-related genes. CD68 and CD3 were also reduced by CD40 silencing. Data are expressed as mean ± s.e.m. a, $P < 0.05$ vs. Veh; b, $P < 0.05$ vs. SC.

Effects of CD40 gene silencing in cold ischemia

The effect of CD40 silencing was also analyzed in our well-established model of renal cold ischemia, with renal transplantation. As shown in Figure 7a, in the syngeneic Lew-Lew studies, the group treated with our siRNA-CD40 improved renal function, as seen by the reduction in creatinine values. In contrast, Veh and SC groups had worse renal function. According to the histopathological evaluation, all ischemic parameters, which were increased in Veh or SC groups, were improved by CD40 gene silencing. Although only acute tubular necrosis was strongly reduced in the treated group, the overall score denoted improvement in the ischemic injury (Figure 7b).

In the allogeneic Wist-Lew studies, there was an initial renal dysfunction peaking on day 1 and decreasing on day 3, indicating the cold ischemic damage. As shown in Figure 8, creatinine values in siRNA-CD40-treated animals were lower than the other groups. From day 5, a further decrease in renal

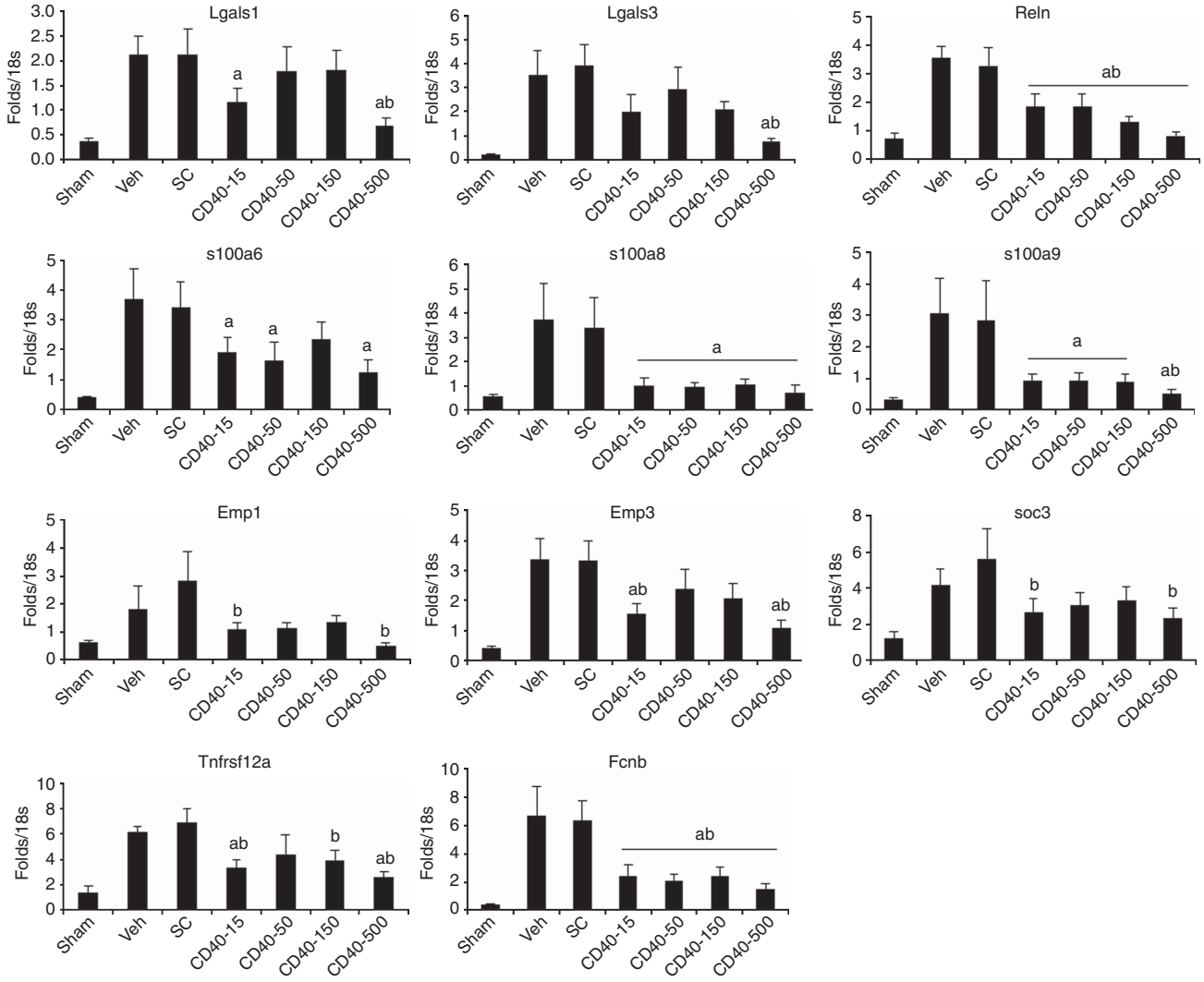

Figure 3 | Kidney gene expression and transduction factors modulated by CD40 gene silencing. Treatment with siRNA CD40 reduced the expression of genes related to ischemia-reperfusion injury (IRI), inflammation, cell cycle, growth, and cell communication. Data are expressed as a mean ± s.e.m. a, $P<0.05$ vs. Vehicle (Veh); b, $P<0.05$ vs. scrambled (SC).

function occurred indicating the acute renal rejection. Thus, in Veh and SC groups, the mean survival time was 12 and 15days due to severe renal dysfunction. In contrast, the renal dysfunction in siRNA-CD40 treated group was less intense with somewhat prolonged survival to 17 days. Animals receiving cyclosporine survived the 21 days of follow-up, with an almost normal renal function. The histological analysis showed severe Banff inflammatory parameters in Veh and SC groups. Those animals that were treated with siRNA-CD40 and survived more than 18 days presented a less severe inflammatory pattern. The cyclosporine animals presented a conserved histological pattern.

DISCUSSION

In vivo protection by co-stimulatory molecule blockade in IRI models has scarcely been reported,[25,26] although promising therapeutic effects on organ function have been suggested. In the present study, we show that IRI induces CD40 overexpression and that CD40 gene silencing *in vivo* prevents IRI in native and grafted kidneys, as made clear by amelioration in renal function, preserved renal integrity, decreased local myelo-monocytic and lymphocytic infiltration, reduced local inflammatory milieu, and distant spleen cell activation. By demonstrating that silencing the CD40-CD154 dyad prevents IRI in rodent models of renal ischemia, our results provide the rationale for novel therapeutic approaches utilizing CD40 gene silencing[17] to maximize donor organ use and function in humans.

We first began studies using mouse warm ischemia models to prove the concept that silencing the CD40 costimulatory pathway was effective in kidney function and morphology protection. In the nineties, Sayegh's group showed that the B7 costimulatory pathway has a critical role in organ dysfunction after renal cold IRI.[26] Later, Kupiec-Weglinski's group[13] modulated cold liver IRI by targeted gene therapy with a CD40Ig-hybrid molecule. Although the effectiveness of the

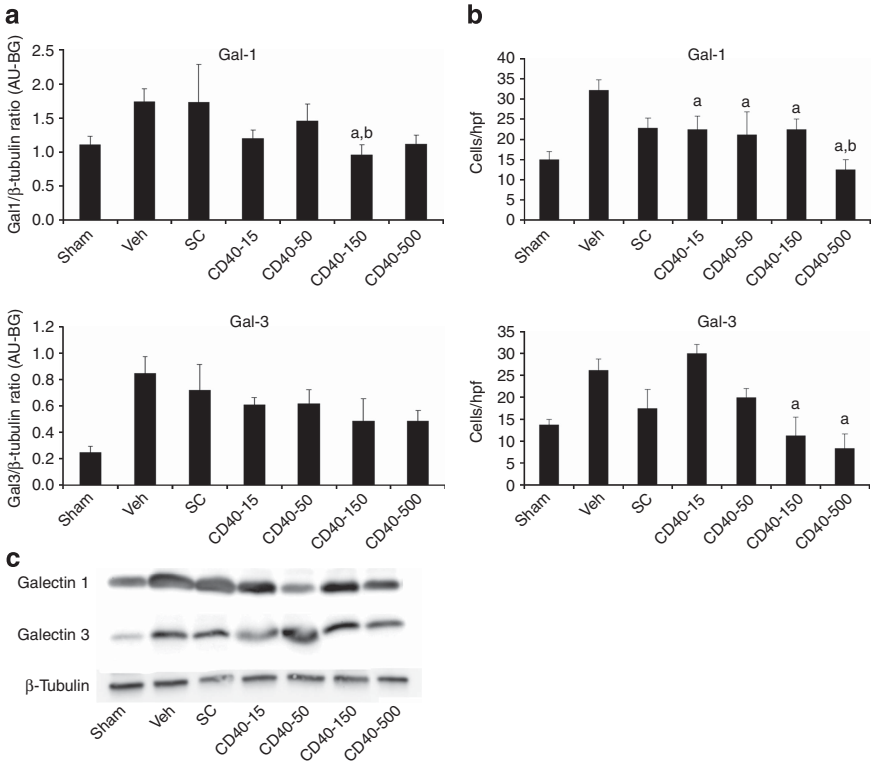

Figure 4 | Galectin 1&3 renal protein expression after siRNA treatment. (**a**) Graphs of mean values of each protein/β-tubulin ratio for each group. (**b**) Representative western blot analysis of galectin 1&3 in all studied groups. Although β-tubulin is similar in all groups, siRNA-treated kidneys show a slight reduction with the highest doses. (**c**) Graphs of mean positive galectin 1&3 cells for HPF. Data are expressed as a mean ± s.e.m. a, $P < 0.05$ vs. Vehicle (Veh); b, $P < 0.05$ vs. scrambled (SC).

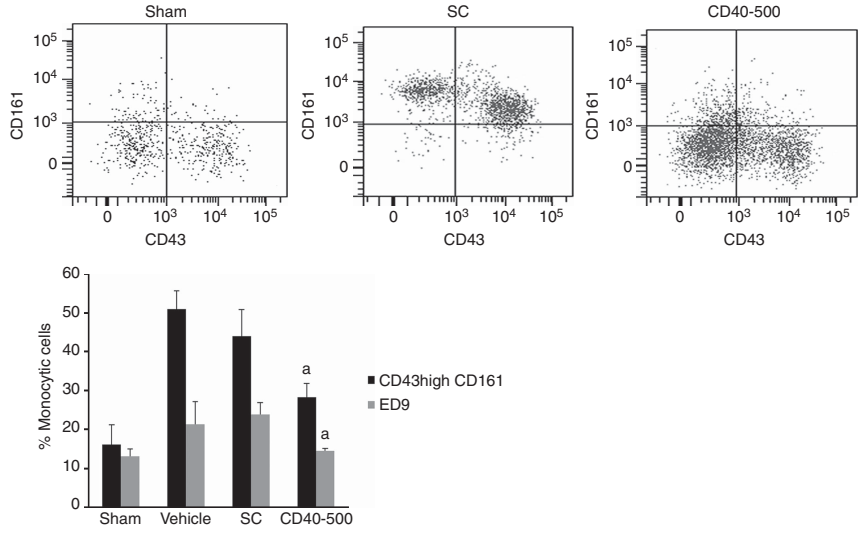

Figure 5 | CD40 silencing distant effects in spleen monocyte population. (**a**) Representative dot plot of sham, scrambled (SC), and CD40-500 groups. (**b**) Monocyte (ED9) and monocyte subpopulation (CD43high/CD161) were quantified by flow cytometry. Both populations were increased due to ischemia-reperfusion injury (IRI) in Vehicle (Veh) and scrambled (SC) groups and were reduced in CD40-500-treated animals. a, $P < 0.05$ vs. Vehicle.

CD40-ligand blockade using monoclonal antibodies has been confirmed on preclinical models, thromboembolic events obscured its translation to the clinic.[27] Our group has recently reported encouraging experimental results that showed that CD40 silencing reduces the progression of lupus nephritis by modulating both local milieu and systemic mechanisms.[24]

We later moved to the rat warm ischemia model aiming to confirm the efficacy and dose dependency of gene silencing in

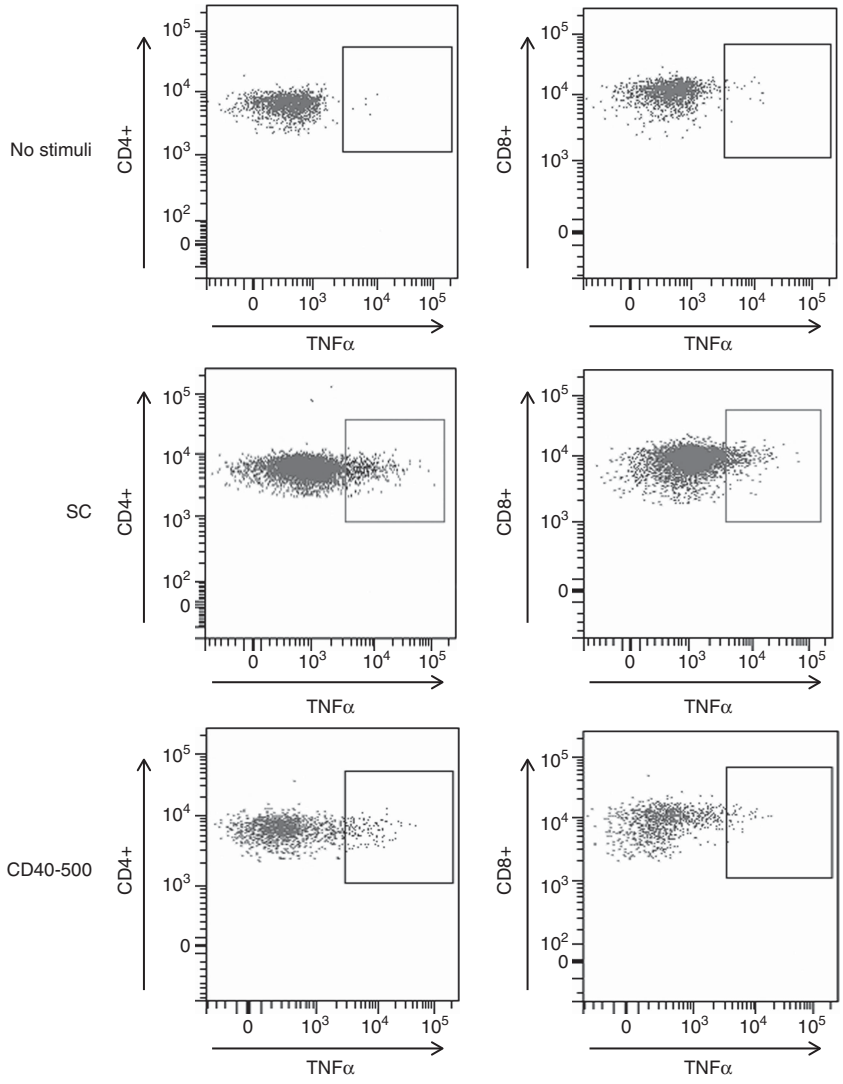

Figure 6 | Determination of TNFα secretion in T lymphocytes. TNFα secretion was determined in nonstimulated and stimulated T cells of scrambled (SC) and CD40-500 groups. siRNA treatment reduced TNFα secretion in CD4+ population (SC: 8.18%; siRNA-500: 6.36%) and in CD8+ population (SC: 5.56%; siRNA-500: 3.74%). The percentage indicates the total amount of CD4+ TNFα+ or CD8+ TNFα+ from the CD3+ lymphocyte population.

another rodent species, using a different siRNA sequence. As stated in the Results section, the highest doses of siRNA exerted the most robust protection in all analyzed functional, morphological, and inflammatory parameters. As the doses were reduced, there was a gradual loss in their beneficial effects, also indicating that gene inhibition completely covered the critical period of post-ischemic inflammatory response.

In liver ischemia, CD40-CD40L signaling, but not IFN-γ signaling, has been reported as important for T-CD4+ cell function without the requirement of *de novo* Ag-specific activation.[28] Our group previously described how CD40 silencing *in vitro* modulates several genes involved in different processes such as innate immune response, signal transduction, and apoptosis.[29] In the present *in vivo* study, we confirmed that CD40 silencing modulated the downstream signal transduction cascade in the ischemic-inflammatory environment. In particular, CD40 silencing modulated TLR expression in kidney tissue. It is known that TLRs participate in IRI, especially TLR 2 and 4, acting as tissue damage sensors, activating innate immunity through pro-inflammatory transcription factors such as NF-kB.[30–32] CD40 silencing was shown to inhibit TLR2 activation selectively and therefore to reduce NF-kB expression.

Chemokines act as potent attractants of inflammatory cells in ischemia injury.[33] CCL2, CCL3, and CCL4 enhance the recruitment of macrophages and neutrophils within the tissue. CCL5 is involved in the migration of T cells, monocytes, natural killer, and dendritic cells. CXCL9, CXCL10, and CXCL11 elicit T-cell chemoattraction by interaction with the chemokine receptor CXCR3. Here we showed that all these chemokines were downregulated by CD40 gene silencing. Therefore, the CD40 signaling pathway proved a key contributor to the inflammatory molecular mechanisms

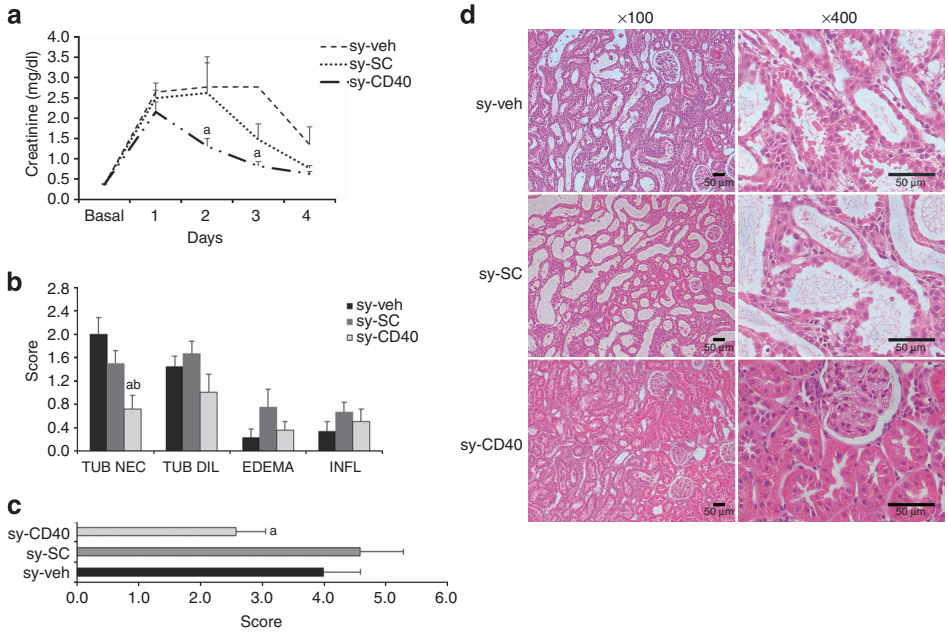

Figure 7 | Renal function and structure in the cold ischemia syngeneic model. Serum creatinine (**a**) levels of rats after 2.5 h renal cold ischemia, follow-up 96 h. The sy-CD40 group showed better renal function compared with sy-scrambled (SC) and sy-vehicle (Veh) groups. Renal histopathology analysis (**b**) showed that injury markers and the overall injury score (**c**) were lower in the sy-CD40-treated group. TUB NEC (tubular necrosis), TUB DIL (tubular dilatation), INFL (interstitial infiltrate). (**d**) Representative hematoxylin and eosin photomicrographs of sy-Veh-, sy-SC-, and sy-CD40-treated groups in two different magnifications (×100 and ×400). Data are expressed as a mean ± s.e.m. a, $P<0.05$ vs. veh; b, $P<0.05$ vs. sy-SC.

induced by ischemia. In particular, the above chemokine modulation could be instrumental in the reduction of infiltrating T cells and macrophages seen in the CD40 siRNA–treated groups.

Furthermore, we report for the first time two new downstream CD40 signaling target families, which might be critical effectors of the pro-inflammatory response to the ischemic insult. Thus, CD40 gen silencing downregulated the expression of S100 and galectin protein families. The Ca^{2+}-binding S100 proteins participate in innate and adaptive immune responses to cell migration, chemotaxis, and inflammation.[34] CD40 silencing knocked down the expression of calcyclin (S100a6) known for its potential role in cell responses to various stress stimuli and apoptosis. Moreover, a recent study has shown its involvement in the regulation of endothelial cell cycle progression and senescence.[35] The downregulation of S100a8 and S100a9 also contributes to inflammatory modulation through reduction in oxidative stress and neutrophil chemotaxis.[36] Galectins are a group of lectins that act as potent immune regulators in inflammatory diseases or acute renal failure.[37–39] In an inflammatory milieu, galectin 1 promotes chemo-attraction and retention of macrophages and neutrophils.[40] The spectrum of galectin 1 and 3 overexpression in our ischemic kidney reflects their activation in the ischemic inflammatory milieu. Their RNA and protein downregulation by CD40 gene silencing eventually amplified the effect of chemokine reduction, with further decrease in immune cell recruitment. Additional genes downregulated by CD40 silencing in ischemic kidneys include the epithelial membrane proteins (Emps) 1 and 3,[41] Fn14 (Tnfrsf12A), which triggers activation of the NF-kB pathways and rises in inflammatory diseases and ischemia,[42] ficolin B (Fcnb), a lectin pathway recognition molecule, and suppressor of cytokine signaling 3 (SOCS-3), a major regulator of inflammation.[43] Indeed, significant upregulation of SOCS-3 in renal tubular cells during IRI has been described, whereas SOCS-3-knockout mice in proximal tubular cells had better renal function, increased tubular cell proliferation, and shifted to M2 macrophages.[44]

Several studies demonstrated that organ ischemia induces distant inflammatory changes in other organs, such as lungs.[45,46] Results showed that, although there was no increase in spleen T–cell frequency, ischemic insult induced an increase in intracellular TNFα production in CD3+ splenocytes from the SC-treated group, an especially great increase in the CD4+ subset. This represents distant T-cell activation pattern after renal ischemia. Similar functional changes have been described in kidney-infiltrating ischemic CD3 cells, without increasing the number of resident CD4+ or CD8+ T cells.[47] Accordingly, intravenous siRNA CD40 delivery reduced TNFα production in CD3+ splenocytes, suggesting that CD40 silencing greatly affects the systemic immune system and consequently the activation of CD3 effector cells.

Concomitantly, an increase in the spleen $CD43^{high}CD161$ monocyte population in the Veh and SC groups was observed. This result corroborates the observations made by Heitbrok's group,[48] who described CD43+ as pro-inflammatory and CD161 as an immune-activator.[49] Reduction in this popula-

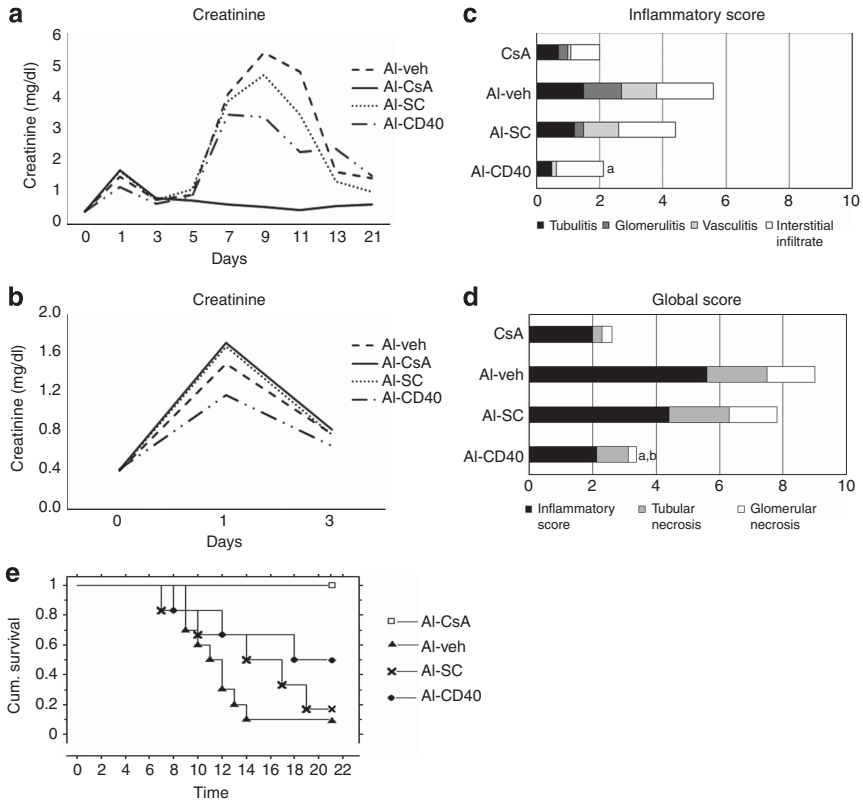

Figure 8 | Survival, renal function, and structure in the cold ischemia allogeneic model. Serum creatinine (**a**) levels of rats after 2.5 h renal cold ischemia, follow-up 21 days. (**b**) Magnification of serum creatinine on days 1 and 3. Al-CD40 group presented better renal function compared with al-scrambled (SC) and al-vehicle (Veh) groups. Renal histopathology analysis (**c**) revealed less inflammatory score, and the overall injury score (**d**) was also reduced in al-CD40 group in contrast to al-veh and al-SC. This overall injury score includes the sum of individual Banff inflammatory scoring with the score of acute tubular necrosis and glomerular necrosis. Al-CsA groups had preserved renal architecture and function. (**e**) Animal survival. Both vehicle and SC groups presented a short survival with an average of 12 and 15 days, respectively. The survival of the Al-CD40 group increased to 17 days. All animals treated with CsA survived the study period. CsA, cyclosporin A.

tion by *CD40* gene silencing also contributed to decreasing the systemic inflammatory response.

Finally, we analyzed the usefulness of *CD40* gene silencing after cold ischemia in the renal transplantation model to approach the clinical application. Results showed a protection both functionally and histologically in the two studied models—allogeneic and syngeneic. To our knowledge, there are no substantial differences from literature in the mechanisms implied in cold or warm ischemia. In contrast, it is still controversial whether allogeneic environment amplifies innate damage in IRI to an adaptive immune response.[50–52] In our allogeneic model, the observed reduction in acute rejection may occur because of a direct inhibition of alloresponse by the CD40 blockade, but this warrants future research.

In summary, knocking down the co-stimulatory CD40 signaling by species-specific siRNAs protects against both warm and cold IRI in rodent kidney models, modulating inflammatory pathways initiated by ischemia. Nowadays, there is a renewed interest in treating ischemic acute renal failure in clinical ground, with many new clinical trials. Our findings may suggest that CD40-gene silencing arise as a new hopeful target to be assayed in patients.

MATERIALS AND METHODS

siRNA design

In this study, two different anti-CD40 siRNA sequences were used, previously described by our group.[17] A mouse-specific siRNA: (sense 5′-GUGUGUUACGUGCAGUGACUU-3′; antisense 3′-GTCACACA AUGCACGUCACUG-5′), and a rat-specific siRNA: (sense 5′-GGC GAAUUCUCAGCUCACUUU-3′; antisense 3′-GTCCGCUUAAG AGUCGAGUGA-5′).

The rat siRNA was chemically stabilized with a partial phosphorothioate backbone and 2′-*O*-methyl sugar modifications on the sense and antisense strands. Moreover, a cholesterol molecule was conjugated to the 3′-end of the siRNA sense strand by means of a pyrrolidine linker (Microsynth, Switzerland). A scrambled siRNA (SC), also chemically stabilized, was used as a control.

Animals and surgical procedures

All procedures followed the Guidelines of the European Community Committee on Care and Use of Laboratory Animals and Good Laboratory Practice. Animals (Charles River, Spain) were housed at constant temperature with a 12 h-light/12 h-dark cycle. Rats were fed standard diet and tap water *ad libitum*. For all rat surgical procedures, a mixture of ketamine (75 mg/kg), atropine (0.05 mg/kg), and valium (5 mg/kg) was used. After surgery, a single intramuscular dose of ciprofloxacin (5 mg) was administered.

In warm studies, Wistar rats received different siRNA doses administered intravenously 1 h before 40 min bilateral renal ischemia, as follows: CD40-15 ($n=6$), 15 μg; CD40-50 ($n=6$), 50 μg; CD40-150 ($n=7$), 150 μg; CD40-500 ($n=6$), 500 μg. Another group received 500 μg ($n=5$) of a nonspecific, scrambled siRNA (SC); PBS-treated animals were used as controls ($n=9$, Veh). Two groups treated with naked scrambled siRNA ($n=4$, SC-naked) and treated with naked siRNA CD40-500 ($n=4$, CD40-naked) were used to compare the efficiency of cholesterol-conjugated siRNAs. The follow-up was 48 h.

For cold ischemia studies, the renal heterotopic transplantation model in male rats was used.[53] In brief for harvesting, a cannula was introduced into the aorta to the ostium of the left renal artery. After a single dose of sodium heparin (1000UI), the kidney was washed with Euro-Collins solution (4 ml, 4 °C, 20 ml/h). The renal artery was excised with an aortic patch, the renal vein cut proximal to its origin and the ureter next to the bladder. Kidneys were preserved for 2.5 h in Euro-Collins solution at 4 °C. For transplantation, the host left kidney was nephrectomized and the ureter cut next to the pelvis. The recipient major vessels were occluded with vascular clamps, and the donor artery and vein were anastomosed to the receptor aorta and cava, respectively. After the vascular clamps were removed, allowing the graft to be reperfused, the ureter was anastomosed end-to-end with four individual 11-0 monofilament stitches. Reanastomosis took no more than 30 min, and total surgical time did not exceed 60 min.[50,51]

The siRNAs (500 μg) were administered 1 h before nephrectomy in the donor and before reperfusion in the recipient. The distribution of the groups was as follows: three groups in the syngeneic model (Lewis–Lewis): sy-Veh (PBS, $n=12$), sy-CD40 ($n=7$), and sy-SC ($n=5$); and four groups in the allogeneic model (Wistar–Lewis): Al-Veh (PBS, $n=9$), Al-CyA (Cyclosporine, $n=5$), Al-CD40 ($n=6$), and Al-SC ($n=5$). The follow-up was 96 h in the syngeneic and 21 days in the allogeneic. The cyclosporine group received a single daily dose (5 mg/kg, in olive oil) by oral gavage (Sandimmun Neoral, Novartis, Spain). Al-CD40 and al-SC received (intravenously) 150 μg of siRNA twice a week.

Blood samples were obtained from the tail vein at time zero and on daily basis. Animals were killed under anesthesia, blood was obtained by aortic puncture, and spleen and kidneys were processed. Serum creatinine and urea measurements were performed following Jaffe's and GLDH reactions (Olympus Autoanalyzer AU400, Hamburg, Germany) in the Veterinary Clinical Biochemistry Laboratory of Universitat Autonoma de Barcelona.

Histological and immunohistochemistry studies

For conventional histology, coronal 1–2 mm thick kidney slices were fixed in buffered 4% formalin, embedded in paraffin, and stained with H&E. Light microscopy sections were examined by a blinded pathologist, evaluating tubular cell necrosis, dilation, interstitial edema, and cellular infiltrates. Abnormalities were graded on a semi-quantitative scale (0–4): 0, no abnormalities; 1, changes $<25\%$; 2, changes 25–50%; 3, changes 50–75%; and 4, changes $>75\%$. An overall score was attained by adding up all the individual parameters. For allogeneic studies sections were analyzed for tubulitis, interstitial infiltration, vasculitis, glomerulitis, tubular necrosis, and glomerular necrosis, following the Banff criteria for acute/active lesions scoring.[54]

Paraffin tissue sections were stained for CD40, L-Galectins 1&3, CD3 (Abcam, Cambridge, UK), and CD68 (AbD Serotec, Raleigh, NC). Sections were immune peroxidase labeled and revealed by diaminobenzidine (Sigma, Madrid, Spain). To measure CD40 expression, a semi quantitative score from 0 to 3 in the kidney compartments (glomeruli, vessels, and interstitium) was used. For measuring CD3, CD68, and galectins at least 10 hpf were counted and the mean value expressed. Immunostained-matched sections without primary antibodies were used as negative control.

Quantification of gene expression in kidneys

Snap-frozen rat kidney was stored at −80 °C. RNA was extracted with the PureLinkTM RNA MiniKit (Invitrogen, Madrid, Spain). All samples had an A260/280 ratio of ~1.8. Five hundred nanograms of RNA was used for reverse transcription with the High-Capacity cDNA reverse Transcription Kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions.

Tissue expression of immune-inflammatory mediators was quantified by TaqMan real-time PCR using the comparative CT method: CD40/TLR2/TLR3/TLR4/NFkβ/IL4/CCL2/CCL3/CCL4/CCL5/CXCL9/CXCL10/CXCL11 (Applied Biosystems).

Moreover, those genes validated in the PROLIGEN study (Supplementary Data) were quantified by Taqman Low Density Array microfluidic cards (ABI-PrismH-7700, Applied Biosystems): Emp1/Emp3/Lgals1/Lgals3/Reln/S100A6/S100A8/S100A9/Socs3/Tnfrsf12a/Fcnb, and eukaryotic 18S as endogenous reference. Controls, which were composed of distilled water, were negative for target and reference genes.

Galectin 1 and 3 western blot analyses in kidneys

Renal tissues were homogenized using a Potter (Sartorius) homogenizer in 10 mM Tris–HCl (pH 7.5) and 100 mM NaCl buffer with protease inhibitors. Subsequently, RIPA detergent was added to the lysis solution and incubated for 15 min, 4 °C. The homogenates were pelleted at 18,000 *g*, 4 °C, 15 min, the supernatant collected and, after quantifying the protein concentration, loaded onto a SDS electrophoretic gel. We then performed a western blot; after blocking, membranes were incubated overnight with antibodies L-galectins 1 and 3. Secondary antibodies were used in 1:10,000 dilutions and revealed with ECL Plus (GE Healthcare, Uppsala, Sweden) in a LAS-3000 (Fujifilm, Tokyo, Japan) apparatus, with the software MultiGauge to visualize and quantify the bands.

Spleen lymphocyte characterization by flow cytometry

Lymphocytes were isolated from spleen by Ficoll (GE Healthcare) density gradient and cryopreserved at −180 °C. Cells were thawed, washed, and recovered by standard methods. Splenocytes were incubated (25 min, RT, dark) with different monoclonal antibodies. The study used a BD FACS Canto II Cytometer and was analyzed by using an FACS DIVA software (BD Biosciences, San Jose, CA). Fluorochrome-conjugated antibodies were appropriately titrated, mixed together, and formulated for optimal staining performance: anti-CD3-FITC (G4.18), anti-CD4-APC (OX-35), anti-CD8a-PerCP (OX-8), anti-CD3-PerCP-efluor710 (G4.18), anti-CD40-APC (HM40-3), anti-CD45R-FITC (HIS24), anti-CD43-PE (W3/13), anti-CD161-AF647 (10/78), and anti-172a-FITC (ED9) (BD Biosciences & Millteny Biotec, Madrid, Spain).

Determination of IFNγ and TNFα secretion in T lymphocytes by flow cytometry

Isolated splenocytes were stimulated with 5 ng/ml of PMA and 500 ng/ml ionomycin in the presence of monensin (10 μg/ml). Samples were incubated for 4 h at 37 °C in a 5% CO_2-humidified atmosphere. The surface of stimulated cells was stained for 20 min at

RT with different mAb. Then, cells were permeabilized with perm/wash solution for 1 h and stained with PE-conjugated mAb anti-TNFα or anti-IFNγ or the appropriate isotype-matched control for 30 min.

The fluorochrome-conjugated anti-rat mAbs used for intracellular flow cytometry analysis were anti-TNFα-PE (TN3-19), anti-IFN-γ-PE (DB-1), PE-conjugated anti-IgG isotype control (eBio299Arm), and PE-conjugated anti-IgG1 isotype control (MOPC-21).

Statistical analysis

For statistical analysis, Student's *t*-test compared two conditions, whereas ANOVA was employed for comparison of multiple conditions. Nonparametric analysis was used as needed. Survival analysis was performed by the Kaplan–Meier's method. A value of $P < 0.05$ was considered significant. Data are given as a mean ± s.e.m.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Mice renal function and histopathology.
Figure S2. Efficiency of siRNA conjugated or not with cholesterol.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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