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ORIGINAL ARTICLE

Absence of donor CD40 protects renal allograft epithelium and preserves renal function

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Summary

Blocking the CD40-CD154 pathway prevents allograft rejection and induces donor-specific tolerance in various experimental models. However, the translation to clinical studies has been hampered by unexpected thromboembolic complications of CD154-blocking antibodies. Thus, blocking CD40 instead is now considered as an alternative strategy. Here, we evaluated the role of donor CD40 in allospecific T-cell responses *in vitro* and in an *in vivo* model for renal transplantation. Fully MHC-mismatched allografts from CD40-deficient donors displayed better renal function than wild type. These functional data correlated with a lower level of apoptosis in renal tubular epithelial cells and higher expression of PD-L1, which is most probably because of a reduced Th17 response in recipients of a CD40-deficient donor. This hypothesis was supported *in vitro*, where donor CD40 expression was important for the induction of direct allospecific T-cell responses. Especially the induction of Th17 cells was critically dependent on donor CD40. IL-17A in conjunction with interferon-γ in turn rendered renal tubular epithelial cells to a more costimulatory state by upregulating CD40 and downregulating PD-L1 expression. In conclusion, CD40 blockade not only reduces the allospecific T-cell responses, but might also lead to protection of tubular epithelium from apoptosis and thereby preserve kidney allograft function.

Introduction

Kidney transplantation is the preferred therapy for end-stage renal disease [1]. Despite improvements in early graft survival, chronic allograft rejection and toxicity of classical immunosuppressant drugs remain major reasons for late graft loss [2]. Thus, there is a need for more specific and less toxic immunosuppression regimens. Blockade of the CD40-CD154-pathway represents a promising target to achieve this goal. It has been shown that blockade of CD154 together with blockade of B7-molecules (CD80 and CD86) leads to long-term graft survival in murine and non-human primate models of skin and renal allografts [3,4]. Furthermore, CD154 blockade is used in several protocols for tolerance induction using donor-specific transfusion [5,6] or bone marrow transplantation [7–9]. Many murine protocols for tolerance induction use a monoclonal antibody directed against CD154 (MR1). However, translation of the anti-CD154 reagent into nonhuman primate and human studies has revealed an unexpected complication of thromboembolic events [10,11], which were because of platelet activation [12]. Thus, CD40 instead of its ligand has become an attractive target [13,14].

Renal tubular epithelial cells (rTECs) are the main target of alloreactive T cells during cellular tubulointerstitial rejection [15]. rTECs function as non-professional antigen-presenting cells (APCs). They express MHC class I and II molecules under inflammatory conditions [16]. The only classical costimulatory molecule expressed on activated rTECs is CD40 [17], whereas CD80 and CD86 are not

expressed. With this surface expression pattern rTECs become targets for both alloreactive CD8 and CD4 T cells.

The T-helper cell population mainly involved in acute rejection is of a Th1 type characterized by IFN-γ production [18]. However, a new subset of proinflammatory T-helper (Th) cells, namely Th17 cells, has come into the focus of transplant research [19]. They are characterized by the expression of the proinflammatory cytokines IL-17A, IL-17F, IL-21 and IL-22 [20]. This T-cell subtype is important for the clearance of pathogens, which cannot be adequately cleared by Th1 or Th2 cells, and it has been described to play a role in various autoimmune diseases [21]. IL-17 has been detected in early renal allograft rejection in rats and humans [22]. Furthermore, human rTECs stimulated with IL-17 become activated and in turn start to secrete chemokines like IL-8 and IL-6 [23]. Nevertheless, the exact role of Th17 cells in allograft rejection is not clear.

In this study we investigated the role of donor CD40 during direct alloreactivity *in vitro* and *in vivo*. We demonstrate an important role of donor CD40 expression for the induction of directly alloreactive cytotoxic T lymphocytes and Th17 cells *in vitro*. Additionally, absence of donor CD40 further reduced tubular cell apoptosis and improved kidney function in fully MHC-mismatched renal allografts.

Material and methods

Mice

Wild type (WT) C57BL/6 (B6, H-2^b), CD40 KO (B6 background, H-2^b), and CBA (H-2^k) mice were housed in specific pathogen-free conditions at the University of Zürich. All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich).

rTEC culture

Preparation and primary culture of rTECs were performed as described [16]. In some experiments primary rTECs were stimulated for 48 h with murine interferon (IFN)-β and IFN-γ at 100 U/ml each and/or murine IL-17A at 50 ng/ml prior to use. All cytokines were purchased from Antigenix America (Huntington Station, NY, USA).

T cell proliferation and cell-mediated lympholysis (CML) assay

T-cell proliferation and CML assays were performed using splenocytes or isolated CD4 and/or CD8 positive T cells as responders. T cells were stimulated with irradiated (30 Gy) splenocytes, CD11c positive dendritic cells (DCs), or splenocytes depleted from CD11c positive cells from allogeneic and syngeneic mice. Splenocytes were sorted by magnetic cell separation (MACS) according to the protocols of Miltenyi Biotec (Bergisch Gladbach, Germany). Purity of sorted cells was confirmed by fluorescence-activated cell sorting (FACS) analysis. Purity for T cells was usually >90% and for DCs >50%.

T-cell proliferation was measured by incorporation of tritium-labeled thymidine (Perkin Elmer, Waltham, MA, USA) on day (d) 4 of culture. Stimulation indices were calculated as follows: Stimulation index = (incorporation allogeneic sample) / (mean incorporation of syngeneic controls).

Cell-mediated lympholysis assays were performed on d 5 of culture: ^{51}chromium (Cr)-labeled, cytokine-stimulated allogeneic rTECs were added to the serially diluted culture for 4 h (killing phase), and allospecific cytotoxicity was assessed by measurement of ^{51}Cr release in the supernatant. Allospecific lysis was calculated as: % specific lysis = (experimental release − spontaneous release)/(total release–spontaneous release) × 100.

FACS analysis

Fluorescence-activated cell sorting analysis was performed on a BD-FACSCanto II (Becton Dickinson, Allschwil, Switzerland). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, CD45R/B220-PE, CD11c-APC, and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Germany).

Cytokines in cell-culture supernatants were quantified using a FlowCytomix™ set purchased from eBioscience (Frankfurt, Germany) according to the manufacturer's manual.

mRNA isolation and quantitative PCR (qPCR)

The mRNA was isolated from either kidney grafts stored in RNase-inhibitor or freshly sorted cells using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). One μg mRNA was transcribed to cDNA using the Omniscript reverse transcription Kit (Qiagen). Predeveloped TaqMan reagents were used for qPCR (Applied Biosystems, Carlsbad, CA, USA) detecting murine programed death-ligand 1 (PD-L1) and the reference 18s rRNA. The expression of candidate genes was normalized to the reference, and fold changes were calculated in relation to the matching controls.

Kidney transplantation and induction of ischemia-reperfusion injury (IRI)

Kidney grafts were performed in a life-supporting manner as described in detail previously [24]. Recipients were sacrificed 1 week after transplantation. In a separate

experiment, we induced IRI by clamping the right renal artery and vein for 22 min.

The left kidney was removed afterwards and used as control. Animals were sacrificed on d 3 and 7 post operation. To assess renal function, plasma urea concentration was measured with the urease/glutamate dehydrogenase method on a Hitachi Modular P autoanalyzer from Roche diagnostics (Rotkreuz, Switzerland). Animal total body weight was determined each day.

Histology and immunohistochemistry

Histologic examination of all kidney grafts was performed by an experienced renal pathologist blinded to the experimental procedures. Tissues were immersion-fixed in 4% phosphate buffered formalin and embedded in paraffin. The thickness of sections was 4 μm. The slides were routinely stained with hematoxylin and eosin (H & E), periodic acid-Schiff stain (PAS), and Elastica-van Gieson (EvG). In selected cases silver methenamine stain and acid fuchsin orange-G stain were added.

Immunohistochemistry for CD3 was performed on paraffin embedded material as previously described using a monoclonal rat anti-CD3 antibody (Clone CD3-12, AbD serotec, Dusseldorf, Germany) [25]. For detection of apoptotic cells by immunohistochemistry the monoclonal antibody F7-26 (Chemicon, International, Inc. Temecula, CA, USA) was used as previously described [26]. F7-26 binds to single-stranded DNA after thermal denaturation. A peroxidase-conjugated monoclonal rat anti-mouse IgM antibody (Zymed, San Francisco, CA, USA) was used as secondary reagent. Dense, apoptotic nuclei positive for single-stranded DNA were quantified in mouse renal allografts in 15 high power fields (original magnification ×250).

Statistical analysis

All statistical comparisons were performed with GraphPad Prism 4. Normally distributed groups were compared using Student's *t*-test. Groups without Gaussian distribution were compared using the Mann–Whitney test. $P < 0.05$ was considered as significant.

Results

Absence of CD40 on renal grafts leads to improved allograft function

To analyze the effect of donor CD40 on renal allograft rejection *in vivo*, we performed life-supporting kidney grafts from WT B6 and CD40 KO donors to fully MHC-mismatched CBA recipients. Syngeneic transplants were used as controls. One week post transplantation allograft function was determined by urea concentration in serum. Recipients of CD40 KO kidney allografts showed an improved kidney function compared with recipients of WT allografts (Fig. 1a). General condition of recipients and survival, as

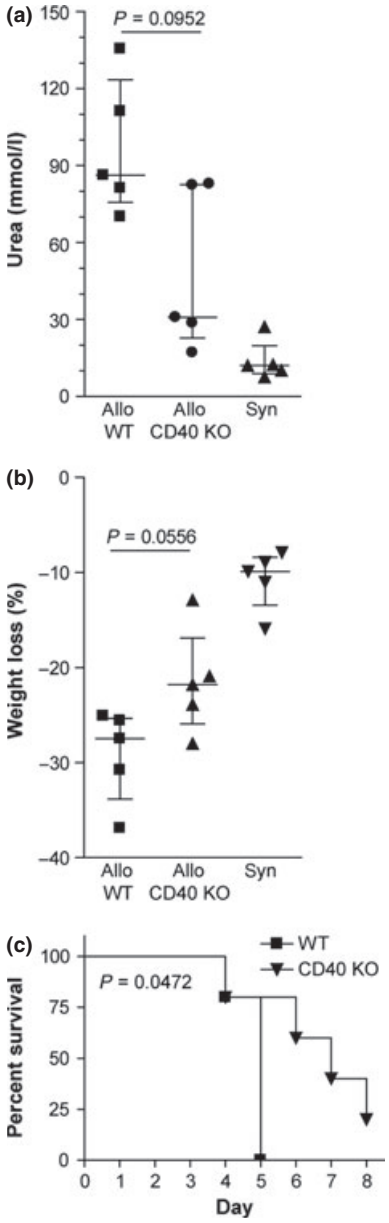

Figure 1 Better renal function of CD40 KO allografts. B6 WT or CD40 KO mice were used as donors for life-supporting kidney grafts to CBA recipients. Syngeneic controls were performed using B6 WT mice as donor and recipient. Organs were harvested after 1 week ($n = 5$ per group). (a) Urea concentration in serum of recipients was determined on harvest day. (b) Total body weight was measured every day after transplantation. Weight loss on harvest day compared with pretransplantation is depicted. Groups were compared using the Mann–Whitney test. (c) Recipients were euthanized, when weight loss exceeded 20%. Survival free of 20% weight loss is depicted in a Kaplan–Meier curve. Groups were compared using a Log-rank test.

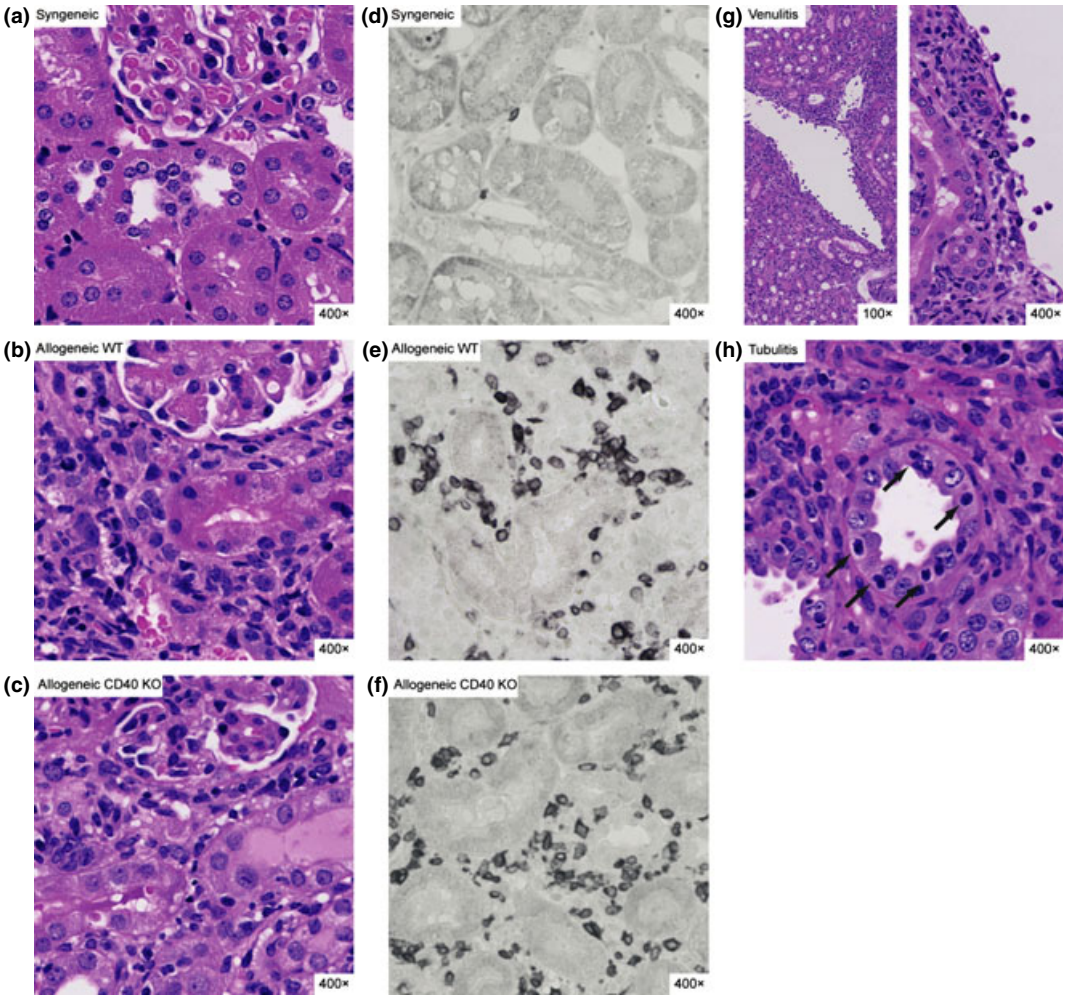

Figure 2 Severity of rejection is comparable between WT and CD40 KO renal allografts. B6 WT or CD40 KO mice were used as donors for life-supporting kidney grafts to CBA recipients. Syngeneic controls were performed using B6 WT mice as donor and recipient. Organs were harvested after 1 week (n = 5 per group). (a) Representative hematoxylin and eosin stain showing normal renal tissue from a syngeneic graft. (b, c) Representative pictures of hematoxylin and eosin stainings showing interstitial infiltrates in allogeneic grafts. (d–f) Representative pictures of immunohistochemistry for murine CD3. (g, h) Representative pictures of venulitis (g) and tubulitis (h) in allografts. Arrows indicate intraepithelial lymphocytes (h).

represented by body weight, were also improved, when the donor organ did not express CD40 (Fig. 1b and c).

To determine severity of rejection, histological analysis was performed on d 7. Despite better renal function of CD40 KO allografts, all allogeneic grafts demonstrated severe interstitial infiltrates and at times prominent tubulitis (Fig. 2b–c and h). These infiltrates showed a perivenular accentuation (Fig. 2g). Infiltrates around arteries were common, but signs of acute vascular rejection (subendothelial infiltrates) were only occasionally seen. CD3 positive cells were as well present as B cells (CD3 staining shown in Fig. 2d–f, B220 staining not shown). The degree of infiltration was comparable between WT and CD40 KO allografts, whereas syngeneic grafts did not show any infiltrates (Fig. 2a and d).

Since we found no difference in the severity of infiltration in the allografts, we tested whether the improved graft function was because of a reduced IRI in CD40 KO allografts. When renal function of WT and CD40 KO mice, whose right kidney was exposed to ischemia-reperfusion, was compared after 3 and 7 days, only minor differences could be detected (Fig. S1a). Also total injury score was not significantly different in both groups (Fig. S1b). On d 3, however, CD40 KO mice showed in tendency higher values for blood urea nitrogen and the kidneys presented with a slightly increased injury score. This implies that CD40 KO kidneys might be slightly more susceptible to early ischemia-reperfusion injury compared with WT. This is in contrast to our results for renal allografts and indicates that the better outcome of CD40 KO renal allografts after 7 days is

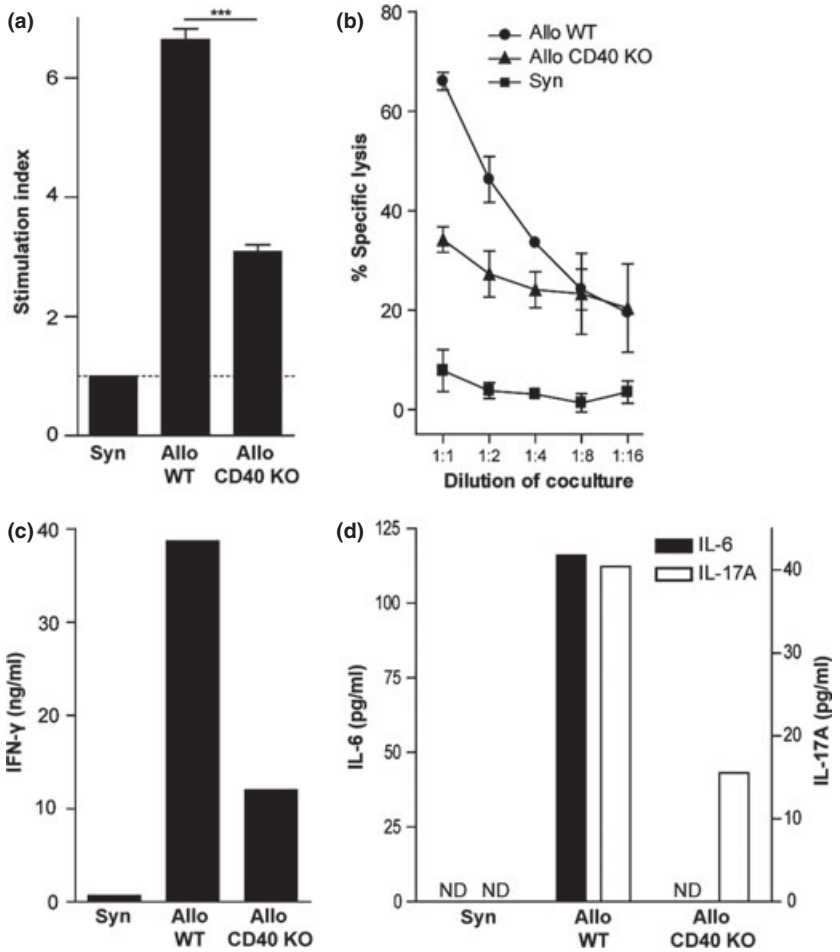

Figure 3 Directly alloreactive T cells depend on donor CD40. CBA CD4 and CD8 T cells were isolated by MACS and cultured in a ratio of 2:1. T cells were stimulated with irradiated allogeneic B6 WT or CD40 KO splenocytes. (a) Allospecific T cell proliferation was measured on d 4 of culture. Mean stimulation indices and standard deviation are depicted. ***$P < 0.0001$ (according to Student's *t*-test). (b) Allospecific cytotoxicity was measured on d 5 in a ^{51}Cr-release assay against IFN-stimulated rTECs from WT B6 mice. Mean killing rates and standard deviation are depicted. (c, d) Cytokine content of coculture supernatants was measured on d 4 using a FlowCytomix assay: (c) IFN-γ, (d) IL-6, and IL-17A concentrations. Mean concentrations are depicted. Standard deviation cannot be displayed because of technical reasons. ND = not detectable. Representative results of at least three independent experiments are shown.

not because of a reduced IRI, but most probably has an immunological reason.

Effector functions of directly alloreactive T cells and Th17 induction are dependent on donor CD40

To understand the processes, which had taken place in renal allograft recipients, we attempted to characterize the direct T-cell alloresponse against CD40 KO stimulators *in vitro*. MACS-isolated T cells from CBA mice (H-2^k) were cocultured with irradiated splenocytes from WT B6 or CD40 KO (both H-2^b) donors. Proliferation was measured by thymidine incorporation, whereas allospecific cytotoxicity was determined by a ^{51}Cr-release assay using IFN-stimulated rTECs as targets. Allospecific proliferation as well as cytotoxicity was significantly reduced, when the T cells did not receive a CD40 signal from the stimulators during stimulation (Fig. 3a and b).

To characterize the subtype of allospecific T-helper cells generated in the cocultures described above, we analyzed cytokine production in the supernatant by multiplex bead assay. A strong induction of IFN-γ production was detected in cocultures of CBA T cells with WT B6 stimulators, which was markedly reduced in the absence of donor CD40 (Fig. 3c). Furthermore, we detected production of IL-6 and subsequently IL-17A. The production of IL-17A was restricted to the CD4 T-cell subset as assessed by ELISA and qPCR (data not shown) and was strongly inhibited in the absence of donor CD40 (Fig. 3d). Th2 cytokines such as IL-4 and IL-10 were not found in any coculture (data not shown).

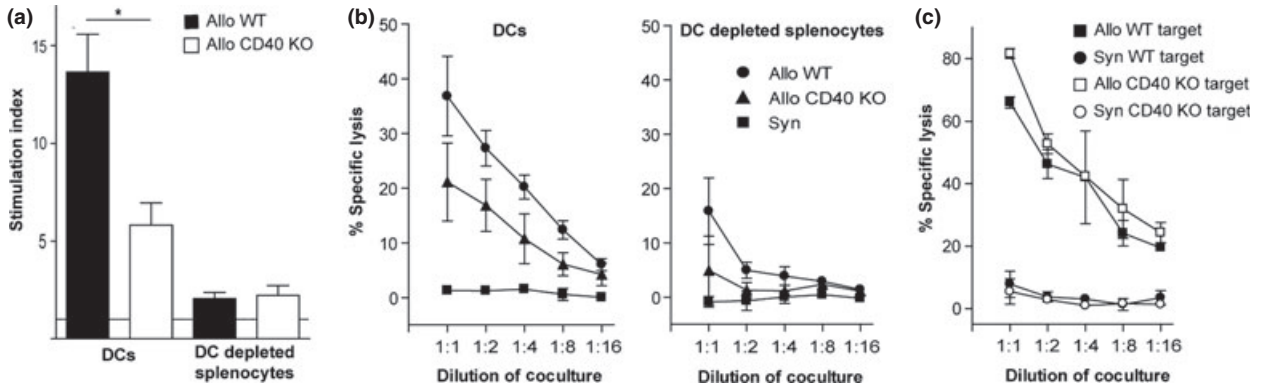

Figure 4 CD40 on allogeneic DCs is critical for cytotoxic T-cell responses *in vitro*. CBA CD4 and CD8 T cells were cultured together in a ratio of 2:1. Splenic DCs from B6 WT or CD40 KO animals were isolated using MACS specific for CD11c. The positive and negative fraction were irradiated and used as stimulators. (a) Proliferation was measured on d 4 of culture. Mean stimulation indices and standard deviation are depicted. *$P < 0.05$ (according to Student's *t*-test) (b) Allospecific cytotoxicity was measured on d 5 in a ^{51}Cr-release assay against IFN-stimulated rTECs from B6 WT mice. Mean killing rates and standard deviation are depicted. (c) CBA CD4 and CD8 T cells were cultured in a ratio of 2:1 and stimulated with splenocytes from B6 WT animals. Allospecific cytotoxicity against B6 WT or CD40 KO rTECs (IFN-stimulated) was measured on d 5 in a ^{51}Cr-release assay. Mean killing rates and standard deviation are depicted. Representative results of at least three independent experiments are shown.

The CD40-mediated effects on *in vitro* allospecific T-cell activation are mediated by DCs

CD40 is expressed on APCs like DCs and B cells. Passenger leukocytes priming the recipient T cells after renal transplantation are mainly DCs. We therefore tested, which APCs are responsible for the observed effects. Using MACS-isolated DCs from B6 and CD40 KO mice as stimulators, allospecific proliferation and cytotoxicity of CBA T cells were induced (Fig. 4a and b) comparably to whole splenocytes (Fig. 3a) and were reduced, when the allogeneic DCs did not express CD40 (Fig. 4a and b). Here, we show the result for CD8 T cells cultured in the presence of CD4 helpers. However, this finding is also true if CD4 or CD8 T cells are stimulated individually (data not shown). In contrast, DC-depleted splenocytes (mainly consisting of B cells) did not induce proliferation or cytotoxicity independent of CD40 expression (Fig. 4a and b).

We further tested the role of CD40 expressed on target rTECs. The ability to express CD40 did not influence allospecific killing of rTECs (Fig. 4c), indicating a minor role for CD40 during the effector phase.

Reduced apoptosis in renal epithelium as an explanation for better renal function in CD40 KO allografts

The fact, that we did not detect differences of T-cell infiltration in renal allografts, indicated a limited effect of donor CD40 on T-cell activation in this *in vivo* setting. We therefore aimed to define further reasons for the functional difference between WT and CD40 KO allografts. By staining for ssDNA we detected a lower amount of apoptotic epithelial cells in CD40 KO allografts (Fig. 5), which indicated that rTECs in CD40 KO allografts may be partially protected from the attack of alloreactive T cells.

Upon IFN-γ stimulation or CD40 triggering, rTECs express higher levels of PD-L1, which may protect them from allospecific T-cell cytotoxicity [27]. Indeed PD-L1 expression detected by RT-PCR was increased in CD40 KO renal allografts compared with WT allografts (Fig. 6a). Since rTECs regulate their surface molecule expression according to the surrounding cytokine milieu (reviewed in [28]), we analyzed the expression of the costimulatory molecule CD40 and the coinhibitory molecule PD-L1 under influence of IFN-γ and IL-17A in *in vitro* cultures of primary rTECs. CD40 expression was induced on WT rTECs under the influence of both cytokines together (Fig. 6b), and its expression was further upregulated upon triggering the CD40 pathway by an agonistic antibody (FGK4.5) (Fig. 6b). In contrast, PD-L1 expression was induced by IFN-γ alone (Fig. 6c). When IL-17A was present in addition, PD-L1 expression was significantly reduced, and this could not be reversed by stimulating the CD40 pathway (Fig. 6c). Thus, a Th1 response concomitant with a Th17 response rendered rTEC surface expression from a rather coinhibitory to a more costimulatory state.

Discussion

In this study, we showed that absence of donor CD40 led to improved function of fully MHC-mismatched renal allografts, despite similar severity of cellular infiltrates. This effect can be explained by a protection of rTECs from apoptosis, which may be mediated by a difference in the cytokine milieu present in WT and CD40 KO renal allografts. We furthermore established a link between CD40

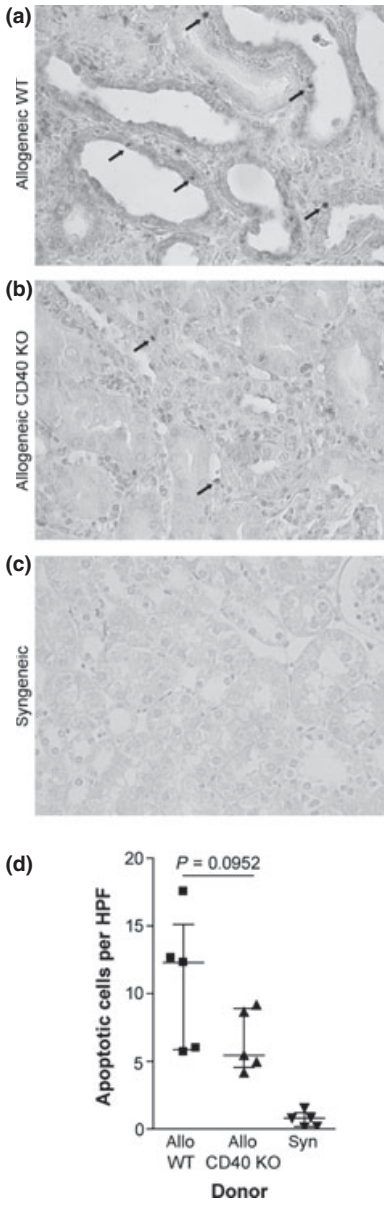

Figure 5 Less apoptosis is detected in tubular epithelium of CD40 KO renal allografts. B6 WT or CD40 KO mice were used as donors for life-supporting kidney grafts to CBA recipients. Syngeneic controls were performed using B6 WT mice as donor and recipient. Organs were harvested at 1 week (n = 5 per group). Immunohistochemistry for ssDNA was performed on all grafts. Representative pictures (magnification: ×400) (a–c) and quantitation (d) are displayed. Arrows indicate apoptotic cells. Groups were compared using the Mann–Whitney test.

signaling and the induction of directly alloreactive Th17 cells *in vitro*.

Directly alloreactive T cells are important effector cells causing acute rejection of solid organ allografts [18]. In this study, we demonstrate a dependence of directly alloreactive T cells on CD40 expressed on donor cells *in vitro*. All effector functions including proliferation, cytokine production, and cytotoxicity were reduced in the absence of donor CD40. Interestingly we found a connection between CD40 signaling and the induction of Th17 cells. It has been shown that the induction of virus-specific Th17 cells is dependent on antigen dose and TLR triggering in the APCs, and this process is boosted by triggering CD40 and completely abolished, when CD40 signaling is blocked [29]. In our study, we show for the first time that the same is also true for alloreactive T cells.

An important role of Th17 cells in response to bacterial pathogens and autoimmune diseases is nowadays widely accepted (reviewed in [30]). However, their importance for solid allograft rejection is still unclear. The most convincing studies showing that IL-17-producing T cells can cause allograft rejection were performed in mice lacking the Th1-specific transcription factor T-bet [31,32]. However, it remains unclear, whether the processes observed in these mice are also taking place in animals sufficient in Th1 responses. When searching for IL-17A expression in acutely rejecting renal allografts, we detected only very low amounts (data not shown). This may be explained by various reasons. First, Iezzi *et al.* have shown that the induction of Th17 responses is dependent on antigen dose and toll-like receptor (TLR) triggering [29]. In our kidney allograft model the amount of donor APCs migrating to the draining lymph node may be rather low in comparison to the amount of APCs used for *in vitro* studies. Furthermore, the APCs in the *in vitro* assays performed here are irradiated and thus might receive a danger signal similar to triggering a TLR. On the other hand in our renal transplant model we did not add any TLR-stimulus. Second, Loong *et al.* detected IL-17-producing mononuclear cells in renal allografts as early as d 2 after transplantation [22]. We harvested the renal allografts only at d 7, and this time point might be too late to detect the described effects. Third, it has been found by Demmers *et al.* in an *in vitro* study that human rTECs do not secrete MIP-3 under inflammatory conditions, a chemokine crucial for the recruitment of Th17 cells [33]. Thus, a low recruitment of Th17 cells to murine renal allografts could explain the rather low mRNA expression of IL-17A.

The direct impact of IL-17 on the modulation of the intragraft immune response is not known. In this study, we show that IL-17A in conjunction with IFN-γ influenced surface expression of CD40 and PD-L1 on murine rTECs *in vitro*. Furthermore, we found higher expression of the coinhibitory ligand PD-L1 in CD40 KO compared with WT allografts *in vivo*. This observation is likely to be because of a difference in the cytokine milieu. We suspect that a higher amount of IL-17A in the allografts early after transplantation in conjunction with IFN-γ leads to lower PD-L1 expression in WT allografts and a lower protection

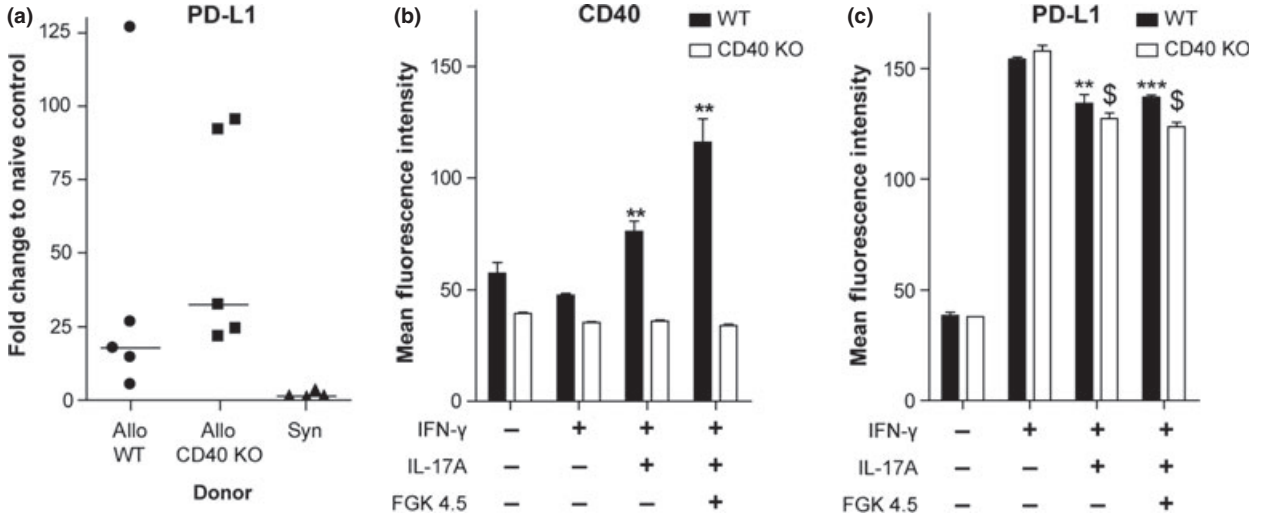

Figure 6 Increased PD-L1 expression protects rTECs in CD40 KO allografts from allospecific cytotoxicity. (a) B6 WT or CD40 KO mice were used as donors for life-supporting kidney grafts to CBA recipients. Syngeneic controls were performed using B6 WT mice as donor and recipient. Organs were harvested after 1 week (n = 5 per group). Intragraft mRNA expression levels of PD-L1 were detected by qPCR. Results are shown as fold change to naïve control kidney samples. Groups were compared using the Mann–Whitney test and no significance was detected between allo WT and allo CD40 KO. (b, c) WT B6 and CD40-KO rTECs were cultured with the indicated stimuli for 48 h (IFN-γ at 100 U/ml, IL-17A at 50 ng/ml, anti-CD40 mAb FGK 4.5 at 20 μg/ml). Surface expression of CD40 (b) and PD-L1 (c) on rTECs was assessed by FACS. Triplicates of mean fluorescence intensities are presented. Groups were compared using Student's t-test: **P < 0.008, ***P < 0.0003 compared to WT cells stimulated with IFN-γ alone; $P < 0.001, $$P < 0.0005 compared to CD40-KO cells stimulated with IFN-γ alone. Representative results of at least three independent experiments are shown.

from allospecific CTL activity. This fits our previous observation that the expression of PD-L1 is responsible for the regulatory effect of human rTECs on allospecific T cells [34].

Renal tubular epithelial cells express CD40 under inflammatory conditions. When CD40 is triggered, this leads to activation and subsequent production of inflammatory molecules, such as IL-6, IL-8, RANTES, MCP-1, IL-15, and PAI-1 [23]. These chemokines and cytokines in turn can cause tubular injury and accelerate renal allograft rejection [35]. The fact, that we found less apoptotic rTECs in CD40 KO allografts, suggests that these cells are partially protected from this process. Similar results were described in a study of chronic proteinuric renal disease, where blockade of CD154 with MR1 led to amelioration of disease [36].

We suppose that these two mechanisms – less activation of rTECs via CD40 and better protection from CTLs via PD-L1 – lead to a better preserved renal function in CD40 KO renal allografts. Whether the surface expression pattern of PD-L1 and CD40 and lower apoptosis level are directly linked in this model needs to be further investigated.

Taken together, our results indicate that blocking donor CD40 not only reduces directly alloreactive cytotoxic T-cell responses and Th17 induction, but might also prevent rTEC activation and killing therefore helping to preserve allograft function. Considering the complications, which blockade of CD154 caused in nonhuman primates and in humans [10], the blockade of CD40 itself becomes a promising strategy to prevent renal allograft rejection.

Authorship

AKK: designed and performed research, analyzed data, wrote the paper. PEC: designed and performed research. AG: performed research, analyzed data. JC: performed research. IE: performed research. RPW: wrote the paper. ML: performed research. SS: analyzed data, wrote the paper. TF: designed research, analyzed data, wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CD40 deficiency does not protect from IRI. The right kidneys of B6 WT and CD40 KO mice were

exposed to IRI by clamping the renal artery and vein for 22 minutes and subsequent reperfusion. Left kidneys were removed afterwards. Animals were euthanized on day 3 and 7 and kidneys as well as serum were harvested. Groups: WT B6, d3 (n = 6); CD40 KO d3 (n = 5); WT B6, d7 (n = 6); CD40 KO d7 (n = 6). (A) Renal function was determined by Urea concentration in serum. Groups were compared using a Mann-Whitney test and no significant differences were detected. (B) Representative PAS-stainings of kidneys 7 days post-operation were blinded and scored for tubular dilatation, loss of brush border, vacuolization, cell detachment and interstitial infiltrates in a range from 0 to 3 (0 = absent, 1 = mild, 2 = intermediate, 3 = severe). The mean score from these five parameters is depicted as total injury score. Groups were compared using a Mann-Whitney test and no significant difference between WT and CD40 KO groups at the according time points could be detected.

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