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Frontline

Tolerance induction towards cardiac allografts under costimulation blockade is impaired in CCR7-deficient animals but can be restored by adoptive transfer of syngeneic plasmacytoid dendritic cells

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Deficiency of transplant recipients for the chemokine receptor CCR7 was originally described to slightly increase the survival time of vascularized solid organ grafts, probably due to a reduced priming of alloreactive T cells. Using a model of allotolerance induction by donor-specific splenocyte transfusion (DST) in combination with anti-CD40L mAb-mediated costimulation blockade (CSB), we show here a striking failure of CCR7-deficient ($CCR7^{-/-}$) recipients to tolerate cardiac allografts. Furthermore, in addition to the recently described lack of Treg, $CCR7^{-/-}$ mice were found to harbor significantly reduced numbers of plasmacytoid dendritic cells (pDCs) within peripheral as well as mesenteric lymph nodes (LNs), but not the bone marrow or spleen. pDCs had previously been suggested to function as tolerogenic APC during allograft transplantation, and a single transfer of syngeneic WT pDCs, but not conventional DCs, was indeed sufficient to rescue graft survival in DST+CSB-treated $CCR7^{-/-}$ recipients in a dose-dependent manner. We therefore conclude that the nearly complete absence of pDCs within LNs of $CCR7^{-/-}$ mice prevents the successful induction of DST+CSB-mediated allotolerance, leading to the observed acute rejection of cardiac allografts under tolerizing conditions.

Keywords: Allotolerance · CCR7 · Donor-specific transfusion · pDC · Vascularized solid organ graft

Supporting Information available online

Introduction

Despite improved immunosuppressive therapy, the chronic rejection of allografts remains a severe problem after vascularized organ transplantation, often limiting long-term graft function and survival [1]. In this context, our understanding of how the chemokine–chemokine receptor network orchestrates crucial immune cell migration events during the induction and maintenance of allotolerance as well as during allograft rejection remains incomplete [2].

Initiation of vascularized allograft rejection or tolerance is thought to mainly occur within secondary lymphoid organs

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(SLOs) of the host, including LNs and spleen (SPL), which provide the environment for APCs to interact with and activate naïve alloreactive T cells [3]. In particular, LNs have been described to play a central role in the induction phase of allograft tolerance [4]. The homing of naïve T lymphocytes into LNs via high endothelial venules (HEVs) as well as the migration of skin and organ-derived DCs towards draining LNs via afferent lymphatics have been reported to crucially rely on the homeostatic chemokine receptor CCR7 [5–7]. Consequently, it had been hypothesized that a delayed allograft rejection might occur in the absence of functional CCR7-signaling mainly due to a reduced priming of alloreactive T cells [8].

Analysis of the survival of cardiac as well as skin allografts in either CCR7-deficient ($CCR7^{-/-}$) or *paucity of LN T cells* (*plt/plt*) recipients (the latter being a naturally occurring mutant lacking expression of CCR7 ligands in lymphoid tissues) has yielded somewhat variable results; while several groups reported the rejection of cardiac allografts to be slightly but significantly delayed in the absence of CCR7-signaling [8–10], others did not find increased survival times for cardiac allografts transplanted into *plt/plt* recipients [11], arguing that the priming of alloreactive host T cells might have primarily occurred within the SPLs of *plt/plt* recipients.

Regarding the question of which APC subsets might primarily participate in the initiation of allotolerance or graft rejection, plasmacytoid dendritic cells (pDCs) have been suggested to be particularly important for the induction of tolerance towards vascularized allografts [4, 12, 13]. Both human and murine pDCs can upregulate the expression of CCR7 and migrate towards CCR7 ligands following in vitro maturation, while different surface expression levels of functional CCR7 have been reported for pDCs under steady-state conditions [14–16]. In this study, we aimed to further elucidate the functional relevance of CCR7-mediated signals during allotolerance induction, using a model of heterotopic transplantation of fully MHC-mismatched vascularized cardiac grafts (CGs) in combination with donor-specific splenocyte transfusion (DST) and costimulation blockade (CSB) with the anti-CD40L(CD154) monoclonal antibody (mAb) MR-1.

Strikingly, $CCR7^{-/-}$ recipients completely failed to establish allotolerance in this setting, with very high numbers of effector T lymphocytes infiltrating the cardiac allograft within few days. As described before [17, 18], the numbers of Tregs within LNs of $CCR7^{-/-}$ animals were largely reduced compared to WT mice, suggesting a possible explanation for the observed failure of allograft tolerance induction. However, while multiple adoptive transfers of wt Tregs significantly prolonged the survival time of cardiac allografts within DST+CSB-treated $CCR7^{-/-}$ recipients, this treatment did not lead to long-term graft acceptance. Analysis of the frequency and organ distribution of pDCs revealed a marked reduction of pDCs within peripheral LNs (pLNs) as well as mesenteric LNs (MLNs), but not bone marrows (BMs) and SPLs of $CCR7^{-/-}$ animals. Importantly, depending on the exact CSB-regimen being used, a single adoptive transfer of variable numbers of syngeneic wt pDCs into $CCR7^{-/-}$ recipients resulted in modest to very substantial increases of the graft survival time in a dose-dependent manner. Taken together, these findings indicate a key role for CCR7 and pDCs in the initiation of allotolerance towards vascularized solid organ grafts.

Results

CCR7-deficiency on recipient T lymphocytes results in a delayed rejection of cardiac allografts

For the present study, we established and validated a mouse model for heterotopic transplantations of fully MHC-mismatched graft hearts (BALB/c graft → C57BL/6 recipient). The observed delay in acute allograft rejection between wt and $CCR7^{-/-}$ recipients thereby confirmed results described previously [8, 9]. Allogeneic graft hearts showed a mean survival time (MST) of 10.4 ± 1.2 days (mean ± SD; $n = 8$) in wt recipients and of 14.2 ± 1.2 days (mean ± SD; $n = 6$) in $CCR7^{-/-}$ recipients (Fig. 1A). In order to test the hypothesis that the acute phase of rejection in this model is mainly mediated by the action of allospecific effector T cells, BALB/c hearts were transplanted into $RAG2^{-/-}$ OT-I recipients of C57BL/6 genetic background. Resulting from the RAG2 deficiency, these mice completely lack normal T (and B) lymphocytes, whereas at the same time the peripheral lymphoid organs are not empty as ovalbumin-specific $CD8^+$ T cells can develop due to the OT-I transgene. Indeed, fully MHC-mismatched graft hearts transplanted into C57BL/6 $RAG2^{-/-}$ OT-I animals were tolerated for more than the observation period of 100 days without overt signs of acute or chronic rejection ($n = 6$; Fig. 1B), indicating that in the absence of alloreactive specificities in the peripheral T-cell pool, neither acute nor chronic rejection of cardiac allografts occurs. In contrast, the adoptive transfer of 1×10^7 polyclonal syngeneic wt T lymphocytes led to a strong reappearance of graft rejection with an MST of only 30.8 ± 11.4 days (mean ± SD; $n = 6$; Fig. 1B). On the other hand, when adoptively transferring 1×10^7 polyclonal syngeneic T lymphocytes from $CCR7^{-/-}$ donors, the MST was 65.9 ± 22.6 days (mean ± SD; $n = 7$; Fig. 1B), suggesting that a CCR7-dependent homing of alloreactive T lymphocytes to SLO contributes to the initiation of allospecific immune responses.

Induction of allotolerance by DST+CSB fails in $CCR7^{-/-}$ recipients of cardiac allografts

Strikingly, however, $CCR7^{-/-}$ recipients perform completely differently in a model of heterotopic heart transplantation after induction of allotolerance by anti-CD40L (CD154) mAb-mediated CSB concomitant with donor-specific transfusion (as described in [19]). While two out of eight wt mice receiving a single 500 μg dose of the mAb MR-1 (anti-CD154) together with a transfusion

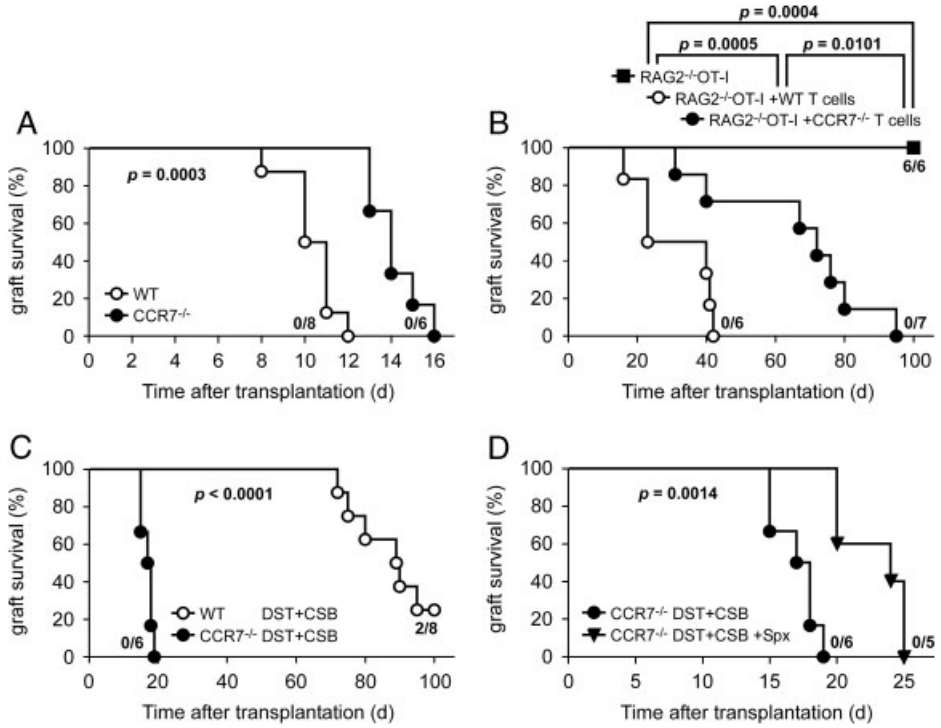

Figure 1. Survival of cardiac allografts in wt and CCR7$^{-/-}$ recipients. (A) Survival of cardiac allografts (BALB/c wt) transplanted into otherwise untreated C57BL/6 wt (open circles, $n = 8$) or CCR7$^{-/-}$ (filled circles, $n = 6$) recipients. (B) Survival of cardiac allografts transplanted into C57BL/6 RAG2$^{-/-}$ OT1 recipients left untreated (filled squares, $n = 6$), or receiving 1×10^7 syngeneic C57BL/6 wt (open circles, $n = 6$) or CCR7$^{-/-}$ (filled circles, $n = 7$) polyclonal T lymphocytes at the day of transplantation. (C) Survival of cardiac allografts transplanted into C57BL/6 wt (open circles, $n = 8$) or CCR7$^{-/-}$ (filled circles, $n = 6$) recipients receiving a single dose of 500 μg MR-1 (anti-CD154 mAb) together with 5×10^6 donor-specific (BALB/c wt) splenocytes (DST+CSB) at the day of transplantation. (D) Survival of cardiac allografts transplanted into DST+CSB-treated C57BL/6 CCR7$^{-/-}$ recipients, with (+Spx, filled inverted triangles, $n = 5$) or without (filled circles, $n = 6$) additionally undergoing splenectomy during operation. Number of allografts functional at day 100 post-Tx is indicated for each group. All p-values were calculated using the Mantel–Haenszel log-rank test.

of 5×10^6 donor splenocytes at the day of transplantation (DST+CSB) tolerated allogeneic heart grafts for more than 100 days (end of observation period, MST of the other six allografts 83.5±9.2 days; mean±SD; Fig. 1C), all grafts within CCR7$^{-/-}$ recipients being treated identically ($n = 6$) were rejected by day 19 post-Tx (MST 17.0±1.7 days; mean±SD; $p<0.0001$). As it has been previously suggested that in the absence of CCR7-signaling, priming of alloreactive T cells might primarily take place in the SPLs [11], we additionally splenectomized DST+CSB-treated CCR7$^{-/-}$ graft recipients during transplantation (+Spx, Fig. 1D), finding only a slight prolongation of graft survival (MST 22.8±2.6 days, $n = 5$ versus 17.0±1.7 days, $n = 6$; mean±SD; $p = 0.0014$).

Rapid allograft rejection in CCR7$^{-/-}$ mice correlates with a pronounced effector T cell infiltration

To characterize the process of acute rejection occurring in CCR7$^{-/-}$ graft recipients despite DST+CSB treatment, we performed histological and flow cytometric analysis of syngeneic and allogeneic graft hearts at day 12 after transplantation (post-Tx). The choice of this time point was determined by the observed kinetics of acute allograft rejection (Fig. 2A). The graft function was monitored daily and graded according to a 0 (no palpable beating of the graft heart) to 4 (strong and fully rhythmic beating) scoring system first introduced by Corry et al. [20]. As displayed in Fig. 2A, graft hearts in DST+CSB-treated CCR7$^{-/-}$ recipients were still fully functional at day 10 post-Tx (all scoring 4, comparable to wt recipients), whereas at day 15 post-Tx some grafts in CCR7$^{-/-}$ recipients had already stopped beating and consequently showed strong signs of tissue necrosis during following dissection. Therefore, in order to correctly address the ensuing process of acute rejection in beating allogeneic CGs, all following analyzes of syngeneic and allogeneic graft hearts were performed at day 12 post-Tx. Histological analysis revealed an increased infiltration by small mononuclear cells within the muscle tissue of cardiac allografts (BALB/c wt) transplanted into C57BL/6 CCR7$^{-/-}$ mice compared to allogeneic hearts grafted into C57BL/6 wt recipients (Fig. 2B and Supporting Information Fig. S1). Quantitative analysis by flow cytometry confirmed this observation, as allogeneic CGs contained an approximately three-fold higher number of infiltrating CD45$^+$ leukocytes at day 12 post-Tx when grafted into CCR7$^{-/-}$ recipients compared to wt controls (Fig. 2C). The number of

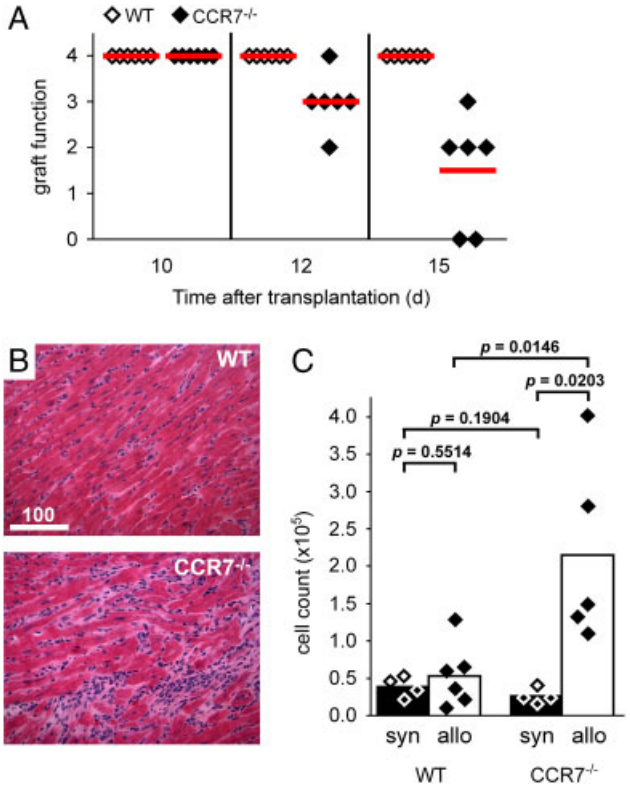

Figure 2. Acute rejection of cardiac allografts in DST+CSB-treated CCR7$^{-/-}$ recipients correlates with an increased lymphocytic infiltration of the graft. (A) Scoring (according to [20]) of cardiac allograft (BALB/c wt) function at days 10, 12 and 15 post-Tx into C57BL/6 wt (open diamonds, $n = 6$) or CCR7$^{-/-}$ (filled diamonds, $n = 6$) recipients, additionally receiving a single dose of 500 μg MR-1 (anti-CD154 mAb) together with 5×10^6 donor-specific (BALB/c wt) splenocytes (DST+CSB) at the day of transplantation. Diamonds represent individual grafts, bars indicate mean values. (B) Representative HE-stained sections of cardiac allografts explanted at day 12 after transplantation into DST+CSB-treated C57BL/6 wt or CCR7$^{-/-}$ recipients. At least four individual grafts per group were analyzed. Scale bar, 100 μm. (C) Absolute numbers of live lymphocytes (gated as DAPI$^-$CD45$^+$ and according to FSC/SSC) isolated from CGs at day 12 after transplantation into C57BL/6 wt or CCR7$^{-/-}$ recipients as determined by flow cytometry. Allograft recipients (allo, filled diamonds+white bars; wt $n = 6$, CCR7$^{-/-}$ $n = 5$) received DST+CSB treatment at the day of transplantation, recipients of syngeneic grafts (syn, open diamonds+black bars; wt $n = 4$ wt, CCR7$^{-/-}$ $n = 4$) were otherwise untreated. Diamonds represent values of individual graft recipients, bars indicate mean values. Data shown are pooled from three independent experiments. All p-values were calculated using the unpaired two-tailed Student's t-test.

leukocytes infiltrating syngeneic CGs from C57BL/6 wt donors was comparably low in both cases.

Further characterizing the infiltrating cells by histology, we found them to be mainly CD4$^+$ and CD8$^+$ T lymphocytes (Fig. 3A). While cardiac allografts in DST+CSB-treated wt recipients contained relatively low numbers of T lymphocytes interspersed within the cardiac muscle tissue at day 12 post-Tx, allografts within CCR7$^{-/-}$ animals harbored higher numbers of T cells, often clustering in patches of lymphocytic infiltration (Fig. 3A and Supporting Information Fig. S1). Flow cytometric analysis again quantitatively confirmed these findings, showing highest numbers of CD4$^+$ as well as CD8$^+$ T cells in allogeneic hearts being grafted into CCR7$^{-/-}$ recipients (Fig. 3B). Furthermore, most of the T lymphocytes infiltrating the CGs were found to be of effector cell phenotype (expressing low levels of L-Selectin (CD62L) and high levels of the effector/memory-marker CD44) in all four groups (Fig. 3B). Consequently, the allografts in CCR7$^{-/-}$ recipients harbored approximately three- to four-fold more effector T lymphocytes at day 12 after Tx compared to allogeneic hearts grafted into wt recipients, suggesting that the acute phase of graft infiltration and destruction by – potentially mostly alloreactive – effector T cells is actually exacerbated in CCR7$^{-/-}$ recipients. This is somewhat unexpected considering the reduced potential of T-cell priming within CCR7$^{-/-}$ animals due to the impaired homing of naive T lymphocytes as well as DC migration to the T-cell zones of LNs and SPLs.

Reduction of LN Treg in CCR7$^{-/-}$ mice might contribute to the impaired DST+CSB-induced allotolerance

Tregs have been shown to play a crucial role mediating graft survival in a number of transplant settings [21]. We therefore hypothesized that an impairment of host Treg function within CCR7$^{-/-}$ recipients might account for the observed failure of tolerance induction towards cardiac allografts by DST+CSB treatment. Consequently, we analyzed different compartments of the graft recipients by immunohistology and flow cytometry for the frequency and localization of Tregs at day 12 post-Tx (Fig. 4).

Immunohistological analysis revealed that cardiac allografts transplanted into DST+CSB-treated CCR7$^{-/-}$ recipients clearly harbored more Tregs than those transplanted into equally treated C57BL/6 wt recipients (Fig. 4A, upper panel), whereas the number of Tregs present within the T-cell zone of pLNs were largely reduced in CCR7$^{-/-}$ animals (Fig. 4A, lower panel), confirming previous reports [17, 18]. To quantify the observed differences, we determined the number of CD4$^+$Foxp3$^+$ Treg-infiltrating cardiac allografts at day 12 post-Tx by flow cytometry (Fig. 4B): the absolute number of Tregs (defined as CD4$^+$CD25$^+$Foxp3$^+$ live lymphocytes) at day 12 post-Tx was much higher in cardiac allografts transferred into DST+CSB-treated CCR7$^{-/-}$ recipients compared to allografts transferred into equally treated C57BL/6 wt recipients ($40.5 \pm 22.9 \times 10^2$, $n = 5$ versus $12.4 \pm 11.6 \times 10^2$, $n = 6$; mean ± SD; $p = 0.0266$). In contrast, syngeneic CGs were observed to generally contain considerably fewer Tregs ($4.2 \pm 6.9 \times 10^2$, $n = 4$ in wt, $6.2 \pm 10.6 \times 10^2$, $n = 4$ in CCR7$^{-/-}$ recipients; mean ± SD; $p = 0.7623$). Importantly, these Treg counts largely reflect the absolute amount of leukocytic infiltration into CGs (see Fig. 2C), indicating that under the experimental conditions tested probably no preferential recruitment of Tregs into CGs is taking place. Furthermore, Treg numbers within pLNs and MLNs of graft recipients were determined by flow cytometry. Paralleling histological observa-

tions, we found C57BL/6 wt recipient mice to generally harbor higher numbers of Tregs in pLNs and MLNs compared to age- and sex-matched congenic CCR7$^{-/-}$ recipients, largely independent of the type of CG (Fig. 4B). This is in line with earlier studies addressing the presence of Tregs in SLO of CCR7$^{-/-}$ animals under steady-state conditions [17, 18].

Importantly, whereas CCR7$^{-/-}$ Tregs were observed to efficiently localize into allografts, they are obviously unable to actually prevent the acute rejection. If this was due to their impaired homing into SLO during the phase of allotolerance induction by DST+CSB treatment, the presence of syngeneic wt Tregs during allotolerance induction should improve the survival of cardiac allografts in DST+CSB-treated CCR7$^{-/-}$ recipients. We thus transferred 1×10^7 MACS-purified C57BL/6 wt Tregs at day −1, +7 and +14 relative to transplantation into DST+CSB-treated CCR7$^{-/-}$ allograft recipients, observing a moderate but significant prolongation of allograft survival (MST 33.0±11.0 days, $n = 4$ versus 17.0±1.7 days, $n = 6$; mean±SD; $p = 0.0037$; Fig. 4C). On the other hand, the transfer of 1×10^7 MACS-purified C57BL/6 wt naive T lymphocytes at the same time points did not improve the survival of cardiac allografts (MST 18.5±9.0 days, $n = 4$ versus 17.0±1.7 days, $n = 6$; mean±SD; $p = 0.7296$; Fig. 4C).

CCR7$^{-/-}$ mice harbour largely reduced numbers of pDCs within LNs, but not SPLs or BMs

As pDCs had previously been implicated in the process of allotolerance induction [4, 22], we next aimed to quantitatively analyze the presence of pDCs within different organs of wt and CCR7$^{-/-}$ graft recipients. Whereas it had been previously reported that pDC numbers within resting lymphatic tissues of wt animals can vary greatly depending on the genetic background [23], to our knowledge their tissue distribution and function in the absence of CCR7-mediated signals has not been addressed so far.

We first analyzed pLNs, MLNs and SPLs of untreated age- and sex-matched wt and CCR7$^{-/-}$ mice of C57BL/6 genetic background by flow cytometry, readily identifying pDCs as a distinct population of 120G8$^+$B220^{int}CD11c^{int} cells (see Supporting Information Fig. S2). Importantly, pDC frequencies (Supporting Information Fig. S2 and data not shown) as well as absolute numbers of pDCs present within pLNs and MLNs, but not SPLs of CCR7$^{-/-}$ animals were clearly reduced in comparison to wt (Fig. 5A), suggesting that CCR7-mediated signals might support the steady-state LN homing of pDCs. Furthermore, pDC numbers in BMs of CCR7$^{-/-}$ mice were unaltered (data not

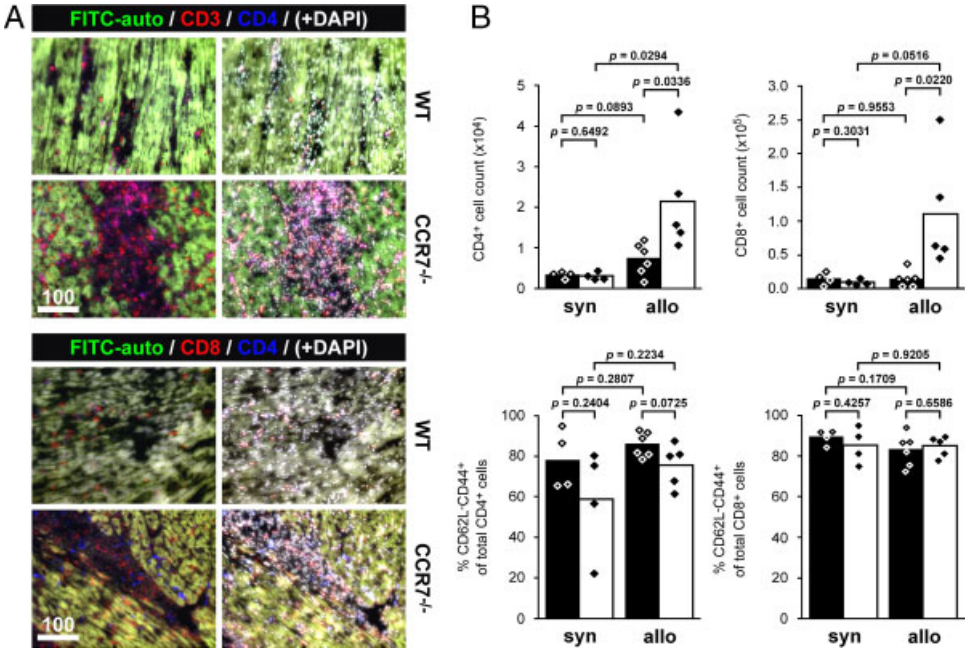

Figure 3. Increased effector lymphocyte infiltration of cardiac allografts in DST+CSB-treated CCR7$^{-/-}$ recipients. (A) Cardiac allografts (BALB/c wt) were transplanted into C57BL/6 wt or CCR7$^{-/-}$ recipients receiving a single dose of 500 μg MR-1 (anti-CD154 mAb) together with 5×10^6 donor-specific (BALB/c wt) splenocytes (DST+CSB) at the day of transplantation. Cryosections were prepared from cardiac allografts explanted at day 12 post-Tx and stained with DAPI (visualization of cell nuclei in the right panel, white) and mAb directed against CD3, CD4 and CD8 (colors as indicated). FITC-channel autofluorescence of cardiac muscle cells (green). Representative fluorescence microscopy images, at least four individual grafts per group were analyzed. Scale bars, 100 μm. (B) Flow cytometric analysis of lymphocytes isolated from CGs at day 12 post-Tx. Absolute numbers of CD4$^+$ and CD8$^+$ T cells (upper panel), as well as percentages of CD62L$^-$CD44$^+$ effector memory T cells of total CD4$^+$ and CD8$^+$ T cells (lower panel) infiltrating syngeneic C57BL/6 wt (syn) or allogeneic BALB/c wt (allo) CGs transplanted into C57BL/6 wt (open diamonds+black bars) or CCR7$^{-/-}$ (filled diamonds+white bars) recipients. Allograft recipients (wt $n = 6$, CCR7$^{-/-}$ $n = 5$) were DST+CSB-treated at the day of transplantation, recipients of syngeneic grafts (wt $n = 4$, CCR7$^{-/-}$ $n = 4$) were otherwise untreated. Diamonds represent values of individual graft recipients, bars indicate mean values. Data shown are pooled from three independent experiments. All p-values were calculated using the unpaired two-tailed Student's t-test.

shown), indicating that the generation and differentiation of pDCs in BMs as well as their export into the circulation were not influenced by the absence of CCR7 signals.

We next checked if this finding also held true under the conditions of our cardiac transplantation model. Analyzing pLNs, MLNs, SPLs as well as CGs at day 12 post-Tx for the presence of pDCs, we

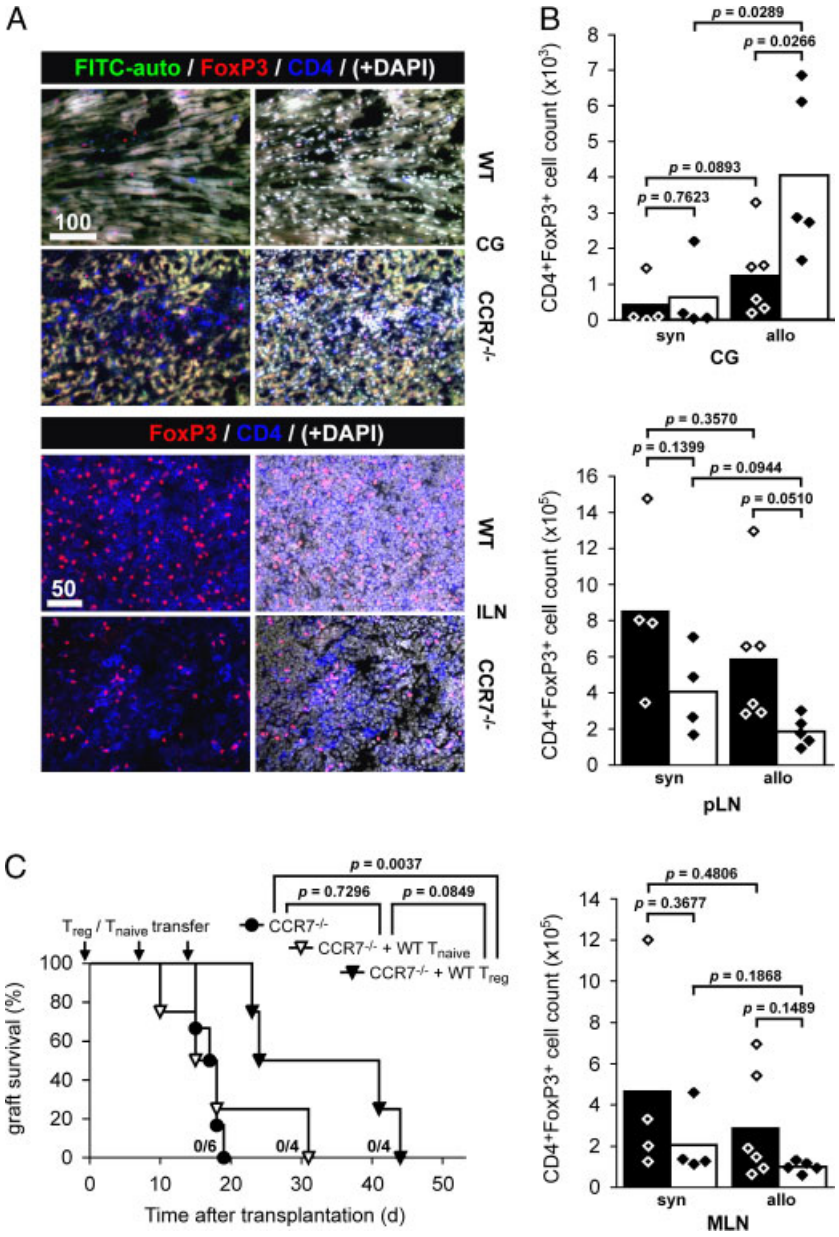

Figure 4. DST+CSB-treated CCR7$^{-/-}$ recipients display higher numbers of CD4$^+$Foxp3$^+$ Tregs within cardiac allografts despite reduced Treg numbers within LNs. Cardiac allografts (BALB/c wt) were transplanted into C57BL/6 wt or CCR7$^{-/-}$ recipients receiving a single dose of 500 μg MR-1 (anti-CD154 mAb) together with 5×10^6 donor-specific (BALB/c wt) splenocytes (DST+CSB) at the day of transplantation. (A) Cryosections were prepared from cardiac allografts (CG) as well as inguinal LNs (ILNs) explanted at day 12 post-Tx and stained with DAPI (visualization of cell nuclei in the right panel, white) and mAb against CD4 (blue)/Foxp3 (red). FITC-channel autofluorescence of cardiac muscle cells (only CG, green). Representative fluorescence microscopy images, at least four individual grafts/LNs of four different animals were analyzed per group. Scale bars, 100 μm (top)/50 μm (bottom). (B) Absolute numbers of CD4$^+$FoxP3$^+$ Tregs present within CGs, pooled pLNs and MLNs explanted at day 12 post-Tx as determined by flow cytometry. Syngeneic C57BL/6 wt (syn) or allogeneic BALB/c wt (allo) CG were transplanted into C57BL/6 wt (open diamonds+black bars) or CCR7$^{-/-}$ (filled diamonds+white bars) recipients. Allograft recipients (wt $n = 6$, CCR7$^{-/-}$ $n = 5$) were DST+CSB-treated at the day of transplantation, recipients of syngeneic grafts (wt $n = 4$, CCR7$^{-/-}$ $n = 4$) were otherwise untreated. Diamonds represent values of individual graft recipients, bars indicate mean values. Data shown are pooled from three independent experiments. All p-values were calculated using the unpaired two-tailed Student's t-test. (C) Survival of cardiac allografts transplanted into DST+CSB-treated C57BL/6 CCR7$^{-/-}$ recipients (filled circles, $n = 6$), additionally receiving three individual doses of 1×10^7 syngeneic CD4$^+$CD25$^-$ naïve (open inverted triangles, $n = 4$) or CD4$^+$CD25$^+$ regulatory (filled inverted triangles, $n = 4$) T lymphocytes at days −1, +7 and +14 relative to transplantation (black arrows). Number of allografts functional at day 100 post-Tx is indicated for each group. All p-values were calculated using the Mantel–Haenszel log-rank test.

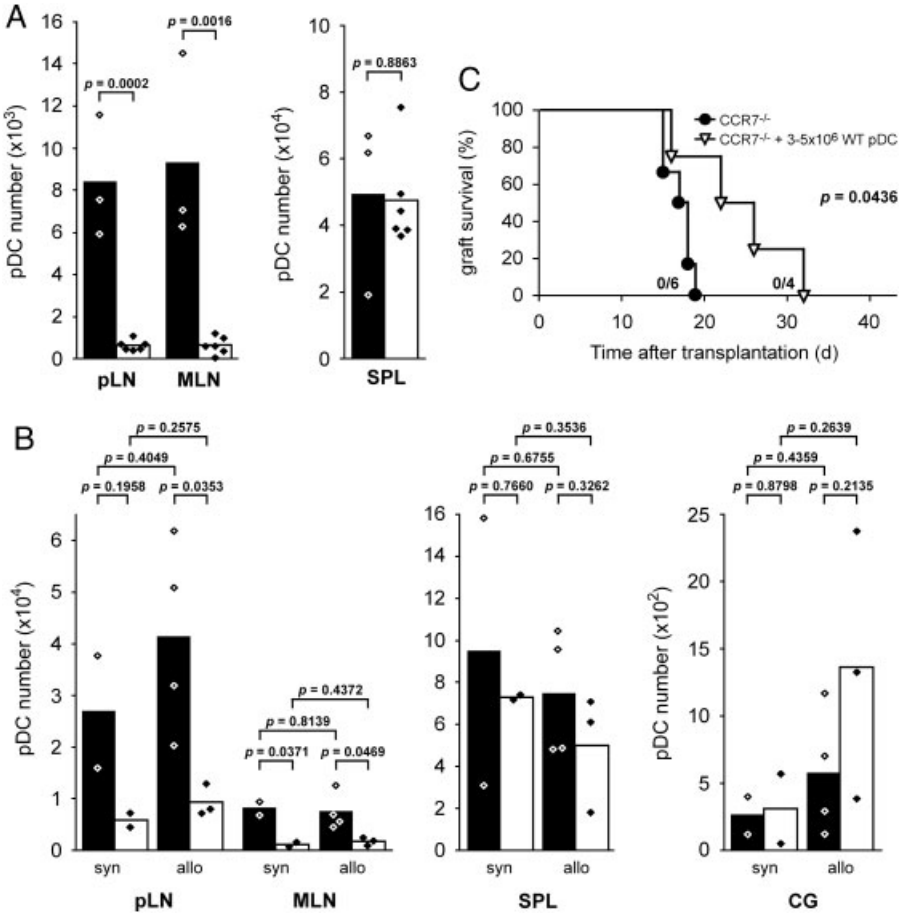

Figure 5. Tissue distribution of pDCs within wt and CCR7$^{-/-}$ mice. (A) Flow cytometric analysis of absolute numbers of 120G8^{+}B220^{int}CD11c^{int} pDCs within pooled pLNs, MLNs and SPLs of untreated C57BL/6 wt (open diamonds+black bars, $n = 3$) or CCR7$^{-/-}$ (filled diamonds+white bars, $n = 6$) animals. (B) Absolute numbers of pDCs within pLNs, MLNs, SPLs and CGs at day 12 after transplantation of syngeneic C57BL/6 wt (syn) or allogeneic BALB/c wt (allo) CG into C57BL/6 wt (open diamonds+black bars) or CCR7$^{-/-}$ (filled diamonds+white bars) recipients. Allograft recipients (wt $n = 4$, CCR7$^{-/-}$ $n = 3$) received a single dose of 500 μg MR-1 (anti-CD154 mAb) together with 5×10^6 donor-specific (BALB/c wt) splenocytes (DST+CSB) at the day of transplantation, recipients of syngeneic CGs (wt $n = 2$, CCR7$^{-/-}$ $n = 2$) were otherwise untreated. In (A, B), diamonds represent values of individual animals, bars indicate mean values. Data shown are pooled from two independent experiments. All p-values were calculated using the unpaired two-tailed Student's t-test. (C) Survival of cardiac allografts transplanted into DST+CSB-treated C57BL/6 CCR7$^{-/-}$ recipients, with (open inverted triangles, $n = 4$) or without (filled circles, $n = 6$) additionally being transfused with 3–5 $\times$ 10^6 C57BL/6 wt pDCs at the day of transplantation. Number of allografts functional at day 100 post-Tx is indicated for each group. p-Value was calculated using the Mantel–Haenszel log-rank test.

found reduced numbers of pDCs within pLNs and MLNs of CCR7$^{-/-}$ recipients independent of the genetic background of the CG (Fig. 5B). pDC numbers in SPLs were largely unchanged, whereas cardiac allografts transplanted into CCR7$^{-/-}$ recipients in some cases harbored higher numbers of pDCs compared to allografts within wt recipients (Fig. 5B). It has to be noted, however, that only extremely low numbers of pDCs were present within CGs at all.

Rescue of DST+CSB-induced allotolerance in CCR7$^{-/-}$ recipients by adoptive transfer of syngeneic pDCs

Assuming that the reduction of pDC numbers within LNs of CCR7$^{-/-}$ animals in the steady state as well as in the transplantation setting contributed to the observed impairment of CSB-induced allotolerance, we speculated that reconstitution of CCR7$^{-/-}$ recipients with syngeneic wt pDCs at the day of transplantation might improve the survival of allogeneic CGs after DST+CSB. To generate large amounts of pDCs for adoptive transfers, C57BL/6 wt animals were subcutaneously injected with Flt3L-secreting tumor cells, thereby massively expanding DCs (and among those pDCs) over the course of 10–14 days. After harvesting LNs and SPLs of tumor-bearing mice, PDCA-1^{+} B220^{int}CD11c^{int} pDCs were sorted by MACS depletion to a purity of 87–95% (Supporting Information Fig. S3) using the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi), and 3–5 $\times$ 10^6 C57BL/6 wt pDCs were i.v. injected into CCR7$^{-/-}$ allograft recipients together with the DST+CSB treatment at the day of transplantation. Also, in comparison to the effect of multiple Treg transfers (Fig. 4C), this single adoptive transfer of pDCs only modestly prolonged the survival time of allogeneic heart grafts (MST 24.0$\pm$6.7 days, $n = 4$ versus 17.0$\pm$1.7 days, $n = 6$;

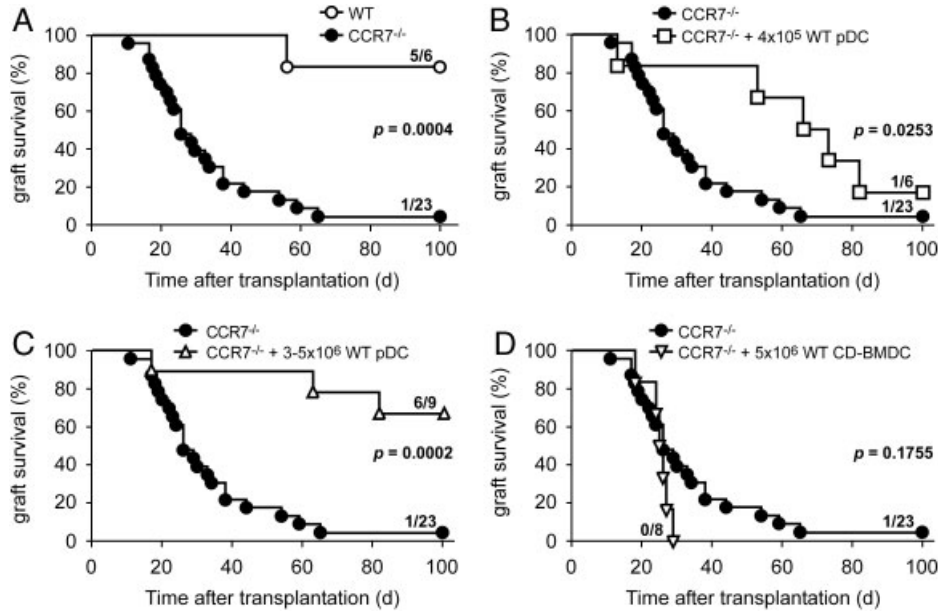

Figure 6. Adoptive transfer of syngeneic wt pDCs, but not BM-derived cDCs, prior to transplantation rescues cardiac allograft survival in DST+CSB-treated CCR7$^{-/-}$ recipients in a dose-dependent manner. (A) Survival of cardiac allografts (BALB/c wt) transplanted into C57BL/6 wt (open circles, $n = 6$) or CCR7$^{-/-}$ (filled circles, $n = 23$) recipients receiving four doses of 250 μg MR-1 (anti-CD154 mAb) at days –7 (together with 1×10^7 donor-specific (BALB/c wt) splenocytes), –4, 0 and +4 relative to transplantation. (B–D) Survival of cardiac allografts transplanted into C57BL/6 CCR7$^{-/-}$ recipients receiving four doses of 250 μg MR-1 at days –7 (together with 1×10^7 donor-specific splenocytes), –4, 0 and +4 relative to transplantation (filled circles, $n = 23$), or additionally being transfused with 4×10^5 syngeneic C57BL/6 wt pDCs (B, open squares, $n = 6$), 3–5 $\times 10^6$ syngeneic C57BL/6 wt pDCs (C, open upright triangles, $n = 9$), or 5×10^6 syngeneic cluster-disrupted BM-derived cDCs (CD-BMDC, D, open inverted triangles, $n = 8$) at day –7 relative to transplantation. Number of allografts functional at day 100 post-Tx is indicated for each group. p-Values were calculated using the Mantel–Haenszel log-rank test.

mean ± SD; $p = 0.0436$; Fig. 5C). Importantly, however, the exact timing of pDC transfer and DST+CSB treatment relative to transplantion might critically influence the ability of pDCs to initiate mechanisms of allotolerance induction [24]. Thus, we tested the tolerogenic potential of wt pDCs adoptively transferred into CCR7$^{-/-}$ recipients prior to the transplantation of the cardiac allograft, using a modified regime of CSB-induced graft tolerance described by Ochando et al. [4]. In this case, 1×10^7 donor-specific splenocytes, together with a first dose of only 250 μg anti-CD154 mAb were administered i.v. already at day 7 before transplantation, with three additional doses of 250 μg anti-CD154 mAb being given at day −4, day 0 (directly after Tx) and day 4 post-Tx. Assessing cardiac allograft survival in wt and CCR7$^{-/-}$ recipients using this new treatment regimen revealed that, whereas the altered timing of DST in combination with the repeated administration of the CSB-mediating anti-CD154 mAb could somewhat ameliorate the acute graft rejection in CCR7$^{-/-}$ recipients, it did not lead to a long-term graft acceptance as observed in wt recipients (Fig. 6A). While the adoptive transfer of a low number of 4×10^5 C57BL/6 wt pDCs at day −7 (together with the donor-splenocyte transfusion and the first dose of anti-CD154 mAb) was found to delay the allograft rejection in CCR7$^{-/-}$ recipients (Fig. 6B), the transfer of 3–5 $\times 10^6$ C57BL/6 wt pDCs massively improved allograft acceptance in this setting, leading to a long-term survival of cardiac allografts in CCR7$^{-/-}$ recipients almost comparable to wt controls (Fig. 6C). Strikingly, the adoptive transfer of comparable numbers of syngeneic so-called 'cluster-disrupted' conventional DC (cDC) derived from GM-CSF-supplemented in vitro cultures of C57BL/6 wt BMs (CD-BMDC) did not improve the survival of cardiac allografts within otherwise identically treated CCR7$^{-/-}$ recipient animals (Fig. 6D). Importantly, these cells did not receive any strong maturation stimulus (such as LPS) during in vitro differentiation, but were treated by 'cluster-disruption', a preparation method described to enhance the tolerogenic potential of BM-derived cDCs [25]. It therefore seems likely that, within this setting of cardiac allotransplantation, pDCs are distinguished from other professional APC populations by functional properties other than a general propensity for tolerance induction, e.g. their potential to home in to LNs from the blood circulation after acquiring alloantigenic material. As a consequence, the pronounced reduction of pDCs within LNs of CCR7$^{-/-}$ recipients might explain the nearly complete inability of these animals to support the long-term survival of cardiac allografts after treatment with DST+CSB. Finally, although Tregs have been previously associated with the long-term maintenance of allotolerance [4, 21, 26], their relevance in this experimental setup remains unclear: analyzing Treg frequencies in pLNs of DST+CSB-treated CCR7$^{-/-}$ allograft recipients at day 12 post-Tx, we did not observe an expansion of CD4$^+$Foxp3$^+$ Tregs following the additional adoptive transfer of wt pDCs at day −7 (Supporting Information Fig. S4A). Aiming to detect the generation of Tregs with higher sensitivity, we used FACS-sorted EGFP$^-$CD4$^+$ T cells

from transgenic Foxp3EGFP donors [27] as reporter cells (becoming EGFP$^+$ when expressing Foxp3) that were transferred into DST+CSB-treated CCR7$^{-/-}$ recipients at day –7. Importantly, while we could clearly identify the population of reporter T cells (being Thy1.1$^+$) within the recipients' LNs and SPLs at day 0, we did not detect any increase of the EGFP$^+$ fraction, irrespective of the co-transfer of large numbers of wt pDCs at day –7 (Supporting Information Fig. S4B). This indicates that, even in the presence of adoptively transferred syngeneic wt pDCs, no substantial de novo generation of Foxp3$^+$ Tregs seems to occur during DST+CSB treatment in CCR7$^{-/-}$ mice. Thus, at least the early phase of a pDC-dependent rescue of allotolerance in CCR7$^{-/-}$ recipients might function independent of Treg induction.

Discussion

In a number of rodent transplantation models, CSB by targeting CD40-CD40L(CD154) interactions, typically combined with donor-specific splenocyte transfusion (DST), has proven successful in preventing rejection of vascularized solid-organ grafts [28, 29], with repeated transfusions with anti-CD154 mAb even further prolonging the survival of skin and cardiac allografts in wt recipients [4, 24]. In striking contrast, both DST+CSB regimes employed in this study almost completely failed to induce long-term acceptance of cardiac allografts in CCR7$^{-/-}$ recipients, and this finding is in line with the rapid rejection of cardiac allografts observed in DST+CSB-treated *plt/plt* mice [30]. Thus, while non-conditioned CCR7$^{-/-}$ and *plt/plt* mice reject skin and cardiac allografts with a similar or even slightly delayed kinetic compared to wt recipients [8–11], absence of CCR7 signaling seems to critically interfere with mechanisms of DST+CSB-mediated transplantation tolerance.

Modes of action being discussed for DST+CSB-induced allotolerance include deletion and/or anergy of allo-reactive T-cell clones, as well as the expansion and/or de novo induction of allo-specific Tregs. Analyzing CCR7$^{-/-}$ graft recipients, we found reduced numbers of Tregs present within pLNs and MLNs, basically confirming earlier results obtained in untreated animals [17, 18]. At the same time, rapid rejection occurred in DST+CSB-treated CCR7$^{-/-}$ recipients while Treg numbers within cardiac allografts were even increased compared to equally treated wt recipients. Thus, although CCR7$^{-/-}$ Tregs have been shown to possess a cell-intrinsic suppressive functionality comparable to that of wt Tregs when tested in vitro [17], their presence within the cardiac allograft is obviously not sufficient to prevent rejection and might rather reflect a functionally irrelevant passive accumulation, either due to rejection-induced inflammatory changes in the graft environment or secondary to the LN homing defect of CCR7$^{-/-}$ T cells (including Tregs). This observation is somewhat in contrast to earlier findings of Lee et al., indicating that the (in their model CCR4-dependent) recruitment of Foxp3$^+$ Tregs into cardiac allografts is relevant for the maintenance of DST+CSB-induced allotolerance in wt recipients [31]. On the other hand, the relative inability of CCR7$^{-/-}$ naïve T cells as well as Tregs to enter LNs during the phase of DST+CSB might prevent the successful expansion of allo-specific protective Treg clones as well as their suppressive action, both presumably taking place primarily within LNs [4, 17]. In this context, it remains largely unknown why the SPL compartment cannot support DST+CSB-mediated tolerogenic processes that are impaired due to lack of CCR7-dependent LN homing. Possible explanations include differences in the repertoire of tolerogenic APCs as well as stromal cells and the cytokine milieu present in the T-cell areas. Importantly, however, the repetitive adoptive transfer of wt Tregs into CCR7$^{-/-}$ allograft recipients was found to confer an intermediate increase of the survival time of CGs, but did not lead to long-term graft acceptance. Although this outcome might have been influenced by the timing and dose of wt Treg transfers (as well as the timing and dose of DST+CSB application [4, 24]), the mere presence of higher numbers of polyclonal CCR7-expressing Tregs was not sufficient to establish and maintain a long-term state of allotolerance within otherwise CCR7$^{-/-}$ recipients of CGs.

Recently, several studies have implicated pDCs as APCs in settings of tolerance induction, including oral tolerance [32] and allograft survival [4, 12, 13, 22]. It was therefore highly intriguing to find that CCR7$^{-/-}$ animals rejecting cardiac allografts despite DST+CSB treatment indeed harbored largely reduced numbers of pDCs within pLNs and MLNs. Although a single i.v. application of wt pDCs actually did not lead to superior allograft survival times compared to multiple transfers of wt Tregs (using in both cases DST plus a single application of anti-CD154 mAb), in a modified treatment regime with four CSB doses, the adoptive transfer of syngeneic wt pDCs was able to almost completely rescue the tolerogenic function of the DST+CSB treatment in CCR7$^{-/-}$ recipients. Taking into account that also resting murine pDCs express small amounts of surface CCR7 and that resting as well as activated CCR7$^{-/-}$ pDCs display a reduced LN homing following adoptive transfer (SS and RF; unpublished observations), this suggests that a homing defect of CCR7$^{-/-}$ pDCs to LNs might be causally involved in the impairment of allotolerance induction observed in CCR7$^{-/-}$ recipients. CCR7 deficiency on pDCs might additionally affect their intranodal positioning in the paracortical T-cell zone, thus impacting on their capacity to exhibit a suppressive activity by interacting with allo-specific T cells. Interestingly, comparing allograft survival times after DST+CSB treatment, the depletion of pDCs in wt recipients using the mAb clone 120G8 [4] seems to be even more detrimental than the constitutive CCR7 deficiency of the graft recipient (mean allograft survival times: approximately 12 days after 4$\times$ 120G8 mAb in DST+CSB-treated wt recipients [4] versus 34 days in DST+CSB-treated CCR7$^{-/-}$ recipients (see Fig. 6A)). Thus, the very small numbers of pDCs present within pLNs and MLNs of CCR7$^{-/-}$ mice seem to actually retain some residual function as tolerogenic APCs, or CCR7$^{-/-}$ pDCs being present in regular numbers in SPLs might partially compensate for the lack of LN pDCs. However, this would be in contrast to the situation reported for DST+CSB-treated wt allograft recipients, in which pDC localization to SPLs was associated with an even stronger priming of allo-reactive effector T cells [4]. Alternatively, whereas in both scenarios tolerogenic pDC-mediated effects are

abolished, the additional LN homing deficiency of naïve T cells as well as of (inflammatory) migratory cDC subsets in CCR7$^{-/-}$ recipients might result in a reduced priming of alloreactive effector lymphocytes, thereby ameliorating the acute rejection without allowing for a long-term survival of the cardiac allograft.

The immature functional status of resting pDCs and pDC precursors (including low expression of MHCII as well as of costimulatory molecules such as CD40, CD80 and CD86) has been proposed to critically determine their tolerogenic potential [22, 33]. On the other hand, some reports have indicated that pDCs, in contrast to cDCs, might possess an intrinsic propensity to drive tolerogenic immune responses, independent of their maturational status or even promoted by maturation [34–37]. The in vivo-expanded primary pDCs used in this study to reconstitute DST+CSB-induced allotolerance in CCR7$^{-/-}$ recipients were phenotypically immature at the time of adoptive transfer, as characterized by their low expression of MHCII, CD40 and CD80 (data not shown); however, we cannot rule out that a subsequent in vivo maturation had occurred, e.g. due to the simultaneous application of DST+CSB or the subsequent transplantation procedure. Interestingly, while Lu and colleagues previously reported a strong tolerogenic effect after administration of donor-specific semi-mature cDCs derived from TGF-β1-supplemented in vitro BM culture plus anti-CD154 mAb treatment [28], we did not observe any graft survival advantage after transfer of host-specific CD-BMDC [25] in combination with DST+CSB, although these cells clearly displayed a semi-mature phenotype and were able to efficiently generate Treg in vitro (data not shown and [38]). Thus, although we did not directly compare the relative tolerogenic potential of primary immature and intentionally in vitro-stimulated mature pDCs in our setup, at least compared to bona fide tolerogenic cDCs, pDCs indeed seem to exhibit a unique tolerogenic potential in the setting of allogeneic vascularized solid-organ transplantation under DST+CSB treatment.

Finally, the tissue origin as well as the environment of the target compartment might largely influence the functionality of APCs. pDCs derived from liver and MLNs have been reported to be endowed with a particularly high intrinsic tolerogenic potential (possibly due to the contribution of liver-resident Treg), and interactions between pDCs and CD4$^+$ T cells particularly within LNs have been proposed to be instrumental for peripheral Treg development and subsequent graft survival, whereas SPL-resident pDCs might rather favor the priming and/or expansion of effector T cells [4, 32, 39]. The rejection of cardiac allografts despite the presence of regular numbers of pDCs within SPLs of DST+CSB-treated CCR7$^{-/-}$ recipients in our cardiac transplantation model indeed supports the idea that LNs represent a crucial and potentially non-redundant compartment for allotolerance induction. Importantly, however, we did not find any evidence for an expansion or de novo induction of Tregs within SLOs following the adoptive transfer of wt pDCs, mirroring other studies analyzing the tolerogenic potential of pDCs [12, 22, 40]. Thus, the exact mode of action of pDCs during induction of allograft tolerance in DST+CSB-treated recipients remains controversial, with at least the early phase of pDC-dependent tolerance induction potentially progressing independent of Treg expansion. Instead, pDCs might function by depleting or anergizing allo-reactive T-cell clones (specifically within LNs, but not SPLs) of DST+CSB-treated recipients, or they might act as specialized 'ferry cells' for LN delivery of allo-antigen that is acquired in the circulation (such as material derived from i.v.-injected donor-specific splenocytes). However, although it had been postulated that pDCs might differ from cDCs especially in their trafficking characteristics, entering SLOs via high endothelial venules from blood, we have evidence that actually few adoptively transferred wt pDCs directly localize to pLNs and MLNs, while most pDCs seem to reside within the liver shortly after i.v. application (SS and RF; unpublished observations).

In summary, rescue of the impaired DST+CSB-induced transplantation tolerance in CCR7$^{-/-}$ recipients by adoptively transferring syngeneic pDCs represents an experimental model system, which will allow to further define requirements of pDC function during allotolerance induction. Since pDCs can be efficiently generated in vitro from BMs of wt as well as various gene-targeted mice, this approach will help to identify molecules and pathways involved in cellular trafficking, antigen presentation and co-stimulation required for the induction and maintenance of long-term tolerance towards vascularized solid-organ allografts.

Materials and methods

Animals

BALB/c, C57BL/6, C57BL/6 CCR7$^{-/-}$ and C57BL/6 RAG2$^{-/-}$ OT-I mice were bred at the central animal facility of Hannover Medical School under specific pathogen-free conditions. BALB/c mice were additionally purchased from Charles River Laboratories. Foxp3EGFP mice [27] were kindly provided by B. Malissen. Animal experiments have been approved by the institutional review board and local authorities.

Heterotopic heart transplantation

Mice were anesthetized by intraperitoneal injection of 50 mg/kg ketamine/10 mg/kg xylazine (Bayer) in normal saline solution. Heterotopic heart transplantation was performed as described earlier [20]. Graft function was checked daily by abdominal palpation and scored from 0 to 4 [20], the end point of graft function was confirmed by laparotomy.

Antibodies and reagents

Anti-B220 (clone TIB146), anti-CD3 (clone 17A2), anti-CD4 (clone RMCD4), anti-CD8β (clone RMCD8-2), anti-CD62L (clone MEL-14), anti-IgD (clone HB250) and anti-Thy1.2 (clone MMT-1) mAb were grown in our labs and conjugated to one of

the following fluorochromes: AlexaFluor488, PacificOrange (Invitrogen), Cy3, Cy5 (Amersham). Further mAb and reagents: Anti-MHCII(I-A^b)-FITC/-biotin (clone AF6-120.1), anti-CD11b-PE-Cy7/-biotin, anti-CD11c-PE/-APC/-biotin, anti-CD40-PE, anti-CD80-PE, anti-Thy1.1-PE, anti-CD4-PerCP-Cy5.5, anti-B220-PerCP-Cy5.5, anti-CD25-PerCP-Cy5.5, StreptAvidin-PerCP/-PE-Cy7 (BD Pharmingen), anti-Foxp3-biotin, anti-B220-APC-AlexaFluor750, anti-CD62L-APC-AlexaFluor750 (eBioscience), anti-CD44-PE, anti-CD86-biotin, anti-CD45-APC (Caltag), anti-mouse PDC/IPC-AlexaFluor488 (clone 120G8.04, Dendritics), anti-mouse PDCA-1-APC (clone JF05-1C2.4.1, Miltenyi), anti-mouse Siglec H-Biotin (clone 440c, Hycult).

Immunohistology

Acetone-fixed 7–8 μm cryosections of LNs and CGs were processed as described earlier [7]. Images were acquired using an upright Olympus BX61 microscope or an inverted Zeiss Axiovert 200 M with motorized xy-table for automated composite image generation.

Isolation and purification of CG-infiltrating lymphocytes

CGs were cut into pieces of 1–2 mm before being transferred into 5 mL digestion media (RPMI1640 containing 25 mM HEPES, 10% FCS, 0.5 mg/mL CollagenaseD and 0.025 mg/mL DNaseI (Roche)). After 60 min incubation at 37°C in a shaking water bath, digestion was stopped by adding 0.5 M EDTA. To obtain single-cell suspensions, the digested tissue was passed through a 70 μm cell strainer (BD Biosciences). After centrifugation and washing, cells were resuspended in 5 mL RPMI1640/10%FCS and overlayed onto 5 mL LympholyteM (Cedarlane Labs). After centrifugation (20 min at 2000 rpm and 20°C without break), the intermediate layer of the LympholyteM-gradient was harvested.

Purification and transfer of polyclonal syngeneic T lymphocytes

Polyclonal syngeneic T lymphocytes were isolated from single-cell suspensions of pooled LN and SPL leukocytes from C57BL/6 wt and $CCR7^{-/-}$ donors using the Dynal Mouse T-cell Isolation Kit (Invitrogen) according to the manufacturer's protocol; 1×10^7 purified T cells (C57BL/6 wt or $CCR7^{-/-}$) were i.v. injected into C57BL/6 $RAG2^{-/-}$ OT-I recipients at the day of transplantation.

Purification and transfer of naïve T cells and Treg cells

Naïve T cell ($CD4^+CD25^-$) and Treg cell ($CD4^+CD25^+$) subsets were enriched from single-cell suspensions of pooled LN and SPL leukocytes from C57BL/6 wt donors using the Treg Isolation Kit in combination with AutoMACS (Miltenyi) and further purified to >99% purity by FACS sorting (FACSAria, BD Biosciences). Three doses of 1×10^7 naïve T cells or Treg cells were transferred into allograft recipients at day −1, +7 and +14 relative to transplantation.

In vivo generation and MACS-purification of pDCs

To expand pDCs in vivo, C57BL/6 wt mice received 5×10^5 to 1×10^6 B16-FL cells, a syngeneic murine melanoma tumor cell line stably expressing murine Flt3L [41], via s.c. injection into both flanks. After 10–14 days, animals were sacrificed and LNs and SPLs harvested. pDCs were isolated by MACS depletion using the Plasmacytoid Dendritic Cell Isolation Kit II (plus additional anti-CD11b-Biotin mAb) in combination with AutoMACS (Miltenyi). The target fraction contained 87–95% PDCA-1^+ $B220^{int}CD11c^{int}$ pDCs after MACS separation.

In vitro generation of 'cluster-disrupted' BM-derived cDCs

To generate so-called 'cluster-disrupted' BM-derived cDC (CD-BMDC) in vitro as described in [25], BMs of C57BL/6 wt donors were cultivated for 7 days in the presence of 100–200 ng/mL GM-CSF produced by a recombinant cell line, with culture medium being exchanged at days 3 and 6. At day 7, cells were gently harvested and replated on new tissue culture plates for 1 additional day without GM-CSF, leading to the differentiation of CD-BMDC expressing a semi-mature phenotype [38].

Flow cytometry

Flow cytometric measurements were performed on FACSCalibur and LSRII instruments (BD Biosciences). BD FACSDiva (BD Biosciences) and FlowJo (Treestar) software were used for data analysis.

Statistical analysis

The unpaired two-tailed Student's *t*-test was used to compare mean cell counts and mean percentages in flow cytometric analysis. To compare the survival curves of different groups of CGs, the Mantel–Haenszel log-rank test was used. *p*-Values of <0.05 were considered as statistically significant.

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Abbreviations: **CCR7$^{-/-}$:** CCR7-deficient · **cDC:** conventional DC · **CG:** cardiac graft · **DST:** donor-specific splenocyte transfusion · **MST:** mean survival time · **pDC:** plasmacytoid dendritic cell · **pLN:** peripheral LN · ***plt/plt:*** *paucity of LN T cells* · **post-Tx:** after transplantations · **SLO:** secondary lymphoid organ

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