



Research Article

Cross-presentation of dead-cell-associated antigens by DNGR-1⁺ dendritic cells contributes to chronic allograft rejection in mice

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The purpose of this study was to elucidate whether DC NK lectin group receptor-1 (DNGR-1)-dependent cross-presentation of dead-cell-associated antigens occurs after transplantation and contributes to CD8⁺ T cell responses, chronic allograft rejection (CAR), and fibrosis. BALB/c or C57BL/6 hearts were heterotopically transplanted into WT, Clec9a^{-/-}, or Batf3^{-/-} recipient C57BL/6 mice. Allografts were analyzed for cell infiltration, CD8⁺ T cell activation, fibrogenesis, and CAR using immunohistochemistry, Western blot, qRT²-PCR, and flow cytometry. Allografts displayed infiltration by recipient DNGR-1⁺ DCs, signs of CAR, and fibrosis. Allografts in Clec9a^{-/-} recipients showed reduced CAR ($p < 0.0001$), fibrosis ($P = 0.0137$), CD8⁺ cell infiltration ($P < 0.0001$), and effector cytokine levels compared to WT recipients. Batf3-deficiency greatly reduced DNGR-1⁺ DC-infiltration, CAR ($P < 0.0001$), and fibrosis ($P = 0.0382$). CD8 cells infiltrating allografts of cytochrome C treated recipients, showed reduced production of CD8 effector cytokines ($P < 0.05$). Further, alloreactive CD8⁺ T cell response in indirect pathway IFN- γ ELISPOT was reduced in Clec9a^{-/-} recipient mice ($P = 0.0283$). Blockade of DNGR-1 by antibody, similar to genetic elimination of the receptor, reduced CAR ($P = 0.0003$), fibrosis ($P = 0.0273$), infiltration of CD8⁺ cells ($p = 0.0006$), and effector cytokine levels. DNGR-1-dependent alloantigen cross-presentation by DNGR-1⁺ DCs induces alloreactive CD8⁺ cells that induce CAR and fibrosis. Antibody against DNGR-1 can block this process and prevent CAR and fibrosis.

Keywords: CD8⁺ T cells · cDC1 · cross-presentation · DNGR-1 · transplantation



Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Chronic rejection ultimately leading to allograft fibrosis and dysfunction constitutes a major constraint to long-term graft survival in transplantation [1–5]. In contrast to early post-transplant acute rejection episodes, which can be effectively treated with standard immunosuppressors to prevent activation and proliferation of alloreactive T lymphocytes, chronic rejection cannot be controlled sufficiently by these immunosuppressive therapies [6–9]. Activation of the immune system in the chronic setting is likely mediated through recognition of non-infectious damaged tissues [10–12]. This connection between cell injury and allograft rejection was proposed many years ago and posits that ischemia-reperfusion injury and other traumas during transplantation results in death of allograft cells, which expose intracellular molecules that trigger defensive immune responses in the host [13]. These intracellular molecules are termed damage-associated molecular patterns (DAMPs) and include molecules such as HMGB-1, ATP, uric acid, DNA, and others, which can in many instances engage innate immune receptors and promote inflammatory and adaptive immune responses [14,15].

DAMPs also include actin filaments, which can be recognized by the DC NK lectin group receptor-1 (DNGR-1, also known as CLEC9A) [16,17]. DNGR-1 is expressed by a subset of DCs in lymphoid and non-lymphoid tissues of mouse and human [18–24] that requires the Batf3 transcription factor for its development. The study of Batf3^{-/-} mice lacking DNGR-1⁺ DCs (known as cDC1) has revealed that the latter play an essential and non-redundant role in CD8⁺ T cell responses against cell-associated antigens such as tumours and allografts, as well as against many viruses [24–26]. Interestingly, DNGR-1 serves not only as a marker for the Batf3-dependent cDC1 subset but appears to be required for its function as DNGR-1-deficient (Clec9a^{-/-}) mice recapitulate many of the functional defects of Batf3^{-/-} mice despite the fact that their cDC1s develop normally. This is likely because F-actin engagement by DNGR-1 promotes cross-presentation of dead cell-associated antigens and cross-priming of cytotoxic CD8⁺ T cells (CTL) [16–19,27–29]. More specifically, F-actin-dependent DNGR-1 signaling facilitates the process whereby antigens associated with dead cell cargo taken up by cDC1 are shuttled into the MHC class I (MHC I) cross-presentation pathway. The latter includes the transporter associated with antigen processing 1 (Tap1) complex, which is essential for antigenic peptide translocation into the ER and, in synergy with other ER chaperones, for loading of the MHC I heavy chain/ β -2-microglobulin (β 2M) light chain complex [30–37].

Given the link between cell death and immunity to allotransplants, the present study aimed to test a possible involvement of DNGR-1 and of cDC1 in cross-priming CD8⁺ T cells against allograft antigens in a model of chronic rejection. Using a combination of genetic approaches and antibody blockade, we report that chronic allograft rejection and fibrosis can be prevented by inhibition of the DNGR-1 receptor and/or loss of DNGR-1⁺ DCs. These findings identify DNGR-1 as an important point of control in immunity to allotransplants that

could be targeted therapeutically to ameliorate chronic allograft rejection.

Results

Cell necrosis in cardiac allografts exposes DAMPs and leads to infiltration by DNGR-1⁺ DCs

To investigate if cardiac allogeneity impacts early immune responses, BALB/c allografts transplanted into CD4⁺ T cell depleted C57BL/6 mice, which is a model of chronic allograft rejection and shows median allograft survival of 27 days, were evaluated by histology and RNA analysis at early times after transplantation (Fig. 1A). Immunohistochemistry revealed early cell infiltration, and necrosis of cells within allografts (nucleus loss in cardiomyocytes, eosinophilic cytoplasm) at day 3 with further increases at day 5 after transplantation in comparison to syngeneic grafts (Fig. 1B; Supporting Information Fig. S1A and B). Additionally, in allografts, expression of death receptor and necrosis related genes such as TNF, myelin-associated glycoprotein (MAG), Fas ligand (FasL), cytochromes, and CD40 was upregulated beginning at day 1 and increasing until day 5 after transplantation (Supporting Information Fig. S1C) [38–42]. This was accompanied by a higher CD11c⁺DNGR-1⁺ DC infiltration of the allografts (BALB/c $\rightarrow$ C57BL/6) in comparison to syngrafts as demonstrated by double immunofluorescence staining (Fig. 1C and D; $p < 0.001$; Supporting Information Fig. S2). In contrast, no difference in DC subset composition was seen in the spleens of mice transplanted with allografts and syngrafts (Supporting Information Fig. S3).

Comparable to the results in mice, human cardiac allografts undergoing rejection showed greater leucocytic cell infiltration and collagen deposition at day 20 after transplantation than allografts without signs of rejection (Fig. 1E; mouse data, Fig. 3B and C). Notably, the human samples from rejecting hearts were infiltrated by CD11c⁺DNGR-1⁺ DCs whereas these DCs were absent in human biopsies from healthy transplants (Fig. 1F and G; $p < 0.001$).

Graft-infiltrating DNGR-1⁺ cDC1s are of recipient origin and take up donor fragments

To elucidate if graft-infiltrating DNGR-1⁺ dendritic cells are of recipient or donor origin, we performed a triple immunofluorescence staining for H2^b (C57BL/6) and H2^d (BALB/c) in combination with CD11c and DNGR-1. This staining showed that graft-infiltrating DNGR-1⁺ DCs are H2^b and not H2^d positive in immunofluorescence staining and therefore are of C57BL/6 recipient origin (Fig. 2A and B). The highest numbers of these graft-infiltrating DNGR-1⁺ DCs can be found on day 5 after transplantation with a decrease till day 20 after transplant (Fig. 2C). At both time points, no H2^d positive DNGR-1⁺ DCs that would indicate passenger DCs from BALB/c origin were detected.

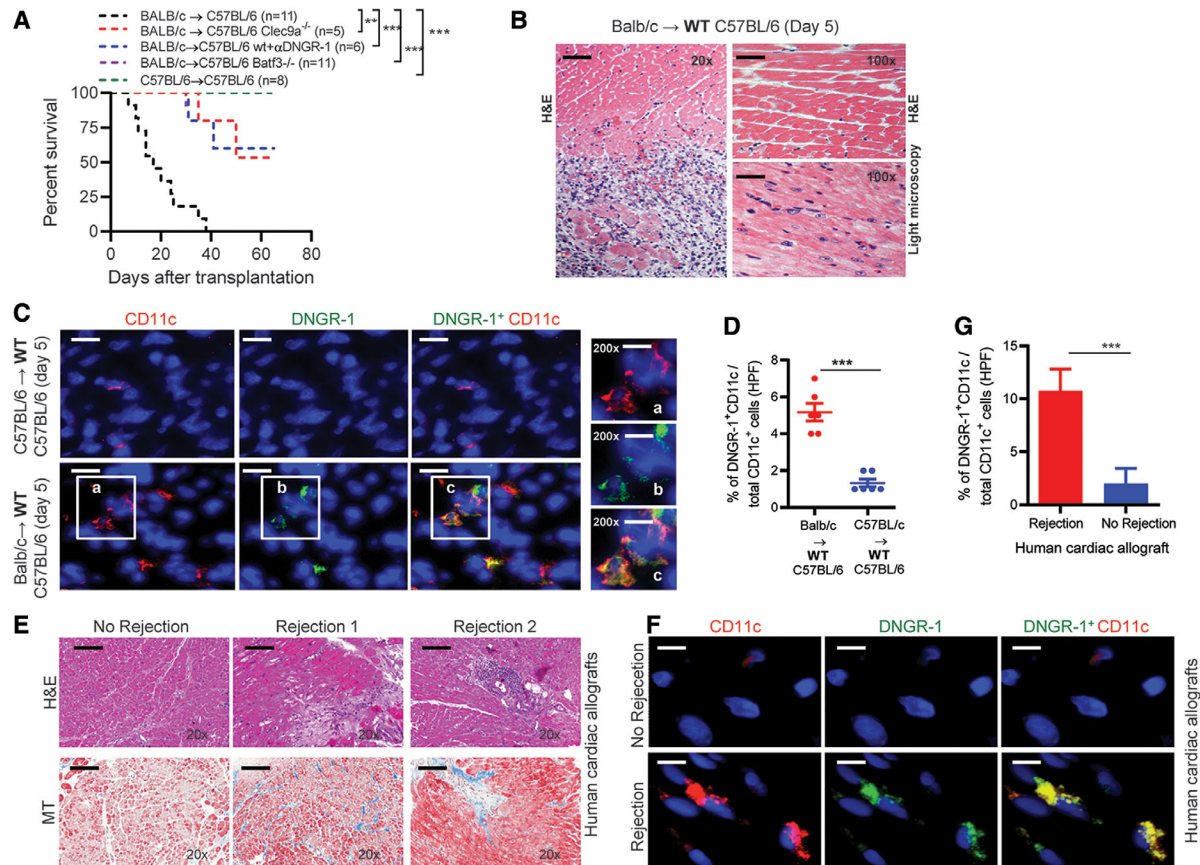


Figure 1. DNDR-1⁺ DCs infiltrate mouse cardiac allografts and human cardiac allografts undergoing chronic rejection. (A) Wild-type (WT) C57BL/6 recipients were transplanted with BALB/c donor hearts (BALB/c → WT C57BL/6, allografts; n = 11) with a median graft survival time of 27 days which was significantly shorter when compared to syngeneic controls (C57BL/6 → WT C57BL/6, syngrafts; n = 8; $p < 0.0001$). CD4⁺ T cells were depleted in all recipients. Further, *Clec9a*^{-/-} (n = 5; $p < 0.01$), *Batf3*^{-/-} (n = 11; $p < 0.001$), and α-DNDR-1 (n = 6; $p < 0.001$) treated C57BL/6 recipients transplanted with BALB/c donor hearts showed significantly prolonged allograft survival. (B) On day 5, cell infiltration (eosinophilia) and necrosis of cells within the allografts (nucleus loss in cardiomyocytes) were evaluated in H&E and light microscopy. Scale bar 100 μm, magnification 20× and 100×. (C) Allogeneic (BALB/c → WT C57BL/6) and syngeneic cardiac transplants (C57BL/6 → WT C57BL/6) were analyzed on day 5 post transplantation. Representative immunofluorescence (IF)-staining shows DNDR-1⁺CD11c cell (DNDR-1⁺ dendritic cell, DNDR-1⁺ DC) within the allografts compared to the syngrafts. The double positive cells for DNDR-1 (green) and CD11c (red) are in yellow; nucleus is stained in blue. Scale bar 50 μm in magnification 40× and Scale bar 10 μm in magnification 200×. (D) Quantification of DNDR-1⁺CD11c⁺/total positive CD11c cells per high power field (HPF) within the allografts (BALB/c → WT C57BL/6) on day 5 compared to syngrafts (C57BL/6 → WT C57BL/6) is depicted (n = 6 per group; shown as mean ± SD; Student's t-test). Error bars represent mean ± SD. (E) Paraffin-embedded human heart allografts (Rejection 1, 2, and No Rejection as control) were obtained. Representative H&E-staining of cell infiltration (upper panel) and Masson's Trichrome (MT)-staining of collagen deposition (in blue, lower panel) within the rejection (1, 2) compared to the no rejected allografts are illustrated. Scale bar 100 μm, magnification 20×. (F and G) Representative IF-staining and quantification of DNDR-1⁺ DCs (DNDR-1⁺ CD11c in yellow; nucleus in blue) in the Rejection (lower panel) compared to the No Rejection groups (upper panel) is illustrated. Scale bar 50 μm, magnification 40×. (A–F) Data are from two experiments with four to six mice per experiment. Reactive oxygen species, ROS; Tumor necrosis factor, TNF; Myelin-associated glycoprotein, MAG; Fas ligand, FasL; cytochromes, Cyba, Cybb; CD40; *** $p \leq 0.001$; paired Student's t-test.

However, we were able to depict H2^d positive BALB/c donor fragments (not DNDR-1 or CD11c positive) in cytoplasm of DNDR-1⁺ DCs (Fig. 2D).

Absence of DNDR-1 reduces chronic rejection, allograft fibrosis, and CD8⁺ T cell infiltration

Since DNDR-1⁺ CDC1s infiltrated allografts but not syngrafts, we tested whether genetic abrogation of the DNDR-1 receptor could prevent chronic allograft rejection and fibrosis. Indeed, *Clec9a*^{-/-} recipient mice that are deficient for the DNDR-1 receptor [27]

showed significantly prolonged allograft survival ($p < 0.01$; Fig. 1A), decreased immune cell infiltration and histology rejection score of allografts compared to WT recipients ($p < 0.0001$; Fig. 3A). This was accompanied by a significant reduction of collagen I deposition in allografts both in Masson's trichrome staining and PCR ($p = 0.0137$; Fig. 3B). Further, profibrotic cytokines such as active TGF-β1 ($p = 0.0043$) and CTGF ($p = 0.0040$) were detected at significantly reduced levels in allotransplanted *Clec9a*^{-/-} compared to WT recipient mice (Fig. 3C). These results were corroborated by immunofluorescent detection of phosphorylated Smad3 in allografts transplanted into WT recipient animals but not into *Clec9a*^{-/-} mice (Fig. 3D).

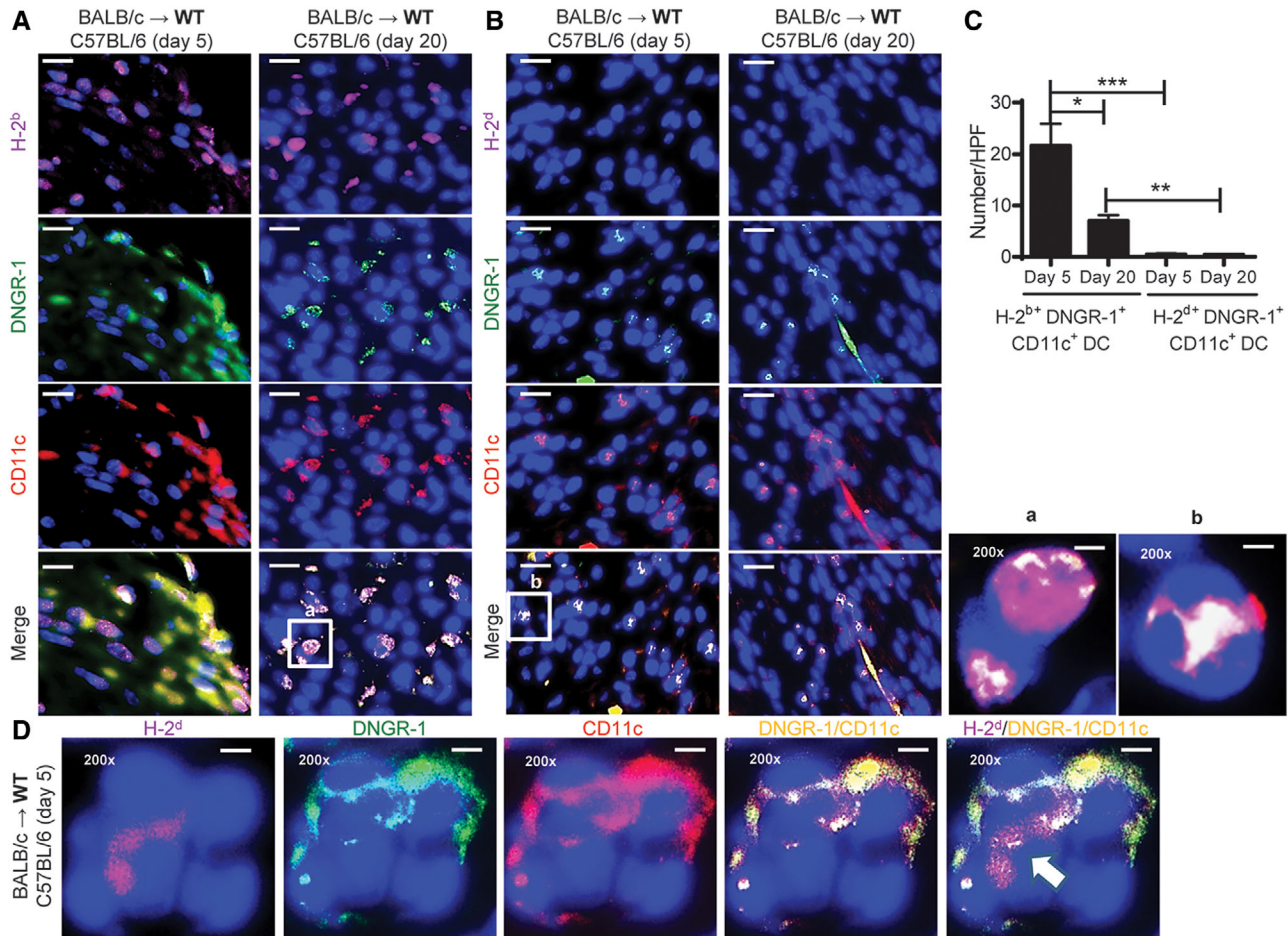


Figure 2. Graft-infiltrating DNDR-1⁺ cDC1s are of recipient origin and take up donor fragments. Wild-type C57BL/6 recipient were transplanted on day 0 with BALB/c donor hearts (BALB/c → WT C57BL/6, allografts). Grafts harvested on day 5 and day 20 were analyzed to determine the origin of DNDR-1⁺ DC (DNDR-1⁺CD11c⁺) within the allografts. (A) Representative IF-staining showing recipient origin of DNDR-1⁺ DCs with positivity for H-2^b (H-2^b DNDR-1⁺ DC, in violet, nuclei in blue; (a) higher magnification (×200)) in allografts at day 5 and day 20 after transplantation. Scale bar 50 μm, magnification 40×. (B) Representative IF-staining showing DNDR-1⁺ DCs (DNDR-1⁺ CD11c⁺ in yellow or orange, nuclei in blue; (b) higher magnification (×200)) with absence of H-2^d (donor BALB/c origin) in allografts at day 5 and day 20 after transplantation. Scale bar 50 μm, magnification 40×. (C) Quantification (mean ± SD; Mann–Whitney U-test) per HPF (graph) of H-2^b DNDR-1⁺ DCs (recipient origin) and H-2^d DNDR-1⁺ DCs (donor origin) within the allografts at day 5 (n = 4) compared to day 20 after transplantation (n = 4). (D) Representative IF-staining showing H-2^d expressing donor fragments (in magenta; white arrow) surrounded by DNDR-1⁺ CD11c⁺ DCs (in yellow); allograft was harvested on day 5. Scale bar 10 μm, magnification 200×. (A–D) Data are from two independent experiments with four to six mice per experiment. *p < 0.05; **p < 0.01; ***p < 0.001.

In *Clec9a*^{−/−} mice, numbers of allograft infiltrating CD8⁺ T cells were significantly reduced in comparison to WT recipients or mice receiving syngeneic grafts (*p* < 0.0001; Fig. 4A). This was accompanied by a significant reduction of CD8 effector cytokines IFN-γ and IL-33 in the *Clec9a*^{−/−} animals (IFN-γ *p* = 0.0151, IL-33 *p* < 0.0001; Fig. 4B) while trafficking of cDC1s into the allograft was not impaired (Supporting Information Fig. S4).

Batf3 is necessary for chronic allograft rejection

It is described previously that the development of DNDR-1 expressing cDC1s is dependent on the transcription factor Batf3 [24, 25]. Indeed, leucocytic cell infiltration was significantly decreased in

Batf3^{−/−} mice receiving allografts when compared to WT recipients (*p* < 0.0001; Fig. 5A; Supporting Information Fig. S4) and allograft survival was prolonged significantly (*p* < 0.001; Fig. 1A). Absence of Batf3 in recipient mice also prevented collagen deposition in the allografts as shown both by Masson's trichrome staining and PCR (*p* = 0.0382; Fig. 5B), as well as the induction of profibrotic cytokines and activation of Smad3 (*p* = 0.0132; CTGF *p* = 0.0352; Fig. 5C and D). As expected, the population of DNDR-1⁺ cDC1s infiltrating allografts was significantly reduced in Batf3^{−/−} mice (Supporting Information Fig. S5A) and was associated with a paucity of activated CD8⁺ T cells (*p* < 0.0001; Supporting Information Fig. S5B) and RT-PCR levels of effector cytokines (IFN-γ *p* < 0.0001, IL-33 *p* = 0.0250; Supporting Information Fig. S5C).

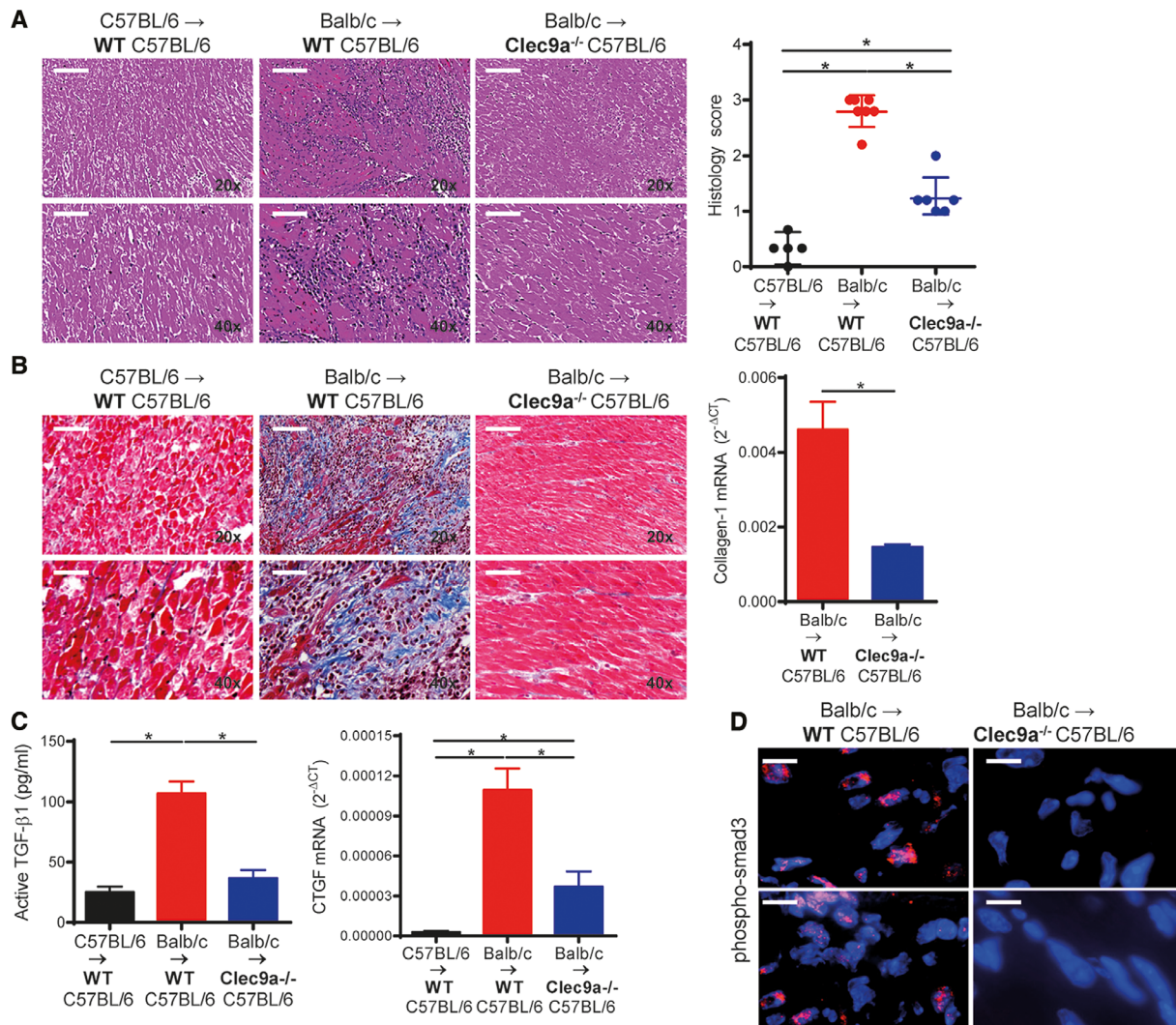


Figure 3. DNDR-1 deficiency in recipient mice prevents chronic rejection of cardiac allografts and fibrosis. Wild-type (WT) and DNDR-1 deficient (Clec9a^{-/-}) C57BL/6 recipients were transplanted on day 0 with BALB/c donor hearts (BALB/c → WT C57BL/6 and BALB/c → Clec9a^{-/-} C57BL/6, allogeneic transplants). Control C57BL/6 recipient were transplanted with C57BL/6 donor hearts (C57BL/6 → WT C57BL/6, syngeneic transplants). Recipient mice were depleted of CD4⁺ T cells with anti-CD4 antibody, i.p. (1 mg/mouse) on days -1, 0, and 7 and mice were analyzed on day 20 (n = 5–10 per group). (A) Representative H&E-staining of cell infiltration within the allografts and histology score of the allogeneic transplants into the Clec9a^{-/-} C57BL/6 recipients compared to the transplants into WT C57BL/6 recipients and syngeneic transplants. Scale bar 100 μm in 20× magnification and Scale bar 50 μm in 40× magnification. (B) Masson's Trichrome-staining shows collagen deposition (in blue) within the allografts into Clec9a^{-/-} C57BL/6 compared to the WT C57BL/6 recipients or syngrafts. Collagen mRNA expression within the allografts into Clec9a^{-/-} C57BL/6 compared to WT C57BL/6 recipients is depicted (graph; n = 6 per group). Scale bar 100 μm in 20× magnification and Scale bar 50 μm in 40× magnification. (C) Determination of active TGF-β₁ and CTGF mRNA expression within the allogeneic transplants into Clec9a^{-/-} C57BL/6 compared to WT C57BL/6 recipients or syngrafts are illustrated (n = 6 per group). (D) Representative IF-staining of phosphorylated smad3 (Phospho-Smad3, in red) within the allografts into Clec9a^{-/-} C57BL/6 compared to WT C57BL/6 is depicted. Scale bar 50 μm, magnification 40×. (A–D) Data are from two independent experiments and are shown as mean ± SD and unpaired one-tailed Student's t-test was applied to compare two groups. *P ≤ 0.05.

Selective apoptosis and DNDR1^{-/-} reduces CD8⁺ T cell allorecognition and allograft rejection

To formally assess the CD8 T cell alloimmune response within our model in vivo, we performed additional experiments with cytochrome C injection of recipient mice. In previous functional studies, both in vivo and in vitro it has been shown that cytochrome c profoundly abrogates OVA-specific CD8 T cell proliferation through its apoptosis-inducing effect on cross-presenting DCs

[43]. In these experiments, in vivo injection of cytochrome C abolished the induction of cytotoxic T lymphocytes to exogenous antigen and reduced subsequent immunity to tumor challenge. Importantly, this model allows assessment of cross-presentation that is totally in vivo that is ideal for our setting of alloimmunity in transplantation [43].

In detail, we injected C57BL/6 recipient mice that were transplanted with BALB/c hearts with three dosages of cytochrome C and analyzed analogous to our original experiments.

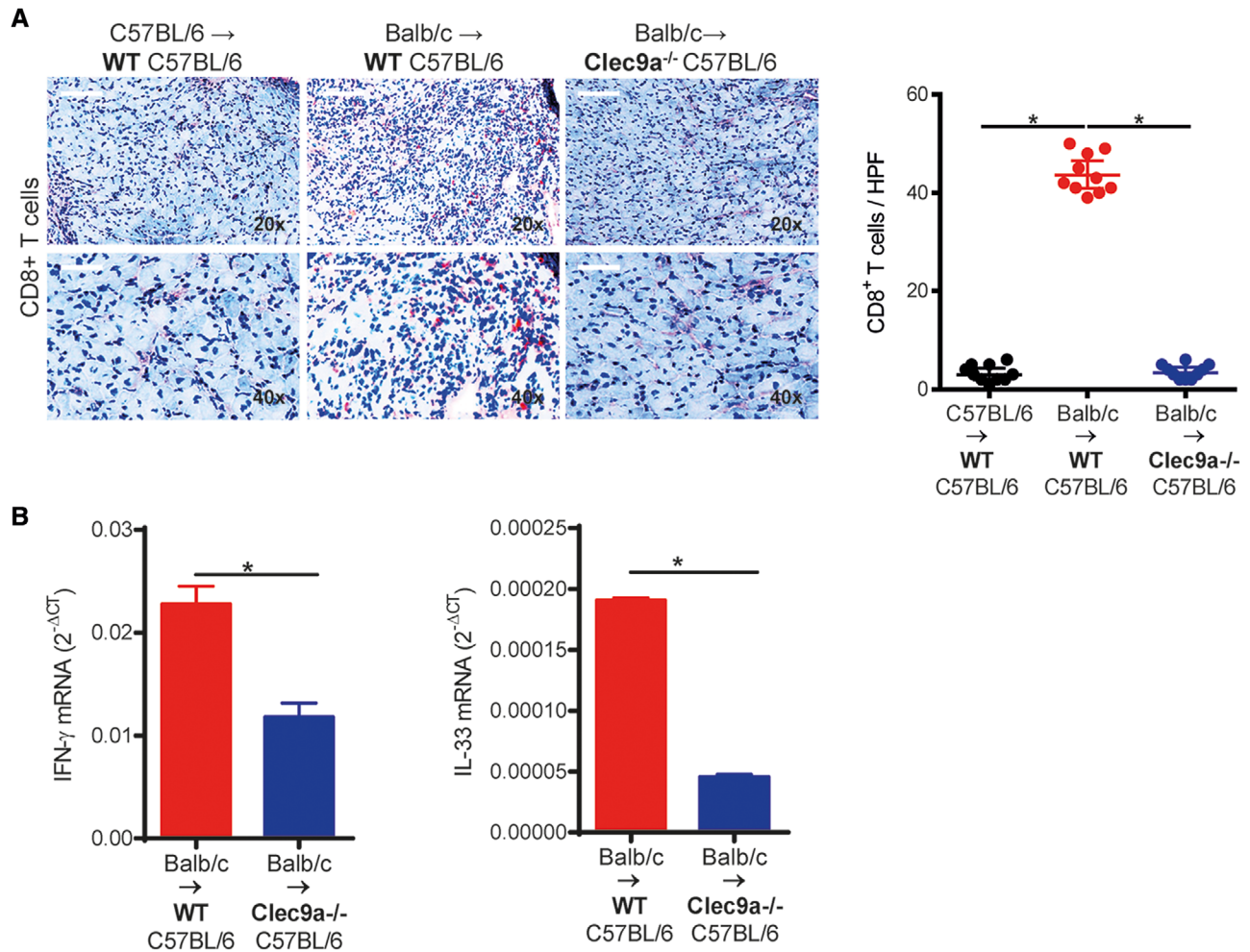


Figure 4. DNCR-1 deficiency in recipient mice prevents infiltration by CD8⁺ T cells. Infiltration by CD8⁺ T cells and effector cytokine expression within heart transplants using the same experimental setup as in Figure 3. (A) Immunohistochemical (IHC) staining showing infiltration of CD8⁺ T cells (positive cells are in brown) and their quantification per high power field (HPF; graph on the right) within allogeneic transplants into *Clec9a*^{-/-} C57BL/6 compared to WT C57BL/6 recipients or syngeneic transplants (*n* = 5–10 per group). Scale bar 100 μm in 20× magnification and Scale bar 50 μm in 40× magnification. (B) Quantification of mRNA expression (normalized against housekeeping gene β-actin) of effector cytokines (IFN-γ and IL-33) within the allogeneic transplants into *Clec9a*^{-/-} C57BL/6 compared to WT C57BL/6 recipients (*n* = 5–10 per group). (A and B) Data are from two independent experiments and are shown as mean ± SD and unpaired one-tailed Student's t-test was applied to compare two groups. **p* ≤ 0.05.

Cytochrome C injected recipient mice showed significantly decreased immune cell infiltration and histology rejection score of allografts compared to WT recipients (*p* = 0.0055; Supporting Information Fig. S6A). This was accompanied by a significant reduction of collagen I deposition in allografts both in Masson's trichrome staining and PCR (*p* = 0.0152; Supporting Information Fig. S6B) and also reduced CTGF levels (*p* = 0.0260; Supporting Information Fig. S6C). These results were corroborated by immunofluorescent detection of phosphorylated Smad3 in allografts transplanted into WT recipient animals but not into cytochrome c injected mice (Supporting Information Fig. S6D).

Further the numbers of DNCR-1⁺ cDC1s infiltrating allografts were significantly reduced in cytochrome C-treated mice (*p* = 0.0116; Fig. 6A) and was associated with decreased numbers of

activated CD8⁺ T cells (*p* = 0.0097; Fig. 6B). CD8⁺ T cells isolated out of allografts transplanted into cytochrome C-treated recipient mice, showed significant reduced production of CD8 T effector cytokines IFN-γ, TNF-α (and IL-2, IL-4, IL-13, and also the cytotoxic marker perforin 1; data not shown; all *p* < 0.05; Fig. 6C). Further, IFN-γ response by CD8⁺ T cells in an indirect pathway Elispot was significantly decreased in *Clec9a*^{-/-} compared to WT recipient mice (*p* = 0.0283; Fig. 6D).

Additionally, in all our experimental groups that show reduced allograft rejection due to either reduced BATF3 dependent DCs or impaired DNCR-1 receptor function (*Clec9a*^{-/-} and DNCR-1 antibody treated mice) reduced IFN-γ levels were detected. This altogether underlines that indeed DNCR-1⁺ DCs cross-prime CD8⁺ T cells and that these primed CD8 T cell effector responses contribute to allograft rejection and fibrosis.

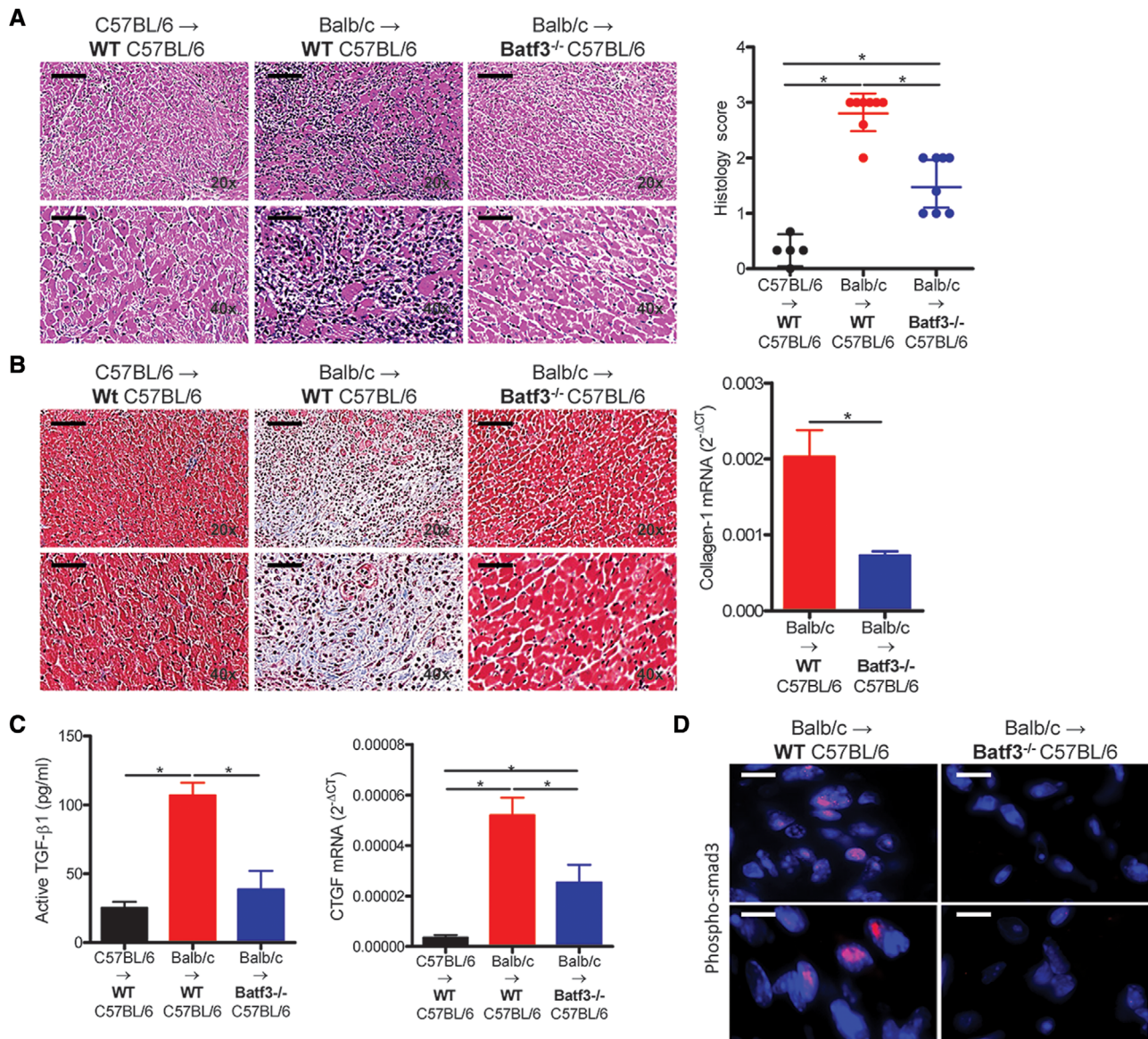


Figure 5. Batf3 deficiency in recipients reduces fibrosis and chronic rejection of allografts. Wild-type and Batf3^{-/-} C57BL/6 recipients were transplanted with BALB/c donor hearts (BALB/c → WT C57BL/6 or Batf3^{-/-} C57BL/6, allogeneic) and with C57BL/6 donor hearts (C57BL/6 → WT C57BL/6, syngeneic) and analyzed on day 20 ($n = 5$ –8 per group). All recipients were depleted of CD4⁺ T cells. (A) H&E staining shows cell infiltration and histology score of allografts of the Batf3^{-/-} C57BL/6 compared to WT C57BL/6 recipients or syngeneic groups. Scale bar 100 μ m in 20 $\times$ magnification and Scale bar 50 μ m in 40 $\times$ magnification. (B) Deposition in MT (in blue) and mRNA expression (normalized against housekeeping gene β -actin) of collagen within the allografts of Batf3 deficient compared to WT recipient or syngeneic transplants are depicted. Scale bar 100 μ m in 20 $\times$ magnification and Scale bar 50 μ m in 40 $\times$ magnification. (C) Levels of active TGF- β 1 and mRNA expression of CTGF within the allografts of Batf3^{-/-} C57BL/6 compared to the WT groups and syngeneic groups are shown. (D) Immunofluorescence-staining of Phospho-smad3 (red, nucleus in blue) in allografts of the Batf3^{-/-} C57BL/6 compared to WT recipients is shown. Scale bar 50 μ m, magnification 40 $\times$. (A–D) Data are from two independent experiments and are shown as mean $\pm$ SD and unpaired one-tailed Student's t -test was applied to compare two groups. * $p \leq 0.05$.

Blockade of DNCR-1 receptor prevents chronic allograft rejection and fibrosis

Finally, blockade of DNCR-1 with specific mAb was tested for the ability to phenocopy genetic loss in allograft recipients and diminish chronic allograft rejection and fibrosis. Notably, mAb treatment significantly prolonged allograft survival ($p < 0.0001$; Fig. 1A),

reduced numbers of allograft-infiltrating cells ($p = 0.0003$; Fig. 7A) and collagen I deposition into allografts ($P = 0.0273$; Fig. 7B). Similar to genetic disruption of Clec9a, the profibrotic cytokines were significantly reduced in mAb treated compared to the control group (active TGF- β 1 $p = 0.0035$; CTGF $p = 0.0267$; Fig. 7C). Further, mAb treatment significantly reduced the infiltration of allografts by CD8⁺ T cells ($p = 0.0006$; Fig. 7D), as well as the

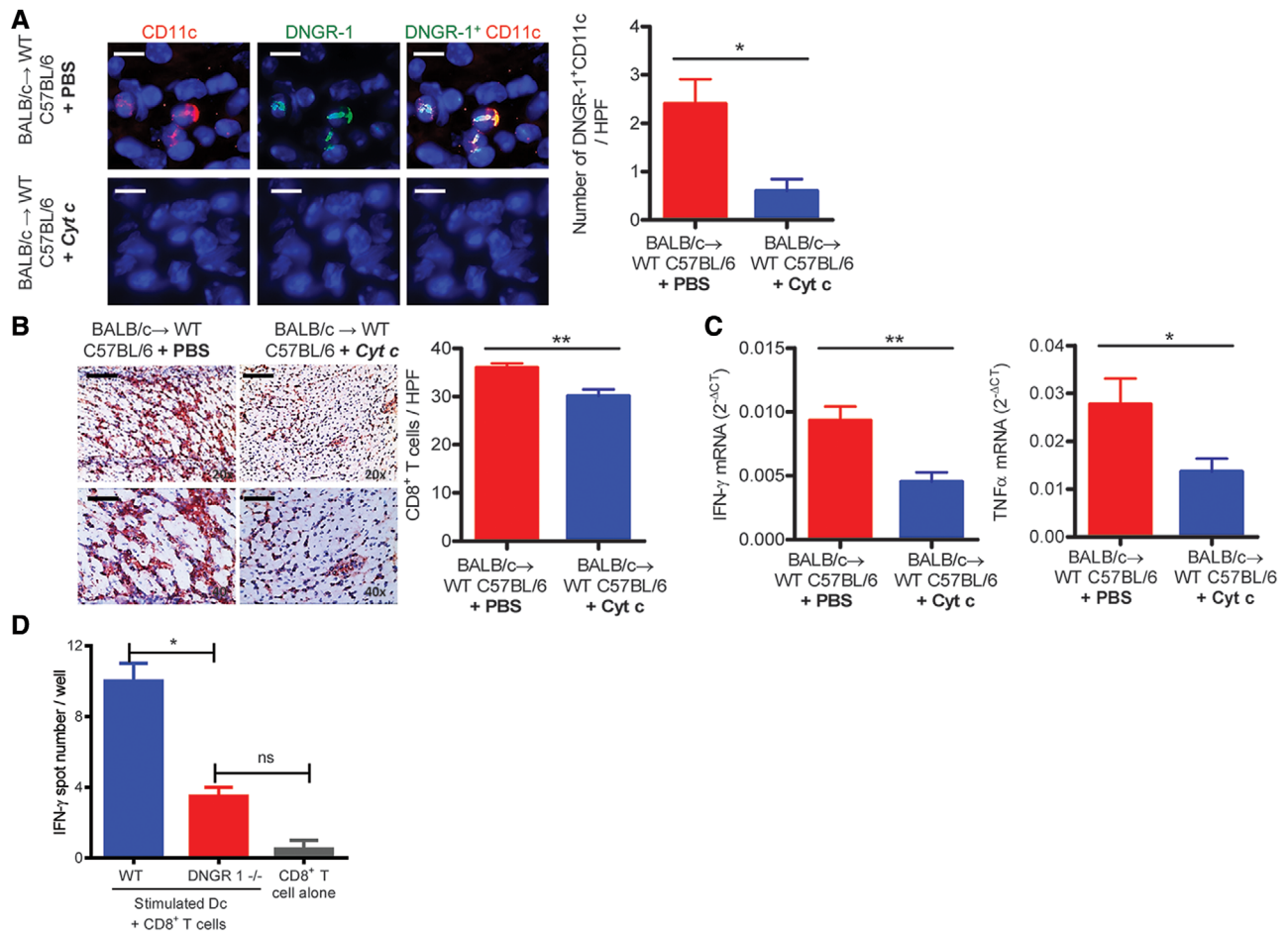


Figure 6. Selective apoptosis and DNDR1^{-/-} in cross-presenting cDC1s reduces indirect CD8⁺ T cell allorecognition and effector cytokine production. Wild-type C57BL/6 depleted of CD4⁺ T cells and treated with cytochrome C (Cyt C) (5 mg/mouse in PBS) on days -1, 2, and 7 relative to the day of transplantation (day 0), were transplanted with BALB/c donor hearts (BALB/c → WT C57BL/6 + Cyt C) and analyzed on day 20 after transplantation ($n = 5$ per group). Allogeneic groups with BALB/c → WT C57BL/6 + PBS were used as control. (A) Representative IF-staining of DNDR-1⁺ DC (DNDR-1⁺ CD11c⁺ in yellow, the nucleus is stained in blue) and their quantification (DNDR-1⁺ DC/total CD11c positive cells (in red) per HPF within the allografts of Cyt C treated compared to PBS control groups is depicted. Scale bar 50 μ m, magnification 40 $\times$. (B) Immunohistochemical staining showing CD8⁺ T cells (positive cells are in brown) and their quantification per HPF within the Cyt C compared to PBS-treated allogeneic transplants. Scale bar 100 μ m in 20 $\times$ magnification and Scale bar 50 μ m in 40 $\times$ magnification. (C) Determination of mRNA expression (normalized against housekeeping gene β -actin) of CD8 T effector cytokines IFN- γ and TNF- α within the allografts of Cyt C treated recipients compared to PBS control groups. (D) Indirect pathway IFN- γ Elispot: CD11c⁺ cells were isolated from spleens of WT and DNDR-1^{-/-} C57BL/6 recipient mice and loaded with alloantigen from allografts (BALB/c → C57BL/6, day 5 post transplantation). Splenic C57BL/6 CD8⁺ T cells were co-incubated for 3 days. Number of spots (IFN- γ expressing cells) per well are compared between WT and DNDR-1^{-/-} group. WT alone group (only C57BL/6 CD8⁺ T cells) was used as negative control. (A–D) Data are from two independent experiments and are shown as mean $\pm$ SD and Mann–Whitney U-test was applied to compare two groups. * $p \leq 0.05$; ** $p \leq 0.01$.

induction of rejection-associated cytokines (IFN- γ $p = 0.0002$, IL-33 $p = 0.0393$; Fig. 7E).

We corroborated these results in the fully immunocompetent Bm12 → C57BL/6 heart transplantation model. In this model, temporary CD4⁺ T cell depletion is not necessary to induce chronic allograft rejection and fibrotic organ remodeling. Antibody blockade of DNDR-1 also resulted in reduction of chronic rejection and fibrosis, similar to the extent seen in the BALB/c into C57BL/6 with CD4⁺ T cell depletion model (Supporting Information Fig. S7A–E). Therefore, DNDR-1 blockade is a useful means of ameliorating chronic rejection in two mouse models of allotransplantation.

Discussion

A transplanted organ experiences various traumas, from physical manipulation to varying oxygen tension, that can cause death of graft cells and consequently cause release of DAMPs [15,44]. DAMPs have been proposed to contribute to the inordinate immunogenicity of allografts through engagement of DAMP receptors on host immune cells but the mechanisms involved remain unclear [12,15]. Here, we establish a connection between DAMP recognition and chronic CD8⁺ T cell-mediated responses leading to cardiac allograft fibrosis. We show that a subset of specialized DCs with a superior capacity to cross-prime CD8⁺ T cells,

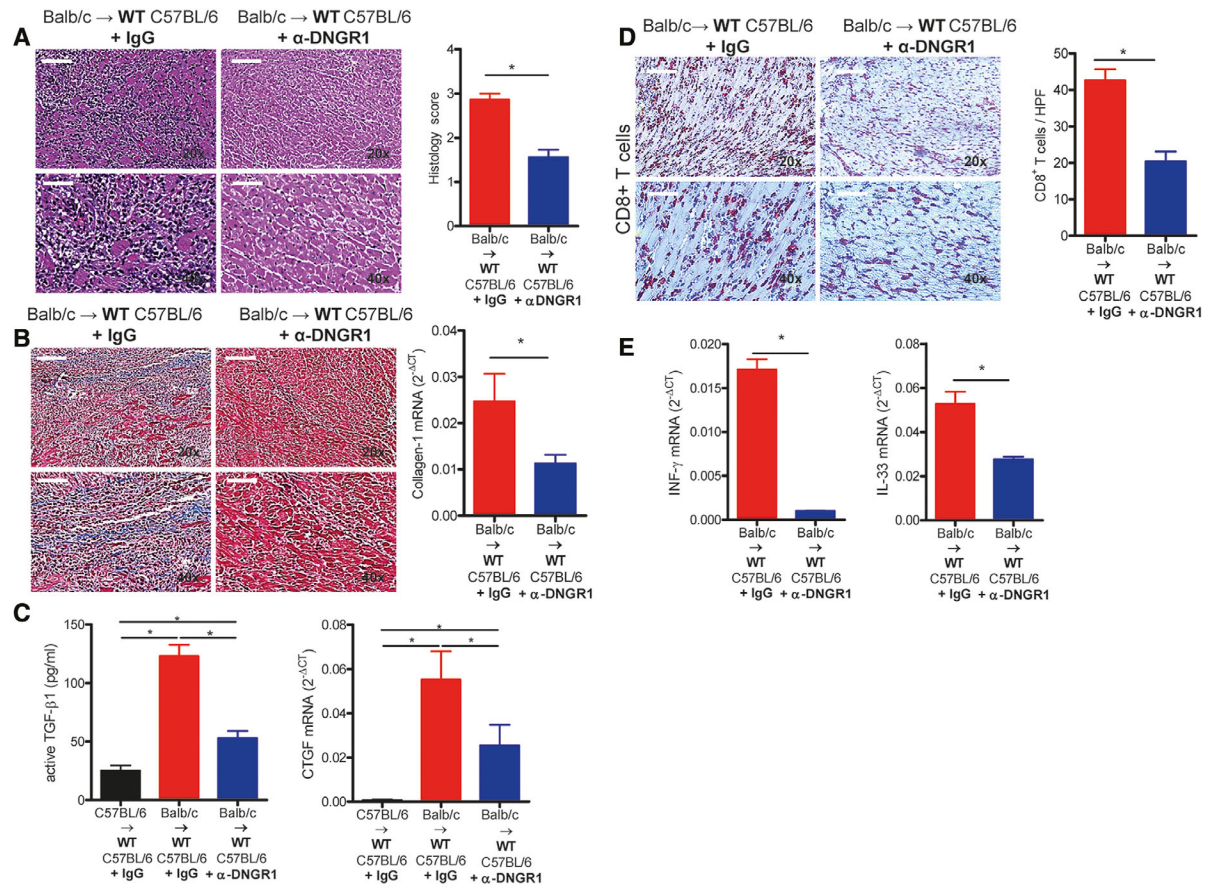


Figure 7. Blockade of DNDR-1 prevents fibrosis and chronic cardiac allograft rejection in BALB/c → C57BL/6 model. Wild-type C57BL/6 depleted of CD4⁺ T cells and treated with anti-DNDR-1 monoclonal antibody, mAb (100 μg i.p. in PBS), or IgG1 as control, on days 0, 3, 10, and 17 relative to the day of transplantation (day 0) were transplanted with BALB/c donor hearts (BALB/c → WT C57BL/6 + α-DNDR-1) and analyzed on day 20 (*n* = 5 per group). Allogeneic groups with BALB/c → WT C57BL/6 + IgG were used as control. (A) Representative H&E-staining of cell infiltration and histology score (graph on the right) of the allografts transplanted into anti-DNDR-1-treated recipients compared to the isotype control groups are illustrated. Scale bar 100 μm in 20× magnification and Scale bar 50 μm in 40× magnification. (B) Representative MT-staining of collagen deposition (in blue) and quantification of mRNA expression (normalized against housekeeping gene β-actin) of collagen (graph) within the allografts transplanted into anti-DNDR-1-treated recipient compared to the group that received IgG are shown. Scale bar 100 μm in 20× magnification and Scale bar 50 μm in 40× magnification. (C) Quantification of active TGF-β₁ and mRNA expression of CTGF within the allografts transplanted into anti-DNDR-1-treated recipients compared to the IgG groups. (D) Immunohistochemical staining showing CD8⁺ T cells (positive cells are in brown) and their quantification per HPF (graph) within the anti-DNDR-1 treated compared to isotype treated allogeneic transplants. Scale bar 100 μm in 20× magnification and Scale bar 50 μm in 40× magnification. (E) Determination of mRNA (normalized against housekeeping gene β-actin) expression of effector cytokine (IFN-γ and IL-33) within the allografts transplanted into anti-DNDR-1 treated recipient compared to control group that received IgG. (A–E) Data are from two independent experiments and are shown as mean ± SD and Mann–Whitney U-test was applied to compare two groups. **p* ≤ 0.05.

cDC1s, is absolutely crucial for CD8⁺ T cell-mediated allograft rejection. Importantly, we further reveal a critical role for the DAMP receptor, DNDR-1, expressed by the cDC1 subset in mediating this rejection and show that blockade of this receptor contributes to allograft acceptance. Our findings help illuminate the immune mechanisms involved in allograft rejection and highlight how increased understanding of such processes can suggest potential avenues for therapeutic intervention.

Allograft rejection is the result of a complex interplay of mechanisms and factors [5]. Rejection episodes are mainly driven by CD4⁺ Th1 cells that produce IFN-γ and TNF [45,46]. In the absence of a Th1-mediated alloimmune responses, CD4⁺ Th17 cells can also drive a pro-inflammatory response that accelerates chronic allograft rejection with IL-17A as a key cytokine [47]. In

this study, we utilized depletion of CD4⁺ cells to reveal an important yet underappreciated role also for CD8⁺ T cells in the development of chronic organ rejection [48–50]. The fact that CD8⁺ T cells can contribute to such rejection highlights their importance as potential targets in immunotherapies aimed at provoking long-term graft acceptance.

The most straightforward interpretation of our experiments is that DNDR-1⁺ cDC1s infiltrate allogeneic grafts and acquire allograft antigens. Some of those cDC1 then migrate to draining lymph nodes where they cross-present the graft antigens on MHC class I and cross-prime CD8⁺ T cells. The primed T cells can then home to the graft and produce effector cytokines such as IFN-γ and IL-33. As a consequence, active TGF-β₁ and CTGF are induced locally and lead to allograft fibrosis. The conundrum is that,

leaving aside the possibility of cross-dressing [51], the primed CD8⁺ T cells are restricted by host MHC and, therefore, cannot be restimulated directly by allograft cells. It is possible, therefore, that they are restimulated within the graft once again by cDC1 locally cross-presenting alloantigens, which would reinforce the observed dependence on that DC subtype and on the DNGR-1 receptor. An analogous role for cDC1 has been proposed in tumor immunity whereby cDC1 prime an antitumor CD8⁺ T cell response in tumor draining lymph nodes but also restimulate effector CD8⁺ T cells within the tumor itself [52–54]. The fact that CD8⁺ T cells would utilize the indirect presentation pathway to respond to alloantigens within grafts may also explain why their primary contribution in this setting appears to be production of cytokines leading to fibrosis rather than direct acute destruction of allograft cell targets as the latter cannot be directly recognized.

DAMP release and inflammation due to transplantation should be the same in syn- and allografts yet, in our study, cDC1 accumulated to a much greater extent in allografts than in syngeneic grafts. One point that remains unclear is why and how allogeneity is detected at these early time points to control accumulation of cDC1s. Early accumulation of alloreactive T-cells could be involved and those cells could produce chemokines such as XCL1/2 that attract cDC1 in a positive feedback cycle [55,56]. However, we saw no difference in cDC1 accumulation in allografts transplanted into mice lacking CD8⁺ T cells (data not shown). Alternatively, allogeneic determinants might be recognized early after transplantation not by T cells but by host NK cells that produce XCL1/2 and CCL5 to recruit cDC1 into grafts as recently demonstrated for tumors [57]. Further experimentation will be necessary to assess these possibilities.

In summary, our experiments suggest that immune activation leading to chronic allograft rejection and fibrosis can be triggered by DAMPs, including F-actin, that are detected by graft-infiltrating host cells. F-actin is recognized by DNGR-1, a receptor that is expressed on cDC1s that develop under the control of the transcription factor Batf3. DNGR-1 is a universal marker of mouse and human cDC1s in lymphoid and non-lymphoid tissues [18–22,24] and targeting antigens to DNGR-1 receptor has been used to induce specific immune responses in vaccination modalities [18,58,59]. In this current study, antibodies to DNGR-1 were used instead to block the receptor and shown to be effective at preventing chronic graft rejection in two different mouse heart transplantation model. These experiments suggest that antibody blockade of DNGR-1 can prevent the immune cascade leading to chronic allograft rejection and fibrosis and could therefore be exploited in humans in a therapeutic setting.

Materials and methods

Mice

Eight to 10 weeks (w) old female BALB/c (H-2^d) and Bm12 (H2-Ab1^{bm12}) as donors and 12–14 weeks old WT and deficient

(-/-) *Batf3*^{tm1Kmm/J} (*Batf3*^{-/-}) and *Clec9a*^{tm1.1CrS/J} (*Clec9a*^{-/-}) recipient female mice on C57BL/6 (H-2^b) background were purchased from Jackson Laboratory (Bar Harbor ME, USA). Mice were then bred in the animal facility of the Department of Surgery (University Medical Center Regensburg, Germany). All mice were housed under pathogen-free conditions and handled according to the local institutional guidelines. The experiments were approved by the local animal committee (TVA DMS-2532-2-102).

Heterotopic heart transplantation

Cardiac allografts from donors BALB/c and Bm12 mice were heterotopically transplanted into WT and C57BL/6^{-/-} as previously established by Corry et al. and adapted in our laboratory [2,5,60,48]. Donor hearts were perfused through the abdominal vena cava with 3 mL of cold 0.9% saline containing 500 IE heparin (Ratiopharm, Ulm, Germany) then harvested and placed in 4°C saline until transplantation. Abdominal palpation was regularly carried out to ensure the beating of the allograft. Functioning hearts were then harvested for analyses. In the BALB/c→C57BL/6 model transient CD4⁺ T cell-depletion was used for generation of chronic allograft rejection [48–50]. This model further allows investigation of allograft rejection in absence of CD4⁺ T cells. The Bm12→C57BL/6 model is a fully immunocompetent model also with the development of chronic allograft rejection.

Human samples

Chronic rejected and control human cardiac graft specimens were obtained from surgical biopsies (Department of Cardiac Surgery, Bad Oeynhausen, Germany). The paraffin-embedded tissue samples were sectioned at 3–4 µm. This was approved by the local ethics committee.

Antibody treatment

CD4⁺ T cell depletion in vivo: Recipient mice were injected intraperitoneally (i.p.; 1 mg/mouse) on day -1, 0, and 7 relative to day of transplantation (day 0) with rat anti-mouse CD4 monoclonal antibody, mAb (IgG2b, clone GK 1.5; Bioxcell, Cologne, Germany) to transiently deplete CD4⁺ T cells as previously described [61].

DNGR-1 antibody blocking in vivo: Rat anti-mouse DNGR-1 (7H11) mAb has been described [18, 27]. Recipient mice received 100 µg of mAb i.p. in PBS or, as control, only PBS on day 0, 3, 10, and 17 after transplantation.

Cytochrome C depletion in vivo: Cytochrome C (Cyt C) is known to be able to induce an Apaf-1-dependent apoptosis selectively in cross-presenting DCs (<https://doi.org/10.1073/pnas.0712394105>). Horse heart Cyt C (C2506, Sigma, St. Louis, MO) was dissolved in PBS at 20–50 mg/mL and stocked at -20°C. All treatments of recipient mice were performed in vivo

and consisted of 5 mg doses of Cyt C in PBS administered intravenously (i.v.) on day 1 prior heart transplantation then subcutaneously (s.c.) on day 2 and 7 post heart transplantation. Recipient receiving only PBS solution (100 μ L/mouse) were used as control groups.

Cardiac cell isolation

Cells were isolated from cardiac grafts as previously described [48]. Briefly, tissue was minced with scalpels in the presence of sterile RPMI 1640 medium containing 10% FCS, 600 U/ml collagenase II (Roche Diagnostics, Mannheim, Germany) and deoxyribonuclease I (DNAse, from bovine pancreas; Sigma, Munich, Germany). The mixture was slowly shaken at room temperature (RT) for 2 h. The supernatant (SN) was obtained and filtered through a 100- μ m-nylon cell strainer. Remaining tissue was again digested in 5 mL of same solution at 37°C and then strained. Filtered material was centrifuged and red blood cells were lysed with ACK lysis buffer (BioWhittaker, Lonza, MD, USA). The pellet containing cells was passed through a 40- μ m-nylon cell strainer. For the CD8⁺ T cell isolation, negative selection procedure was performed as recommended by manufacturer (MACS, Miltenyi Biotec: mouse CD8a⁺ T Cell Isolation Kit). After elution of MicroBeads conjugated cells through magnetic MACS LS column, the fraction containing CD8⁺ T cells was recovered and washed by centrifugation.

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded (mouse and human) or frozen (mouse) cardiac graft specimens were prepared and sectioned (3–4 μ m). For hematoxylin and eosin staining, paraffin-embedded sections were deparaffinized and fixed successively in Roti and ethanol solutions. Slides were washed with water and stained in hematoxylin for 13 s, washed again, and immersed in eosin for 2–4 min. Slides were returned with washing to the fixation buffers beginning from ethanol to Roti and mounted with Roti[®]-Histokitt mounting medium (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for microscopy. For immunohistochemistry (IHC), after deparaffinization and fixation (immersion into Roti and ethanol for paraffin-embedded and in methanol-ethanol solution for frozen sections), slides were blocked with PBS+20% goat serum (Sigma-Aldrich, St Louis, MO, USA) for 20 min at RT. After incubation overnight (O/N) in humid chamber at 4°C, slides were stained with monoclonal rat anti-mouse CD8a antibody (Ab25478; Abcam, Cambridge, UK; dilution 1:50) in PBS + 1% goat serum (GS). After washing with PBS, slides were stained with the secondary goat anti-rat-Fab2 (sc-3822; Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at RT in PBS+ 1% GS. Then, sections were incubated with SensiTek HRP solution (ScyTec Laboratories, Logan, UT, USA) and positive signals were visualized using an AEC+ High Sensitivity Substrate Chromogen kit (Dako, Hamburg, Germany). Slides were finally mounted using Aquatex[®] aqueous mounting agent (Merck

KGaA, Darmstadt, Germany) and images were taken with Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany). For the evaluation of cardiac allograft rejection, the revised 2004 ISHLT grading system was used [23]. Graft-infiltrating CD8⁺ cells were manually counted using ImageJ software version 1.48 (<http://imagej.nih.gov/ij/>), in ten high power fields (HPFs) at 20 $\times$ magnification. For analysis, at least 10 high-power fields (HPF) were selected per slide. All results are presented as the mean $\pm$ SEM and all analysis was performed in a blinded fashion.

Immunofluorescence

Slides were stained as described in immunohistochemistry section until primary Ab step. The primary antibodies: Armenian Hamster monoclonal antibody (ab119342; Abcam, Cambridge, UK) against CD11c (mouse and human), Rat purified IgG1 (BioLegend, Fell, Germany) against DNCR-1 (mouse), Mouse purified IgG1 (BioLegend) against DNCR-1 (human), and rabbit monoclonal antibody (ab52903) against phosphorolysed Smads3, Phospho-Smad3 (mouse and human) were used (at dilution 1:50 in PBS+ 1% goat serum) and samples were incubated O/N at 4°C. Secondary (Cy2 or Cy3) conjugated goat anti-rabbit, goat anti-rat, goat anti-mouse, or goat anti-Armenian Hamster IgG were used for detection. For the H-2b and H-2d positive cells detection, primary antibodies PE-conjugated anti-mouse H-2b (eBioscience, clone: AF6-88-5.5.3) and anti-mouse H-2d (BioLegend, clone: SF1-1.1) were used at dilution 1:150. Polyclonal rabbit anti-PE (Novus Biologicals) as secondary antibody was used at dilution 1:150, then Cy5 conjugated goat anti-rabbit (Dianova) was used for the detection. DNA was labelled with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; dilution 1:2000-5000 in PBS+1% goat serum) for 2–3 min. Reaction was stopped using distilled water and slides were mounted with Fluorescence Mounting Medium (Dako, Hamburg, Germany). Images were taken using an Axio Visio microscope (Carl Zeiss, Oberkochen, Germany). For the analysis, at least 10 high-power fields were selected per slide in a blinded fashion.

Masson's trichrome staining

Formalin-fixed and paraffin-embedded graft tissue samples (mouse and human) were deparaffinized and fixed as described above in H&E staining. To improve staining quality, sections were fixed in a Bouin's solution (Sigma-Aldrich) for 30 min at 56°C. Successively, slides were stained for 10–15 min in Weigert's iron hematoxylin working solution, Biebrich scarlet-acid fuchsin and differentiated in phosphomolybdic-phosphotungstic acid solution (Sigma-Aldrich) for each solution. Then, slides were transferred into aniline blue solution (Sigma-Aldrich) and briefly differentiated in 1% acetic acid. After returning to the ethanol and Roti solution, slides were mounted with Roti[®]-Histokitt mounting medium and observed using Axio Visio microscope.

Flow cytometry (FACS)

After collagenase digestion of harvested grafts and production of a single-cell solution graft-infiltrating cells were stained using fluorochrome-conjugated mouse-specific antibodies (all Abs were from eBioscience, Frankfurt, Germany). Data were acquired using a FACS Canto II flow cytometer (BD, Heidelberg, Germany) and analyzed using FlowJo software (version 8.8.6) in adherence to Guidelines for the use of flow cytometry and cell sorting in immunological studies [62]

RNA isolation, RT-PCR, and PCR array

Total RNA was extracted from whole allografts or isolated CD8⁺ T cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the AffinityScript™ QPCR cDNA Synthesis Kit (Aiglent Technologies, Böblingen, Germany). Quantitative Real-time PCR (qRT-PCR) assay was performed to quantify collagen 1, CTGF, TGF- β 1, IFN- γ , and IL-33 expression using the QuantiTect SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany) and the Roche LightCycler480 System. The following primer sequences were used: forward (sense) 5'-TGTTTCAGCTTTGTGGACCTC-3', reverse (anti-sense) 5'-TCAAGCATACCTCGGGTTTC-3' (mouse collagen, procollagen 1a); forward 5'-AGAGGGAAATCGTGCGTGAC-3', reverse 5'-CAATAGTGATGACCTGGCCGT-3' (mouse β -actin); forward 5'-TTGCTTCAGCTCCACAGAGA-3', reverse 3'-TGGTTGTAGAGGGCAAGGAC-5' (mouse TGF- β 1); forward 5'-GGAAACATTAAGAAGGGAAAA-3', reverse 3'-CCGCAAGCTTAGCCCTGTA-5 (mouse CTGF); Forward 5'-ACTGGCAAAGGATGGTGAC-3', Reverse 3'-CTCCAGTTGTTGGGTGTCCA-5' mouse IFN- γ); and Forward 5'-CACATTGAGCATCCAAGGAA-3', Reverse 5'-AACAGATTGGTCATTGTATGTACTCAG-3' (mouse IL-33). Relative gene expression was determined using the 2^{- Δ CT} method (normalized to the mean of the expression level for the housekeeper β -actin). Mouse Necrosis/apoptosis related-gene expression was evaluated using a mouse Necrosis RT² Profiler PCR Array kit (SA Biosciences, Hilden, Germany) using LightCycler 480 Real-Time PCR System (Roche). Mean of triplicated well values and fold change in expression relative to control were considered for each sample.

Indirect pathway IFN- γ -Elispot

WT and Clec9a^{-/-} recipient's APCs were isolated using mouse CD11c MicroBeads UltraPure isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and loaded for 3 day in cultures with 500 μ g/mL of alloantigen. Alloantigen (protein lysate) was isolated after sonication in PBS 1 $\times$ solution of allografts (Balb/c $\rightarrow$ C57BL/6) harvested on day 5 after heart transplantation. Protein concentration in the lysate was measured using

Bradford protein assay (B6916, Sigma-Aldrich). Note that 200 μ L/well of culture medium containing 300 000 of CD11c⁺ DCs and 500 μ g/mL of alloantigen was added and incubated for 3 days at 37°C and 5% CO₂. Then, splenic C57BL/6 WT CD8⁺ T cells were isolated using mouse CD8a⁺ T cell isolation Kit (Miltenyi Biotec). Elispot was performed using a MB Multi-screen PVDF plate (MAIPS4510, Millipore). Plates were pre-coated with an IFN- γ antibody (3321-2A, Mabtech) for 2 h at 37°C and 5% CO₂. Antigen-loaded DCs (as stimulators) were added to the plates with splenic C57BL/6 WT CD8⁺ T cells (as effectors) at a ratio of 50 000:150 000 and incubated for 3 days at 37°C and 5% CO₂. Following removal of the cells and washing of the plate wells, biotinylated specific detection antibodies (3321-2A, R4-6A2, Mabtech) were added to the wells. The resulting antibody complex was thus detected by addition of Streptavidin-ALP-labeled conjugate (3321-2A, Mabtech) and 1-Step NBT/BCIP substrate solution (34042, ThermoFisher Scientific). Spots were counted with a specific Elispot reader (EliSpot Robotic System ELROB05i, AID Advanced Imaging Devices GmbH Straßberg).

Statistics

All groups are shown as mean $\pm$ SD of the mean and were compared using a one-tailed Student's *t*-test or Mann-Whitney *U*-test (GraphPad Prism, version 5.00). The log-rank test was used to compare graft survival between the groups. Difference among groups was considered statistically significant (*) if *p* was < 0.05.

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Abbreviations: CAR: chronic allograft rejection · CTL: cytotoxic CD8⁺ T cell · DAMP: damage-associated molecular pattern · DNGR-1: DC NK lectin group receptor-1 · MAG: myelin-associated glycoprotein

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