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The Transcription Factor NFATc1 Supports the Rejection of Heterotopic Heart Allografts

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1 The immune suppressants cyclosporin A (CsA) and tacrolimus (FK506) are used worldwide in transplantation medicine to suppress graft rejection. Both CsA and FK506 inhibit the phosphatase calcineurin (CN) whose activity controls the immune receptor-mediated activation of lymphocytes. Downstream targets of CN in lymphocytes are the nuclear factors of activated T cells (NFATs). We show here that the activity of NFATc1, the most prominent NFAT factor in activated lymphocytes supports the acute rejection of heterotopic heart allografts. While ablation of NFATc1 in T cells prevented graft rejection, ectopic expression of inducible NFATc1/ α A isoform led to rejection of heart allografts in recipient mice. Acceptance of transplanted hearts in mice bearing NFATc1-deficient T cells was accompanied by a reduction in number and cytotoxicity of graft infiltrating cells. In CD8⁺ T cells, NFATc1 controls numerous intracellular signaling pathways that lead to the metabolic switch to aerobic glycolysis and the expression of numerous lymphokines, chemokines, and their receptors, including Cxcr3 that supports the rejection of allogeneic heart transplants. These findings favors NFATc1 as a molecular target for the development of new strategies to control the cytotoxicity of T cells upon organ transplantation.

Keywords: NFATc1, transplantation, heterologous, CD8⁺ T cells, ChIPseq, metabolism

INTRODUCTION

The worldwide application of cyclosporin A (CsA) and tacrolimus (FK506) as immune suppressants in transplantation medicine saved the lives of thousands of patients. CsA and FK506 prevent organ rejection by inhibiting the activity of phosphatase calcineurin (CN) that orchestrates the activation of peripheral lymphocytes upon antigen contact (1). In cells, CsA binds to CypA, a low molecular

Abbreviations: Ab, antibody; AICD, activation-induced cell death; BAC, bacterial artificial chromosome; CsA, cyclosporin A; CN, Calcineurin; ECAR, extra cellular acidification rate; FK506, tacrolimus; GICs, graft-infiltrating cells; NFATs, nuclear factors of activated T cells; NGS, next-generation sequencing; OXPHOS, oxidative phosphorylation; qRT-PCR, quantitative real-time polymerase chain reaction; T + I, 12-O-tetradecanoylphorbol-13-acetate (TPA) + Ionomycin; TF, transcription factor; tg, transgenic; WT, wild type.

weight immunophilin, and these complexes bind to CN and suppress its enzymatic activity (2). However, due to the numerous side effects caused by CsA and FK506, sustained applications of both immune suppressants not only lead to severe complications in transplant patients, in particular to nephrotoxicity, but also to neurotoxicity, hypertension, fibrosis, and cancerogenesis (3, 4). Although CsA and FK506 inhibit in lymphocytes primarily the activation of nuclear factors of activated T cell (NFAT) transcription factors (TFs), NFATs are not the only TFs whose activity is affected by CsA. Since CN supports the induction of NF- κ B factors upon TCR stimulation, CsA interferes also with their TCR-mediated activation (5, 6).

Due to the many deleterious side effects of CsA and FK506 in therapy, numerous searches have been undertaken for novel CN inhibitors, or for inhibitors that suppress directly the activation of NFATs. One set of such inhibitors are cell-penetrating peptides that interfere with the interaction between CN and NFATs (7). Based on the affinity-driven selection of peptides corresponding to the main interaction site of NFATs with CN (8), several peptide versions of general structure P_xI_xIT (where x represents any natural amino acid) were shown to inhibit the interaction between CN and NFATs (9). They interfere with the binding of CN to an NFAT peptide that is highly conserved between all NFAT proteins and, thereby, those inhibitory peptides do not distinguish between individual NFAT members. Since, however, the individual NFAT proteins differ remarkably in their activity *in vivo* (10), for therapeutic interventions inhibitors have to be identified that allow the specific block of individual NFAT proteins.

Among the treatments that suppressed the rejection of allogeneic transplanted hearts in mice (3) there are several therapies that affect the activation of NFATs and/or their targets in T cells. Apart from the use of CN inhibitors CsA and FK506, metabolic inhibitors and the inactivation of NF- κ B or *Cxcr3*, a chemokine receptor (6), led to acceptance of transplanted allogeneic hearts (11–13). We showed previously that NF- κ B factors support the induction of short NFATc1/ α A in B lymphocytes (14). We will show here (13) NFATc1 ablation prolongs allograft survival, impairs the metabolic switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis in activated CD8⁺ T cells and the expression of chemokine receptor *Cxcr3* in CTLs. These data indicate NFATc1 as a key factor in activated T cells that controls the rejection of transplanted allogeneic hearts.

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MATERIALS AND METHODS

Mice and Isolation of T Cells

Male C57BL/6J (B6, H-2^b) and BALB/c (H-2^d) wild-type (9) mice were purchased from Janvier (France). *Nfatc1^{flx/flx}* and *Nfatc2^{-/-}* mouse lines have been described previously. *Nfatc1^{flx/flx}* mice were crossed with *CD4-cre* mice for inactivating the *Nfatc1* gene in all T cells, and with *mb1-cre* mice in all B cells (15–20). In *dlck-cre x Nfatc1^{flx/flx}* mice, the *Nfatc1* gene is inactivated in peripheral T cells. In mice of (11) *dlck-cre x Nfatc1^{flx/flx} x caNfatc1-STOP^{flx/flx}* line, a constitutively active version of NFATc1/ α A is expressed from the *Rosa26* locus upon removal of a “floxed” STOP sequence and inactivation of endogenous *Nfatc1* gene in peripheral T cells

(21, 22). *Nfatc1P2A* mice carry an *Nfatc1 P2* promoter deletion and, due to a CMV-promoter-driven cre are deficient for P2-directed transcripts [see Figure S1 in Supplementary Material and Ref. (23)]. Bacterial artificial chromosome (BAC) transgenic (tg) mice expressing NFATc1/A-Bio protein [and BirA, the biotin-ligase (4) from *E. coli* (24)] have been described previously in Ref. (25). All mice were maintained in the Central Animal Facility of the Medical Faculty (ZEMM), University of Wuerzburg, according to the institutional guidelines (acceptance AKZ 55.2-2531.01-80/17) from 22.10.2010.

For CD8⁺ T cell isolation, the “CD8 (Ly2) microbeads, mouse” kit (Miltenyi Biotech) was used. For α CD3/CD28 stimulation, 5 μ g CD3 ϵ (clone 145-2C11) and 2 μ g CD28 (clone 37.51) (both BD Pharmingen) were used to coat multi well plates. T cells were also stimulated with 10 ng/ml tetradecanoylphorbol-13-acetate and 0.5 μ M ionomycin (normally for 5 h).

Heterotopic Murine Heart Transplantation

Abdominal heterotopic heart transplantation into mice was performed as described previously in Ref. (26).

Isolation of Graft-Infiltrating Cells (GICs)

Heart grafts cut into small pieces were incubated in 100 U/ml collagenase at 37°C for 30 min. Cells were washed with phosphate-buffered saline (PBS), counted, and purified on a Ficoll-Hypaque gradient.

Histologic and Immune Histochemical Analysis

Freshly explanted heart grafts were fixed in 4% paraformaldehyde and embedded in paraffin. For hematoxylin–eosin staining, 4- μ m sections were de-paraffinized with xylene, rehydrated in absolute ethanol, stained in hematoxylin solution, and counter-stained with eosin. For immune histochemical staining, de-paraffinized and rehydrated sections (1 μ m) were heated for antigen unmasking in 10 mM sodium citrate buffer (pH 6.0), and stained with α CXCR3 (CD183, #bs-2209R, Bioss Antibodies, Inc. MA, USA), diluted 1:200 in antibody (Ab) diluent (DAKO, Hamburg, Germany) at 4°C overnight. Sections were washed in PBS and incubated with 1:100 diluted horseradish-labeled goat anti-rabbit IgG (DAKO, P0448) at room temperature for 1 h. Staining was developed by adding 3,3'-diaminobenzidine (DAB ready to use, DAKO) and counterstaining was done with hematoxylin.

Confocal Microscopy of CD8⁺ T Cells

Upon isolation, splenic CD8⁺ T cells were stimulated with α CD3/CD28 Abs for 24 h, attached to poly-L-Lysin-coated chamber μ -slides, fixed in 4% formaldehyde, permeabilized with 0.2% Triton-X100, and blocked with 5% BSA. Samples were incubated with primary mouse anti-NFATc1 Ab 7A6 in 1% BSA at 4°C overnight.

RNA Seq Transcriptome Analysis

Graft-infiltrating cells were isolated from heart grafts at day 5 after transplantation. Purification of RNA from GICs and transcriptome assays were performed at TRON GmbH (Mainz, Germany) as described previously in Ref. (27).

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ChIP Seq Assays

CD8⁺ T cells from BAC tg mice expressing BirA, or from tg mice expressing BirA and Nfatc1/A-Bio, a Bio-tagged version of NFATc1/ α A [25]; Figure S4 in Supplementary Material] were differentiated to CTLs *in vitro*. Upon re-stimulation with T + I (5 ng/ml and 0.5 μ M) for 5 h, chromatin from 1×10^7 fixed cells was prepared and sonicated, followed by precipitation of chromatin on streptavidin beads (M-280, 1205D, Thermo Fisher Scientific, MA, USA) (28). After cDNA library preparation (using a NEBNext[®] Ultra[™] I₅A Library Prep Kit for Illumina[®]) sequencing was performed as a 50-bp single read run using an Illumina HiSeqTM2500 (Illumina, San Diego, CA, USA) using a TruSeq[®] Rapid SBS Kit v2 and a HiSeq Rapid Flow Cell v2. 50 bp sequence reads that passed the Illumina quality filtering were aligned to the mouse genome assembly version of July 2007 (CB I37/mm9), using the map with Bowtie for Illumina 1.1.2. Results were visualized with the “Integrative Genomics Viewer” IGV version 2.3.81 (29).

Immunoblotting

Western blots were performed using the NFATc1-specific mAbs 7A6 and NFATc2 mAb (# 556602 and 5062574, BD Pharmingen) for NFAT detection.

Flow Cytometry

Lymphocyte stainings were performed by incubation for 20 min on ice using conjugated the following mAbs (eBioscience, San Diego, CA, USA): CD8-FITC (#11-0081-82), CD4-FITC(#MCD0401), CXCR3-APC (#17-1831-82), CCR5-PE (#12-1951-81), CD62L-PE (#12-0621-81), CD44-APC (#17-0441-81), CD25-APC (#17-0251-82), B220 FITC (#11-0452-82), CD5-PE (#12-0051-82), CD23-APC (#MCD2305).

Cytotoxicity Assays

The mineral oil induced murine MOPC-315.BMP2.FUGLW55 plasmacytoma cell line (H2^b) expressing eGFP and luciferase was used as target cell line. To measure luciferase during coculture the assays were performed by cultivating MOPC-luciferase target cells together with equal numbers of GICs isolated from WT B6 or *Nfatc1^{flx/flx} x CD4-cre* recipient mice during the acute rejection phase (d6, effector/target ratio 7, followed by 20 or 36 h incubation). After centrifugation, the cell pellet was washed once with PBS and finally re-suspended in 100 μ l harvesting buffer to lyse cell membranes. 50 μ l of supernatant was transferred into a white, non-transparent 96-well plate. The LUMIstar Omega was primed with 1 ml of ready-to-use luciferin solution before luciferase-activity measurement was performed. 50 μ l of luciferin solution per well were automatically added to the sample and measurement was performed.

Extracellular Flux Assays

The extra cellular acidification rate (ECAR) of CD8⁺ T cells was measured in a XF96 analyzer (Seahorse Bioscience) as described previously in Ref. (25).

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Statistical Analysis

Student's *t*-test or the Mann–Whitney *U* test was used for statistical analysis with the software GraphPad Prism 6. *P* values above 0.05 were considered not significant.

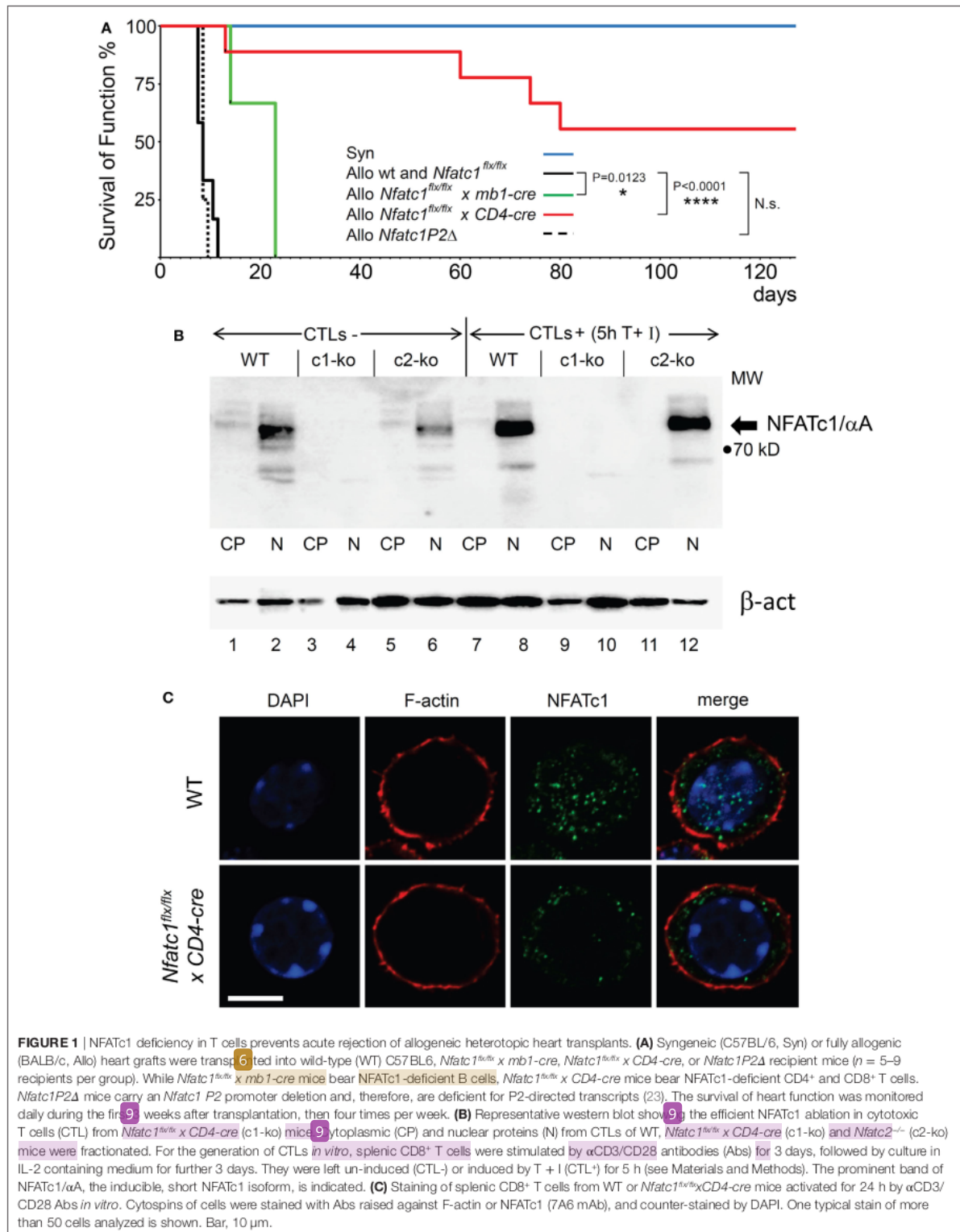
RESULTS

NFATc1 Supports the Rejection of Allogeneic Heart Transplants

In a previous report on the survival of heterotopic heart and skin transplants, in *Nfatc2*- or *Nfatc3*-deficient recipient mice a moderate prolongation of allogeneic transplant survival was described Ref. (30). These observations prompted us to investigate whether NFATc1, the most prominent NFAT factor in activated peripheral T and B cells, plays a role in the rejection of allogeneic organ transplants. In a model of heterotopic heart transplantation, we used *Nfatc1^{flx/flx}* mice crossed with the *CD4-cre* or *mb1-cre* mouse lines to ablate the expression of NFATc1 in peripheral T or B lymphocytes, respectively (18, 20, 22). Fully allogeneic hearts from donor BALB/c mice transplanted into WT B6 recipient mice are normally rejected within 7 days by the recipient's immune system. However, BALB/c heart allografts transplanted into *Nfatc1^{flx/flx} x CD4-cre* mice bearing both NFATc1-deficient CD4⁺ and CD8⁺ T cells survived significantly longer for more than 50 days, and more than 50% of the allografts for more than 120 days. In contrast, transplantation of allogeneic hearts into *Nfatc1^{flx/flx} x mb1-cre* mice bearing *Nfatc1^{-/-}* B cells had only a minor positive effect on the survival of transplanted allogeneic hearts (Figure 1A).

In lymphocytes, the *Nfatc1* gene is expressed in six individual α - and β -isoforms at the RNA and protein levels. These are generated under the control of two alternate promoters, P1 and P2, and polyA sites, pA1 and pA2, and, therefore, differ in their N- and C-terminal peptides (Figure S1 in Supplementary Material) (31). In T cells from *Nfatc1^{flx/flx} x CD4-cre* mice, the expression of all isoforms should be ablated (Figure 1B and unpubl. data), but upon staining of immunoblots or cytopins prepared with NFATc1^{-/-} CD8⁺ T cells using the 7A6 NFATc1 mAb various shorter protein bands or stained dots were detected. However, these NFATc1-like proteins were not imported into the nucleus upon activation of NFATc1^{-/-} T cell cells (Figure 1C) and, therefore, should play a minor, if any role in transcriptional regulation.

To identify the role of individual NFATc1 isoforms in transplant rejection, we crossed *Nfatc1-P2^{flx/flx}* mice created in our laboratory (32) with CMV-cre mice for the deletion of P2 promoter and exon 2 sequences. In such *Nfatc1P2 Δ* mice, the generation of NFATc1/ β isoforms was inhibited [see Figure S1 in Supplementary Material and Ref. (23)]. *Nfatc1P2 Δ* mice were born at expected Mendelian ratios, were viable and developmentally indistinguishable from their *Nfatc1P2^{flx/flx}* littermates, and their lymphoid compartments remained unaffected. Both T and B cells from *Nfatc1P2 Δ* mice could be normally activated *ex vivo* and did not reveal any defects in proliferation or induction of Activation-Induced Cell Death (Figure S2 in Supplementary Material). However, the rejection of allogeneic hearts upon



transplantation into *Nfatc1^{IP2Δ}* mice was detected on the same day as upon transplantation into WT B6 mice (Figure 1A). This led us to conclude that NFATc1/β proteins play a minor role in graft rejection.

In order to demonstrate that—as we assumed—the induction of the short NFATc1 isoform NFATc1/αA, the most prominent NFATc1 protein in cytotoxic T cells (Figure 1B) (25), plays an important role in the rejection of allogeneic heart transplants, we used *caNfatc1-STOP^{flx/flx}* mice as recipients. Those mice express a constitutive active (ca) version of NFATc1/αA (caNFATc1) from the murine *Rosa26* locus upon cre-mediated removal of a “floxed” STOP sequence (21). However, to our surprise (over-) expressing caNFATc1/αA in thymocytes of *CD4-cre* mice was not tolerated since all T cells expressing solely caNFATc1/αA were eliminated during positive selection and did not appear in peripheral organs of mice (unpubl. data). Therefore, we crossed *caNfatc1-STOP^{flx/flx}* mice with *dclck-cre x Nfatc1^{flx/flx}* mice that express cre under the control of distal *lck* promoter and, thereby, caNFATc1/αA in peripheral T cells (but no endogenous NFATc1). In peripheral CD8⁺ T cells from all *dclck-cre x Nfatc1^{flx/flx} x caNfatc1-STOP^{flx/flx}* mice that we investigated, we observed caNFATc1/αA expression. In addition, each transplantation of allogeneic BALB/c hearts into *dclck-cre x Nfatc1^{flx/flx} x caNfatc1-STOP^{flx/flx}* mice led to acute rejection around day 9 (mean survival time, MST), comparable to MST observed in C57BL/6 WT mice expressing endogenous NFATc1 (Figures 2A,B).

Compared to the sustained survival of allogeneic transplanted hearts in the majority of *Nfatc1^{flx/flx} x CD4-cre* recipient mice for 60 days and longer (Figure 1A), we detected a long-term survival (>60 days) of heart allografts in two out of five *dclck-cre x Nfatc1^{flx/flx}* recipient mice (Figure 2A). In immunoblots using protein of activated CD8⁺ T cells from five *dclck-cre x Nfatc1^{flx/flx}* mice, we detected numerous, albeit weak bands with the same or a similar size of the endogenous NFATc1 (Figure 2C). This might be due to an incomplete ablation of endogenous NFATc1 in splenic CD8⁺ T cells of *dclck-cre x Nfatc1^{flx/flx}* mice. A deletion efficiency of approximately 80% has been reported for the *dclck-cre* line in CD8⁺ T cells, whereas a markedly higher efficiency rate was detected for the murine *CD4-cre* line (33).

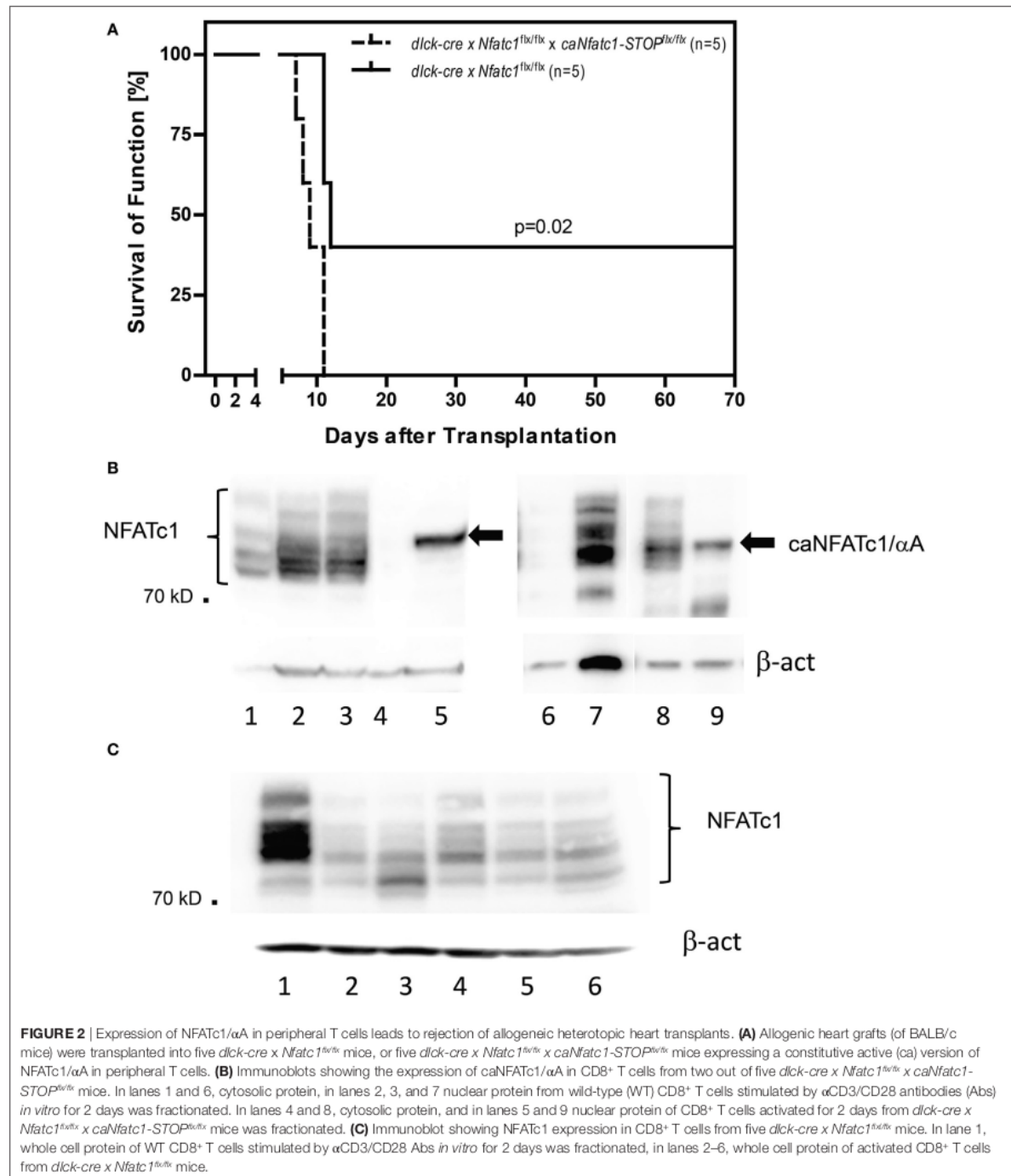
NFATc1 Ablation in T Cells Leads to a Decrease in Transcripts of Genes of T Cell Activation Signature in GICs

In allogeneic BALB/c hearts, we observed a threefold to fivefold increase of GICs at days 5 and 6 during the acute rejection phase, compared to the number of GICs in transplanted syngeneic hearts (Figures 3A,B). However, approximately the same number of GICs we detected in allogeneic hearts transplanted into *Nfatc1^{flx/flx} x CD4-cre* mice (Figure 3B), and in long-term accepted heart transplants (>100 days after transplantation) a further decrease in number of GICs was detected (Figure 3B). Compared to GICs isolated from rejected hearts, the cytotoxicity of GICs from accepted hearts of *Nfatc1^{flx/flx} x CD4-cre* mice was significantly impaired, reaching approximately one-third of cytotoxicity of GICs from rejected hearts (Figure 3C).

To define molecular targets of NFATc1 in GICs of transplanted hearts, we determined by next-generation sequencing (NGS) the transcriptomes of GICs isolated from syngeneic and allogeneic grafts on day 5 after transplantation, i.e., during the acute rejection phase of allogeneic grafts, and from allogeneic grafts of *Nfatc1^{flx/flx} x CD4-cre* mice. Some of the B6 WT recipients of allogeneic grafts were treated with CsA ($n = 5$). In GICs from untreated allografts, the transcript levels of T cell signature genes (e.g., of *Cd3g,d,e*, *Cd4*, *Cd8a,b*, and *Il2ra,rb* genes) and of genes coding for signal molecules in T cells (of *Lck*, *Zap70*, and *Fyn* genes) were found to be increased, compared to GICs from syngeneic mice. In GICs from *Nfatc1^{flx/flx} x CD4-cre* and CsA-treated WT recipients the transcript levels were lower, compared to GICs from untreated allogeneic recipients (Figure 4A). This is also true for the *Gzmb* and *Prf1* genes encoding the most prominent granzymes and perforins in CTLs.

The rejection of heart allografts is executed by various subsets of cells of adaptive and innate immune system. The population of GICs has been characterized by flow cytometry (34) but not at the transcriptome level. We hypothesized that the transcription of numerous genes that control signaling pathways for the activation, survival, and migration of GICs become activated in the majority of GICs, and suppressed by CsA. Analyses of signaling pathway activity (Figure 4B) revealed the expected activation and CsA-sensitivity of several T cell activation pathways, including the signatures of NK cell activity and activation of CD28 co-stimulatory pathway (35). Among them are the NF-κB and NFAT signatures that were found to be activated during the acute rejection phase. However, while NF-κB signaling remained unaffected, NFAT signaling in GICs was markedly suppressed by CsA treatment and in *Nfatc1^{flx/flx} x CD4-cre* mice (Figure 4B). From these data, one may conclude that NFAT signaling is a major CsA-sensitive transcriptional pathway activated in GICs during acute rejection phase of fully allogeneic heart transplants.

Among the genes whose transcript levels increased upon transplantation and decreased by CsA treatment or NFATc1 ablation, there were numerous genes that encode enzymes of aerobic glycolysis (Figure 4C). This metabolic switch from OXPHOS to aerobic glycolysis is a typical sign of activated lymphocytes which have a high demand for energy and cellular building blocks, such as nucleotides and amino acids (36). To elucidate whether NFATc1 indeed plays a role in the control of aerobic glycolysis, we studied glycolysis by extracellular flux analysis in WT and *Nfatc1^{-/-}* CD8⁺ T cells from *dclck-cre x Nfatc1^{flx/flx}* mice that we used in transplantation experiments (Figure 2A). As seen in Figure 4D, NFATc1 ablation in peripheral T cells by *dclck-cre* led to a marked decrease in ECAR which reflects the metabolic switch to glycolysis upon T cell activation. However, when—on the background of deficiency of endogenous NFATc1—the ca version of NFATc1/αA was expressed (Figure 4B), ectopic expression of NFATc1/αA exerted an overall increase in ECAR (Figure 4D), reflecting a stimulation of glycolysis and glycolytic capacity of CD8⁺ T cells by NFATc1/αA. These data suggest that NFATc1 supports the switch from OXPHOS to aerobic glycolysis during activation of peripheral CD8⁺ T cells. They are in line with the observation that inhibiting aerobic glycolysis in T cells suppressed the rejection of allogeneic heart transplants (37).



We were also interested in elucidating whether the transplantation of an allogeneic heart at d6, i.e., during the acute rejection phase, affected the peripheral T cell compartment in WT mice, in WT mice treated with CsA and in *Nfatc1^{flx/flx} x CD4-cre* mice

bearing NFATc1-deficient T cells. However, upon transplantation we detected only subtle differences in the composition of splenic T cells from these three types of mice (Figure S3 in Supplementary Material).

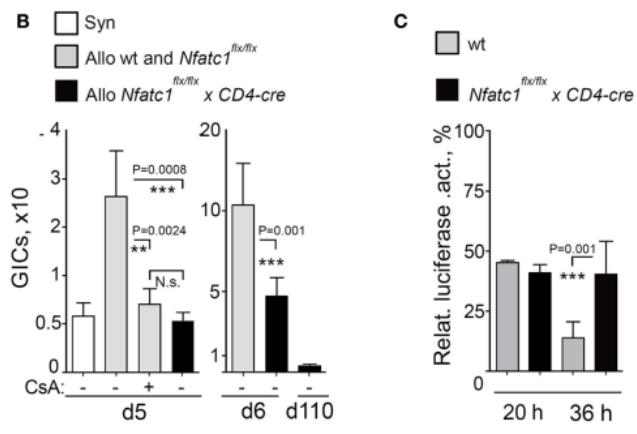
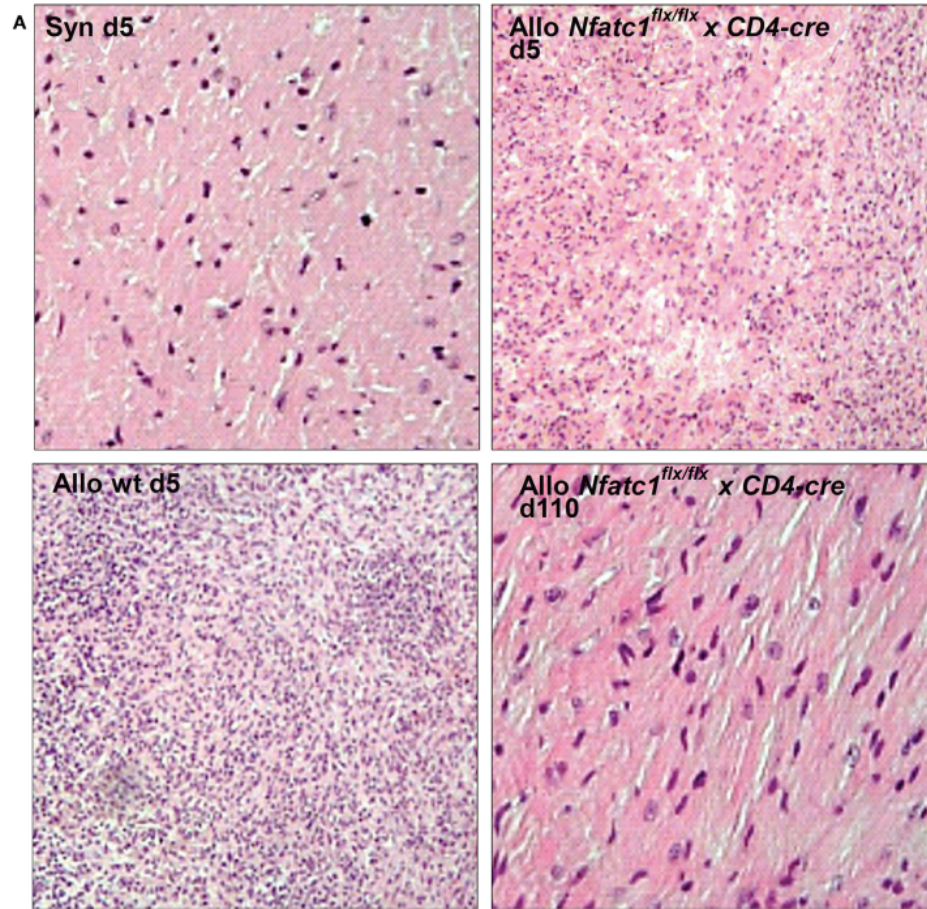


FIGURE 3 | Graft-Infiltrating Cells (GICs) in transplanted hearts. **(A)** Representative hematoxylin-eosin stains of grafts from syngeneic and allogeneic wild-type (WT) recipients, and from *Nfatc1*^{flx/flx} x *CD4-cre* recipients during the acute rejection phase (d5), and from *Nfatc1*^{flx/flx} x *CD4-cre* recipients at d110. Bar, 300 μm. **(B)** Quantification of GICs in transplanted allogeneic hearts with (+) and without (-) cyclosporin A (CsA) treatment of animals. **(C)** Cytotoxicity of GICs. Luciferase assays were performed after cultivation of MOPC-luciferase target cells together with equal numbers of GICs isolated from WT or *Nfatc1*^{flx/flx} x *CD4-cre* recipient mice during the acute rejection phase (d6, effector/target ratio 4/1, followed by 20 or 36 h incubation).

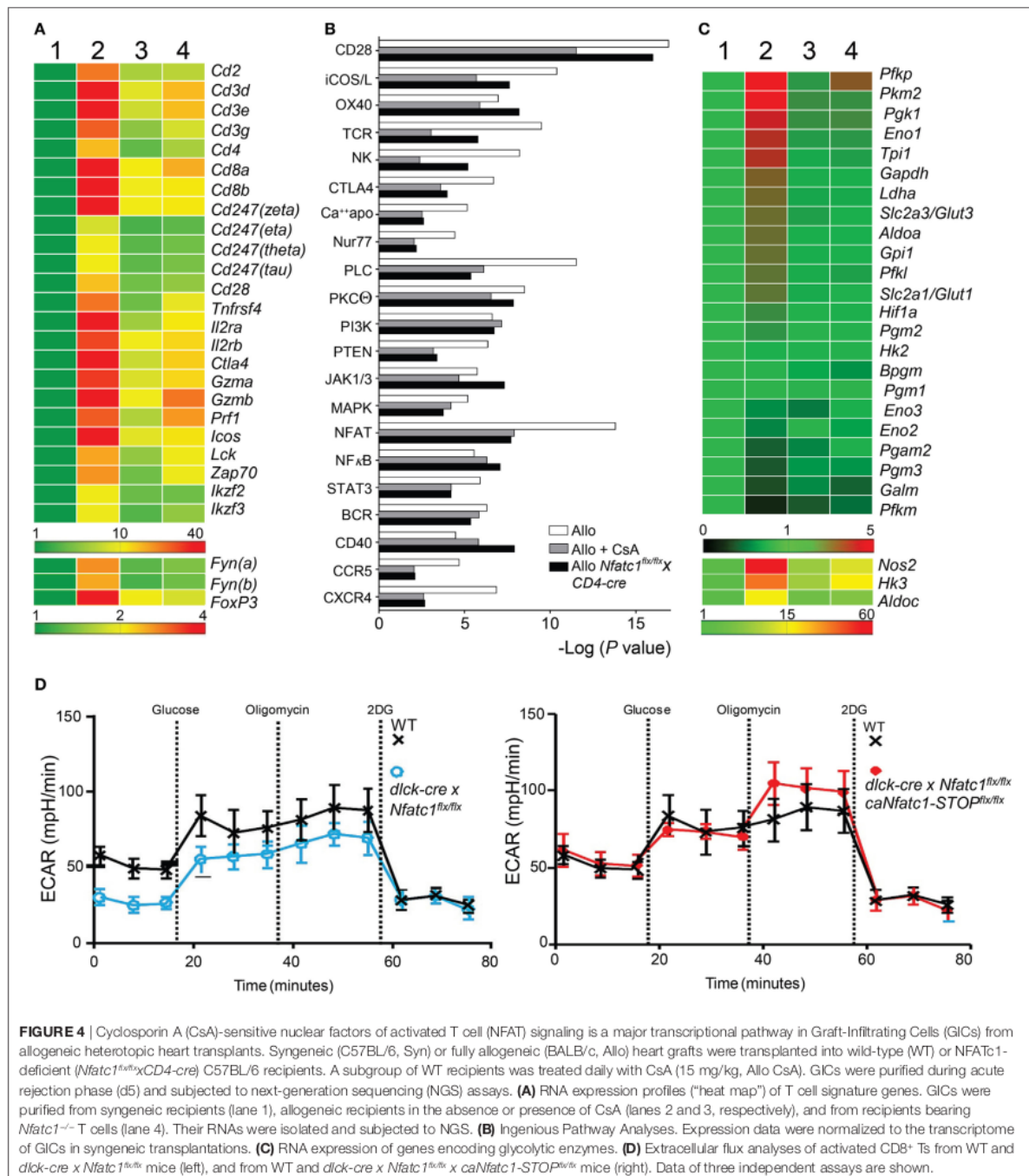


FIGURE 4 | Cyclosporin A (CsA)-sensitive nuclear factors of activated T cell (NFAT) signaling is a major transcriptional pathway in Graft-Infiltrating Cells (GICs) from allogeneic heterotopic heart transplants. Syngeneic (C57BL/6, Syn) or fully allogeneic (BALB/c, Allo) heart grafts were transplanted into wild-type (WT) or NFATc1-deficient (*Nfatc1^{flx/flx}* x *CD4-cre*) C57BL/6 recipients. A subgroup of WT recipients was treated daily with CsA (15 mg/kg, Allo CsA). GICs were purified during acute rejection phase (d5) and subjected to next-generation sequencing (NGS) assays. **(A)** RNA expression profiles ("heat map") of T cell signature genes. GICs were purified from syngeneic recipients (lane 1), allogeneic recipients in the absence or presence of CsA (lanes 2 and 3, respectively), and from recipients bearing *Nfatc1^{flx/flx}* T cells (lane 4). Their RNAs were isolated and subjected to NGS. **(B)** Ingenuity Pathway Analyses. Expression data were normalized to the transcriptome of GICs in syngeneic transplantations. **(C)** RNA expression of genes encoding glycolytic enzymes. **(D)** Extracellular flux analyses of activated CD8⁺ Ts from WT and *dclk-cre* x *Nfatc1^{flx/flx}* mice (left), and from WT and *dclk-cre* x *Nfatc1^{flx/flx}* x *caNfatc1-STOP^{flx/flx}* mice (right). Data of three independent assays are shown.

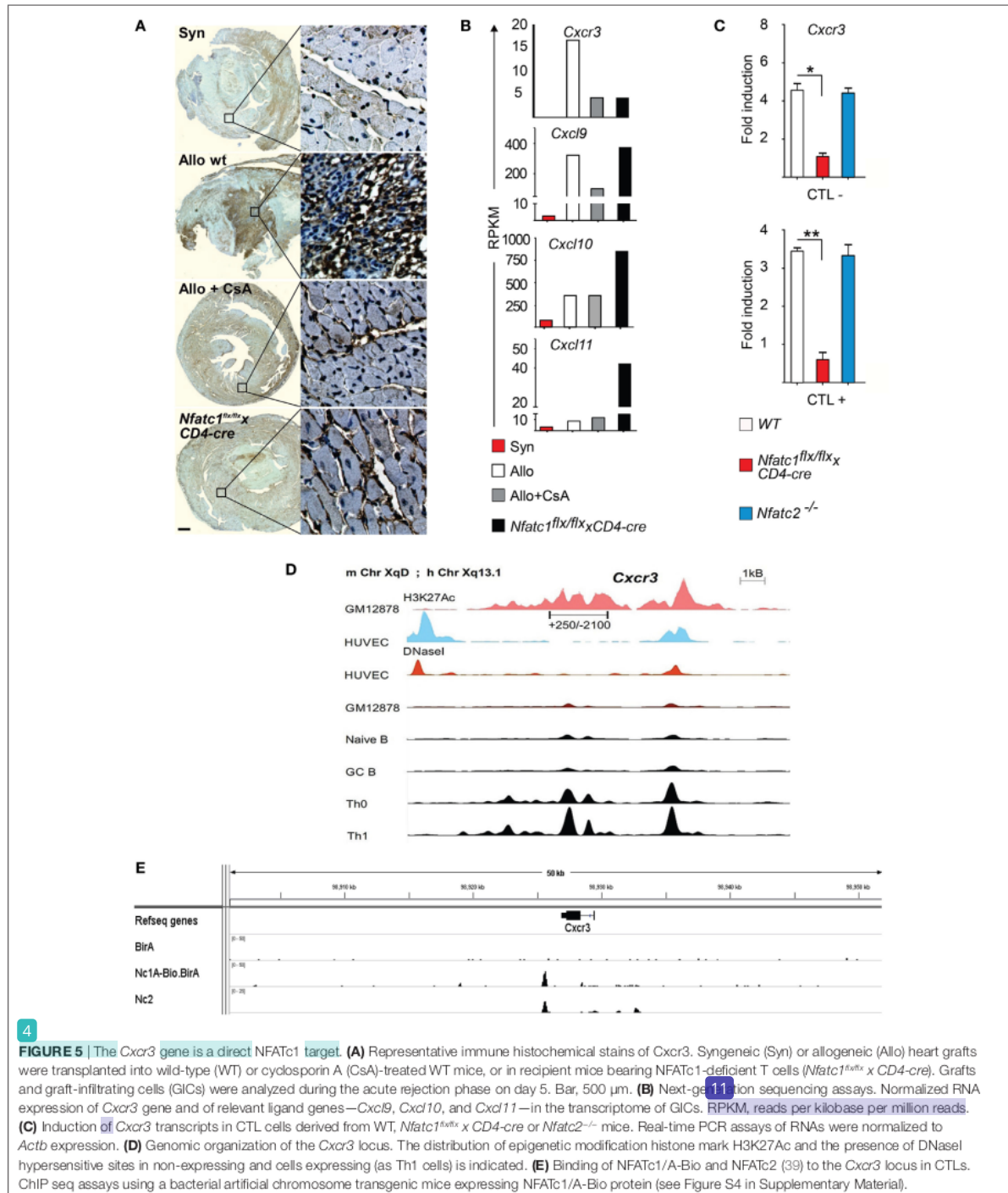
NFATc1 Controls the Expression of *Cxcr3* Gene

The expression of chemokines and chemokine receptors plays a crucial role in the migration of peripheral T cells to inflamed

organs. *Cxcr3* expression was shown to determine the balance between effector and memory T cells (38) and to be required for acute allogeneic heart rejection (12). Histochemical stains of *Cxcr3* expression revealed high levels of *Cxcr3* in cells of

allogeneic heart transplants whereas a strong decrease in *Cxcr3* levels was observed in heart allografts of CsA-treated B6 WT or in *Nfatc1^{flx/flx} x CD4-cre* mice (Figure 5A). In addition, the

data of transcriptome assays showed a strong increase in *Cxcr3* transcripts in GICs isolated from allogeneic heart transplants and much less *Cxcr3* transcripts in GICs isolated from hearts



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FIGURE 5 | The *Cxcr3* gene is a direct NFATc1 target. **(A)** Representative immune histochemical stains of *Cxcr3*. Syngeneic (Syn) or allogeneic (Allo) heart grafts were transplanted into wild-type (WT) or cyclosporin A (CsA)-treated WT mice, or in recipient mice bearing NFATc1-deficient T cells (*Nfatc1^{flx/flx} x CD4-cre*). Grafts and graft-infiltrating cells (GICs) were analyzed during the acute rejection phase on day 5. Bar, 500 μ m. **(B)** Next-generation sequencing assays. Normalized RNA expression of *Cxcr3* gene and of relevant ligand genes—*Cxcl9*, *Cxcl10*, and *Cxcl11*—in the transcriptome of GICs. RPKM, reads per kilobase per million reads. **(C)** Induction of *Cxcr3* transcripts in CTL cells derived from WT, *Nfatc1^{flx/flx} x CD4-cre* or *Nfatc2^{-/-}* mice. Real-time PCR assays of RNAs were normalized to *Actb* expression. **(D)** Genomic organization of the *Cxcr3* locus. The distribution of epigenetic modification histone mark H3K27Ac and the presence of DNaseI hypersensitive sites in non-expressing and cells expressing (as Th1 cells) is indicated. **(E)** Binding of NFATc1/A-Bio and NFATc2 (39) to the *Cxcr3* locus in CTLs. ChIP seq assays using a bacterial artificial chromosome transgenic mice expressing NFATc1/A-Bio protein (see Figure S4 in Supplementary Material).

transplanted into CsA-treated or *Nfatc1^{flx/flx} × CD4-cre* mice (Figure 5B). These findings are supported by quantitative real-time polymerase chain reaction assays of RNAs from murine CTLs that show a marked decrease of *Cxcr3* transcripts in *Nfatc1^{-/-}* CTLs (Figure 5C).

In murine T cells, the *Cxcr3* locus is characterized by numerous DNase I hypersensitive chromatin sites in front of the *Cxcr3* gene, and extended H3K27 acetylation both in front and within the gene (Figure 5D). However, we were unable to trans-activate the immediate upstream promoter region (spanning the nucleotides from +250 to -2100) cloned in front of a luciferase reporter gene by NFATc1 (over-) expression in Jurkat T cells (data not shown). ChIP seq assays of CTLs that were generated *in vitro* from splenic CD8⁺ T cells of a novel tg mouse line expressing NFATc1/α-Bio protein (Figure S4 in Supplementary Material) (25) revealed strong NFATc1 binding downstream of the *Cxcr3* gene, approximately 1 kb 3' from its poly A addition site (Figure 5E, below). These data suggest that NFATc1 plays a critical role in the expression of *Cxcr3* gene in T cells (Figure 5A). Moreover, the data suggest that in part the effect of NFATc1 on heart allograft rejection is mediated by *Cxcr3* expression.

DISCUSSION

The findings of our study presented here showed that ablation of *Nfatc1* induction in T cells prevented the rejection of heterotopic heart transplants. In contrast, the expression of a ca version NFATc1/αA in T cells supported heart graft rejection indicating an important function for NFATc1/αA in the T cell-mediated immune reaction against allogeneic heart grafts.

The deficiency of NFATc1 in recipient mice resulted in an impaired infiltration of graft and decreased cytotoxicity of GICs. While during acute heart rejection GICs correspond to lymphocytes, monocytes, and neutrophils (40), organ graft rejection is a T cell-dependent process (41, 42) and a massive CD8⁺ T cell infiltration and a high ratio of CD8⁺/CD10 cells was reported for heart allograft rejection in mice (43). The fate of organ transplants is determined to a large part by the number of effector T cells. Regulatory T cells (Tregs) that are known to control the activation and expansion of effector T cells can also affect the fate of a transplanted organ. In a MHC class II- mismatched cardiac allograft model, acute rejection of the donor heart grafts was inhibited by CD4⁺CD25⁺ Tregs that restricted the clonal expansion of alloreactive T cells (44). While we did not observe an effect of NFATs on the generation of thymus-derived naturally occurring regulatory T cells (nTreg), the generation of peripherally induced Treg by TGF-β was highly dependent on NFAT expression (15). However, neither inactivation of NFATs did impair the suppressive capacity of Tregs (15, 45), nor did activation of Tregs lead to any NFATc1/αA induction (46). These findings prompted us to conclude that Tregs play only a minor, if any role during induction of transplant tolerance in mice bearing NFATc1-deficient T cells.

One may hypothesize that due to the MHC I and II mismatches of allogeneic graft, NFATc1 expression is rapidly induced in T lymphocytes of peripheral lymphoid organs, in particular in

spleen (47). This might lead to the fast re-stimulation of effector CTLs and their migration into the allograft in which CTLs destroy the graft (48, 49). Among the “best” target genes of NFATc1 that we determined by NGS and ChIP seq assays of murine CTLs generated *in vitro* we detected numerous lymphokine, chemokine, and chemokine receptor genes (25). We showed here that the expression of *Cxcr3* gene, which was shown previously to play an important role in acute allograft rejection (12) is controlled by NFATc1 in T cells. However, *Cxcr3* is not the only molecule of chemokine network that affects heart transplants (50). Ablation of *Ccr4*, a further chemokine receptor, led to prolonged survival of allogeneic murine hearts in a chronic transplant model (51), and priming of T cells through the receptor of hepatocyte growth factor, *c-Met*, *in vitro* resulted in the generation of “T cell cardiotropism,” including *Cxcr4* and *Ccr4* expression (52). The priming through *c-Met* affected also the expression of *Ccl3* and *Ccl4*, two ligands of chemokine receptor *Ccr5* that are strongly induced by NFATc1 in murine CTLs (25). Among the direct targets immediately downstream of NFATc1 in T cells is the *Irf4* gene (25). The TF IRF4 has been shown recently to control the rejection of allogeneic murine hearts upon heterotopic transplantation (53).

In summary, our data show that ablation of *Nfatc1* induction in T cells is sufficient to prevent the rejection of allogeneic heart transplants, whereas (over-) expressing NFATc1/αA led to fulminant rejection. These findings favor NFATc1/αA as molecular target to prevent graft rejection upon heart transplantation.

ETHICS STATEMENT

Animal experiments were performed according to project licenses (acceptance AKZ 55.2-2531.01-80/10 from 22.10.2010 and AKZ 55.2-2531.01-63/11), which are approved and controlled by the Regierung von Unterfranken, Würzburg.

AUTHOR CONTRIBUTIONS

JB, CO, US, SK-H, KhM, TP, KrM, RW, C-TG, IK, and NM performed experiments, ES and AA designed the study, performed experiments, analyzed data, and wrote the manuscript, along with CO.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01338/full#supplementary-material>.

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