Targeting the orphan nuclear receptor NR2F6 in T cells primes tumors for immune checkpoint therapy

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Abstract

Background: NR2F6 has been proposed as an alternative cancer immune checkpoint in the effector T cell compartment. However, a realistic assessment of the in vivo therapeutic potential of NR2F6 requires acute depletion.

Methods: Employing primary T cells isolated from Cas9-transgenic mice for electroporation of chemically synthesized sgRNA, we established a CRISPR/Cas9-mediated acute knockout protocol of Nr2f6 in primary mouse T cells.

Results: Analyzing these Nr2f6 CRISPR/Cas9 knockout T cells, we reproducibly observed a hyper-reactive effector phenotype upon CD3/CD28 stimulation in vitro, highly reminiscent to Nr2f6−/− T cells. Importantly, CRISPR/Cas9-mediated Nr2f6 ablation prior to adoptive cell therapy (ACT) of autologous polyclonal T cells into wild-type tumor-bearing recipient mice in combination with PD-L1 or CTLA-4 tumor immune checkpoint blockade significantly delayed MC38 tumor progression and induced superior survival, thus further validating a T cell-inhibitory function of NR2F6 during tumor progression.

Conclusions: These findings indicate that Nr2f6 CRISPR/Cas9 knockout T cells are comparable to germline Nr2f6−/− T cells, a result providing an independent confirmation of the immune checkpoint function of lymphatic NR2F6. Taken together, CRISPR/Cas9-mediated acute Nr2f6 gene ablation in primary mouse T cells prior to ACT appeared feasible for potentiating established PD-L1 and CTLA-4 blockade therapies, thereby pioneering NR2F6 inhibition as a sensitizing target for augmented tumor regression.

Keywords: Immune checkpoint NR2F6; transcriptional repressor of CD3+ effector T cell functions, CRISPR/Cas9 genetically modified cell therapy, Combinatorial treatment regimens

Background

Solid tumors are infiltrated by effector T cells with the potential to control or reject them; however, the tumor immune microenvironment (TIME) has the ability to restrict the function of these cells at the tumor site and thereby promote tumor growth. An understanding of the interaction between T cells and tumor cells can help unleash the therapeutic anti-tumor activity of effector T cells [1–10]. This concept has led to the successful development of checkpoint blockade immunotherapy targeting either CTLA-4 or PD-1/PD-L1 interaction. These immune checkpoint blockade therapies have proven to be effective in treating several malignancies, including non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), melanoma, colorectal cancer, head and neck cancer, liver cancer, bladder cancer and Hodgkin’s lymphoma [11–19]. The percentage of responders, however, even though encouraging, is limited, thus highlighting the need for innovative sensitizer approaches mediating improved tumor regression in the clinic.

Particularly, weak tumor-associated inflammatory responses and clinical T-cell dysfunction in inflamed tumors, the latter as a consequence of cancer-mediated
immune evasion, remain major hurdles to broader effectiveness of cancer immunotherapy.

Clinical trials suggest that targeting multiple immunosuppressive pathways may better antagonize such tumor immune-therapy resistance and significantly improve patient survival. Therefore, on-going clinical approaches are combining several strategies [20, 21]. Among others, adoptive T cell transfer (ACT) with genetic modifications represents a particularly attractive personalized therapy regimen [22–24], that, in combination with antibody blockade therapy, is likely to achieve more effective remission with long-term tumor control.

Our group identified the orphan nuclear receptor NR2F6 (nuclear receptor subfamily 2, group F, member 6; alias Ear2 and COUP-TFIII) as an intracellular immune checkpoint candidate fine-tuning adaptive immunity [25–30]. Nr2f6-deficient mice show an increased tendency for experimentally-induced neuroinflammation [25, 26] as well as improved intratumoral effector T-cell response resulting in strongly decelerated tumor growth in different spontaneous as well as transplantable mouse tumor models [29, 30]. Mechanistically, lymphatic NR2F6 acts as a negative-regulatory signaling intermediate downstream of the antigen receptor and sets the threshold of TCR/CD28 activation-induced effector functions by acting as a transcriptional repressor that directly antagonizes the DNA accessibility of activation-induced NFAT/AP-1 transcription factors at cytokine gene loci such as Il2 and Ifng [29, 30].

Particularly, in light of an advantageous phenotypical effect of a combinatorial PD-L1/NR2F6 inhibition [30], we here explore the concomitant inhibition of these distinct immune checkpoints in the murine MC38 cancer model. In the present work, we have employed ex vivo CRISPR/Cas9-mediated gene ablation of Nr2f6 prior to therapeutic adoptive transfer, in order to determine whether acute inhibition of NR2F6 gene function indeed enables improved therapeutic anti-cancer activity by the approved PD-L1 or CTLA-4 immune checkpoint therapy in vivo and thus could be a useful dual strategy to elicit meaningful and host-protective tumor immunity.

**Methods**

**Mice**

Nr2f6-deficient mice [29–31] back-crossed eight times on C57BL/6 background were used. The Cas9 transgenic mice were purchased from the Jackson Laboratory, stock no. 028555). Mice were maintained under SPF conditions. All animal experiments were performed in accordance with national and European guidelines and reviewed and authorized by the committee on animal experiments (BMWFUW-66.011/0064-WF/V3b/2016). Investigations were strictly gender-stratified and not blinded. Experimental mice were randomly chosen from litters with a minimal sample size of three.

**Ex vivo T cell analysis**

CD3⁺ or CD4⁺ T cells were isolated using either the mouse CD3 or CD4 T Cell Isolation Kit II (Miltenyi Biotec). CD3⁺ or CD4⁺ T cells were activated in complete RPMI medium in the presence of plate-bound mouse 2C11 (αCD3, 5 μg/ml, BioXcell, BE0001–1) and soluble mouse αCD28 (1 μg/ml, BioXcell, BE0015–1). Cells were harvested at the indicated time points.

**T-cell activation and electroporation**

CD3⁺ or CD4⁺ T cells from Cas9 transgenic mice were isolated as described above. The procedure of T cell activation, transduction, and analysis was always the same as outlined in Fig. 2a. Isolated T cells were activated with 5 μg/ml 2C11 and 1 μg/ml αCD28 for 2 days. On day 2, cells were electroporated. The optimized electroporation condition for mouse T cells was Amxax program X01 with the Amaxa Mouse T Cell Nucleofector® kit t from Lonza (VPA-1006). After electroporation, cells were rested overnight in nucleofector media supplemented with 20 ng/ml hIL-2. Next day cells were spread on an anti-CD3 coated 96-well plate with addition of hIL-2.

The CD3 stimulus on day 6 and further cultivated in RPMI solely with hIL-2 until FACS analysis and gDNA isolation was performed.

**sgRNA application**

crRNAs for each target gene were purchased from Dharmacon. crRNA target sequences are listed in Table 2. To prepare the crRNAs, they were dissolved in 1x siRNA buffer (100 μM) from Dharmacon and mixed in a 1:1 ratio with tracrRNA to increase stability (crRNA:tracrRNA constructs represent the term sgRNA). The mix was denatured at 95 °C for 5 min and cooled down to anneal at room temperature before stocks were frozen. After annealing, 1 μg of sgRNAs, alone or as a pool of up to five, were electroporated on day 2 after T-cell activation of isolated Cas9 transgenic CD3⁺ or CD4⁺ T cells as described above.

**T7 cleavage assay**

Electroporated T cells were harvested at indicated time points, and pellets snap frozen at −80 °C. gDNA was extracted using the PureLink® Genomic DNA Mini Kit (10053293). The T7 cleavage assay was performed as follows: briefly, targeted regions of CD44 or NR2F6 were PCR-amplified from genomic DNA. The PCR product was denatured and re-anneled in NEBuffer (NEB) using a thermocycler. Hybridized PCR products were digested with T7 Endonuclease I (NEB, M0302S) for 15 min and
separated by a 1.5% agarose gel. Primers for PCR are listed in Table 1.

Takara kit
The efficiency of sgRNA cleavage was tested on gDNA from wild-type thymocytes with the Guide-it™ sgRNA Screening Kit from Takara (632639). As for the T7 cleavage assay, targeted regions of CD44 or NR2F6 were PCR amplified. The recombinant Cas9 nuclease (500 ng/μl) was added together with sgRNA (50 ng/μl). The cleavage reaction was accomplished following the manufacturer's instructions and analyzed 1.5% agarose gel.

Tumor induction
5×10⁵ B16-OVA, 5×10⁵ MC38 tumor cells (kindly provided by Maximilian Waldner, University of Erlangen, Germany) were injected s.c. into the left flank of 8- to 12-week-old wild-type, or Nr2f6⁻/⁻ mice. The high tumor load was applied to ensure robust tumor growth along with therapy (anti-PD-L1, anti-CTLA-4). Tumor growth was monitored three times a week by measuring tumor length and width. Tumor volume was calculated according to the following equation: ½(length × width²). For survival analysis, mice with tumors greater than the length limit of 15 mm were sacrificed and counted as dead. Cell lines were tested negative for mycoplasma (GATC, Konstanz, Germany).

| Table 1 | Sequences of primers for PCR amplification of targeting sites |
|--------|---------------------------------------------------------------|
| Primer | Primer Sequences (5' to 3') | Amplicon (bp) |
| CD44.03.F | TGCCTCTGCTCCGTCTTCATC | 561 |
| CD44.03.R | CCTGTGCACTCCTCATCTCC | 545 |
| CD44.01.02.F | GAAGTTGCTGGGGTGGGAAT | 513 |
| CD44.01.02.R | ACACGGATGGGTTTGCAAAA | 555 |
| CD69.01.F | CCCCTCCCAGTGTTTTCTGT | 934 |
| CD69.01.R | AGTGCCTGATGAGCTCTCA | 580 |
| CD69.02–04.F | TGTTCATGCCCCATCCTCG | 745 |
| CD69.02–04.R | CTGATGGTATACCCATG | 764 |
| Nr2f6.01.05.F | CTCTATCGCCTGGACCTCTG | 505 |
| Nr2f6.01.05.R | AGGAAGTTGCCGCAAAACT | 505 |
| Nr2f6.02.F | AGGCCAGAGACAGAAGCTGA | 934 |
| Nr2f6.02.R | AGGACAGAGACAGAAGCTGA | 580 |

In vivo antibody blockade
Mice were injected s.c. with 5×10⁵ B16-OVA melanoma cells or 5×10⁵ MC38 tumor cells and administered either 0.5 mg (B16-OVA) or 0.25 mg (MC38) of anti-mouse PD-L1 (Clone10F.9G2; BE0101), anti-mouse CTLA4 (Clone 9H10, BE0131), corresponding IgG2b (LTF-2; BE0090) or polyclonal Syrian hamster IgG (BE0087) control (all from BioXCell, USA) every 3 days starting from day 3 of tumor challenge according to ref. [30, 32].

CRISPR/Cas9-mediated Nr2f6 knockout and adoptive cell transfer
5×10⁵ MC38 tumor cells were injected s.c. into C57BL/6 wild-type recipients. Two adoptive cell transfers (ACT) of sgRNA.NTC or sgRNA.Nr2f6.04 electroporated CD3+ T cells from Cas9 transgenic mice into wild-type mice were carried out three and 10 days after tumor induction by injecting intra-peritoneally 1×10⁷ MACS sorted CD3+ T cells (viability > 95%) using the Pan T Cell Isolation Kit II mouse (Miltenyi Biotech 130–095-130). Antibody treatment with 0.25 mg anti-mouse PD-L1 (Clone10F.9G2; BE0101) or anti-mouse CTLA-4 (Clone 9H10, BE0131) with corresponding control antibodies as described above was administered i.p. on day 3, 5, 7, 10, 12 and 14. Tumor growth was subsequently measured as described above.

Western blotting
Cells were washed and lysed in lysis buffer. Whole-cell extracts were electrophoresed on NuPAGE gels (Invitrogen) and transferred to PVDF membranes. Protein lysates were subjected to immunoblotting with antibodies against aFlag (Sigma, F1804-200UG, 1:1000), and Actin (Santa Cruz Biotechnology Inc., USA: sc-1615, 1:1000).

Flow Cytometry
Splenocytes or bone marrow cells were depleted of erythrocytes using an erythrocyte lysing buffer and, like lymph node cells or thymocytes, mashed through a 100-μm filter. Splenocytes, thymocytes, lymph node, and bone marrow cells were incubated with FcR Block (BD Biosciences, 553,142) to prevent nonspecific antibody binding before staining with appropriate surface antibodies for 30 min at 4 °C, washed with PBS+ 2% FCS, and used for FACS analysis. For intracellular cytokine staining, cells were stimulated with 50 ng/ml phorbol 12, 13-dibutyrate (PDBu, Sigma, P1269), 500 ng ionomycin (Sigma, I0634) and GolgiPlug (BD Biosciences, 555,029) for 4–5 h. After fixation (cytokines: Biolegend fixation buffer (420801), 20 min, 4 °C; transcription factors: eBioscience FoxP3 staining buffer set (Invitrogen, 00–5523-00), > 30 min, 4 °C), cells were permeabilized with the fixation/permeabilization kit (BioLegend, 421,002).
for cytokines and the eBioscience Foxp3-staining buffer set (Invitrogen, 00–5523-00) for transcription factors, incubated with FcR Block (BD Biosciences, 553,142) before staining with specific cell surface or intracellular marker antibodies. Data were acquired on a FACS Calibur, or FACS Canto cell analyzer (Becton Dickinson). Data were analyzed using FlowJo software (version 10). The following antibodies were used for flow cytometry: CD4–V500 (BD, 560783), CD4–PE (BD, 553049), CD8a–APC (BD, 553035), CD25–PE (BD, 553866), CD44–PE–Cy7 (BioLegend, 103,030), CD62L–APC (BD, 553152), IL-2–APC (BD, 554429), CD8a–PE (eBiosciences, 12–0081-82), IFNγ–PE–Cy7 (eBiosciences, 25–7311-82), CD45–APC (eBiosciences, 17–0451-81), CD3–PE (eBiosciences, 12–0031-83), CD8a–bv421 (BioLegend, 100,738), CD25–bv421 (BioLegend, 102,034), CD69–APC (Ebiosciences 17–0691-80), CD11b–PE (eBiosciences, 12–0112-83), Gr1–APC (BioLegend, 108,412), CD19–PE (BD, 557399).

### Statistics
Data were analyzed using Prism 5.03 software (GraphPad Software). Experiments were repeated at least two times with a minimum sample size (n) of three. Data are represented as indicated (either the mean ± SEM or ± SD) for all figures in which error bars are shown. Overall survival was expressed using the Kaplan–Meier method, and differences between groups were determined using the log-rank test. The p-values were assessed using two-tailed unpaired Student’s t-test, or two-way ANOVA. A p value of < 0.05 was considered statistically significant. *p < 0.05; **p < 0.01; ***p < 0.001.

### Results
**Efficient CRISPR/Cas9-mediated mutagenesis in primary mouse T cells**

The CRISPR/Cas9 technology has opened new avenues to physiologically validate alternative and potentially additive and/or synergistic immune checkpoint candidates in preclinical mouse models systems of cancer immunotherapy. In order to avoid the requirement of having to deliver Cas9 to primary mouse T cells, we used the previously established Cas9 transgenic Rosa26-Cas9 mouse line B6(C)-Gt(Rosa26Sor™/CAG-ca

1b). A T7 mismatch detection assay has been typically used for the detection and quantification of insertions and deletions (indel) mutations created by the CRISPR/Cas9 system in intact cells [34]. In brief, genomic DNA was isolated from cells 5 days after sgRNA transfection and screened for the presence of site-specific gene modification by PCR amplification of regions surrounding the target sites followed by the T7 cleavage assay. As shown in Fig. 2a, transfection of CD3+ T cells from Cas9 transgenic mice with synthetic sgRNA.Nr2f6.04 resulted in detectable indels at the chosen target site as observed by the cleavage bands. This result validated the gene-specific cleavage mediated by CRISPR/Cas9 in primary mouse T cells derived from Cas9 transgenic mice and demonstrated that the selected sgRNAs worked effectively in intact cells.

For subsequent experiments, we used this validated sgRNA.Nr2f6.04, which is highly sequence specific within the whole mouse genome as established encoding a perfect match only for the Nr2f6 gene. To establish the robust efficiency of mouse genome editing using synthetic sgRNA, selected surface marker genes such as CD44 and CD69 have been tested as positive controls in primary mouse CD3+ T cells, as they are easily detectable by flow cytometry. Employing this method (Fig. 2a), we reproducibly achieved high efficiencies for bi-allelic gene ablation in primary CD3+ T cells derived from the Cas9 transgenic mouse line between 50 and 90% of surface receptors CD44 and CD69 used as convenient positive controls, respectively (sgRNA target sequences in Table 2).

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Transfected T cells were subsequently stimulated, as shown in Fig. 2a and described below: We started with a pool of up to five different crRNAs per gene, these crRNAs were coupled to a tracrRNA to allow guidance and stability and subsequently designated as sgRNAs (crRNA:tracrRNA). We then isolated CD4⁺ T cells from Cas9 transgenic mice and activated them for 2 days with CD3/CD28 crosslinking. On day 2, we performed the electroporation with the sgRNAs, following continued stimulation with αCD3 and IL-2 for three more days. Three days after transfection, we switched to an IL-2 only culture and started FACS analysis of surface marker knock-out (Fig. 2b,c) and gDNA extraction for the T7 cleavage assay. For sgRNAs against CD44, a high bi-allelic knockout efficiency of about 70% was reproducibly achieved (Fig. 2b,c). Similar targeting of CD69 resulted in about 50% of the T cells showing bi-allelic knockout (Fig. 2b,c); however, the viability of CD69 CRISPR/Cas9 knockout T cells was reproducibly impaired (data not shown).

This elaborated methodology enabled us to establish acute gene knockouts upon transfection with the given sgRNAs. For the NR2F6 protein, no direct knockout efficiency testing is feasible due to a lack of high-affinity anti-NR2F6 antibodies and low protein expression levels in primary T cells. Instead, we analyzed cytokine production responses from Nr2f6CRISPR/Cas9 knockout as an established surrogate marker of NR2F6 function (see [25, 26, 29, 30]) and - reminiscent of germine Nlr2f6⁻/⁻ T cells - reproducibly observed strongly increased cytokine activation response levels for IL-2 and IFNγ (Fig. 2d, e, and data not shown). This indicates that the CRISPR/Cas9-mediated acute Nr2f6 gene ablation is effective. Consistent with this observation, acute Nr2f6 gene editing in mouse T cells reduced the thresholds of

| Gene | Target Sequence | Order # |
|------|----------------|---------|
| Cd44.01 | TATGTAACCGGTCCATCGA | CR-041132-01-0020 |
| Cd44.02 | GTTGGCTGACAGATAGCCG | CR-041132-02-0020 |
| Cd44.03 | TCTGATCCGGTGCTAAG | CR-041132-03-0020 |
| Cd44.04 | CATGGAATACACCTGCGTAG | CR-041132-04-0020 |
| Cd44.05 | ATGTATCCTCGTCATCTGGA | CM-057343-01-0020 |
| Cd44.06 | CATCTCGAGTCGCAAAA | CM-057343-02-0020 |
| Cd44.07 | CATCTCCACCAACAAAGG | CM-057343-03-0020 |
| Cd44.08 | GCAATTGTACTTGCCCGG | CM-057343-04-0020 |
| Cd44.09 | CTCCGATGGGATGTCCG | CM-057343-05-0020 |
| Cd44.10 | AATGGTCGGGCTGCGGTC | CM-045088-01-0020 |
| Cd44.11 | GCCTCTCGAGTCGCG | CM-045088-02-0020 |
| Cd44.12 | TCCCGGATGAGGGTCTCGAT | CM-045088-03-0020 |
| Cd44.13 | CTCAAGAAGTGCTTCCGGG | CM-045088-04-0020 |
| Cd44.14 | AGGTTAGCTGAGATTCG | CM-045088-05-0020 |
antigen receptor signaling and led to hyper Responsiveness of T cells in vitro.

**Germline Nr2f6 deletion in T cells synergizes with CTLA-4 blockade therapy**

In previous studies [28–30] we showed a T cell-intrinsic tumor rejection phenotype by the hyper-reactive effector T cell compartment of whole body Nr2f6−/− mice using, among others, the transplantable B16-OVA subcutaneous mouse tumor model. Especially in combination with the established immune checkpoint therapy of PD-L1 blockade, we observed a host-protective tumor immunity effect in Nr2f6-deficient mice [30]. Additionally, as a therapeutic approach, we previously have demonstrated that adoptively transferred Nr2f6 siRNA-silenced polyclonal CD3+ T cells act as an adjuvant for the established αPD-L1 immune checkpoint in the mouse B16-OVA tumor model [30]. To strengthen our hypothesis of synergy between immune checkpoint therapy and Nr2f6 inhibition as intracellular immune checkpoint target, we next tested the αCTLA-4 treatment in wild-type and Nr2f6−/− mice injected with B16-OVA cells showing superior tumor rejection and strongly enhanced survival in the double treatment group (Fig. 3a-d). Of note, 60% long-term survivors were seen in the αCTLA-4 treatment group of Nr2f6−/− mice (as a combinatorial cancer treatment setting) when compared with 25% in the wild-type monotherapy group (Fig. 3c).
Acute Nr2f6 inhibition via CRISPR/Cas9-mediated mutagenesis in T cells is sufficient for priming superior tumor immunity upon CTLA-4 and PD-L1 blockade

With the ultimate goal of developing an innovative immunotherapy-based combinatorial approach, we aimed to confirm our hypothesis that NR2F6 inhibition could vastly enhance T cell effector responses specific for tumor antigens as well as confer protection from the immunosuppressive TIME in a relevant murine tumor model system. Particularly because there is a significant correlation of lymphatic PD-1 or CTLA-4 with lymphatic NR2F6 expression in human NSCLC patients [30], we wanted to determine whether acute Nr2f6 inactivation enables anti-cancer activity in vivo. Pursuing this concept, and mirroring in principle a pharmacological treatment, therapeutic adoptive transfer (ACT) of acute Nr2f6 gene-edited autologous CD3+ T cells into tumor-bearing mice in a combinatorial CTLA-4 or PD-L1 blockade setting was carried out (Fig. 4a). Fully immunocompetent wild-type mice received a high dose of MC38
Fig. 4 (See legend on next page.)
knockout CD3+ T cells act as a robust system into primary mouse T cells from Cas9 transgenic in combination with established checkpoint blockade. Similarly elicits superior anti-cancer immune responses evidence that acute manipulation of lymphatic NR2F6 mary T cells, we here provide strong pre-clinical evi-

CRISPR/Cas9-mediated mutagenesis technology in pri-

ally relevant cancer models as well as in T cell biology has been firmly established [29, 30, 36–38]. Employing CRISPR/Cas9-mediated mutagenesis technology in primary T cells, we here provide strong pre-clinical evidence that acute manipulation of lymphatic NR2F6 similarly elicits superior anti-cancer immune responses in combination with established checkpoint blockade. Employing a robust sgRNA transfection-based knockout system into primary mouse T cells from Cas9 transgenic mice, efficient CRISPR-mediated Nr2f6 gene editing for cancer immunotherapeutic purpose has been established. It has previously been shown that Nr2f6−/− T cells are hyper-reactive in regard of cytokine production (IL-2, IFNγ, TNFα) as those cytokines are direct target genes of NR2F6-dependent transcriptional repression [25, 26, 28–30] leading to an improved anti-tumor immune contexture at the tumor site [29, 30]. Consistent with this working hypothesis, the phenotype of Nr2f6CRISPR/Cas9 knockout T cells - as an acute genetic loss-of-function approach employed in this study – similarly induced hyper-responsiveness, thereby resembling the germline Nr2f6 knockout immune phenotype in the effector T cell compartment. Of note, siRNA-mediated NR2F6 silencing in human T cells similarly reduced the thresholds of antigen receptor signaling and induced hyper-responsiveness in polyclonal T cells ([30] and Fig. 2d, e).

Tumor cells upregulate the expression of PD-L1, which indicates the induction of adaptive resistance. Our previous data even indicated that PD-1 is strongly upregu-

lated in Nr2f6-deficient T cells and that a combinatorial blockade of both the PD-L1/PD-1 and the NR2F6 pathways is effective in delaying tumor growth and improving long-term survival with complete tumor regression [30]. Next, in order to validate our working hypothesis that NR2F6 inhibition could vastly enhance T-cell effector responses specific for tumor antigens in vivo as well as confer protection from the immunosuppressive TIME in a relevant preclinical murine tumor model system, acute Nr2f6 gene editing in combination with the established immune checkpoint blockade was performed. In the present study, we investigated whether blockade of PD-L1 together with acute CRISPR/Cas9-mediated Nr2f6 deletion can cause rejection of tumors that otherwise do not respond to anti-PD-L1 monother-

apy. This was also investigated with the combination of CTLA-4 blockade/acute CRISPR/Cas9-mediated Nr2f6 deletion. Remarkably, we found that blockade of either PD-L1 or CTLA-4 upon ACT of Nr2f6CRISPR/Cas9 knockout CD3+ T cells decelerated tumor growth. Strikingly, we discovered that complete regression of established tumors could be achieved in 37.5% of mice using tumor cells and were treated with therapeutic ACT twice on days 3 and 10 using CD3+ T cells from Cas9 transgenic mice transfected with control sgRNA (sg. RNA.NTC) or sgRNA.Nr2f6.04, in combination with either αPD-L1 (Fig. 4b–e) or αCTLA-4 (Fig. 4f–i) checkpoint blockade, respectively. Therapeutic adoptive transfer of Nr2f6CRISPR/Cas9 knockout polyclonal CD3+ T cells was sufficient for causing a significant delay in tumor growth in this dual treatment setting when compared to mice receiving CD3+CRISPR.NTC control cells (Fig. 4b–f). As a remarkable result, 66.67% or 8 out of 12 mice receiving CD3+CRISPR.Nr2f6−/− with αCTLA-4 (Fig. 4h, i) and 37.5% (3/ 8) with αPD-L1 (Fig. 4d, e) therapy survived the tumor burden. With αPD-L1 treatment alone, none of the control mice survived (Fig. 4c, e), whereas one-third or 4 out of 12 of mice receiving control ACTs treated with αCTLA-4 (Fig. 4g, i) survived the tumor challenge. Thus, these data provide an independent confirmation of the critical NR2F6 function in T cell-mediated cancer immunity, strongly suggesting that in combination with either of the approved PD-L1 and CTLA-4-targeted immune checkpoint therapies, T cell-based ACT therapies have increased efficacy from modulation of the NR2F6 inhibitory signaling pathway.

Taken together, adoptively transferred Nr2f6−/− CRISPR/Cas9 knockout CD3+ T cells act as a robust “sensitizer” for both the established αPD-L1 and αCTLA-4 immune checkpoint blockade in the mouse MC38 tumor model, significantly improving immune-activating cancer therapy outcomes.

Discussion

The physiological relevance of NR2F6 function in clinically relevant cancer models as well as in T cell biology has been firmly established [29, 30, 36–38]. Employing CRISPR/Cas9-mediated mutagenesis technology in primary T cells, we here provide strong pre-clinical evidence that acute manipulation of lymphatic NR2F6 similarly elicits superior anti-cancer immune responses in combination with established checkpoint blockade. Employing a robust sgRNA transfection-based knockout system into primary mouse T cells from Cas9 transgenic
combined aPD-L1 therapy and in 66.67% of mice with combined aCTLA-4 therapy (Fig. 4e, i). Thus, combining adoptive transfer of Nr2f6 CRISPR/Cas9 genetically modified T cells showed synergistic effects with both the established PD-L1 and the CTLA-4 checkpoint blockade to promote tumor regression and increase survival in a subcutaneous tumor mouse model.

Taken together, this data suggest that disruption of lymphatic Nr2f6 converts tumor-infiltrating T cells into IFNγ- and IL-2-hypersecreting effector cells, apparently sufficient to prime the TIME for immune checkpoint therapy to more effectively control tumor growth. NR2F6 has been defined as a negative master switch of therapy to more effectively control tumor growth. During adoptive transfer of Nr2f6 CRISPR/Cas9 genetically modified T cells, the unique feature of lymphatic NR2F6 as an alternative intracellular immune checkpoint may influence combinatorial cancer therapies in the future.

**Conclusion**

In summary, these findings are in line with our previous data from germ-line knockout studies and indicate that the Nr2f6/Crispr/cas9 T cells are comparable to germline Nr2f6-deficient T cells, a result providing an independent confirmation of the cancer immune checkpoint function of lymphatic NR2F6. As a pre-clinical proof of concept, this establishes NR2F6 as a promising cancer therapeutic candidate target and NR2F6 inhibition as a sensitizing concept for next-generation immune-oncology regimens. From a clinical perspective, if valid, such combinatorial immunotherapies including NR2F6 gene-edited ACTs are likely to strengthen the portfolio of precision medicine applications for the successful development of personalized cancer immune therapy for improving patient survival.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12964-019-0454-z.

**Additional file 1: Figure S1** Characterization of Cas9 transgenic mice. (A) FACS analysis of GFP expression in CD4+ T cells, CD19+ B cells, Gr1+ granulocytes, and CD11b+ monocytes derived from the Cas9 transgenic mouse on a B6 background [33]. Cells were isolated from LN (first column), spleen (middle) or thymus/BM (third column). Cells from wildtype C57Bl/6 mice (grey) and Cas9 transgenic mice (pink) are shown in the same FACS plots. (B) Western blot analysis of lysates prepared from isolated CD3+ T cells of wildtype or Cas9 transgenic mice using a Flag antibody. (C) Representative histogram of GFP expression in wildtype, single transgenic Cas9, or double transgenic Cas9 mice.

**Additional file 2: Figure S2** Comparison of Cas9 and wildtype mice in regard of immune cell subsets. Percentages of the indicated immune cell populations within all cells in the lymph node (A), the bone marrow (B), the spleen (C), and the thymus (D) of wildtype (black) or Cas9 transgenic mice (pink). Each mouse is represented by one dot. Results shown are derived from two independent experiments. (A-D) Results reach no statistical significance.

**Abbreviations**

ACT: Adoptive cell transfer; BM: Bone marrow; bp: base pairs; Cas9: CRISPR associated protein 9; CD: Cluster of differentiation; CRISPR: Clustered regularly interspaced, short palindromic repeats; crRNA: CRISPR RNA; CTLA-4: Cytotoxic T lymphocyte associated antigen 4; d: day; FACS: Fluorescence-activated cell sorting; gDNA: genomic DNA; GFP: Green fluorescent protein; IFNγ: Interferon gamma; IL: Interleukin; KO: Knock-out; LN: Lymph node; NR2F6: Nuclear receptor subfamily 2 group F member 6; NTC: Non-targeting control; PAM: Protoscaler adjacent motif; PCR: Polymerase chain reaction; PD-1: Programmed cell death 1; PD-
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