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DAP12-Based Activating Chimeric Antigen Receptor for NK Cell Tumor Immunotherapy

Katrin Töpfer,* Marc Cartellieri, † Susanne Michen,* Ralf Wiedemuth,* Nadja Müller,* Dirk Lindemann, ‡ Michael Bachmann, ‡ Monika Füssel, ‡ Gabriele Schackert,* and Achim Temme*,#

NK cells are emerging as new effectors for immunotherapy of cancer. In particular, the genetic engraftment of chimeric Ag receptors (CARs) in NK cells is a promising strategy to redirect NK cells to otherwise NK cell–resistant tumor cells. On the basis of DNAx-activation protein 12 (DAP12), a signaling adaptor molecule involved in signal transduction of activating NK cell receptors, we generated a new type of CAR targeting the prostate stem cell Ag (PSCA). We demonstrate in this article that this CAR, designated anti–PSCA-DAP12, consisting of DAP12 fused to the anti-PSCA single-chain Ab fragment scFv(AM1) confers improved cytotoxicity to the NK cell line YTS against PSCA-positive tumor cells when compared with a CAR containing the CD3ζ signaling chain. Further analyses revealed phosphorylation of the DAP12-associated ZAP-70 kinase and IFN-γ release of CAR-engineered cells after contact with PSCA-positive target cells. YTS cells modified with DAP12 alone or with a CAR bearing a phosphorylation-defective ITAM were not activated. Notably, infused YTS cells armed with anti–PSCA-DAP12 caused delayed tumor xenograft growth and resulted in complete tumor eradication in a significant fraction of treated mice. The feasibility of the DAP12-based CAR was further tested in human primary NK cells and confers specific cytotoxicity against KIR/HLA-matched PSCA-positive tumor cells, which was further enhanced by KIR-HLA mismatches. We conclude that NK cells engineered with DAP12-based CARs are a promising tool for adoptive tumor immunotherapy.

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binding of the low-affinity FcγRIII (CD16) to IgG-opsonized targets, thereby inducing Ab-dependent cellular cytotoxicity. Likewise, the activation of CD3ζ-based CARs led to an Ab-dependent cellular cytotoxicity–like activity of CAR-modified NK cells when engaging target cells (21, 23). To establish a CAR that is not involved in CD3ζ-signaling and that provides an alternative route to activate NK cells, we focused on DNAX-activation protein 12 (DAP12) as a signaling domain. Because DAP12 contains only one ITAM, it was also of special interest, whether a DAP12 domain can provide sufficient signaling to induce NK cell activation, compared with a CAR containing the CD3ζ-chain with three ITAMs. In the immune system, DAP12 is found in cells of the myeloid lineage, such as macrophages and granulocytes, where it associates, for instance, with the triggering receptor expressed on myeloid cell members (TREM) and MDL1 (myeloid DAP12-associated lectin 1/CLEC5A), both involved in inflammatory responses against pathogens like viruses and bacteria (for review, see Ref. 24) (25).

In the lymphoid lineage, DAP12 is expressed in NK cells and associates with activating receptors such as the C-type lectin receptor NKG2C (26), the natural cytotoxicity receptor Nkp44 (27), and the short-tailed KIR3DS1 (28) and KIR2DS1/2/5, respectively (29–31). In particular, NKG2C is the dominant activating NK cell receptor for controlling CMV infection in both humans and mice (32–34). Therefore, we hypothesized that in NK cells a DAP12-containing CAR should generate sufficient activating signals upon cross-linking with its Ag.

In this study, we generated a DAP12-based CAR, designated anti–prostate stem cell Ag (PSCA)–DAP12, for redirecting NK cells toward PSCA-positive tumor cells. As a PSCA-binding moiety, we used our recently described single-chain fragment variable scFv(AM1) (35) derived from the hybridoma 7F5 (35). PSCA represents a prostate- and prostate cancer–associated GPI-anchored cell surface Ag (36–39) that is predominantly expressed in normal prostate-specific tissue and overexpressed in prostate cancer specimens, including high-grade prostatic intraepithelial neoplasia and androgen-dependent/-independent tumors (39). Recently, PSCA was also found to be expressed in prostate cancer metastases (40) and prostate-unrelated cancers such as renal clear cell carcinoma (41), pancreatic adenocarcinoma (42), and glioblastoma (43). PSCA has been successfully used as target molecule for various immunotherapeutic approaches (37, 38, 44–46). In this article we show that the DAP12-based CAR led to ZAP-70 phosphorylation upon cross-linking with its Ag and therefore efficiently reprogrammed the cytotoxicity of YTS NK cells toward otherwise resistant PSCA-positive tumor cells. Interestingly, YTS-NK cells modified with the DAP12-based CAR showed a gradually improved specific cytotoxicity when compared with NK cells expressing a CD3ζ-based CAR. Furthermore, we demonstrate also that human primary NK cells can efficiently be modified with a anti–PSCA-DAP12 CAR and therefore acquire specific cytotoxicity against KIR/HLA-matched PSCA-positive tumor cell lines in vitro. In conclusion, our results suggest that DAP12-based CARs are suitable for the development of a NK cell–based immunotherapy for solid tumors.

Materials and Methods

Cells and generation of PSCA* target cell lines

The human embryonic kidney cell line 293T, the prostate cancer cell line PC3, and the glioma cell line H4 were engineered to express PSCA by lentiviral gene transfer. The resulting cell lines were designated 293TPSCA, PC3PSCA, and H4PSCA. The 293T cells were maintained in DMEM (PAA, 10 mM HEPES (PAA), 100 U ml⁻¹ penicillin, and 10 mg ml⁻¹ streptomycin (PAA)). H4 cells were cultured in BME Life Technologies (Life Technologies, Darmstadt, Germany) with 10 mM HEPES (PAA), 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin (PAA), and 1× MEM NEAA (nonessential amino acids; PAA). The PSCA-positive bladder carcinoma cell lines RT4 and HT1376 were grown in DMEM (PAA) supplemented with 10% v/v heat-inactivated FBS (PAA), 2 mM l-glutamine (Biochrom, Berlin, Germany), 10 mM HEPES (PAA), 100 U ml⁻¹ penicillin, 1 mg ml⁻¹ streptomycin (PAA), and 1× MEM NEAA (nonessential amino acids; PAA). The resulting cell lines were subjected to selection with 37°C and 5% CO₂ in a humidified incubator.

Virus production and transduction of NK cells

Lentiviral particles for transduction of YTS cells and primary NK cells were produced by a transient three-vector packaging protocol (50). Briefly, 4 × 10⁶ 293T cells were transfected using polyethylenimine (Polysciences, Warringtom, PA), 5 µg pCDN/BL-BH (50), pczSVS-G (51), and lentiviral vector, respectively. After 20 h, 293T cells were incubated with 10 mM sodium butyrate (Sigma-Aldrich, Taufkirchen, Germany) for 6 h. At 24 h after the replacement of the sodium butyrate by fresh medium, the supernatant was removed from cells and passed through a 0.45-µm filter, mixed with 8 µg ml⁻¹ Polybrene (Sigma-Aldrich) and used to transduce NK cells. To enhance the expression of transgenes, YTS cells were selected in 5 mg ml⁻¹ Zeocin (Invitrogen, Karlsruhe, Germany) for 1 wk. The use of human NK cells was approved by the local ethical committee (#EK242102007) of the Medical Faculty Carl Gustav Carus, Technische Universität Dresden. Human PBMCs were isolated from buffy coats of the Medical Faculty Carl Gustav Carus, Technische Universität Dresden. Human PBMCs were isolated from buffy coats of the Medical Faculty Carl Gustav Carus, Technische Universität Dresden.
negative NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), we isolated NK cells from human PBMCs. Staining with anti-CD3 and anti-CD56 Abs routinely confirmed >90% purity of CD56+ and depletion of CD3+ cells. The NK cells were cultured overnight in complete CellGro medium (CellGenix) supplemented with 500 U/ml IL-2, 200 U/ml IL-15, and 10 ng/ml TNF-α, washed twice with PBS, and plated in a round-bottom 96-well plate (5 × 10^3 cells per well). CAR-modified YTS cells and NK cells, as well as controls, were added to labeled target cells at various target to effector ratios. After 18 h, 25 μl cell supernatant was mixed with 150 μl of scintillation solution OptiPhase SuperMix (Wallac Scintillation Products, Turku, Finland) in a 96-well plate by shaking for 3–5 min at room temperature. The chromium release was measured using a Wallac 1450 Microbeta Trilux Liquid Scintillation and Luminescence Counter (PerkinElmer). Maximal and minimal releases were measured by treating target cells with 5% Triton X-100 (Serva, Heidelberg, Germany) and medium alone, respectively. Incubations of YTS cells and primary human NK cells with isogenic PSCA-negative cells were included for comparison. Percentage of specific lysis was calculated using the standard formula: 100 × (cpm release target cells – cpm minimum release) / (cpm maximum release – cpm minimum release). The experiments were performed three to four times using YTS cells and at least two times using primary NK cells from three donors, each with similar results.

**Experimental immunotherapy of xenografted mice**

NMRI-Foxn1nu/Foxn1nu mice were obtained from the animal facility of the University of Düsseldorf. Mice were housed under standardized pathogen-free conditions with ad libitum access to food and water. Experiments were approved by the Landesdirektion Düsseldorf under the auspices of the German Animal Protection Law. To establish tumors, 100 μl PBS containing 4 × 10^6 293TPSCA cells were s.c. injected into the left flank of female NMRI nu/mice (10 mice per group). At day 4 the tumor reached an average size of ~10 mm^2, and i.v. injections with 5 × 10^6 nonmodified YTS cells, YTSmyc-DAP12 cells, and YTS anti–PSCA-DAP12 cells were started. YTS cells were injected via the tail vein every 48 h or 72 h over a period of 8 wk. As a control for in vivo tumor cell growth, one group was not treated. The tumor experiment was repeated three times with, in total, 25 or 26 mice per group, with similar results. Tumors were measured in two dimensions one or two times per week, using a digital caliper. Once the tumor exceeded 18 mm in any of the three perpendiculars or animals appeared to be in distress, mice were euthanized. The tumor area was calculated according to the formula of ellipse area: 1/4 × π × (a × b). For the analysis of PSCA expression in 293TPSCA xenografts treated with CAR-modified YTS cells, we compared Ag expression levels of tumors in all treatment groups. Therefore, randomly selected mice having tumors exceeding 18 mm were sacrificed, and tumors were excised at days 22, 22, 22, and 24 for non-treated tumors; days 22, 22, 28, and 28 after start of YTS treatment; days 22, 22, 24, and 34 after the start of YTSmyc-DAP12 treatment; and days 31, 42, 45, and 50 after the start of YTS anti–PSCA-DAP12 treatment. The 293TPSCA cells of the excised tumors were prepared using the Nucleic Acid Dissection Kit (Miltenyi Biotec) according to the instructions of the provider and were plated on 10-cm cell culture dishes for 24 h. After extensive washing with PBS, the remaining adherent cells were trypsinized; simultaneously stained for HLA-A, -B, and -C (HLA-ABC, clone W6/32; BD Biosciences) and PSCA or corresponding isotype controls; and analyzed by flow cytometry. In a further experiment, 293TPSCA xenografts were grown to a size of ~20 mm^2, the experimentally two mice per group were i.v. injected with 5 × 10^6 YTS-DAP12 cells or YTS anti–PSCA-DAP12 cells every 24 h, for a total of three times. At 24 h after the last injection, the mice were sacrificed and tumors were removed and transferred into embedding molds containing tissue-freezing medium (Leica, Wetzlar, Germany) and then snap frozen in dry ice. For microscopic examination, 10-μm slices were prepared with a microtome (Jung CM1800; Leica) and counterstained with DAPI. Digital images of tumors were acquired with the fluorescence microscope Axioskop 2 mot plus (Carl Zeiss, Göttingen, Germany; original magnification, ×200) and the AxiosVision software version 8.0 (Carl Zeiss). For quantification of infiltrating EGF-marked YTS cells, 12 randomly selected fields were counted for nuclei and EGF-positive cells, and the percentage of infiltrating YTS cells was calculated.

**Statistical analyses**

The results of IFN-γ and chromium release assays were expressed as mean with SEM and analyzed performing a one-way ANOVA (p < 0.05) combined with Bonferroni’s multiple comparison test (GraphPad Software, San Diego, CA). As a reference, the results were used for comparison of tumor-infiltrating YTS cells. A log-rank test was used for analyses of the survival data. All statistical analyses were performed with Prism software version 6.0 (GraphPad Software, La Jolla, CA).
Results

Generation of YTS cells expressing the CAR anti–PSCA-DAP12

The design of the anti–PSCA-DAP12 chimeric NK cell Ag receptor and lentiviral vector regions is depicted in Fig. 1A. The DAP12-based CAR was generated by fusion of the recently described PSCA-specific single-chain fragment variable scFv(AM1) (35) to the extracellular domain of human DAP12. An internal SFFV U3 promoter allowed transgene expression in combination with expression of an EGFP-ZeoR reporter gene via an internal IRES. Furthermore, we included an extracellular c-myc-tag for detection of CAR surface expression (Fig. 1A). To investigate a nonspecific activation of NK cells owing to an ectopic expression of the signaling adaptor protein DAP12, a control consisting of Igkappa SP fused to c-myc and DAP12 was included and referred to as myc-DAP12. As further control, we constructed a CAR containing a phosphorylation-defective ITAM (anti–PSCA-DAP12mut).

The NK cell line YTS, which is devoid of KIR expression (47, 53), was transduced with anti–PSCA-DAP12 CAR, anti–PSCA-DAP12mut control, and myc-DAP12 control, respectively. After antibiotic selection, the modified YTS cells expressed the CAR or control constructs, as shown by Western blot analyses (Fig. 1B). Protein bands consistent with the estimated molecular mass of ~46 kDa for anti–PSCA-DAP12 CAR and the signaling-deficient anti–PSCA-DAP12mut control, as well as 18 kDa for myc-DAP12 control, were detected using an anti–c-myc Ab (Fig. 1B). Furthermore, a strong surface expression of the CAR and of the control constructs, respectively, was detected in nearly all EGFP-marked YTS cells (Fig. 1C), which remained stable in a continuous culturing period of ≥5 mo (data not shown).

Specific killing of PSCA-positive tumor cell lines of different origin, using YTS cells engineered with anti–PSCA-DAP12 CAR

To investigate cytotoxicity of CAR-modified YTS cells, we used target cell lines with ectopic expression of PSCA but also chose HT1376 and RT4 cells with endogenous PSCA expression for our experiments. Flow cytometry analyses demonstrated robust endogenous expression of PSCA on RT4 and HT1376 bladder carcinoma cells and a high expression on PC3PSCA, H4PSCA, and 293TPSCA cells.
transduced with a vector encoding PSCA. Endogenous PSCA expression was not detectable in PC3, H4, and 293T wt cells (Fig. 2A). We performed chromium release assays to investigate the specific cytotoxicity of CAR-modified YTSanti–PSCA-DAP12 against PSCA-positive tumor cells. As anticipated, PSCA-positive tumor cells were significantly lysed by YTSanti–PSCA-DAP12 cells at different target to effector ratios when compared with control cells (***p < 0.0001; Fig. 2B). YTSanti–PSCA-DAP12 cells showed an average tumor cell lysis ranging from 60% at a target to effector ratio of 1:2.5 to ~80% and higher at a target to effector ratio of 1:10. Of note, YTSanti–PSCA-DAP12 cells killed the different PSCA-positive tumor cells at almost the same efficiency regardless of their origin and level of PSCA expression. As expected, incubation of YTSanti–PSCA-DAP12 cells with PSCA-negative isogenic tumor cells did not lead to tumor cell lysis. Moreover, unmodified YTS cells, YTSmyc-DAP12 cells, and YTSanti–PSCA-DAP12mut cells lysed neither PSCA-positive nor PSCA-negative tumor cells. Taken together, these results clearly demonstrate that the anti–PSCA-DAP12 CAR confers specific cytotoxicity against PSCA-positive target cells from different tumor entities.

ZAP-70 phosphorylation and IFN-γ release in NK cells upon CAR cross-linking

The biochemical events accompanying stimulation of DAP12-associated receptors in NK cells are still not well characterized, but involve ZAP-70 and Syk protein tyrosine kinases, as shown for the activating human KIR2DS2 (31). We hypothesized that cross-linking of anti–PSCA-DAP12 CAR might also lead to an ITAM phosphorylation of the DAP12 portion within the CAR, leading to recruitment and phosphorylation of ZAP-70. Subsequently, activated ZAP-70 should trigger downstream signaling, which essentially contributes to NK cell activation and cytokine release (54). To test the capability of anti–PSCA-DAP12 CAR to induce ZAP-70 phosphorylation and cytokine release, we cocultured YTSanti–PSCA-DAP12 cells with 293TPSCA cells and 293T wt cells, respectively, and analyzed phosphorylation of ZAP-70. As additional controls, YTS and YTSmyc-DAP12 were included in the experiments. The Western blot analyses revealed equal steady state protein expression of total ZAP-70 protein in YTSanti–PSCA-DAP12 cells as well as in control cells. Notably, we observed a robust phosphorylation of ZAP-70 only in YTSanti–PSCA-DAP12 cells when incubated with 293TPSCA cells, whereas phosphoZAP-70 was absent in cell lysates of YTS cells and YTSmyc-DAP12 cells (Fig. 3A). Moreover, when cocultured with PSCA-negative 293T wt cells, phosphoZAP-70 was not detectable in YTSanti–PSCA-DAP12 cells or in YTS and YTSmyc-DAP12 cells, indicating that phosphorylation occurs only after cross-linking of the DAP12-based CAR with its Ag.

To assess whether phosphorylated ZAP-70 correlates to increased cytokine release (54), we analyzed secreted IFN-γ levels

![FIGURE 2. Specific cytotoxicity of anti–PSCA-DAP12 CAR-expressing YTS cells against PSCA+ tumor cells. (A) PSCA-positive tumor cells (upper panels) and isogenic control cells (lower panels) were stained using a PSCA-specific 7F5 mAb and secondary Cy3-labeled anti-mouse Ab (gray histograms). An isotype control staining is included (open histograms). (B) Gene-engineered and parental YTS cells were cocultured with sodium chromate 51–loaded PSCA-expressing tumor cells and isogenic PSCA-negative control cells at different target to effector ratios for 18 h. The mean of specific tumor cell lysis and SD of triplets of one representative chrome release assay are shown. Note the strong tumor cell lysis mediated by YTS-anti–PSCA-DAP12 cells. ***p < 0.0001.](http://www.jimmunol.org/DownloadedFrom)}
CD3+ cells subjected to Western blot analysis. Phosphorylated ZAP-70 was detected only in YTSanti–PSCA-DAP12 cells after incubation with PSCA-positive target cells. Equal ZAP70 expression was confirmed after stripping of the protein-loaded membrane and reprobing with an anti–ZAP-70 Ab. Tubulin staining was included as an additional loading control. (B) YTS, YTSmyc-DAP12, and YTSanti–PSCA-DAP12 cells were incubated with PSCA-positive tumor cell lines PC3PSCA, H4PSCA, 293TPSCA, RT4, or HT1376. In addition, nontransduced YTS cells were included in the experiment. After 6 h of incubation, cell-free supernatant was harvested and the amount of released IFN-γ was measured by sandwich ELISA. Mean IFN-γ release and SD of triplets are shown. ***p < 0.0001.

Comparison of DAP12-CAR and CD3ζ-CAR formats

Because it was of special interest whether a DAP12-based CAR signals as efficiently as the mostly used NK-CAR format containing the CD3ζ-chain, we sought to compare the DAP12-based CAR with a CD3ζ-based CAR. We therefore constructed anti–PSCA-CD3ζ (Fig. 4A) and generated YTSanti–PSCA-CD3ζ cells. As control, we included mock-transduced YTS cells expressing only the signaling adaptor CD3ζ. The analyses of CAR expression revealed equal levels of anti–PSCA-CD3ζ and anti–PSCA-DAP12 molecules on the surface of the transduced YTS cell lines (Fig. 4B). To compare the different types of CARs, we performed IFN-γ release assays and chromium release assays and included 293TPSCA target cells and, as control, 293T wt cells. Furthermore, we used YTS cells, YTSmyc-DAP12 cells, and YTSmyc-CD3ζ cells to exclude any nonspecific side effect arising from ectopically expressed ITAM-containing protein domains. Of interest, YTSanti–PSCA-DAP12 and YTSanti–PSCA-CD3ζ released similar amounts of IFN-γ cells when confronted with 293TPSCA cells at a target to effector ratio of 10:1, which was recapitulated using other PSCA-positive target cell lines (Supplemental Fig. 1). In a chromium release assay, both the DAP12-based CAR and the CD3ζ-based CAR specifically lysed PSCA-positive target cells, whereas 293T wt cells were not affected (Fig. 4C). In contrast, YTS cells, YTSmyc-DAP12 cells, and YTSmyc-CD3ζ cells showed no cytotoxicity against PSCA-positive or PSCA-negative target cells, which again confirms that ectopically expressed DAP12 and CD3ζ signaling proteins do not induce nonspecific NK cell cytotoxicity. Notably, we constantly observed an increased specific cytotoxicity (*p < 0.05) of YTSanti–PSCA-DAP12 when compared with YTSanti–PSCA-CD3ζ at the lower target to effector ratios of 1:2.5 and 1:5. Therefore, it appears that a DAP12-based CAR is slightly superior when compared with a CAR containing the CD3ζ signaling domain.

Antitumor effects of YTSanti–PSCA-DAP12 cells injected into tumor-bearing mice

The in vivo antitumor effect of PSCA-specific YTS cells on established solid tumors was analyzed in a mouse xenograft tumor model. For this procedure, 293TPSCA cells were s.c. injected into the left flank of female NMRI-Foxn1nu/Foxn1nu mice. Once the tumor developed, YTS cells, YTSmyc-DAP12 cells, and YTSmyc-CD3ζ cells, respectively, were given via the tail vein every 48 h or 72 h over a period of 8 wk, and the behavior and weight of animals were monitored daily. We chose this regimen because our initial experiments revealed only a transient and moderate control of tumor growth after a single injection of CAR-modified YTS cells or after prolonging the intervals between YTS injections (data not shown). That the CAR-modified YTS cells as well as the YTSmyc-DAP12 control cells reached the tumor was confirmed using DoRed-marked 293TPSCA/dsRed xenografts for better visualization. The experiments revealed an inhomogeneous and moderate infiltration and no differences in the numbers of tumor-infiltrating YTSanti–PSCA-DAP12 cells when compared with YTSmyc-DAP12 cells (Supplemental Fig. 2). Through applying the aforementioned continuous treatment protocol, 14 of 26 mice receiving YTSanti–PSCA-DAP12 cells showed a complete or near complete tumor eradication at day 45 after transplantation of the tumors, whereas no such effect was observed in the control groups. Overall, YTSanti–PSCA-DAP12-treated mice showed a significant decrease in tumor growth when compared with all control groups (**p < 0.0001; Fig. 5A). In addition, the treated mice...
showed neither significant loss of weight nor altered behavior during the treatment. Of note, the injection of YTSanti–PSCA-DAP12 cells caused a statistically significant increase in median survival time of 99 d in comparison with control groups (without YTS cells: 63 d; with YTS cells: 52 d; with YTSmyc-DAP12 cells: 86 d; \( p < 0.001 \)).

Moreover, 31% of mice with YTSanti–PSCA-DAP12 treatment showed a complete and stable tumor regression and remained tumor free at the termination of the experiments at day 155 (Fig. 5B). To exclude a loss of PSCA-Ag as a potential immune evasion mechanism, randomly selected tumors were prepared to determine PSCA surface expression levels. Yet, tumor growth controls, tumors treated with control YTS, YTSmyc-DAP12, and YTSanti–PSCA-DAP12, displayed no decrease in expression of PSCA (Supplemental Fig. 3). To investigate engraftment but also to exclude negative effects of YTS NK cells in mice (e.g., development of lymphoproliferative disease), the peripheral blood and bone marrow of three survivors that had received treatment with YTSanti–PSCA-DAP12 cells were tested after termination of the experiment for circulating EGFP-positive YTS cells. In all cases, YTS cells were absent in the blood or bone marrow of analyzed mice (Supplemental Fig. 3). Likewise, additional analyses of spleens and livers revealed no residual EGFP-positive YTS cells (data not shown).

Taken together, these data indicate, on the one hand, a profound antitumor effect of continuously administered YTSanti–PSCA-DAP12 cells but, on the other hand, demonstrate the inability of YTS cells to stably engraft in NMRI-Foxn1nu/Foxn1nu mice.

Primary NK cells genetically engineered to express a DAP12-CAR kill PSCA-expressing target cells

To assess whether a DAP12-based CAR is suitable for potential clinical use, we sought to generate primary NK cells from healthy donors with expression of anti–PSCA-DAP12 CAR and myc-DAP12 as a control. In many transduction efforts, using lentiviral vectors with the p6NST50 backbone, we obtained mean transduction efficiencies in NK cells, 20%, and so we decided to use shorter and modified pHATtrick lentiviral vectors for transduction of primary NK cells (see Materials and Methods).
of EGFP+ NK cells expressed the CAR and the myc-DAP12 or control cells measured at day 45 after tumor transplantation. Note that a Mean tumor growth of living mice injected with YTSanti–PSCA-DAP12 cells showed improved survival. Shown data summarize overall survival of nontreated mice, mice injected with YTSanti–PSCA-DAP12 or YTSmyc-DAP12 cells, and mice receiving control cells. Mice treated with YTSanti–PSCA-DAP12 or YTSmyc-DAP12 cells and mice receiving control cells. Mice treated with YTSanti–PSCA-DAP12 cells showed improved survival. Shown data summarize three independent experiments with similar results (n = 25 for YTS-treated mice; n = 26 for nontreated, YTSmyc-DAP12-treated, and YTSanti–PSCA-DAP12-treated mice). ****p < 0.0001.

pHATtrick contains a T2A-endoproteolytic cleavage site and EGFP after the CAR construct and myc-DAP12 control, respectively, as depicted in Fig. 6A. Flow cytometry analyses of EGFP expression levels revealed that transduced PBMC-derived NK cells from seven different donors expressed a mean of 50% (±10%) myc-DAP12 control or 48% (±16%) anti–PSCA-DAP12 CAR (Fig. 6B), with stable expression for ≥5 wk (data not shown). Yet, we constantly observed weakened mean fluorescence intensities when using myc-DAP12– and CAR-encoding vectors when compared with an empty vector control, which inversely correlates to the insert size of the lentiviral vectors. However, further analyses using an anti–c-myc-tag Ab revealed that <75% of EGFP+ NK cells expressed the CAR and the myc-DAP12 control construct on the cell surface, respectively (Fig. 6C). To predict a possible allogeneic reactivity, the HLA genes of donors and target cells were genotyped (Supplemental Fig. 4B). We calculated that NK cells from our donors were fully compatible with H4 mismatch in the GvH direction. Of note, only PSCA-positive target cells stimulated NK anti–PSCA-DAP12 controls from the same donors did not react against PC3PSCA. H4PSCA and also did not kill isogenic PC3 and H4 wt cells devoid of PSCA expression. Although PC3PSCA and H4PSCA cells expressed comparable levels of PSCA (Fig. 2A), the CAR-modified NKanti–PSCA-DAP12 cells developed a stronger cytotoxic response against H4PSCA, irrespective of whether the donors showed an HLA-C match and Bw4 mismatch (donors no. 1 and no. 3) or were HLA-B/C matched (donor no. 2). Intriguingly, when we probed CAR-engineered NK cells from donor no. 3 against RT4 and HT1376 cells, which both expressed lower levels of PSCA but had Bw6/C1 and Bw4/C2 mismatches, respectively, we revealed very strong cytotoxic reactions. Yet, no such cytotoxic reactions were monitored when using nontransduced NK cells and NKmyc-DAP12 cells. It therefore might be conceivable that in our experimental setting the HLA-C/KIR-mismatches to RT4 and HT1376 cells likely resulted in a decreased activation threshold of NK cells, which enabled an enhanced cytotoxic reaction of DAP12-CAR–modified NK cells when encountering PSCA+target-cells.

**Discussion**

Initial clinical trials with naturally occurring tumor-reactive T cells have proven that adoptive immunotherapy is a feasible and promising approach for cancer treatment (55). Limitations of this approach, such as HLA restriction or defective Ag presentation on tumor cells, and difficulties in raising sufficient numbers of tumor-reactive native T cells from patients, can be solved by the use of genetically modified autologous T cells with tumor-peptide TCRs or CARs (56, 57). So far, CARs have been designed for the redirection of genetically modified autologous T cells with tumor-peptide TCRs or CARs (56, 57). HER-2 on ovarian and breast cancer cells (60), TARP on Hodgkin lymphoma cells (58), CEA on colorectal cancer cells (59), HER-2 on ovarian and breast cancer cells (60), TARP on prostate and breast cancer cells (61), and EGFRvIII and IL13R on glioblastoma cells (62–64). However, concern has been raised about genetically modified autoreactive T cells, which might cause undesirable side effects after infusion into patients. NK cells, in contrast, do not possess TCR-like molecules that might...
cause these unwanted immunological side effects. A number of studies have demonstrated that native NK cells are reactive against tumor cells in vitro and in vivo. Moreover, it has been shown that the NK cell lines NK92 and YT, as well as primary NK cells, can be engineered with CARs (21, 41–43, 65–68).

Whereas other approaches focused on the CD3ζ-chain as a signaling subunit in NK-redirecting CARs, our newly developed PSCA-specific CAR incorporated the signaling adaptor protein DAP12, which is involved in the signal transduction of activating NK cell receptors, in particular, NKG2C (26). Yet, some reports describing DAP12-deficient mice indicate that DAP12 associated with TREMs under special circumstances also transmits negative signals in cells of the myeloid lineage, such as macrophages and granulocytes (69). It has been proposed that a low-avidity interaction of TREMs with their so far unknown ligands might lead to incomplete DAP12 phosphorylation and somehow increases inhibitory signals (70). However, in our experimental settings using lymphoid NK cells, DAP12 was proven to efficiently induce cellular cytotoxicity when used as the signaling domain of a CAR, as discussed below.

To our knowledge, we report the first NK cell CAR containing DAP12 as an intracellular signaling domain. Additional costimulatory signaling fragments, which are frequently integrated into CARs (71), were not included owing to the anticipation that they are not necessary for DAP12-mediated NK cell activation.
interest, it appears that the DAP12-based CAR conferred a gradual improved cytotoxicity to YTS cells when compared with a CD3ζ-based CAR. So far it is tempting to speculate why a DAP12-based CAR containing only one ITAM might be at least as efficient in downstream signaling as a CD3ζ-based CAR containing three ITAMs. Yet, we suggest that ITAM phosphorylation of DAP12 might directly form a docking site for downstream signaling, whereas in the case of the CD3ζ-chain different phosphorylation grades might influence docking sites and eventually regulate the strength and different effector mechanisms of the NK cell response. That the ITAM domain of DAP12 was indispensable for the activation of NK cells was demonstrated using a phosphorylation-defective DAP12-ITAM, which fails to induce NK cell cytotoxicity after confrontation with PSCA-positive target cells.

Along with a strong and stable CAR expression of anti–PSCA-DAP12 transduced YTS cells, we observed that the cross-linking of the CAR with PSCA caused an enhanced phosphorylation of the tyrosine protein kinase ZAP-70. As a likely consequence of ZAP-70–mediated signal induction, CAR-modified YTS cells released significantly increased amounts of IFN-γ when compared with controls. At the same time, no obvious correlation between the amounts of IFN-γ secretion by YTS cells and target Ag expression was detectable, which resembles results obtained by CD3ζ-CAR–modified NK92 cells engaging different ErbB2/HER-2-positive target cell lines (67). In line with the aforementioned observation, also the strength of the cytotoxic reaction as well as the IFN-γ release of CAR-modified primary NK cells was not directly correlated to the level of PSCA expression on target cells. Hence, we suggest that the observed dissimilar IFN-γ release after cocultivation of CAR-engineered YTS cells and primary NK cells with tumor cells not only is due to the level of PSCA expression but also might depend on the composition and expression levels of cell adhesion molecules and, in particular for primary NK cells, is linked to the lack of inhibitory HLA-B and -C molecules on target cells.

According to the results of the IFN-γ release assays, we revealed that YTS cells engineered with anti–PSCA-DAP12 CAR caused a highly specific lysis of only PSCA-positive target cells originating from prostate cancer, bladder carcinoma, and glioblastoma, whereas PSCA-negative tumor cells were not affected. Therefore, it can be concluded that the observed cytotoxic activity of PSCA-redirected YTS cells was exclusively due to the interaction of the PSCA-specific CAR with its Ag. In subsequent experimental NK immunotherapy, we demonstrated an antitumor effect of our DAP12-based CAR in a preclinical tumor model. To our knowl-

**FIGURE 7.** CAR-engineered primary human NK cells recognize and kill PSCA+ tumor cells. Expanded primary NK cells from three different donors were transduced with anti–PSCA-DAP12 CAR or myc-DAP12 control. The gene-engineered NKmyc-DAP12, NKanti–PSCA-DAP12, and nontransduced control cells were cocultured with PSCA-expressing tumor cells (PC3PSCA, H4PSCA) and isogenic PSCA-negative control cells (PC3, H4) at different target to effector ratios for 18 h. HLA-mismatch predicting for a KIR-mismatch in the GvH direction is indicated. The mean of specific tumor cell lysis and SD of triplets are shown. Gene-engineered NK cells from donor no. 3 were also incubated with the bladder carcinoma cell lines RT4 and HT1376, which show endogenous expression of PSCA. *p < 0.05.
edge, we treated for the first time established solid tumors with CAR-modified NK cells, whereas other groups favored mixing of NK cells and target cells prior to tumor transplantation (23, 65–67) or chose an experimental setting that most likely confronts injected tumor cells and CAR-modified NK cells in the bloodstream or lung capillaries (67). In this article, we demonstrate that treatment with YTS\textsuperscript{anti–PSCA-DAP12} NK cells resulted in a significantly delayed growth of PSCA-positive tumors when compared with tumors treated with YTS wt cells and YTS\textsuperscript{myc-DAP12} controls, respectively. However, this significant antitumor effect was accomplished only by continuous injection of YTS\textsuperscript{anti–PSCA-DAP12} cells. After termination of YTS\textsuperscript{anti–PSCA-DAP12} treatment, 31% of mice remained tumor free, but in the remaining mice tumor growth accelerated, suggesting that injected YTS\textsuperscript{anti–PSCA-DAP12} cells are short lived. This idea is further supported by our results showing lack of YTS\textsuperscript{anti–PSCA-DAP12} engraftment in cured mice. Furthermore, our data suggest only a moderate tumor infiltration of YTS cells, which, in conjunction with other factors (i.e., differences in the vascularization of the tumors), might have resulted in the appearance of weak responders and nonresponders. In the future it might therefore be worthwhile to genetically modify NK cells with chemokine receptors to enhance selective tumor infiltration. That this kind of engrafted chemotaxis is beneficial was previously demonstrated by CXCR2- and CCR2-modified T cells, which showed improved tumor infiltrations and eradication in tumor xenograft models (72, 73).

In line with the results obtained with YTS NK cells, CAR-modified primary NK cells also showed a high and specific cytokotoxicity toward PSCA-positive tumor cells, which, as mentioned above, was further enhanced by KIR ligand mismatches, particularly demonstrated using RT4 and HT1376 target cells having moderate PSCA expression levels. Although it is well known that in HLA-B and -C mismatches are important for the development of GvH disease in allogeneic hematopoietic stem cell transplantation (10, 11), we observed in our experiments that KIR/ligand mismatches in the GvH direction did not lead to increased cytokotoxicity of nontransduced NK cells and genetically engineered NK\textsuperscript{myc-DAP12} donor cells. Hence we assume that our 18-h protocol (i.e., differences in the vascularization of the tumors), might have provided the NK cell line YTS and F. Zachow, B. Goldberg, and K. Robel

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Disclosures
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