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# Costimulation by chimeric antigen receptors revisited: the T cell antitumor response benefits from combined CD28-OX40 signalling

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**The therapeutic success of adoptive therapy with chimeric antigen receptor (CAR) engineered T cells depends on the appropriate costimulation of CD3ζ to induce full T cell activation. Costimulatory endodomains of the CD28 family are therefore fused with CD3ζ in a dual signalling CAR. Serious adverse events in two most recent trials; however, highlight the need to analyse in more detail the impact of each costimulatory endodomain on individual effector functions of redirected T cells. We therefore performed a thoroughly controlled side-by-side comparison of the most frequently used endodomains with respect to their impact on $CD4^+$ and $CD8^+$ T cell effector functions. CD28 reinforced T cell proliferation and is mandatory to induce IL-2. In the absence of added IL-2, CD28 and OX40 (CD137) but not 4-1BB (CD134) enhanced specific cytolysis. While CD28, 4-1BB and OX40 similarly improved pro-inflammatory cytokine secretion, OX40 most efficiently prevented activation induced cell death of $CD62L^-$ effector memory T cells. CD28 was superior to initiate the T cell response, OX40 and 4-1BB sustained the response in long term with OX40 being most effective. We consequently combined the beneficial functions in a 3rd generation CD28-OX40 CAR which substantially improved the antitumor response without loosing specificity.**

Chimeric antigen receptors (CARs) with an antibody derived binding domain for MHC-independent antigen recognition redirect cytotoxic T lymphocyte (CTL) activation in a predefined fashion for producing potent antitumor effects.[1] The first generation prototype CAR mediates activation of modified T cells *via* the CD3ζ signalling domain leading to secretion of pro-inflammatory cytokines, T cell proliferation and tumor cell lysis *in vitro* and eradication of transplanted tumors *in vivo* as demonstrated in various mouse models.[2,3] Clinical trials, however, showed modest efficacy in patients with lymphoma, neuroblastoma or ovarian and renal cell carcinoma[4–6] as a result of limited expansion and persistence *in vivo*. This is thought to be due to incomplete T cell activation and lack of costimulation because tumors frequently do not provide ligands for costimulatory molecules on T cells. This gives the rationale to combine CD3ζ with the endodomain of a costimulatory molecule in a dual signalling CAR.[7–9] These 2nd generation CARs incorporating CD28, OX40 (CD134) or 4-1BB (CD137) within the endodomain improved antigen-specific expansion of engineered T cells.[7,10] CD28-ζ CAR redirected T cells secreted IL-2 which in turn acts as an autocrine and paracrine factor to improve T cell proliferation and survival.[9,11] Once activated T cells down-regulate CD28 and up-regulate a second set of "late" costimulatory molecules including OX40 and 4-1BB which furthermore modulate T cell activation including clonal expansion and cytokine secretion.[7,12,13] While the overall benefit of costimulation has been clearly demonstrated,[14] it is not clear how early and late costimulation might differentially affect cellular functions of different engineered T cell subsets. Costimulatory CARs are currently evaluated in more than 10 clinical trials, as listed in ClinicalTrials.gov, with some encouraging preliminary evidence of clinical efficacy. However, two fatal serious adverse events were most recently reported enrolled in trials using costimulatory CARs.[15–17] Although the CAR redirected adoptive immune response is considered to be highly specific some concerns are raising about costimulation induced effector functions of these 2nd generation CARs to produce potent activation. It is particularly unclear which effector functions in particular are modulated by the individual costimulatory signals and how they contribute to the overall T cell response. To address this issue we revisited in a thoroughly controlled side-by-side analysis cytokine secretion, proliferation, prevention from apoptotic cell death and cytolysis which all will be of major impact on the redirected

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antitumor attack by both $CD4^+$ and $CD8^+$ T cells. Using T cells on the same preactivation level we revealed unique consequences of CD28, OX40 and 4-1BB costimulation along with redundant ones on CAR mediated T cell activation. Based on these data, a CAR with CD28 and OX40 costimulation ideally combines beneficial effector functions induced by early and late costimulation and improves T cell antitumor reactivity *in vitro* and in a mouse model *in vivo* without loosing specificity.

## Material and Methods

### Cell lines and reagents

293T cells are human embryonic kidney cells that express the SV40 large T antigen.[18] The CEA-expressing colon carcinoma cell lines LS174T (ATCC CCL 188) and SW948 (ATCC CCL 237) and the CEA-negative cell line Colo320 (ATCC CCL 220.1) were obtained from ATCC, Rockville, MD. The anti-BW431/26 anti-idiotypic mAb BW2064/36 was described earlier.[19] OKT3 (ATCC CRL 8001) is a hybridoma cell line that produces the anti-CD3 mAb OKT3. All cell lines were cultured in RPMI 1640 medium, 10% (v/v) FCS (all Life Technologies, Paisly, UK). OKT3 mAb was affinity purified from hybridoma supernatants utilizing goat anti-mouse IgG2a antibodies (Southern Biotechnology, Birmingham, AL) which were immobilized on *N*-hydroxy-succinimid-ester-(NHS)-activated sepharose (Amersham Biosciences, Freiburg, Germany). The goat anti-human IgG antibody and the biotin-, FITC- or PE-conjugated $F(ab')_2$ derivatives were purchased from Southern Biotechnology. The anti-human IFN-γ mAb NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3, the anti-IL-2 mAb 5344.111 and the biotinylated anti-human IL-2 B33-2 mAb, the anti-human IL-10 mAb 4D5 and the biotinylated anti-human IL-10 mAb 6D4/D6/G2 were purchased from BD Bioscience, San Diego, CA.

### Generation and expression of recombinant anti-CEA CARs

The generation of the retroviral expression cassettes for the anti-CEA BW431/26-scFv-Fc-CD3ζ and the BW431/26-scFv-Fc-CD28-CD3ζ CARs were described in detail.[9,20] The BW431/26-scFv-Fc-CD3ζ-OX40 and BW431/26-scFv-Fc-4-1BB-CD3ζ CARs were generated as follows. The cDNA coding for the endodomains of OX40 and 4-1BB were amplified by RT-PCR utilizing cDNA of activated T cells as template and the primer oligonucleotides 1-4 (Table 1). The PCR product was inserted into the vector pCR2.1 (Invitrogen, Karlsruhe, Germany). To generate the cDNA coding for the chimeric 4-1BB-CD3ζ and CD3ζ-OX40 signalling domains, the cDNAs of 4-1BB, OX40 and CD3ζ were amplified by PCR utilizing oligonucleotides 8 and 9 or 5 and 11 (CD3ζ), 4 and 7 (OX40) and 6 and 10 (4-1BB), respectively, thereby flanked with overlapping CD4 transmembrane sequences (Table 1). The DNAs for the respective domains were assembled by PCR. The DNAs for the chimeric endodomains were reamplified by PCR utilizing oligonucleotides 10 and 11 for 4-1BB-CD3ζ and 4 and 9 for CD3ζ-OX40, respectively (Table 1). To generate the cDNA coding for the chimeric CD28-CD3ζ-OX40 signalling domain, the cDNAs of CD28-CD3ζ and OX40 were amplified by PCR utilizing oligonucleotides 8 and 14 (CD28ζ) and 4 and 7 (OX40), respectively (Table 1). The DNAs were assembled by PCR and reamplified with oligonucleotides 4 and 14, respectively. Amplified DNAs were flanked by BamHI and XhoI or SalI restriction sites and inserted into the retroviral expression vector pBULLET. The sequences coding for the extracellular scFv binding and constant IgG1 Fc domains were amplified by PCR utilizing the BW431/26-scFv-Fc-ζ cDNA as template and oligonucleotides 12 and 13 introducing NcoI and BglII restriction sites. The PCR product was digested with NcoI and BglII and inserted into the NcoI and BamHI restriction sites of the retroviral expression vector pBULLET containing the cDNA

**Table 1.** Primer oligonucleotides used to generate CARs[1]

| |
|---|
| 1. 4–1BBS (sense) |
| GCGAGGGATCCAGGACACTCTCCGCAGATC |
| 2. 4–1BBAS (antisense) |
| TCAGTCGACCTCGAGTCACAGTTCACATCCTCCTTCTTC |
| 3. OX40S (sense) |
| CCCGGGGATCCGCGTGCGGTTGCCGCCATCCTG |
| 4. OX40AS (antisense) |
| TCAGTCGACCTCGAGTCAGATCTTGGCCAGGGTGGAGTG |
| 5. 4–1BBzeS (sense) |
| GAAGAAGGAGGATGTGAACTGCTGAGAGTGAAGTTCAGCAGGAGCGCAGAC |
| 6. 4–1BBzeAS (antisense) |
| GTCTGCGCTCCTGCTGAACTTCACTCTCAGCAGTTCACATCCTCCTTCTTC |
| 7. 5zetaOX40 (sense) |
| CACATGCAGGCCCTGCCCCCTCGCAGGGACCAGAGGCTGCCCCCCGATGCC |
| 8. 3zetaOX40 (antisense) |
| GGCATCGGGGGGCAGCCTCTGGTCCCTGCGAGGGGGCAGGGCCTGCATGTG |
| 9. SCD4TmZ-BamHI (sense) |
| TACTGGATCCTCAGCCAATGGCCCTGATTGTGCTGGGGGGCGTCGCCGG CCTCCTGCTTTTCATTGGGCTAGGCATCTTCTTCTGTGTCAGGAGAGTGA AGTTCAGCAGGAGCG |
| 10. SCD4Tm41BB-BamHI (sense) |
| TACTGGATCCTCAGCCAATGGCCCTGATTGTGCTGGGGGGCGTCGCCGGC CTCCTGCTTTTCATTGGGCTAGGCATCTTCTTCTGTGTCAGGAAACGGGGC AGAAAGAAACTCCTG |
| 11. 3zebulneu (antisense) |
| GGCAGATCTGTCGACCTGTTAGCGAGGGGGCAG |
| 12. Lkappa-NcoI (sense) |
| CTACGTACCATGGATTTTCAGGTGCAGATTTTC |
| 13. hIgG1Fc-BglII (antisense) |
| CCCACCCAGATCTTTTTTACCCAGAGACAGGGAGAGGCTCTTCTG |
| 14. TMcd28bam BamHI (sense) |
| CTGGATCCCAAATTTTGGGTGCTGGTGGTGGTTG |

[1]Recognition sites for restriction endonucleases are underlined.

sequences for 4-1BB-CD3ζ, CD3ζ-OX40 and CD28-CD3ζ-OX40, respectively. Retroviral transduction of T cells with recombinant receptors was described in detail elsewhere[18,21] and receptor expression was monitored by flow cytometric analyses. Recombinant receptors were also expressed in 293T cells after transfection of the vector DNA utilizing Polyfect™ transfection reagent (Quiagen, Hilden, Germany) (1.5 μg DNA/2x10$^5$ cells).

### Cell sorting

Peripheral blood lymphocytes from healthy donors were isolated by density centrifugation and monocytes were depleted by plastic adherence. Nonadherent lymphocytes were washed with cold PBS containing 0.5% (w/v) BSA, 1% (v/v) FCS, 2 mM EDTA. CD3$^+$, CD4$^+$ and CD8$^+$ T cells were isolated by magnetic activated cell sorting (MACS) utilizing the "CD3, CD4 and CD8 T cell isolation kit" (Miltenyi, Bergisch Gladbach, Germany) resulting in purities of routinely >95%. MACS purified CD3$^+$ naive and memory T cells were isolated by fluorescence activated cell sorting utilizing FITC-conjugated anti-CD45RA, APC-conjugated anti-CD62L and PE-conjugated anti-CD45R0 mAbs (all BD Bioscience) and a FACSAriaIII cell sorter (BD Bioscience). Sorted cells were activated and retrovirally engrafted with CARs as described above.

### Immunofluorescence analyzes

CAR engineered T cells were stained with PE-conjugated F(ab′)$_2$ anti-human IgG1 antibody (0.1 μg/ml) and a FITC-conjugated anti-CD3 mAb (UCHT-1, 1:20) and identified by flow cytometry using a FACScan™ cytofluorometer equipped with the CellQuest research software (Becton Dickinson, Mountain View, CA).

### CAR mediated activation of engineered T cells

T cells were engineered with anti-CEA CARs and cultivated in microtiter plates (Polysorb, Nunc, Roskilde, Danmark) (2.5x10$^4$ receptor grafted T cells/well) precoated with purified anti-idiotypic mAb BW2064/399 or an IgG1 mAb for control (each 0.01-10 μg/ml). After 48 hrs cell proliferation was determined utilizing the 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III (Roche Biochemicals, Mannheim, Germany). In a second set of experiments CAR engineered T cells were cocultivated for 48 hrs in 96-well round bottom plates with CEA$^+$ LS174T and CEA$^-$ Colo320 tumor cells (each 2.5x10$^4$ cells/well). After 48 hrs culture supernatants were analysed by ELISA for IFN-γ, IL-2 and IL-10, respectively. Briefly, cytokines in the supernatants were bound to the solid phase capture antibodies (each 1 μg/ml) and detected by the biotinylated detection antibodies (each 0.5 μg/ml). The reaction product was visualized by a peroxidase-streptavidin-conjugate (1:10,000) and ABTS®. Specific cytotoxicity of engineered T cells against target cells was monitored by a XTT based colorimetric assay according to Jost *et al.*[22] Briefly, receptor-engineered T cells (10$^4$ cells per well) were coincubated with 2.5 x 10$^4$ tumor cells for 48 h. The number of totally T cells was adjusted to same numbers with nonmodified T cells. Viability of tumor cells was monitored using the "Cell Proliferation Kit II" (Roche Diagnostics, Mannheim, Germany) and reduction of XTT to formazan by viable tumor cells was monitored colorimetrically. Values were calculated as means of six wells containing tumor cells only substracted by the mean background level of wells containing RPMI 1640 medium, 10% (v/v) FCS. Nonspecific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells. The number of viable tumor cells was calculated as follows: viability (%) = [OD(experimental wells—corresponding number of effector cells)]/[OD(tumor cells without effectors-medium)] x 100. Cytotoxicity (%) was defined as 100 – viability (%).

### Activation induced cell death

CAR engineered T cells (2.5 x 10$^6$ total cells) were cultivated in presence of 0.5 μg/ml of the anti-idiotypic mAb BW2064/399 and 0.25 μg/ml of an goat anti-mouse IgG antibody (Southern Biotechnology), respectively. After 72 hrs cells were removed and stained for CAR and CD62L expression with a FITC-conjugated F(ab′)$_2$ anti-human IgG1 (Southern Biotechnology) and PE-conjugated anti-CD62L antibody (Immunotools, Friesoythe, Germany), respectively. Cell viability was recorded by staining with APC-conjugated Annexin V (Immunotools) and 7-AAD, respectively. T cells were analysed by flow cytometry and the mean fluorescence intensity (mfi) of CD62L expression and numbers of CAR$^+$ and CAR$^-$ AnnexinV$^+$ cells were determined.

### CAR mediated suppression of tumor growth

NIH-3 mice (N:NIH- bg-nu- xid) (Charles River, Sulzfeld, Germany) (4-7 animals/group) were subcutaneously transplanted with CEA$^+$ C15A3 tumor cells (5 x 10$^5$ cells/animal). Eight days after tumor transplantation a single dose of CAR modified T cells was injected intravenously (1x10$^5$ CAR engineered T cells/animal). The number of totally injected T cells was adjusted to same numbers with nonmodified T cells. T cells without CAR served as control. Tumor growth was recorded every 2-3 days and tumor volume determined. Statistical significance between groups was determined by Student's t test.

## Results

We generated a panel of CEA specific CARs of the same format but different signalling endodomains, *i.e.,* CD3ζ alone or combined with CD28, 4-1BB, or OX40 costimulatory domains, respectively (Fig. 1). CARs were expressed by retroviral gene transfer in both CD4$^+$ and CD8$^+$ T cells with similar efficacies (data not shown). To explore the impact of the individual costimulatory domains we recorded in a side-by-side comparison the effector functions of redirected CD4$^+$

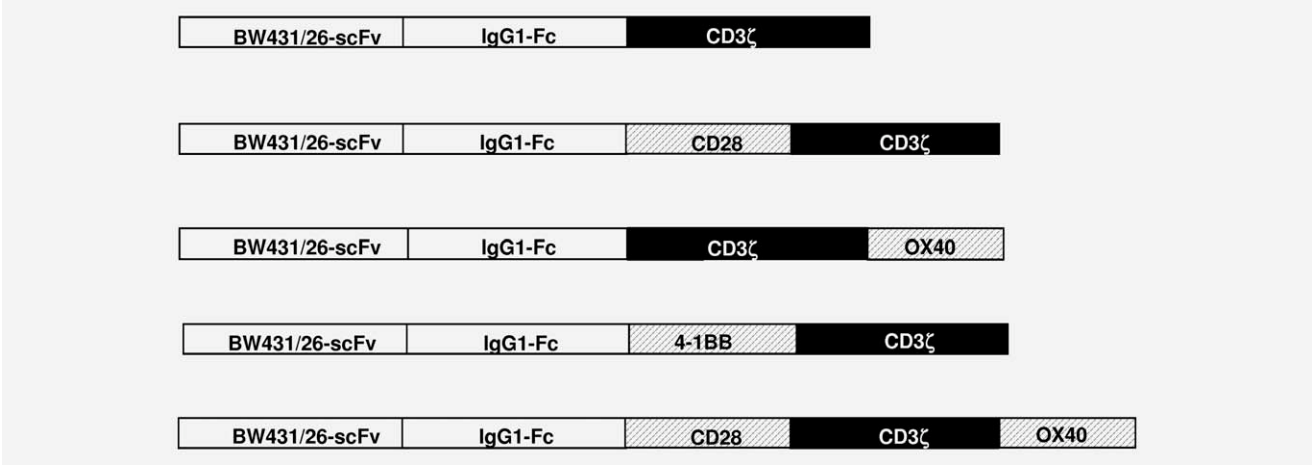


**Figure 1.** Schematic diagram of the expression cassettes for the anti-CEA CARs with different endodomains.

and $CD8^+$ T cells, including proliferation, secretion of pro-inflammatory cytokines, and target cell lysis.

For specific activation CAR engineered $CD4^+$ and $CD8^+$ T cells were incubated with the immobilized anti-idiotypic antibody BW2064 as surrogate antigen which is specific for the BW431/26 scFv CAR binding domain. Engineered $CD4^+$ and $CD8^+$ T cells were induced to proliferate upon CAR engagement of the anti-idiotypic mAb whereas an isotype matched control mAb did not induce T cell amplification (Fig. 2). Proliferation of both $CD4^+$ and $CD8^+$ T cells was strongly enhanced by CD28 cosignalling. CAR costimulation *via* OX40 and 4-1BB, in contrast, had no impact on CD3ζ driven proliferation of engineered $CD4^+$ T cells; proliferation of $CD8^+$ T cells was slightly enhanced by the 4-1BB, but not by the OX40 costimulatory CAR. The data demonstrate that redirected T cell amplification is predominantly improved by a CD28 but not by an OX40 or 4-1BB costimulatory CAR.

CAR triggered T cells require CD28 costimulation for the induction of IL-2 secretion.[9] We therefore asked whether OX40 or 4-1BB can substitute CD28 to induce IL-2 along with the improvement of other pro-inflammatory cytokine secretion including IFN-γ. Upon CAR engagement of immobilized antigen, CD28, OX40 and 4-1BB costimulation substantially enhanced IFN-γ secretion compared to CD3ζ signalling only (Fig. 2). In contrast, IL-2 secretion by engineered $CD4^+$ and $CD8^+$ T cells was restricted to the CD28 costimulatory CAR. We basically obtained similar results with engineered $CD4^+$ and $CD8^+$ T cells. Cytokine induction was specific because incubation of engineered T cells with an isotype matched control mAb was ineffective (Fig. 2). We also asked whether costimulation may impact the induction of immunosuppressive cytokines and analysed supernatnats for IL-10 secretion. Similar to IL-2 we found IL-10 secretion restricted to CD28 costimulation in $CD4^+$ T cells but no IL-10 secretion upon 4-1BB and OX40 costimulation (Fig. 2). Taken together, OX40 and 4-1BB CARs improve the overall T cell activation as indicated by increased IFN-γ secretion but cannot substitute CD28 for the induction of IL-2.

We compared the redirected cytolytic activity of engineered T cells upon engagement of $CEA^+$ target cells. In addition to $CD8^+$, $CD4^+$ T cells were also included in the study since circumvention of MHC restriction by the antibody-derived CAR binding domain allows both T cell types to execute cytolysis.[23] Since CD28 mediated IL-2 may have an impact, which is obviously absent in OX40 and 4-1BB CAR engineered T cells, we analysed CAR redirected cytolytic activity in presence and absence of added IL-2. In the presence of IL-2, CAR redirected cytolysis of $CEA^+$ target cells was similarly efficient for all CARs tested (Fig. 3*a*). Target cell lysis by $CD4^+$ and $CD8^+$ T cells is antigen-dependent and more efficiently executed by $CD8^+$ than by $CD4^+$ T cells. In the absence of added IL-2, however, the CD28-CD3ζ CAR was most effective whereas CD3ζ and CD3ζ-OX40 CARs mediated cytolysis by $CD8^+$ T cells with similar efficacy (Fig. 3*b*). The 4-1BB-CD3ζ CAR, however, was less effective compared to a CD3ζ CAR. In contrast, CD28-CD3ζ and CD3ζ-OX40 CARs induced cytolysis with similar efficacy in $CD4^+$ T cells whereas CD3ζ and 4-1BB-CD3ζ signalling were less effective. Taken together, in the presence of IL-2 all CARs mediated high cytolytic activities in $CD4^+$ and $CD8^+$ T cells while they have different impact in the absence of IL-2.

Sustained antitumor reactivity by CARs depends on (i) the efficient induction of cytolytic effector T cells that are predominantly found in the $CD62L^-$ memory T cell subset and are directed into the periphery[24,25] and (ii) protection of those cells from activation induced cell death (AICD) to sustain the CAR redirected T cell response upon antigen encounter. We asked whether "early" CD28 costimulation can benefit from additional "late" OX40 costimulation and generated a CAR which combines the CD28-CD3ζ with the OX40 endodomain. The rationale is that an OX40 CAR proved to

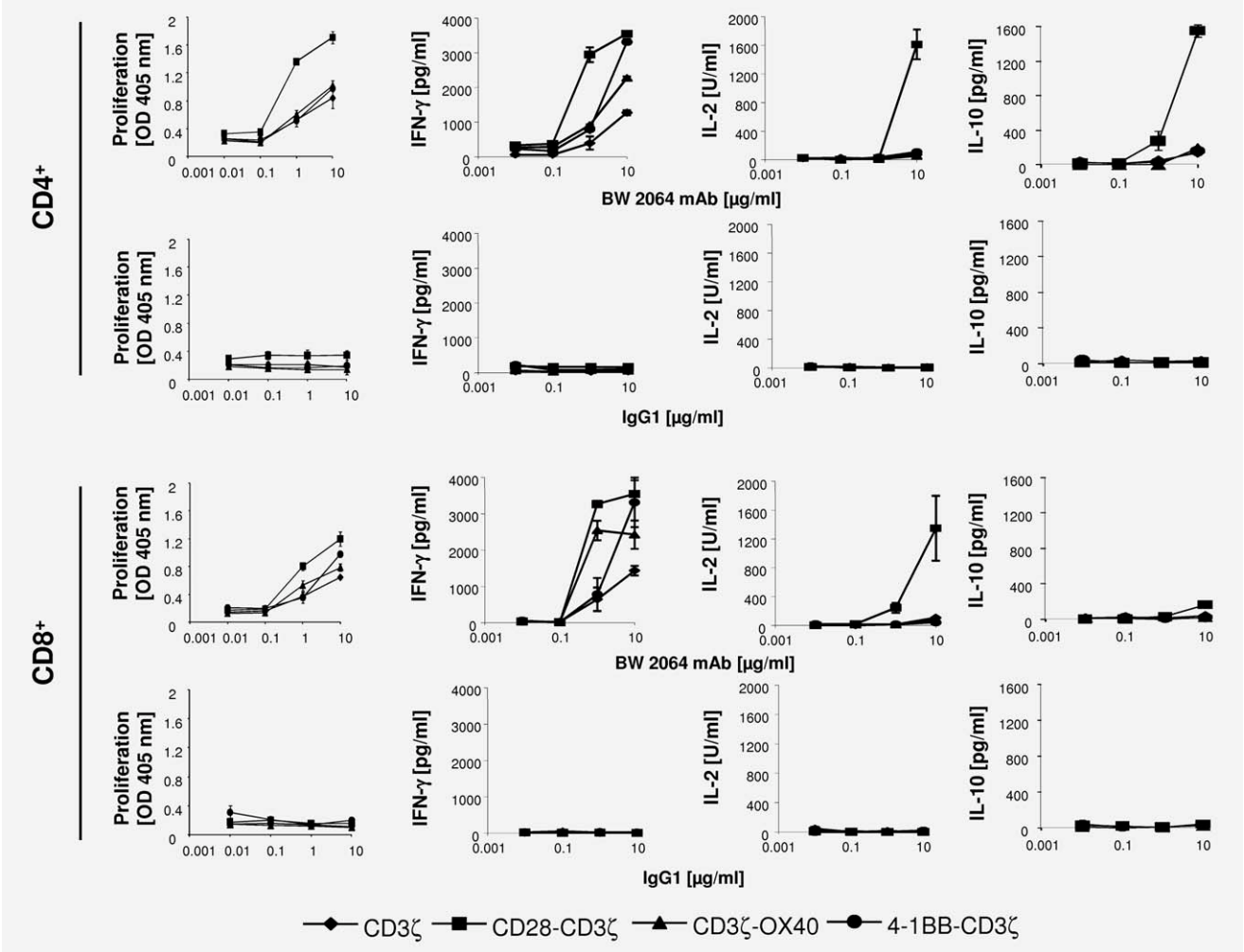


**Figure 2.** Different costimulatory endodomains in a CAR differentially impact proliferation and cytokine secretion of $CD4^+$ and $CD8^+$ T cells. Serial dilutions of the anti-idiotypic mAb BW2064, which is directed to the CAR anti-CEA scFv domain, or an isotype control mAb IgG1 were coated onto microtiter plates (0.01–10 µg/ml). $CD4^+$ and $CD8^+$ T cells were engineered with the respective CEA-specific CAR and adjusted to equal numbers of CAR expressing cells by adding nonmodified T cells of the same donor. T cells were incubated for 48 hrs in coated microtiter wells ($2.5 \times 10^4$ CAR engineered T cells/well). Cell proliferation was determined by BrdU incorporation and IFN-γ, IL-2 and IL-10 secretion, respectively, recorded by ELISA. The assays were performed in triplicates and mean values ± SEM are shown.

be more efficient than a 4-1BB CAR to modulate the primary CD3ζ signal upon antigen encounter (cf. Fig. 1). We used sorted naïve $CD45RA^+CD62L^+$ and memory $CD45R0^+$ T cells to explore the impact of CAR-mediated costimulation on the respective naive and memory T cell population. As summarized in Figure 4*a*, all CARs redirected naive $CD45RA^+CD62L^+$ T cells to cytolysis of $CEA^+$ target cells whereas CD28-CD3ζ and CD28-CD3ζ-OX40 CARs were most effective in redirecting $CD45R0^+$ memory T cells. CARs with an OX40 endodomain, however, induced higher IFN-γ secretion in memory T cells whereas CD28 costimulation alone induced higher IFN-γ in naive T cells. Taken together, data indicate that early costimulation *via* CD28 works preferentially in naïve T cells in contrast to late costimulation which works better in memory T cells. Accordingly, we asked whether CAR-mediated late costimulation impacts the generation and survival of $CD62L^-$ memory T cells. CAR engineered T cells were stimulated by the soluble anti-idiotypic mAb BW2064/399 and the number of $CD62L^-$ T cells with CAR and with and without Annexin $V^+$ staining was determined. As summarized in Figure 4*b*, CAR-mediated activation is accompanied by CD62L down modulation with combined CD28-OX40 costimulation to be most effective. Moreover, OX40 costimulation alone or in combination with CD28 prevents apoptosis of $CD62L^-$ T cells most efficiently whereas 4-1BB and CD28 costimulation were less effective. For control, T cells without CAR expression did neither down-regulate CD62L nor undergo enhanced apoptosis

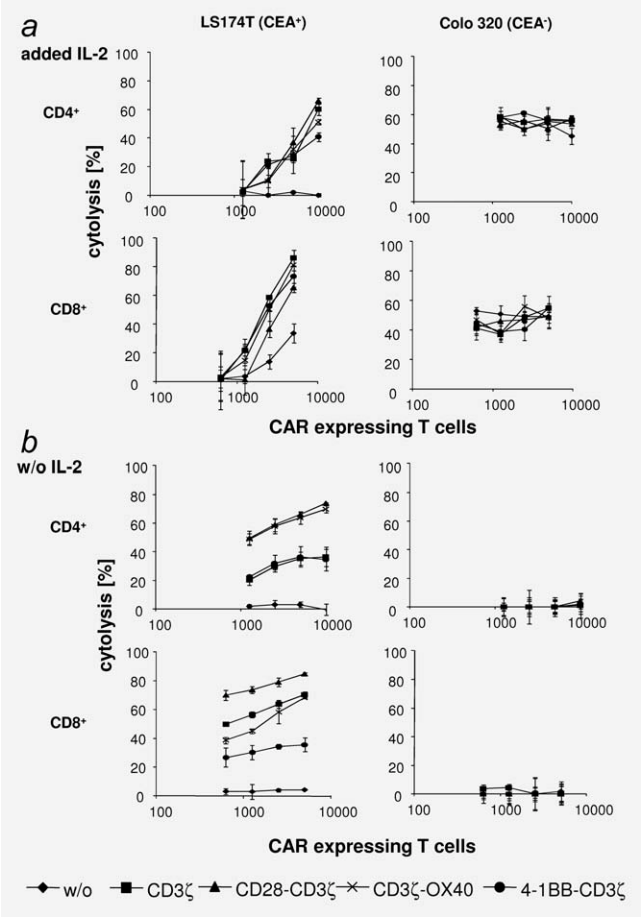


**Figure 3.** The absence of IL-2 reveals different impact of costimulation on redirected cytolysis by $CD4^+$ and $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells were engineered to express anti-CEA CARs with different endodomains domains and coincubated ($1.25 \times 10^3$–$1 \times 10^4$ and $0.625 \times 10^3$–$5 \times 10^3$ CAR T cells/well, respectively) with $CEA^+$ LS174T or $CEA^-$ Colo320 tumor cells (each $2.5 \times 10^4$ cells/well) for 48 hrs in the presence (*a*) or absence (*b*) of added IL-2 (500 U/ml). T cells were adjusted to equal numbers of CAR engineered cells by adding nonmodified T cells of the same donor. For control, T cells in identical numbers, but without CAR were used (nonmodified). The effector: tumor cell ratio was 1:2.5–1:20 ($CD4^+$) and 1:5–1:40 ($CD8^+$), respectively. Cytolysis of tumor cells was determined colorimetrically by a tetrazolium salt based XTT-assay. Numbers represent the mean of triplicates ± SEM.

upon antigen engagement. Our data indicate that combined CD28-OX40 costimulation induces $CD62L^-$ memory T cells and efficiently rescues those cells from AICD.

Since CD28 and OX40 costimulation appears to be complementary we asked whether redirected nonfractionated $CD3^+$ T cells that will most probably used for therapeutic application may benefit from a triple signalling CAR. As summarized in Figure 5*a*, the triple signalling CAR induced substantially higher tumor cell lysis compared to CARs with the CD28-CD3ζ or CD3ζ-OX40 endodomain only. CD28-CD3ζ CAR mediated IFN-γ and IL-2 secretion was furthermore enhanced by additional OX40 signalling (Fig. 5). The data demonstrate the benefit of combining OX40 with CD28-CD3ζ in a combined costimulatory CAR for redirected T cell effector functions. To prove for antitumor activity *in vivo* NIH-III mice lacking T, B and NK cells were transplanted with C15A3 tumor cells modified to express human CEA. To reveal differences in the antitumor activity of anti-CEA CAR redirected T cells, a single dose of engineered T cells was intravenously applied 8 days after tumor inoculation which is obviously not effective to completely prevent tumor growth. T cells with the triple endodomain CAR, however, suppressed tumor growth more significantly than T cells with CD28-CD3ζ CAR alone (Fig. 5*b*). This demonstrates that OX40 signals provided by a combined CAR cannot substitute for CD28 but CAR mediated CD28 costimulation benefits from additional OX40 costimulation in a third generation CAR providing improved antitumor response.

## Discussion

The overall benefit of costimulation in redirecting T cells was previously demonstrated for individual 2nd generation CARs;[7,14,26] the benefit of each costimulatory endodomain remained to be revealed. We here performed a side-by-side comparison of different costimulatory CARs on the individual effector functions of various T cell populations. For clinical application engineered T cells are routinely expanded ex vivo, which requires preactivation, before being infused into the patient; to allow a side-by-side comparison we here prestimulated engineered T cells *via* CD3 in presence of IL-2. This is in some contrast to the physiological situation where T cell costimulatory molecules act in a sequential order. While the "early" costimulatory molecule CD28 is constitutively expressed on most resting T cells, "late" costimulatory molecules like OX40 and 4-1BB occur after the initiation of T cell activation when early signals are down-regulated. In contrast, CARs artificially combine the costimulatory endodomain with the primary TCR signal to deliver both signals to freshly activated T cells. CD28 modulates the primary TCR/CD3ζ signal in a different fashion than the late costimulatory elements OX40 and 4-1BB: CD28 improves the initiation of the T cell immune response and induces IL-2 which is required for this step, while OX40 and 4-1BB amplify and modulate activation of $CD4^+$ and $CD8^+$ T cells to perpetuate the ongoing T cell response without IL-2 induction. Accordingly, CD28-CD3ζ engineered CARs show improved and rapid T cell expansion while CAR cosignalling *via* OX40 and 4-1BB, respectively, does not substantially induce IL-2 or enhance proliferation. On the other hand late costimulation by OX40 signalling induces propagation and survival of $CD62L^-$ effector memory cells most efficiently. In this context it is of major relevance that in presence of added IL-2 the CAR induced cytolytic activity of engineered T cells is not modulated by the individual costimulatory domains and is similar to that of CD3ζ CAR T cells. This is in accordance

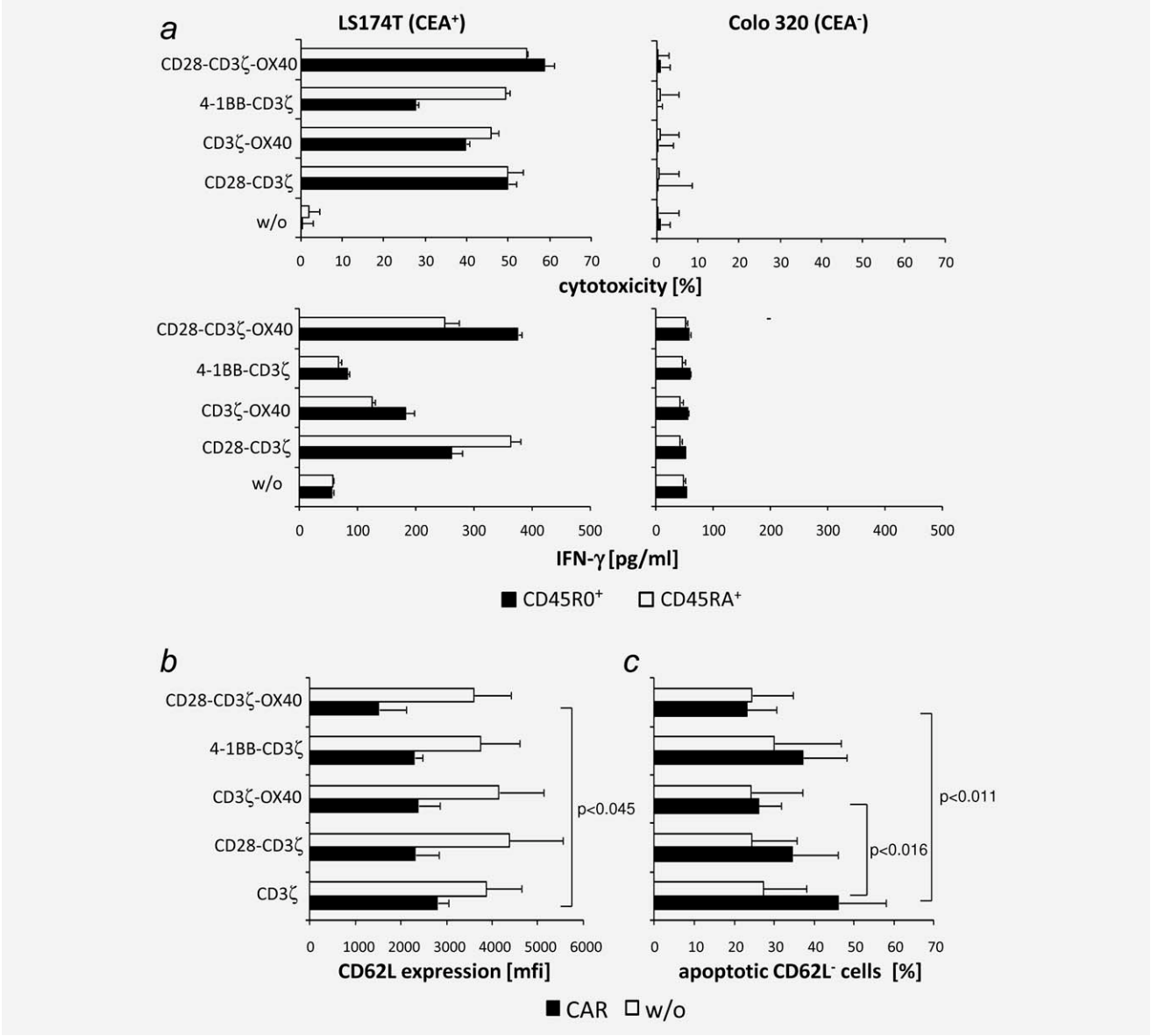


**Figure 4.** Costimulation affects differentially the generation, activation and apoptosis of CAR engineered memory T cells. (*a*) $CD45RA^+CD62L^+$ and $CD45R0^+$ T cells were FACS-sorted and engineered to express anti-CEA CARs with different endodomains domains. Cells were coincubated ($1 \times 10^4$ CAR T cells/well) with $CEA^+$ LS174T or $CEA^-$ Colo320 tumor cells (each $2.5 \times 10^4$ cells/well) for 48 hrs. T cells were adjusted to equal numbers of CAR engineered cells by adding nonmodified T cells of the same donor. The effector: tumor cell ratio was 1:2.5. For control, T cells in identical numbers, but without CAR were used (nonmodified). Viability of tumor cells and IFN-γ secretion was determined as described above. Numbers represent the mean of triplicates + SEM. (*b*) The anti-idiotypic mAb BW2064/399 (0.5 μg/ml) and an anti-mouse antibody (0.25 μg/ml) were added to $CD3^+$ T cells engineered to express CARs with different endodomains ($2 \times 10^5$ T cells/ml). Cells were cultured for 96 hrs, stained with anti-human IgG-FITC and anti-CD62L-PE antibodies, respectively and analysed by flow cytometry. CAR-expressing T cells and nonmodified T cells were gated and the mean fluorescence of CD62L expression determined. Numbers represent the mean of three independent donors + SEM. (*c*) $CD3^+$ T cells expressing CARs with different endodomains were cultured in the presence of the anti-idiotypic mAb BW2064/399 as described for 96 hrs and stained with anti-human IgG-FITC, anti-CD62L, Annexin V-APC and 7-AAD, respectively, analysed by flow cytometry and the number of $CD62L^-$Annexin $V^+$ cells with and without CAR determined. Numbers represent the mean of five independent donors + SEM. *p* values were determined by Student's t test.

to a previous report.[9] In the absence of IL-2, however, the individual costimulatory endodomains differentially impact cytolysis. CD28-CD3ζ and CD3ζ-OX40 most effectively improved target cell lysis whereas 4-1BB did not improve cytolytic activities compared to CD3ζ CAR engineered $CD8^+$ T cells. IFN-γ secretion, however, is similarly increased by

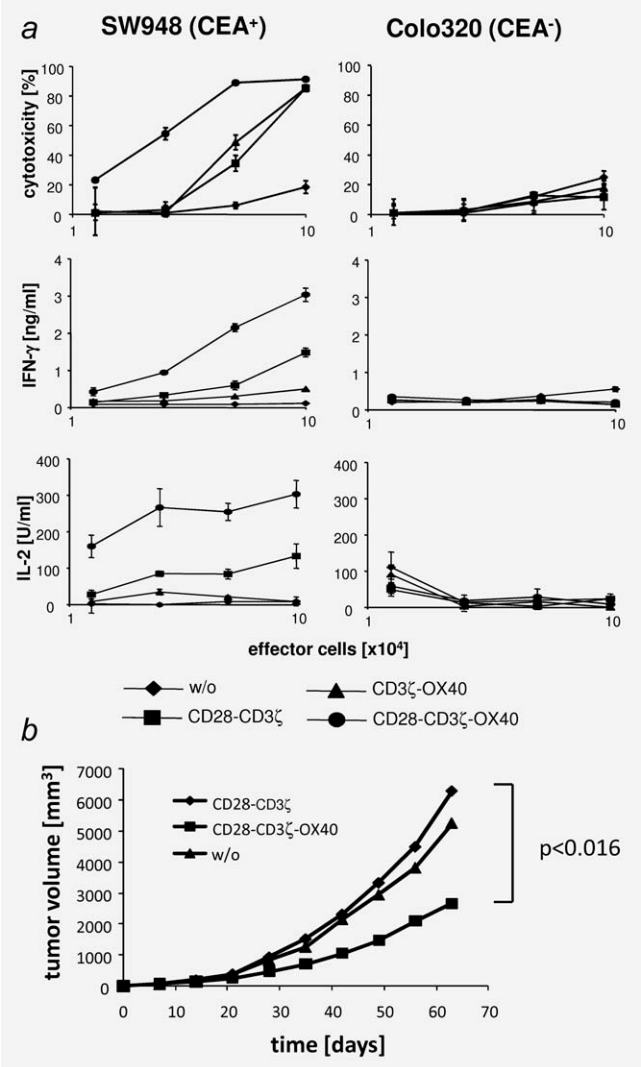


**Figure 5.** OX40 improves CD28-mediated costimulation of unfractionated T cells in a CD28-CD3ζ-OX40 signalling CAR. (*a*) $CD3^+$ T cells were engineered with anti-CEA CARs with either CD28-CD3ζ, CD3ζ-OX40 or CD28-CD3ζ-OX40 endodomains and coincubated ($1.25 \times 10^4$ –$10 \times 10^4$ CAR T cells/well) with $CEA^+$ SW948 or $CEA^-$ Colo320 tumor cells (each $2.5 \times 10^4$ cells/well) for 48 hrs. Cells were adjusted to equal numbers of CAR engineered T cells by adding nonmodified T cells of the same donor. The effector: tumor cell ratio was 4:1–0.5:1. For control, T cells in identical numbers without CAR were used (w/o). Tumor cell lysis was determined and the culture supernatants recorded for IFN-γ and IL-2 secretion, respectively. The assay was performed in triplicates and mean values ± SEM are shown. (*b*) NIH-III mice were transplanted with $CEA^+$ C15A3 tumor cells ($5 \times 10^5$ cells per mouse). At day 9, when the tumor is fairly established, T cells with CEA specific CAR ($5 \times 10^5$ CAR expressing T cells/mouse) and nonmodified T cells for control, respectively, were once intravenously injected (4–7 animals/group). The numbers of injected T cells were adjusted with nontransduced T cells. Tumor growth was recorded every 2–3 days and the volume determined. Mean values are shown; *p* values were determined by Student's t test.

the costimulatory CARs. This reflects the physiological situation which is characterized by a functional dichotomy of late OX40 and 4-1BB costimulation on the T cell response.[27,28] OX40 rather increases the T cell response whereas 4-1BB costimulation can be a negative regulator of some T cell functions.[27] Accordingly, 4-1BB did not show superior to CD28 costimulation with respect to enhancing the cytolytic antitumor attack. 4-1BB, in contrast, is a potent enhancer of clonal $CD8^+$ T cell expansion to amplify the specific T cell response[29,30] which results, when integrated into the 4-1BB-ζ CAR, in enhanced persistence of CAR $CD8^+$ T cells *in vitro* and *in vivo*.[26]

Adoptive immunotherapy by engineered T cells requires tuning of effector functions in a predictive way to induce an optimized antitumor response. A major outcome of our comparative study is that CD28 cosignalling over all improves the T cell effector functions whereas OX40 costimulation, in contrast to 4-1BB, in a combined CAR enforces cytolysis in absence of IL-2 and improves differentiation and survival of memory T cells. This was the rationale to combine CD28 and OX40 in a third generation CAR which improved CD28-mediated effector functions in both naive and memory $CD4^+$ and $CD8^+$ T cells to enforce the cytolytic antitumor attack. CD28-OX40 redirected T cells, in contrast to OX40 CAR T cells, moreover secrete IL-2 sustaining T cell expansion. Taken together the CD28-CD3ζ-OX40 CAR combines the beneficial effector functions induced by early and late costimulation and improves T cell antitumor reactivity in a mouse model without loosing specificity.

Our analysis is of particular clinical relevance since costimulatory CARs are currently explored in phase I trials, some with encouraging preliminary evidence of clinical efficacy.[4,15,17,31] However, two fatal serious adverse events occurred after infusion of T cells with 2nd and 3rd generation CARs.[15,16] In one trial the events were attributed to "on-target off-organ" activation of engineered T cells, potentially due to extensive costimulation, resulting in fatal autoimmunity. Side-by-side comparison of different costimulatory endodomains will help to combine the appropriate costimulatory domains to impact T cell effector functions in a predictive way. Forced CD28 costimulation as it occurs by combined CD28 and 4-1BB costimulation may predestine for a cytokine storm as observed in the NIH trial.[15,16] An alternative approach in providing the costimulatory signal is to express a first-generation CAR without costimulation on virus-specific T cells which receive costimulation by antigen engagement through their native TCR on antigen-presenting cells (APCs). The strategy is supported by a recent trial in which selected Epstein-Barr virus-specific T cells modified with a neuroblastoma associated antigen-specific CAR showed longer *in vivo* persistence than unselected T cells with the same CAR which do not receive costimulation by EBV antigen presenting APCs.[32] Apart from that, it remains unresolved whether costimulation lowers the CAR activation threshold facilitating "off-target" activation of engineered T

cells by engaging cells with low antigen levels. We recently demonstrated that a low affinity CAR can discriminate between tumor cells and normal cells with high and low target antigen expression, respectively, whereas CARs with high binding affinity do not.[33,34] Altered activation threshold may result in loss of tumor selectivity and, finally, in T cell activation by physiological antigen levels leading to "on-target off-organ" activation.

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