Antigen-specific T-cell activation independently of the MHC: chimeric antigen receptor-redirected T cells

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Adoptive T-cell therapy has recently shown promise in initiating a lasting anti-tumor response with spectacular therapeutic success in some cases. Specific T-cell therapy, however, is limited since a number of cancer cells are not recognized by T cells due to various mechanisms including the limited availability of tumor-specific T cells and deficiencies in antigen processing or major histocompatibility complex (MHC) expression of cancer cells. To make adoptive cell therapy applicable for the broad variety of cancer entities, patient’s T cells are engineered ex vivo with pre-defined specificity by a recombinant chimeric antigen receptor (CAR) which consists in the extracellular part of an antibody-derived domain for binding with a “tumor-associated antigen” and in the intracellular part of a T-cell receptor (TCR)-derived signaling moiety for T-cell activation. The specificity of CAR-mediated T-cell recognition is defined by the antibody domain, is independent of MHC presentation and can be extended to any target for which an antibody is available. We discuss the advantages and limitations of MHC-independent T-cell targeting by an engineered CAR in comparison to TCR modified T cells and the impact of the CAR activation threshold on redirected T-cell activation. Finally we review most significant progress recently made in early stage clinical trials to treat cancer.

Keywords: chimeric antigen receptor, T-cell receptor, adoptive cell therapy, antibody, antigen-presenting cell

TUMOR-SPECIFIC T CELLS FOR ADOPTIVE CELL THERAPY

Experimental and clinical evidences indicate that the immune system is capable of identifying and destroying cancer cells in a specific fashion; tumor-infiltrating lymphocytes (TILs), expanded ex vivo and re-administered to the patient, exhibit a powerful anti-tumor response and induce an acute inflammatory reaction which attracts a second, antigen-independent wave of immune cell invasion into the same lesion. Adoptive TIL therapy has shown some success in the treatment of chemotherapy resistant melanoma, even in advanced stages of the disease (1). The procedure, however, is technically challenging since it involves the isolation of T cells from melanoma biopsies and their amplification ex vivo to therapeutic numbers; not every melanoma biopsy provides TILs and allows sufficient expansion. Moreover, the range of TIL bearing malignant lesions, apart from melanoma, is small limiting the application of the strategy to a broad variety of cancer entities.

The implementation of redirected T cells in cancer therapy is based on engineering T cells with pre-defined specificity to target virtually every cancer cell and on the production of engineered T cells in therapeutic numbers. To provide specificity peripheral blood T lymphocytes were ex vivo engineered with a recombinant T-cell receptor (TCR) of known specificity which recognizes cognate peptide-loaded major histocompatibility complexes (pMHC) of a so-called tumor-associated antigen (TAA). Such TCR engineered T cells showed promise in clinical trials (1, 2). Some conceptual deficits, however, limit the broad application of TCR engineered T cells including the HLA restriction, the dependency on adequate major histocompatibility complex (MHC) expression by tumor cells, the limited number of peptide-MHC complexes identified so far which can be used for screening and the potential mispairing with the endogenous TCR producing novel, unforeseen specificities which might induce severe auto-immunity after adoptive transfer (3).

Whereas the T-cell therapy using ex vivo expanded patients’ TILs leads to significant clinical effect in patients with metastatic melanoma (1), difficulties are arising when engineering T cells with a recombinant TCR, in particular when non-immunogenic tumor-associated self-antigens are targeted (4). In a pre-clinical tumor model the treatment with TCR engineered T cells alone was without effect whereas the combination of vaccination with TCR modified T-cell transfer was synergistic.

In this situation, Zelig Eshhar, Weizmann Institute, proposed to redirect T cells by a recombinant receptor molecule, a chimeric antigen receptor (CAR), which in the extracellular part consists of an antibody with pre-defined binding specificity to a broad variety of targets and in the intracellular part of a T-cell activation domain (5). Such CAR modified T cells became generally known as “T-bodies” (5). In contrast to the TCR, the archetypical CAR is composed of one polypeptide chain (Figure 1). The binding domain is mostly a recombinant antibody in the single chain format consisting of the variable domain of the heavy and light chain linked by a short synthetic peptide (scFv). The extracellular part of a receptor molecule, for instance the NK cell-derived NKG2D ligands (6) and the surface NKp-30 (7) receptor, were also successfully integrated into the conventional CAR structure instead of the classical antibody-derived binding domain. The CAR intracellular
FIGURE 1 | Modular composition of the chimeric antigen receptor (CAR) compared to the T-cell receptor (TCR). The TCR binds to cognate peptide-loaded MHC (pMHC) by the TCR α and β chains, forms the immunological synapse by clustering accessory components including CD3ζ and CD28, and initiates the downstream signaling pathway for T-cell activation through phosphorylation of the CD3ζ ITAM motives. The CAR, in contrast, is composed of one polypeptide chain; the extracellular single chain fragment of variable region (scFv) antibody domain binds to the target antigen in a MHC-independent fashion. Upon CAR clustering, the intracellular CD3ζ chain, with or without costimulation through members of the CD28 family, initiates the downstream signaling for T-cell activation. Co-receptors may modulate CAR activity. In contrast to a first generation (1°) CAR, second (2°), and third (3°) generation CARs harbor in addition one or more costimulatory moieties in their intracellular part.

signaling domain is preferentially derived from the CD3 ζ-chain of the TCR/CD3 complex or, alternatively, from the γ-chain of the high affinity IgE Fc receptor-I (FcεRI). Binding with cognate antigen on the tumor cell surface results in CAR clustering on the engineered T-cell with the consequence that the immunoreceptor tyrosine-based activation motifs (ITAMs) of the signaling moiety become phosphorylated and initiate a downstream signaling cascade which finally induces T-cell amplification, cytokine secretion, and cytolytic activity of the CAR T-cell toward the cognate tumor cell.

CAR T cells overcome some limitations of the TCR-based strategy by targeting cells in an MHC- and dendritic cell (DC)-independent fashion. The properties of a CAR and TCR differ substantially in respect to target binding and subsequent T-cell activation. For instance these are in particular the TCR avidity for a given pMHC, the number of MHC molecules, the availability of co-receptors and the moderate TCR affinity for the cognate MHC peptide complex compared to the high affinity of the antibody in a CAR. On the one hand the use of antibody-based CAR T cells enables the targeting of antigens of different composition and structure such as peptides, carbohydrates, or inorganic compounds, and on the other hand, the TCR recognizes peptide antigens exclusively in the context of the particular MHC and thereby faces a limited variability. TCRs are moreover inherently cross-reactive toward endogenous antigens (8). The potential CAR targets thereby far outnumber their MHC-presented counterparts which can be recognized by TCR modified T cells. In this report we review some advantages and limitations of MHC-independent target recognition by CAR T cells and review most significant progress recently made in early stage clinical trials to treat cancer.

THE CAR STRATEGY: ANTIBODY-MEDIATED, MHC-INDEPENDENT ANTIGEN RECOGNITION BY ENGINEERED T CELLS

The design of the antibody-derived CAR differs in several major features from the TCR which physiologically mediates target recognition by T cells (Figure 1). By using an antibody for binding, T cells gain antibody-defined specificity: (i) T cells without CAR or equipped with a CAR of different specificity are not activated by the target cells; (ii) the cognate antigen needs to be on the cell surface to trigger CAR T-cell activation, intracellular antigens are not recognized by the CAR; and (iii) CAR-mediated T-cell activation can be specifically blocked by an antibody directed toward the CAR binding domain (9).

As a consequence of using an antibody for binding, CARs can redirect T cells toward targets of any chemical composition or conformation as far as an antibody is available. Indeed, CARs were engineered which target T cells toward carbohydrate antigens like CA19-9 (10–12). The TCR, in contrast, is restricted to the recognition of specific peptides presented by the particular MHC. Antibody-mediated target recognition by CARs, however, does not exclude targeting MHC presented antigens. Using an antibody which recognizes NY-Eso-1 peptide (157–165) in the context of HLA-A*0201, Stewart-Jones et al. engineered a CAR recognizing the MHC presented peptide analog SLLMWITQV (13). The antibody domain used for CAR targeting was optimized by
modification of the individual amino acids which interact between the antibody and the peptide providing an antibody with 20-fold improved affinity, exceeding the affinity of the respective TCR by about 1000-fold. The high affinity antibody when engineered as recombinant CAR on T cells conferred specific killing of HLA-A*0201/NY-ESO-1<sub>157-165</sub> target cells as do T cells modified with the corresponding TCR.

**BOTH CD8<sup>+</sup> AND CD4<sup>+</sup> T CELLS CAN BE REDIRECTED IN A MHC-INDEPENDENT FASHION**

By bypassing MHC class I and class II restriction by an antibody-derived binding domain, CAR engineered T cells of both CD8<sup>+</sup> and CD4<sup>+</sup> subsets can be recruited for redirected target cell recognition (9, 14, 15). Equipped with a CAR, CD4<sup>+</sup> T cells showed as cytolytic as do CD8<sup>+</sup> T cells toward CAR-defined target cells. While human CD8<sup>+</sup> T cells predominantly use two pathways in executing cytolysis, i.e., perforin and granzyme exocytosis and to some extend death receptor signaling via Fas/Fas-ligand (Fas-L) or TNF/TNF-receptor (TNF-R) (16), the mechanism of CAR-mediated lysis by redirected CD4<sup>+</sup> T cells was a matter of debate for some time. Investigations utilizing mutant and knock-out mice report that non-modified human CD4<sup>+</sup> T cells lyse TNF-resistant target cells. The observation is in accordance to a perforin mediated process whereas death receptor signaling induces cytolysis of the target cells. In contrast to murine cells, CAR redirected human T cells mediate cytolysis predominantly by granule exocytosis and not by the Fas/Fas-L system (14). The extent in redirected cytolysis correlates with the amount of cytolytic effector molecules; CAR CD4<sup>+</sup> T cells which harbor about half amounts of perforin and granzyme B required about twice the number of effector cells to achieve the same cytolytic efficacy compared to CAR redirected CD8<sup>+</sup> T cells. CD4<sup>+</sup> T cells rapidly lyse their targets in a short term in vitro cytotoxicity assay as do engineered CD8<sup>+</sup> T cells which is in accordance to a perforin mediated process whereas death receptor signaling induces cytolysis of the delayed type. CAR engineered CD4<sup>+</sup> T cells lyse both Fas- and TNF-resistant target cells. The observation is in accordance to a report that non-modified human CD4<sup>+</sup> T cells execute cytolysis predominantly by granule exocytosis and not by the Fas/Fas-L system (20). In contrast to CAR modified cells, CD4<sup>+</sup> T cells engineered with a MHC class I restricted TCR were reported to lyse exclusively those target cells that are susceptible for death receptor signaling (21). Both studies, however, differ in several issues including the use of a MHC class I-dependent TCR vs. a MHC-independent CAR for redirecting T cells. As a consequence for adoptive cell therapy, CAR engineered patient’s CD4<sup>+</sup> T cells can efficiently provide help upon CAR-mediated activation and can eliminate tumor cells in a direct fashion and independently from MHC class II restriction.

**“AFFINITY CEILING” OF ANTIBODY-MEDIATED CAR T-CELL ACTIVATION**

CAR-mediated T-cell activation is thought to depend on and to increase with the binding affinity to cognate antigen; however, the interaction is likely of higher affinity than binding of the physiological TCR to peptide loaded MHC. Two studies addressed in detail the situation (22, 23). The Chmielewski study (22) made use of a panel of CARs of the same backbone and same epitope specificity but with different binding affinities. The affinities were in the broad range of $10^{-7}$–$10^{-11}$ M and were obtained upon mutation of the parental antibody while preserving the binding specificity. CAR T-cell activation correlated with the affinity of the antibody binding domain when the target antigen is present in an immobilized fashion coated onto surfaces. In contrast, when the cognate antigen is present on the surface of the target cell, the CAR-mediated cytotoxic effect on target cells and the release of IFN-γ and IL-2 did not increase with the binding affinity above threshold, which was in that example about $K_D = 10^{-8}$ M. While the conditions that define the activation threshold, however, are so far not understood on the molecular level, the study makes clear that furthermore increase in affinity above threshold does not improve the redirected T-cell attack toward target cells but may result in antigen-independent T-cell activation.

The Hudecek study (23) evaluated scFv’s of different affinities and CARs with different backbones with respect to their efficacy in redirecting T cells. The CAR with higher binding affinity conferred maximum T-cell activation with respect to cytokine release and proliferation compared to the CAR with lower affinity. The redirected cytolytic activity, however, was nearly the same. Although the study confirms previous observations that increase in affinity does not necessarily improve all T-cell effector functions, the comparison of the CARs is alleviated in that two binding domains targeting different epitopes, although in the same domain of the targeted ROR1 molecule, were used.

A recent study explored the situation for TCR modified T cells to determine the affinity threshold with respect to the optimal balance between anti-tumor efficacy and auto-immunity (24). Similar as for CAR modified T cells, TCR redirected anti-tumor activity shows a plateau at a defined TCR affinity, likely due to diminished contribution of TCR affinity to avidity above the threshold. Additional differences probably lie in the ability of different affinity interactions eliciting different effector functions at different antigen concentrations. The observations are in accordance to the CAR situation and strongly suggest that a relatively low affinity threshold is mandatory to avoid self-damage; that high affinity TCRs do not necessarily improve efficacy given the close relationship between anti-tumor activity and auto-immunity.

**THE POSITION OF THE TARGETED EPITOPE MATTERS: MEMBRANE PROXIMAL VS. DISTAL EPITOPES AS TARGETS FOR CAR ENGINEERED T CELLS**

By using an antibody for targeting, CAR engineered T cells can be redirected toward a variety of epitopes of the same antigen as far as the epitope is accessible to the respective antibody. The various epitopes of a given membrane-bound molecule, however, are not equally good targets for efficient T-cell activation. This was shown when membrane distal and proximal epitopes of the same membrane-bound molecule were targeted by CARs. For instance, when targeting carcinoembryonic antigen (CEA) expressed on gastrointestinal carcinoma cells, a higher degree of T-cell activation was obtained when epitopes closer to the cell membrane
were targeted (25). The epitope itself is not the cause of the phenomenon since the isolated, solid phase bound CEA induces T-cell activation independently of the epitope position but dependent of the antibody binding affinity. The distal epitope when expressed in a more membrane proximal position activated CAR T cells with higher efficiency than in the distal position indicating that the position effect of the targeted epitope has, at least in this example, prominent impact on T-cell activation. The accessibility of the epitope for binding additionally impacts the efficiency in CAR-mediated T-cell activation; in the case of CEA targeting, however, the epitope accessibility seems not to be limiting because the distal epitope, which is thought to be more accessible than the more proximal epitope, is superior in binding but less capable in mediating CAR activation. This is in accordance with another report which analyzed the impact of the position of the target epitope on the structural requirements of the CAR (26). Basically the same observation was made when targeting B-cell lymphoma associated CD22 by CAR T cells (27, 28). To explain the observation, a kinetic-segregation model, initially proposed by Davis and van der Merwe (29) and hypothesized also to occur in CAR engineered T cells, is currently favored. The model suggests that targeting membrane distal epitopes increases the size of the CAR-ligand clusters, which in turn permits large phosphatase molecules such as CD45 to enter the synapse and to repress TCR signaling which is less the case when targeting the membrane proximal epitope. The model, however, does not exclude that accessibility and flexibility of the targeted epitope itself may also contribute to some extent.

The best suitable target epitope and binding affinity for optimal CAR T-cell activation remains so far to be empirically evaluated in each case. This is of major relevance given the broad variety of potential targets for a CAR in contrast to the TCR, the specificity of which is restricted to MHC presented peptides.

CD28 costimulation provided by second generation CARs: major differences to stimulation through APCs

First generation CARs provide exclusively only one signaling domain such as CD3ζ-ε, -γ, or FcεRI γ-chain to initiate redirected activation of pre-stimulated T cells upon CAR binding with antigen. To prevent engineered T cells undergoing activation-induced cell death and anergy, CD28 costimulation simultaneously to CD3ζ signaling is required. CD28 is the prototype of a family of costimulatory molecules that is physiologically engaged on T cells by binding to the respective ligands on antigen-presenting cells (APCs). The agonistic CD28 ligands B7.1 (CD80) and B7.2 (CD86), physiologically expressed on APCs, are missing on most cancer cells with the consequence that the CD3ζ CAR upon binding to cancer cells does not provide the costimulation required for full activation. The limitation was overcome by linking the intracellular signaling domain of CD28 to CD3ζ in one polyepitope chain of the same CAR (30–33). In this so-called “second generation” CAR the artificial fusion of the CD28 and CD3ζ signaling domains facilitates Lck-mediated CD28 phosphorylation that binds and activates phosphatidylinositol 3-kinase for downstream signaling, resulting in full T-cell activation and IL-2 release. Other costimulatory molecules of the TNF-receptor family including 4-1BB (CD137) and OX40 (CD134) can also be integrated into the same CD3ζ CAR molecule or combined with CD28 in a “third generation” CAR. This type of CAR has the advantage that T-cell costimulation occurs in an APC-independent fashion and is accompanied by suppressing inhibitory and/or strengthening stimulatory signals, each costimulatory signal modulating the T-cell effector function in a specific fashion (34). CD28 costimulation is integrated into most currently used CARs because CD28 sustains survival and prolongs polyclonal expansion of engineered T cells without the need of B7–CD28 engagement (35). CD28 co-signaling induces IL-2 that is used in an autocrine fashion by redirected T cells to increase their amplification (36). CD28-CD3ζ CAR signaling moreover counteracts transforming growth factor-β1 (TGF-β1)-mediated repression in T-cell amplification (37). Both prevention from AICD and increased amplification produce prolonged T-cell persistence and an improved anti-tumor attack. Other beneficial properties and some draw backs were recently discussed in more detail (38). Taking advantage of CD28 of other costimulatory moieties like 4-1BB, second generation CARs are currently being explored in early phase clinical trials.

The impact of CAR provided CD28 costimulation on the threshold of antigen-dependent, APC-independent T-cell activation was addressed by using a panel of CARs targeting T cells in the absence of agonistic CD28 ligands (39). CAR provided CD28 costimulation increases cytokine secretion but does not impact the activation threshold or “affinity ceiling,” above which an increase in affinity does not increase T-cell activation. CD28 did not increase sensitivity toward target cells with intermediate or low densities of the respective target antigen. Additional CD28–B7 engagement did not further alter CD28-CD3ζ CAR-mediated T-cell activation. In the presence of a CD3ζ CAR, however, B7 engagement increased IFN-γ secretion indicating that the physiological CD28 costimulation through APCs cooperates with CAR-driven T-cell activation.

Another aspect concerns the fact that most target antigens for adoptive immunotherapy are not exclusively expressed on tumor cells but broadly present on a variety of healthy tissues, although frequently at lower levels. Since CAR provided CD28 costimulation in the absence of APCs does not alter the activation threshold, costimulation does not impact the selectivity of a redirected T-cell attack in peripheral tissues, does not lower the affinity ceiling and antigen-dependent threshold of CAR redirected T cells and thereby protects healthy cells with physiological levels of antigen from a T-cell attack.

With respect to costimulation, there are some fundamental differences in the physiologic vs. CAR-mediated T-cell activation. To induce full T-cell activation, the peptide loaded MHC has to interact with the TCR in a form that allows the appropriate synapse formation on the T-cell and to recruit costimulatory molecules which increase stability during early stages in this process. CD28 recruitment by B7 engagement on APCs sustains formation of the immunological synapse which is accompanied by lower amounts of antigen required for T-cell activation. During T-cell–APC interactions, in particular during early activation events, CD28–B7 binding potentiates synapse formation by increasing the density of the synapse components through approximation of the interacting membranes (40); increased clustering integrates the TCR with costimulatory signaling which can compensate for weak TCR
signals (41). The TCR binding threshold exhibits a sharp cutoff between full T-cell activity and no activity; the activation efficiency correlates with the TCR binding to the cognate peptide–MHC on APCs (42). Optimal CD28 costimulation occurs upon high-avidity engagement of dimeric B7.1, followed by dimer dissociation, CD28 down-regulation, and B7.1 internalization (43). CD28-B7 interactions with APCs sustain synapse formation which facilitates T-cell signaling upon low affinity target engagement depending on the extend of supra-molecular clustering (44). This mechanism is in contrast to CD28 CAR-mediated T-cell activation, in particular, the avidity of CAR binding is generally higher than of physiological TCR–MHC interactions. Whether the CAR synapse is formed in the same way as the TCR recruits additional components is so far not resolved. There are, however, some cooperative interactions between the CAR and downstream signaling molecules since additional B7.1-CD28 costimulation improves cytokine secretion initiated by CAR signaling.

**CAR BASED ADOPTIVE CELL THERAPY GAINED SUBSTANTIAL SUCCESS IN RECENT EARLY PHASE TRIALS**

Adoptive cell therapy with CAR engineered T cells is currently being evaluated in a number of early phase trials, some of them initiated by CAR signaling.

**Table 1 | Recent adoptive cell therapy trials using CAR engineered T cells.**

| Target antigen | Disease | CAR signaling domain | Clinical Trial.gov identifier | Clinical center |
|----------------|---------|----------------------|------------------------------|-----------------|
| CD19           | B-CLL   | CD28-CD3ζ            | NCT00466531                  | MSKCC           |
| CD19           | B-ALL   | CD28-CD3ζ            | NCT01044069                  | MSKCC           |
| CD19           | Leukemia | CD28-CD3ζ             | NCT01416974                  | MSKCC           |
| CD19           | Leukemia/lymphoma | CD28-CD3ζ             | NCT00924326                  | NCI             |
| CD19           | Leukemia/lymphoma | CD28-CD3ζ             | NCT01067294                  | NCI             |
| CD19           | Leukemia/lymphoma | CD28-CD3ζ vs. CD3ζ   | NCT0086391                  | BCM             |
| CD19           | B-NHL/CLL | CD28-CD3ζ vs. CD3ζ   | NCT00802870                  | BCM             |
| CD19           | Advanced B-NHL/CLL | CD28-CD3ζ vs. CD3ζ | NCT0709033                  | BCM             |
| CD19           | ALL post-HSCT | CD28-CD3ζ          | NCT00840853                  | BCM             |
| CD19           | Leukemia/lymphoma | CD137-CD3ζ            | NCT01029366                  | UP              |
| CD19           | B-lymphoid malignancies | CD28-CD3ζ          | NCT00968760                  | MDACC           |
| CD19           | B-lineage malignancies | CD28-CD3ζ         | NCT01362452                  | MDACC           |
| CD20           | Mante cell lymphoma/indolent B-NHL | CD28-CD137-CD3ζ | NCT00621452                  | FHCRC           |
| PMSA           | Prostate cancer | CD28-CD3ζ             | NCT0140373                  | MSKCC           |
| CEA            | Breast cancer | CD28-CD3ζ             | NCT00673829                  | RWMC            |
| CEA            | Colorectal cancer | CD28-CD3ζ          | NCT00673322                  | RWMC            |
| Her2/neu       | Lung cancer | CD28-CD3ζ             | NCT00889954                  | BCM             |
| Her2/neu       | Osteosarcoma | CD28-CD3ζ            | NCT00902044                  | BCM             |
| Her2/neu       | Glioblastoma | CD28-CD3ζ            | NCT01090995                  | BCM             |
| Kappa light chain | B-NHL and B-CLL | CD28-CD3ζ vs. CD3ζ | NCT00881920                  | BCM             |

MSKCC, Memorial Sloan-Kettering Cancer Center; NCI, National Cancer Institute; BCM, Baylor College of Medicine; RWMC, Roger Williams Medical Center; UP, University of Pennsylvania; MDACC, M.D. Anderson Cancer Center; FHCRC, Fred Hutchinson Cancer Research Center.
CD19⁺ CLL patient with CD28–CD3ζ CAR T cells was attributed to an extravasation of a latent bacterial infection subsequent to lymphodepletion (51). Despite the observed severe adverse events, MHC-independent targeting of cancer cells by CAR modified T cells showed promise in controlling CD19⁺ leukemia in the long-term; currently initiated and future trials will address whether solid cancer lesions will also successfully be targeted and controlled by CAR T cells.

ACKNOWLEDGMENTS

Work in the author’s laboratory was supported by the Else Kröner-Fresenius Stiftung, Wilhelm Sander-Stiftung, the Deutsche Krebshilfe, the European Union (European Regional Development Fund – Investing in your future), the German federal state North Rhine-Westphalia (NRW), and the Fortune program of the Medical Faculty of the University of Cologne. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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Chmielewski et al. Chimeric antigen receptor-redirected T cells
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 September 2013; accepted: 29 October 2013; published online: 11 November 2013.

Citation: Chmielowski M, Hombach AA and Abken H (2013) Antigen-specific T-cell activation independently of the MHC: chimeric antigen receptor-redirected T cells. Front. Immunol. 4:371. doi: 10.3389/fimmu.2013.00371
This article was submitted to Tumor Immunity, a section of the journal Frontiers in Immunology.

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