An MHC-restricted antibody-based chimeric antigen receptor requires TCR-like affinity to maintain antigen specificity

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Chimeric antigen receptors (CARs) are synthetic receptors that usually redirect T cells to surface antigens independent of human leukocyte antigen (HLA). Here, we investigated a T cell receptor-like CAR based on an antibody that recognizes HLA-A*0201 presenting a peptide epitope derived from the cancer-testis antigen NY-ESO-1. We hypothesized that this CAR would efficiently redirect transduced T cells in an HLA-restricted, antigen-specific manner. However, we found that despite the specificity of the soluble Fab, the same antibody in the form of a CAR caused moderate lysis of HLA-A2 expressing targets independent of antigen owing to T cell avidity. We hypothesized that lowering the affinity of the CAR for HLA-A2 would improve its specificity. We undertook a rational approach of mutating residues that, in the crystal structure, were predicted to stabilize binding to HLA-A2. We found that one mutation (DN) lowered the affinity of the Fab to T cell receptor-range and restored the epitope specificity of the CAR. DN CART cells lysed native tumor targets in vitro, and, in a xenogeneic mouse model implanted with two human melanoma lines (A2+/NYESO+ and A2+/NYESO−), DN CART cells specifically migrated to, and delayed progression of, only the HLA-A2+/NY-ESO-1+ melanoma. Thus, although maintaining MHC-restricted antigen specificity required T cell receptor-like affinity that decreased potency, there is exciting potential for CARs to expand their repertoire to include a broad range of intracellular antigens.

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INTRODUCTION

Chimeric antigen receptors (CARs) are high-affinity ligand or single-chain variable fragment (scFv)-based receptors used to redirect T cells to recognize and lyse tumor cell targets. CD19-specific CAR T cells are emerging as a powerful clinical therapy, showing impressive outcomes in non-Hodgkin lymphoma, chronic lymphocytic leukemia, and acute lymphoblastic leukemia. CARs are generally limited to native surface antigen targets. However, most of the known “high-priority” tumor antigens ranked by a National Cancer Institute panel, which includes universal tumor antigens and cancer-testis antigens, and most neoantigens, are intracellular proteins and are thus only subject to immune surveillance as cell surface peptides borne by MHC molecules. Expanding the repertoire of CARs to target intracellular antigens would open new avenues for cancer immunotherapy.

The T cell receptor (TCR) is the natural biologic receptor employed by the immune system for surveillance of cytoplasmic antigens. TCRs are typically low-affinity receptors (micromolar kD) that bind to peptide epitopes in the context of host MHC molecules. Several groups have explored retroviral transduction of native TCRs with the goal of redirecting T cells to intracellular antigens. However, these transduced T cells have the potential to express four different T cell receptors (native-alpha/beta, transduced alpha/beta, and native/transduced heterodimers), which is problematic for two crucial reasons: (i) the native/transduced heterodimers have unknown specificity and potential autoimmune consequences, and (ii) there is dilution of the signal transduction apparatus, since the availability of CD3 complex molecules is limiting. Nevertheless, this approach has been applied in early clinical studies without mispairing-related adverse events. In an effort to resolve the potential mispairing issues, some investigators have also designed single-chain TCRs.

Immunoglobulin structures share structural homology with TCRs and may mimic TCR recognition of HLA/peptide complexes. CARs incorporating such recognition elements would be advantageous in that they do not directly compete with the native TCR, and would further provide, in the case of second generation CARs, supportive costimulation to the transduced T cells. Phage display libraries have enabled the rapid isolation of human Fab fragments highly specific to peptide/MHC molecules. In early attempts, these types of antibodies were found to derive most of their affinity from binding to the MHC alpha helix, in a conformation quite different from the binding footprint of TCRs. More recently, a high-affinity Fab
Directed mutations based on the crystal structure of the T1 scFv specifically reduce binding to HLA-A2.

Based on the crystal structure of the T1 Fab binding to HLA-A2 presenting NY-ESO-1\textsubscript{157-165} the amino acid residues in the light chain of the T1 scFv at positions D53 and Y34 were predicted to be essential candidates in stabilizing the binding of the T1 scFv to the HLA A2 alpha helix (Figure 2a). Breaking the salt bridge at D53 was predicted to have a significant impact on binding. Mutating this residue to an asparagine (N) would preserve the steric properties but reduce the salt bridge between the aspartic acid (D53) residue and the basic arginine residue (R65) of MHC. The Y34 ring forms part of an aromatic cluster, while the OH group of tyrosine (Y) hydrogen-bonds to the carbonyl group (C0) at MHC R65. Mutation of this Y34 to a phenylalanine (F) would preserve the aromatic cluster but not maintain the hydrogen bonding. Using a panel of linkers in the T1-28z retroviral construct sequence, we made the D53N and Y34F mutations alone and in combination, expecting to break one salt bridge and decrease hydrogen bonding while preserving the steric properties important for the stability of the complex. A mutation in the heavy chain of the T1 scFv, at the K65 position, was predicted to have a smaller impact on affinity because it is largely solvent-exposed. This residue was mutated to T to retain some of the Ca/Cb stalk that is packed against the CDR2 Y60 in the heavy chain. This mutation was evaluated separately for technical ease of generating the mutants.

T cells transduced with the T1-28z CAR incorporating the light chain mutations DN, YF, or both (DNYF) were evaluated for pentamer binding by fluorescence-activated cell sorting (FACS) (Figure 2b) and for cytotoxicity against peptide-pulsed T2 cells (Figure 2c). Based on the mean fluorescence intensity of pentamer binding under the same conditions, it was clear that the DN mutation had a significant impact in lowering the affinity of the T1 CAR (Figure 2b). The YF mutation had no significant impact, while the DNYF mutant had a moderate effect on pentamer binding. *In vitro* cytotoxicity assays against T2 cells pulsed with either NY-ESO-1 or flu peptide revealed distinct separation of the curves with the DN-transduced T cells, with preserved lysis of antigen-bearing targets. The YF mutation had no discernable effect in this *in vitro* functional assay. The heavy chain mutation K65T alone had no significant effect on pentamer binding or cytotoxicity, and did not appear to have additive effect when combined with the DN mutation (DNKT) (data not shown). On these data, the DN mutant version of the T1-28z CAR (DN/28z) became our leading candidate. The K65T mutation had negligible impact compared with the DN mutation (Supplementary Figure S1).

BIACore measurement of the DN mutation in T1 reveals significant decrease in affinity.

To measure the affinity of the DN mutant directly, we generated Fabs with the DN mutation and performed surface plasma resonance (BIACore). Two versions of the NY-ESO-1 peptide were examined (9C and 9V), because the naturally occurring cysteine (C) at the ninth position in the peptide epitope can sometimes form disulfide bonds that interfere with the binding measurements of interest. The binding of DN Fab to the 9C and 9V peptides was measured over a range of five different concentrations; the kD was calculated at 672 nmol/l (9C) and 715 nmol/l (9V) (Figure 3). The kD of the parental T1 Fab was 2 nmol/l (9C) – 4 nmol/l (9V). Neither Fab bound to flu peptide at all (flat line, data not shown). In a study comparing BIACore affinities of antiviral TCRs (A2 Tax, A24 EBV, B27 flu, B8 EBNA, and A2 flu) to the tumor-associated antigen TCRs (A2 mel, A2 gp100, and A2 telomerase), the average kD of a viral antigen TCR was 9 ± 7 µmol/l,
independent lysis observed at high E:T ratios previously. In con-



bindung to CD80, ICAM, and LFA3). We noted that T1-transduced



whereas the average Kd of Class I-restricted tumor-associated anti-
gen-specific TCRs was 21 ± 12 μmol/l.26 Specifically, the 1G4 HLA-A2/
NY-ESO-1 specific TCR is in the 6–13 μmol/l range, which is slightly
higher than the published BIacore studies of the affinity of the JM22
TCR specific for HLA-A2/flu.27 Thus, the antigen-recognition region
of the DN Fab has an affinity that is significantly lower than a high-
affinity antibody, but slightly higher than naturally occurring TCRs
specific for viral antigens (Table 1).



The DN mutation restores specificity to antigen in transduced
T cells



Figure 1 T1/28z CAR construct. (a) SFG vector indicating LTRs, packaging signal (ψ), splice donor and splice acceptor sites, leader sequence, single-chain variable fragment of the T1 (HLA-A2/NYESO1-specific) antibody fused to human CD28 and human CD3zeta signaling domains. The ires-hrGFP domain is 3′. (b) Primary human T cells 5 days after transduction with T1/28z retroviral vector, stained with HLA-A2/NYESO pentamer. T cells are gated on FSC/ SSC only. Chromium release cytotoxicity assay using T1/28z-transduced T cells as effectors against (c) T2 cells pulsed with 10 μg/ml of either NYESO1 or flu peptide. E:T ratios normalized to pentamer+ cells or (d) human melanoma lines SK Mel 37, SK Mel 23, or SK Mel 52. GFP, green fluorescent protein; E:T, effector:target.



Next, we tested the parental T1-28z CAR and DN-28z CAR T cells for their ability to lyse target cells in an antigen-concentration-dependent manner. We found that T1-28z CAR T cells lysed NY-ESO-1-peptide pulsed T2 cells independent of peptide concentration (ranging from 0 to 10 μg/ml, Figure 4a), confirming the peptide-independent lysis observed at high E:T ratios previously. In con-



dependent manner, indicating restored antigen specificity with the lower affinity receptor (Figure 4b). Because the potential clinical application of these A2/NY-ESO-1 CARs only applies to patients who have the HLA-A2 genotype, we also examined the growth of the T1 and D53N CARs when transduced into T cells from an individual with the HLA-A*0201 genotype. The CAR T cells were rested for a week after transduction and then stimulated in vitro with mouse-fibroblast-based artificial APCs28 transduced with HLA-A*0201, β2-microglobulin, and full-length NY-ESO-1 (in addition to CD80, ICAM, and LFA3). We noted that T1-transduced T cells expanded less than DN-CAR T cells over 5 days, suggesting ongoing fratricide in the HLA-A2+ cultures (Figure 4c). Similarly, when HLA-A2+ T cells were stimulated with natural tumor HLA-A2+ NY-ESO-1+ tumor cells (multiple myeloma U266), or peptide-pulsed T2 cells, the proliferative response of T1-28z CAR T cells was impaired compared with CART cells transduced with DN-28z CARs. This effect was most pronounced at high levels of antigen expression (U266 and T2+NY-ESO-1) rather than lower levels of antigen expression



(SK Mel 37). Interestingly, this could not be solely explained by HLA expression levels (Supplementary Figure S2).



We attempted to test the safety of the T1-28 and DN-28z CAR T cells in HLA-A2 transgenic NSG (nonobese diabetic, SCID, gamma-chain knockout) mice,29 hypothesizing that severe GvHD directed against HLA-A2 would develop in the T1-28z CART cell-treated mice. To avoid an allogeneic anti-HLA-A2 response, we used T cells from donors with the HLA-A*0201 genotype. Preliminarily, we found that the expression of HLA-A2 was several logs higher in the human donor T cells than in any of the NSG/A2 transgenic mouse blood cells or tissues (data not shown). Mice were injected with a single dose of either T1-28z or DN-28z CAR T cells, and sacrificed at predetermined intervals for histopathologic analysis of organs that were most likely to be targets of an anti-HLA-A2 response (granulocytes, lungs, liver, and skin). None of the mice developed clinical signs of GvHD-type disease (skin ruffling, weight loss, or loss of fur) over 3 weeks, but they all developed hair loss at 6 weeks; this is typi-



cased for NSG mice injected with human T cells, and is thought to be



mediated by a xenogeneic response of human T cells directed to



mouse MHC molecules. At days 14 and 22, there were higher levels
of T cell infiltration into the lungs and livers of mice that received
the T1-28z-CAR-T compared with those that received either DN-28z-
CAR-T or mock-transduced-T (Supplementary Figure S3), but both
forms of CAR T cells resulted in greater lymphocyte infiltrate than
untransduced T cells. T cell infiltrates were quantified on a 0–4 grad-
ing system developed by expert veterinary pathologists, but these
assessments were more qualitative overall than strictly quantifiable
(Supplementary Table S1). There was no effect on the white blood
cell counts in the mice (data not shown). Interestingly, a greater effect was imparted on the persistence of the HLA-A2+ CD3+ GFP+ human T cells, which had a much higher level of HLA-A2 expression than the mouse tissues. Although all the mice were injected
with the same number of CD3+ GFP+ T cells, the DN-28z CART T cells
achieved higher circulating levels of CD3+ T cells in the blood at day
15, again suggesting some degree of fratricide among the T1-28z-
transduced HLA-A2+ donor T cells (Figure 4e). Taken together, these
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Figure 2  Rationally targeted mutations designed to decrease binding of T1 to HLA-A2 alpha helix. (a) Crystal structure of T1 Fab binding HLA-A2/NYESO1, with highlighting of targeted amino acids. (b) A2/NYESO1 pentamer stains of primary human T cells 5 days after transduction with parental (T1), D53N mutant, Y34F, and DNYF mutations in the CAR. Fluorescence-activated cell sorting (FACS) plots are gated on FSC/SSC only. (c) Chromium release assays of corresponding CAR-transduced effectors against T2 cells pulsed with either flu or NYESO peptide as targets. Effector to target ratios are normalized to pentamer+ cells. CAR, Chimeric antigen receptor.

Figure 3  Direct measurement of affinity of the DN mutation in the Fab antibody by Biacore. Sensograms of DN Fab run over a range of concentrations (18.5 nmol/l – 1187 nmol/l) of HLA-A2 presenting (a) native NYESO1 (SLLMWITQC) or (b) mutant C9V NYESO1 (SLLMWITQV) peptide, which avoids disulfide bonding. Data were fitted (red) in a Biaevaluation 3.0 following a simple 1:1 Langmuir binding model.
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data suggest that the DN mutation has a significant impact on specifically detargeting the HLA-A2 molecule.

DN-28z CART cells lyse tumor targets as effectively as T1-28z in vitro. Having improved the in vitro and in vivo “safety profile” of the T1 scFv with the D53N mutation, we turned our attention again to the efficacy of the lower affinity DN-28z CART cells toward lysing native tumor targets. T cells were transduced with the T1-28z or DN-28z CARs in preparation for the subsequent in vitro and in vivo studies. Here again we confirmed the lower affinity of the DN mutant by mean fluorescence intensity of pentamer staining; T1-28z-transduced T cells had an MFI of 596, while the DN-28z-transduced T cells had an mean fluorescence intensity of 170, after gating on GFP+ cells (Figure 5a,b). About 1 week after transduction, DN-28z CART cells

| Analyte          | Peptide         | $K_{on}$ /mol/second | $K_{off}$/second | $K_D (K_{off}/K_{on})$, nmol/l | Reference                  |
|------------------|-----------------|----------------------|-----------------|-------------------------------|----------------------------|
| DN               | SLLMWITQC; SLLMWITQV | 90,100; 56,000       | 0.0606; 0.0401   | 672; 715                      | Stewart-Jones PNAS (2009)18|
| T1               | SLLMWITQC; SLLMWITQV | 138,375; 249,000     | 0.000674; 0.000835 | 2; 4                          | Chen JEM (2005)27          |
| 1G4 TCR          | SLLMWITQC; SLLMWITQV | 12,200; 11,800       | 0.17; 0.049      | 13,300; 5,700                 | Cole JI 2007; Wilcox Immunity (1999) |
| JM22 TCR         | GILGFVFTL; GILGFVFTL | 18,000; 40,000       | 0.03; 0.2        | 1,000; 6,000                  | Nicholson Mol Imm (1997)   |
| FMC63 (aCD19)    | Antibody; scFv   |                      |                 |                               |                            |

scFv, single-chain variable fragment; TCR, T cell receptor.

Figure 4  DN mutation restores epitope specificity of T1-antibody-based CAR-transduced T cells. Chromium release assays using (a) T1-28z CAR-transduced or (b) DN-28z CAR-transduced T cells as effectors against target T2 cells pulsed with titrating concentrations of NYESO1 peptide. (c) Expansion of T1/28z and DN/28z CAR-transduced T cells derived from an HLA-A2+ donor. T cells were rested for one week after transduction and then stimulated with artificial APCs. Growth of CD3+GFP+ cells was calculated as total number of lymphocytes × frequency of CD3+GFP+ cells as determined by fluorescence-activated cell sorting (FACS) staining. (d) Expansion of HLA-A2+ T cells in response to antigen. Data are shown as fold-increase over unstimulated cells. Mel 37 is the SK-Mel 37 tumor cell line (HLA-A2+, NY-ESO-1+); U266 is the multiple myeloma cell line (also HLA-A2+, NY-ESO-1+). Proliferation was measured 4 days after restimulation. (e) Absolute number of CD3+GFP+ T cells per µl of blood at two timepoints after injection of NSG-HLA-A2 transgenic mice with T1/28z-, DN/28z-, or untransduced HLA-A2+ T. CARs, Chimeric antigen receptors; GFP, green fluorescent protein.
were able to lyse NY-ESO-1-peptide pulsed T2 cells and native tumor targets (SK-Mel-37, HLA-A2+/NY-ESO-1+) as well as the T1-28z CAR T cells. Lysis of flu-pulsed T2 targets and HLA-A2+ NY-ESO1- targets (SK Mel 23), however, was much reduced (Figure 5c,d).

A2/NYESO-CAR (DN)-transduced T cells specifically infiltrate and delay progression of an NY-ESO-1 expressing tumor in vivo

Next, we set up a xenogeneic mouse model utilizing the NSG strain that permits long-term engraftment of human cells. 30 To address whether human T cells transduced with the DN-28z CAR could specifically home to a NY-ESO-1+ tumor, NSG mice were injected subcutaneously on day 0 with 3×10⁶ SK-Mel-37 human melanoma cells on one flank and 3×10⁶ SK-Mel-23 human melanoma cells on the other. DN-28z CAR T cells were infused at four different doses in each group of five mice. An intermediate dose of 2×10⁶ 19-28z CAR T cells (using the same vector backbone) was infused in a group of control mice. Tumors were measured until day 33. At the time of sacrifice, the tumors were excised and analyzed by immunohistochemistry for infiltration by human CD3+ T cells. All the subcutaneous tumor implants were noted to exhibit central necrosis, but in the mice injected with DN-28z-transduced T cells, the rim of SK-Mel-37 (NY-ESO-1 positive) tumors demonstrated infiltration by human CD3+ T cells (Figure 6a, c); the total number of T cells per mm² of tumor was dose-dependent (Figure 6a). In contrast, the SK-Mel-23 (HLA A2+/NYESO-negative) tumor in the same mice was not infiltrated with T cells (Figure 6b,d). In mice that were injected with CD19 CAR-transduced T cells, there was no infiltration of human T cells into either tumor (data not shown).

In addition to infiltrating the NY-ESO-1+ tumors, DN28z CAR T cells were detectable in the peripheral blood and spleen of tumor-bearing mice past day 30 (Supplementary Figure S3). At the lowest dose of T cells, DN-28z CAR T cells were detected at a frequency of 5 cells per µl of blood; at the highest dose, the frequency was more variable but ranged from 5 to 30 cells per µl of blood. DN-28z CAR T cells were also detectable in the spleens at similar frequencies at all doses. No CD19-28z CAR T cells were detectable in blood or spleen past day 30, consistent with prior data indicating the need for continued antigen stimulation to maintain 28z-based CAR-transduced T cells.31

Compared with treatment with T cells transduced with the irrelevant 19-28z CAR, the DN-28z CAR T cells significantly delayed growth of only the NY-ESO-1+ tumor (SK-Mel-37). This was most clearly evident at the highest dose of DN-28z-CAR-T cells (Figure 6e,f). Unfortunately, disease-specific survival could not be measured in this model for two reasons: first, the NY-ESO-1-negative tumor (SK-Mel-23) was much more clinically aggressive, and was not impacted by the DN-28z CAR T cells, making the survival data from these bilateral-tumor experiments not evaluable. Second, the survival of NSG mice injected with human T cells is confounded by a xenogeneic graft-vs-host disease (GVHD) effect, which begins to clinically affect the mice 4–6 weeks after T cell infusion (data not shown and ref. 32). This model is not
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aggressive enough to cause lethality in that time frame, and any possible survival data even with a single tumor implant would be confounded by the effect of this xenogeneic GVHD.

DISCUSSION
We sought to extend the applicability of antibody-based CARs to intracellular antigens because this approach has the potential to open access to an unlimited repertoire of monoclonal antibodies, while circumventing the problem of mispairing of transduced receptors with the endogenous TCR α/β chains, and extending the benefits of costimulatory engineering of CARs to HLA-peptide targeting. We found that maintaining the specificity of a CAR-based receptor for an intracellular antigen to be significantly more complex than targeting other cell surface molecules. Specifically, we found that the affinity of the antibody directed to an intracellular antigen and binding to HLA need to be carefully evaluated upon conversion to a CAR and expression in T cells. Minimal binding to HLA may cause loss of CAR specificity, likely owing to the increased avidity resulting from CAR clustering at the cell surface.

Currently, there are three examples of CARs based on antibodies that bind HLA/peptide that appear to have high specificity and cytotoxicity in vitro and in xenogeneic mouse models: one is specific to HLA/EBNA3C and resulted in high cytotoxicity when introduced into NK cells; another is specific for HLA-A2/gp100 and was found to suppress melanoma progression in a xenograft model when introduced as a CAR into human T cells, and one is specific for HLA-A2/WT1 and appears to have high specificity and cytotoxicity in vitro and in xenogeneic mouse models of leukemia.

Our study and others, however, have further scrutinized the “background activity” of engineered T cells, not just of the scFv. In...
one such study, an HLA-A2/WT1-specific CAR based on a single-chain variable fragment showed decreased cytotoxicity and poor specificity compared with a low-affinity TCR specific to the same antigen, despite the higher affinity of the scFv.16 In another study, antibodies specific to MHC/peptide complexes were isolated after immunization of HLA-A2 transgenic mice, and their variable fragments used to design CARs, but these required >100-fold higher antigen density to exert cytotoxicity compared with a cognate T cell clone bearing a natural TCR.37

The optimal affinity of an antigen receptor that is MHC-restricted and specific to a peptide epitope has not been definitively defined. TCRs target these complexes efficiently but generally with low binding affinity.39 The range of affinities of naturally-occurring TCRs, even for viral epitopes, is in the micromolar range.40 Indeed, engineered high affinity TCRs often result in decreased specificity.21–23 Furthermore, there is evidence that for TCRs, there is a maximum affinity threshold that limits T cell function (kD ~ <5 µmol/l), beyond which higher affinities do not increase clustering, signaling, lysis or growth. CAR-based receptors that are directed to surface molecules typically have high affinities (in the low nmol/l range), but affinity studies are not required to optimize specificity. On the other hand, very low affinity CARs specific for cell surface proteins may display poor efficacy, independent of epitope location on the target antigen.41

The minimal affinity of the antigen binding domain of a CAR required to trigger effective signaling has not yet been determined. We found that the low-affinity DN-28c CAR was sufficient to trigger specific lysis of peptide-pulsed targets, and natural HLA-A2-bearing, NY-ESO-1 expressing tumor cells (SK-Mel-37). Interestingly, the decreased HLA binding of the DN CAR relative to T1 also resulted in decreased T cell fratricidal activity. Although our SK-Mel-37 xenogeneic mouse model was not optimized, we were disappointed to find that the tumors were not eradicated despite infiltration of the DN CAR T cells and their persistence in the blood for over 1 month. This is a new xenogeneic model, and it is not clear how sensitive it is to adoptive cell therapies. Nevertheless, we interpret this outcome to reflect insufficient signal strength provided by the low-affinity CAR to effectively eliminate macroscopic tumor deposits. This finding points to the contradictory affinity requirements to achieve T cell potency while maintaining specificity.

The signal strength of physiological TCRs is augmented by engagement of additional molecules. Thus, the TCR/MHC interaction is stabilized by MHC binding to CD8 and consequent signaling through Ικκ; the engagement of additional costimulatory receptors, including B7 and TNF receptor family members, trigger separate signaling cascades that influence the function of the responding T cell. The second-generation CAR described here included a CD28 signaling domain, which may be suboptimal in the setting of very low affinity such as with the DN CAR. We previously demonstrated that the in vivo effects of low-activity CARs can be rescued through T-cell encoded costimulatory ligand expression42,43 or the use of chimeric costimulatory receptors.44,45 These two approaches may be valuable to enable the use of HLA-restricted CARs to target intracellular proteins.

### MATERIALS AND METHODS

#### Cell lines and cell culture

Blood samples were collected from donors after giving written informed consent. High-resolution HLA-typing was previously known in HLA-A2 donors, and only confirmed by antibody staining for HLA-A2. Normal human peripheral blood mononuclear cells were prepared from healthy donors by ficoll gradient centrifugation. T2 cells and the melanoma tumor cell lines SK-Mel-23 and SK-Mel-37 were provided by the Ludwig Center for Immunotherapy at Memorial Sloan-Kettering Cancer Center. For transduction with CARs, peripheral blood mononuclear cells were stimulated with PHA at 2 µg/ml on day 0 and transduced with viral supernatants on day 2. Recombinant hIL-2 was added at 50 IU/ml starting on day 2 and replenished every 2–3 days during in vitro culture. T cells were cultured in Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum, glutamine, and antibiotics. To assess T cell growth, transduced T cells were cultured at a 10:1 ratio with artificial APCs generated in our laboratory and previously described16; these were additionally transduced with full-length NY-ESO-1 protein using a retroviral vector. Expression was confirmed by intracellular staining with the E978 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

#### Generation of retroviral T1-CAR constructs and mutants

The single-chain variable sequence (heavy chain—GSG linker—light chain) for the parental T1 construct16 was cloned into the SFG-28xires-GFP vector previously generated in our laboratory.24,46 The D53N, Y34F, and DNYF mutants were generated by a series of linkers and standard molecular biology techniques. All constructs were verified by sequencing (Genewiz). The RD30-HEK293 packaging line was transfected with the construct of SFG DNA, and viral supernatants were collected at days 2 and 3; these supernatants were used to make stable Gal9- HEK293 producer cells, from which viral supernatants were collected from confluent dishes to transduce T cells. Packaging cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine, and antibiotics.

#### Staining and flow cytometry

Transduced T cells were analyzed for GFP expression and expression of cell surface molecules by standard staining and flow cytometry techniques. HLA-A2/NYESO1 pentamers were purchased from Proimmune (Oxford, UK). Antibodies to CD3 and HLA-A2 were purchased from BD Biosciences (San Jose, CA). Blood samples of 50 µl from mice were ACK-lysed and stained for 20 minutes at 4 °C with CD3 antibody; T cells were quantified with Count Bright counting beads (Invitrogen, Grand Island, NY).

#### Chromium release assays

In vitro cytotoxicity assays were performed according to standard techniques. Briefly, target cells were labeled with 115Cr (sodium chromate) for 90 minutes, then washed and co incubated for 4–6 hours in 96-well round bottom plates in triplicate with diluting ratios of effector T cells. Supernatants were harvested, transferred to Luma Plates (Perkin Elmer, Shelton, CT) and acquired on a Perkin Elmer Top Count. Percent specific lysis was calculated with the formula (x spontaneous)/(maximal spontaneous), Peptide pulsing was performed where indicated either overnight or simultaneous with chromium labeling. HLA-A2 restricted influenza matrix protein (Flu, GILGFVFTL) and NY-ESO-1,157-165 (SLLMWITQC) peptides were purchased from Proimmune.

#### Surface plasmon resonance measurements of affinity

Surface plasmon resonance measurements of affinity SPR studies were performed using a Biacore 3000 (Biacore AB) as previously described.18 HLA-A*0201-SLLMWITQC and HLA-A*0201-SLLMWITQV were enzymatically biotinylated by BirA enzyme on the C-terminal biotinylation site and immobilized to CM5 sensor chips via covalently coupled streptavidin. Sensograms were measured over seven concentrations of D53N Fab. Kinetic constants were derived using the curve-fitting facility of the BIAevaluation program (version 3.0; Biacore AB) and rate equations derived from the simple 1:1 Langmuir binding model.

#### Mouse studies

NSG (stock # 005577-NOD.Cg-Pkdckscid Ii2gtrm1Wjl/SzJ) and HLA-A2-transgenic NSG (stock#009617-NOD.Cg-Pkdckscid) mice were purchased from Jackson laboratories (Bar Harbor, ME). Mice were injected subcutaneously in each flank with human melanoma cells on day 0. Tumors were measured every 2–3 days with calipers and tumor volume was calculated with the formula (length × width × width × π/6). Mice were sacrificed and tumors were excised and stained by standard immunohistochemistry for CD3 to evaluate T cell infiltration.
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