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Adoptive transfer of T cells genetically modified to express cancer-specific receptors can mediate impressive tumor regression in terminally ill patients. However, T cell function and persistence over time could be hampered by the activation of inhibitory costimulatory pathways, such as programmed death 1 (PD1)/programmed death ligand 1, leading to T cell exhaustion and providing tumor cells with an escape mechanism from immunosurveillance. In addition, the lack of positive costimulation at the tumor site can further dampen T cell response. Thus, as T cell genetic engineering has become clinically relevant, we aimed at enhancing T cell antitumor activity by genetically diverting T cell–negative costimulatory signals into positive ones using chimeric costimulatory retargeting molecules and which are composed of the PD1 extracellular domain fused to the signaling domains of positive costimulatory molecules such as CD28 and 4-1BB. After characterizing the optimal PD1 chimera, we designed and optimized a tripartite retroviral vector that enables the simultaneous expression of this chimeric molecule in conjunction with a cancer-specific TCR. Human T cells, transduced to express a PD1/28 chimeric molecule, exhibited enhanced cytokine secretion and upregulation of activation markers upon coculture with tumor cells. These engineered cells also proliferated better compared with control cells. Finally, we tested the function of these cells in two xenograft models of human melanoma tumors and show that PD1/28-engineered human T cells demonstrated superior antitumor function. Overall, we propose that engineering T cells with a costimulatory retargeting molecule can enhance their function, which bears important implications for the improvement of T cell immunotherapy.

engineered T cells can mediate impressive tumor regression in advanced cancer patients (16, 17). The latter approach led to tumor regression in up to 13–50% of the melanoma patients treated, as well as 66% of the synovial cell sarcoma ones (18, 19). In addition to the use of genes to endow T cells with a new specificity (either TCR or chimeric Ag receptors), several reports have dealt with the qualitative improvement of T cell function [reviewed in (20)]. Indeed, costimulation could be manipulated to enhance T cell function, cytokine secretion, and survival, as follows: for instance, restoration of CD28 (21) has enhanced T cell function and proliferation, but, as CD28 ligands are seldom expressed by tumor cells (22), this approach might be of limited applicability in cancer-related therapies. Thus, tumors can escape T cell surveillance by preventing the expression of positive costimulatory ligands such as B7 and/or by overexpressing inhibitory costimulatory ligands (23).

In the present report, we aimed at enhancing T cell antitumor activity by genetically diverting T cell–negative costimulatory pathways into positive ones using retargeting molecules that we also termed costimulatory converters, which are composed of the extracellular domain of PD1 fused to the signaling domains of positive costimulatory molecules. Expression of such a molecule, PD1/28, in human T cells endowed them with improved cytokine secretion and upregulation of activation markers upon coculture with tumor lines, which translated into superior antitumor function in cytotoxic assays of human melanoma tumors using a chick embryo chorioallantoic membrane (CAM)–based model as well as a xenograft model in mice.

Materials and Methods

Patient PBMCs and cell lines

All of the PBMCs used in this study were from normal donors obtained from the Israeli Blood Bank (Sheba Medical Center, Tel-Hashomer, Israel). Melanoma cell lines HLA-A2/MART-1(+) (526, 624, 624.38) and HLA-A2/MAPK(+) (888, 938) were generated at the Surgery Branch (National Cancer Institute, National Institutes of Health, Bethesda, MD), as described previously (23). The 938/A2 is a HLA-A2–transduced line derived from 938. SK-MEL-23 is a HLA-A2+ melanoma cell line (24). p53+/HLA-A2+/MART-1(+) cells were generated at the Surgery Branch (National Cancer Institute, National Institutes of Health, Bethesda, MD), and maintained in a 37˚C and 5% CO2 incubator. Lympocytes were cultured in BioTarget medium (Biological Industries) supplemented with 10% heat-inactivated FBS. Cells were washed in FACS buffer, stained for phosphorylated ERK (pERK) expression, and analyzed by flow cytometry.

PD1 chimeras and retroviral constructs

The α- and β-chains from the previously characterized TCRs specific for MART-1(+)26,60, termed F4 (or DMF4) (18, 27) and for p53/938.272 (25) were subcloned into the pGEM-4Z/64A vector, as described previously (28). The chimeras PD1/28 and PD1/28BB were created by overlapping PCR, and their amino acid composition is indicated in Fig. 1A. A truncated version of PD1, PD1-Stop was produced by amplifying and cloning the PD1 cDNA with tumor lines, which translated into superior antitumor function in cytotoxic assays of human melanoma tumors using a chick embryo chorioallantoic membrane (CAM)–based model as well as a xenograft model in mice.

Transduction of PBLs

For virus production, transfection of 2 × 10^6 293GP cells with 9 μg DNA of MGSV1-based retroviral construct and 4.5 μg envelope plasmid (vesicular stomatitis virus-G) was performed using JetPrime transfection reagent (Polyplus). After 4 h, the medium was replaced. Retroviral supernatant was collected 36 h after the DNA transfection. Freshly isolated PBLs were stimulated for 48 h in the presence of 50 ng/ml OKT3 (eBioscience, San Diego, CA) before transduction. Following stimulation, lymphocytes were transduced with retroviral vectors by transfer to nontreated tissue culture dishes (Nunc, Rochester, NY) that had been precoated with RetroNectin (Takara) and retroviral vectors, as previously described (31).

FACS analysis and Abs

PBL cultures were tested for reactivity in cytokine release assays using commercially available ELISA kits for IFN-γ, IL-2, and TNF-α (R&D Systems, Minneapolis, MN). For these assays, 1 × 10^5 responder cells (PBL) and 1 × 10^4 stimulator cells (tumor cells) were incubated in a 0.2 mL culture volume in individual wells of 96-well plates. Stimulator cells and responder cells were cocultured for 18 h. Cytokine secretion was measured in culture supernatants diluted to be in the linear range of the assay. As a control for T cell activity, we incubated the different T cell cultures with PMA/ionomycin at a concentration of 50 ng/ml and 1 μM, respectively.

Phosphorylated ERK staining

Following a 15-min coculture of 7 × 10^4 transduced T cells with 3 × 10^5 melanoma targets, the cells were fixed with 5% formaldehyde and permethilized using ice-cold 90% methanol for 30 min. The cells were washed in FACS buffer, stained for phosphorylated ERK (pERK) expression using a specific Ab for phospho-p44/42 MAPK (clone D13.14.4E; Cell Signaling Technology, Danvers, MA), and analyzed by flow cytometry, gated on the lymphocyte population.

Cell separation

CD8+ cells were separated using a magnetic beads–based approach for both negative and positive selection of those subsets (Milenyi Biotec, Auburn, CA).

Cell proliferation assay

T cells were labeled with 1 μM CFSE (eBioscience, San Diego, CA) for 6 min and then cultured with 9 × 10^5 recombinant PD1-Fc chimera (R&D Systems). On day 3 after stimulation, cell fluorescence was analyzed by flow cytometry.

Cell-mediated cytotoxicity assay

Target cells were labeled with 2 μM CFSE (eBioscience) for 6 min and then cocultured with transduced lymphocytes at 37˚C for 4 h, at E:T ratio of 4:1. After the coculture, 1 μM propidium iodide (Sigma-Aldrich, Rehovot, Israel) was added for assigning the ratio of cell death, and the samples were analyzed by flow cytometry.

Chicken CAM model system

Fertilized white chicken eggs obtained from a local farm were incubated at 37˚C for 4 d, and a small perforation (2 mm diameter) was performed in the upper side of the shell to create an artificial air pocket. At embryonic day E8–9, a 1-cm^2 window was cut in the shell above the air pocket. A total of 5 × 10^5 human melanoma cells, resuspended in 25 μl Matrigel (Sigma-Aldrich), was inoculated on the CAM in a plastic ring of 4 mm diameter for engraftment, and the shell was sealed with adhesive tape. One day after the tumor cell graft, 100 μl 1:10 stock antibiotic solution of penicillin-streptomycin-nystatin (Biological Industries) was added on the CAM. On E12–13, the presence of tumors ~5 mm in diameter was assessed visually. Using a light source approximated to the side of the egg, a large blood vessel was located and a small 10 × 5-mm window was cut,
with care not to damage the shell membrane. A total of $5 \times 10^6$ human T lymphocytes, resuspended in 100 µl lymphocyte medium, was adaptively transferred i.v. using a 29G insulin syringe, and the excised shell was replaced and reattached by tape. On E17–18, the upper shell was removed and the tumors were excised, measured, and weighed (in a blinded way). For single suspension, CAM-grown tumors were sliced with a scalpel into small pieces, ∼2–3 mm$^3$ in size. Enzymatic digestion of the pieces was done for 30–60 min, 37°C, in lymphocyte media containing collagenase, hyaluronidase type V, and DNase I type IV (Sigma-Aldrich), as previously described (32). The obtained single-cell suspension was passed through a cell strainer and washed twice with PBS.

**Winn assay**

Six-week-old athymic nude-Foxn1nu female mice (Harlan, Jerusalem, Israel) were inoculated in the flank with a mixture of $10^6$ melanoma cells, and $2 \times 10^6$ transduced lymphocytes were resuspended in 40 µl Biotarget medium. Tumor size was measured every 3 d using a caliper in a blinded fashion. All of the procedures were performed according to the guidelines of the university committee for animal welfare.

**Results**

**Design and expression of PD1-chimeric constructs**

Costimulation can greatly influence T cell function, and thus, in the current study, we designed and evaluated two costimulatory retargeting molecule constructs, based on the fusion of the PD1 extracellular domain to either transmembrane and intracellular portion of the CD28 molecule (PD1/28) or to the latter followed by the 4-1BB signaling domain (PD1/28BB) (Fig. 1A). The cDNA encoding these molecules was cloned into the MSGV1 retroviral vector, and we tested their expression in primary lymphocytes following transduction. As seen in Fig. 1B, we were able to express both constructs in human T cells efficiently without any selection. However, it seems that the addition of the 4-1BB signaling domain considerably impaired PD1/28BB surface expression levels compared with the PD1/28 chimera, as follows: 45.2% (mean fluorescence intensity [MFI] = 43) versus 89.8% (MFI = 284) of positive cells. In both cases, this level of expression by transduced PBLs cultured in vitro was sustained for >30 d without selection (data not shown).

PD1 (B7-H1), a ligand for PD1, has been shown to be expressed by tumor lines (5, 6). Thus, we also tested its level of surface expression by different melanoma lines. As seen in Fig. 1C, all the melanoma lines tested expressed detectable levels of PD1 (ranging from MFI = 6 to 13; p < 0.05). Thus, the expression of PD1 is likely to provide a potential source for costimulation via PD1 chimeras.

**Selection of an optimal PD1-based costimulatory converter**

Next, we aimed at testing the function of PD1/28 and PD1/28BB chimeras in primary human T cells in an Ag-specific setting. To do so, one has to express concomitantly a TCR with a defined specificity. Thus, human T cells, retrovirally transduced to express the PD1 chimeras, were electroporated with mRNA encoding the CD8-dependent MART-1–specific TCR, F4 (31), previously used in TCR gene transfer clinical trials (18). These cells were co cultured with different melanoma cell lines, and cytokine secretion was measured in the supernatant. As seen in Fig. 2A, T cells expressing the PD1/28 were able to secrete higher levels of cytokines compared with the GFP-transduced control (IFN-γ, 1150 versus 280 pg/ml; IL-2, 2375 versus 1183 pg/ml in coculture with 624.38, respectively; p < 0.05). As observed for the surface expression data (Fig. 1B), the PD1/28 chimera outperformed the PD1/28BB chimera, which minimally influenced cytokine secretion. Thus, we selected the PD1/28 chimera for subsequent work. No significant cytokine secretion was observed in cocultures with the control melanoma line 888 or without TCR, which underscores the dependence on TCR signaling for the costimulatory chimeric molecules to improve cytokine secretion.

To demonstrate that this effect was independent of the TCR employed, we replaced the MART-1–specific TCR with a p53–specific one (p53TCR) we previously characterized (25). As expected, PD1/28 triggered more IL-2 secretion (Fig. 2B; H2087 cell line, 1242 pg/ml) than the GFP control (278 pg/ml). Similar results were observed for IFN-γ secretion (data not shown). Additionally,
to ascertain the role of CD28-signaling moiety, we generated a truncated version of the PD1 receptor (PD1-Stop) that was used in these coculture experiments. As seen in Fig. 2B, the lack of the CD28-signaling domain led to a statistically significant decrease in cytokine secretion (from 1190 to 710 pg/ml in coculture with Saos2/143). Similar results were observed in conjunction with the F4 receptor (data not shown). This confirms that the CD28-signaling domain is necessary for the improved cytokine secretion by transduced cells. In conclusion, expression of the PD1/CD28 chimera can improve cytokine secretion in an Ag-specific manner.

**FIGURE 2.** Function of PD1 chimeras. (A) Transduced PBLs expressing either PD1/28, PD1/28BB, or GFP (control) were electroporated with the F4 TCR and were cocultured with different melanoma lines, as indicated. IFN-γ (upper panel) or IL-2 (lower panel) secreted in the coculture supernatant was measured by ELISA. These results are representative of four independent experiments, performed with two different donors (*p < 0.05, calculated using a Student paired t test). (B) PBLs that were transduced with either PD1/CD28, a truncated version of PD1 (PD1-Stop), or GFP (control) were electroporated with a p53-specific TCR and were cocultured with different p53+ tumor lines, as indicated. IL-2 secreted in the coculture supernatant was measured by ELISA. These results are representative of three independent experiments, performed with two different donors (*p < 0.05, calculated using a Student paired t test).

**Design and expression of tripartite vectors that include PD1/28 in human T cells**

To adequately study the antitumor potential of the PD1/28 chimera from a more translational perspective, we decided to generate tripartite retroviral constructs in which PD1/28 is expressed conjunctively with the F4 TCR chains. In control vectors, PD1/28 was replaced by a truncated version of the low-affinity NGFR (33). We tested two possible vector configurations, as follows (Fig. 3A): either by placing the TCR chains upstream (linked by a 2A sequence) followed by the PD1/28 (or NGFR) under the control of an IRES (vectors 1 and 2, respectively) or the other way around (vectors 3 and 4). We produced retroviral supernatant from the aforementioned vectors and transduced human PBLs. Whereas both configurations led to the expression of the introduced genes (Fig. 3B), higher levels of PD1/28 (and NGFR) were observed using vectors in which these molecules were expressed upstream to the TCR chains (84.5% positive cells and MFI = 144 for vector 3 versus 57.5% positive...
cells and MFI = 18 for vector 1). In comparison, we observed a modest decrease in TCR-β surface expression levels when using vector 3 instead of vector 1 (63.9% positive cells and MFI = 32 versus 71.8% positive cells and MFI = 39, respectively). Thus, PD1/28-F4 was chosen as the optimal configuration for the concomitant expression of the chimera and the TCR chains.

**Functional assessment of the tripartite vector PD1/28-F4 in human T cells**

We then evaluated the function of human T cells transduced with PD1/28-F4 or NGFR-F4 in coculture with MART-1–expressing cell lines. Similar levels of TCR expression were assessed by Vβ12 staining to negate any possible bias in the results resulting from differential TCR expression (Fig. 3B). These cells were cocultured overnight with different human melanoma lines. As shown in Fig. 4A–C, HLA-A2+/MART-1+ melanoma tumors (526, 624, 624.38, and SK-MEL23) stimulated T cells to secrete three cytokines that are important for T cell antitumor function (34), that is, IFN-γ, IL-2, and TNF-α; in these assays, the PD1/28 population secreted higher levels of cytokines than the NGFR-transduced T cells (e.g., 702 pg/ml versus 434 pg/ml IFN-γ and 923 versus 169 pg/ml IL-2, respectively, in coculture with 624;...
No significant cytokine secretion was noted in cocultures with control HLA-A2888 melanoma cells. TGF-β is an immunosuppressive cytokine present in the tumor microenvironment that can dampen critically T cell function and IFN-γ secretion (35). As PD1/28-transduced T cells demonstrated an increased IFN-γ secretion (Fig. 2A), we sought to test how they would perform in the presence of TGF-β. We set up an overnight coculture with SKMEL23 cells in the presence (or not) of 10 μg/ml TGF-β and measured the IFN-γ secretion in the supernatant. As expected, IFN-γ secretion was reduced in the presence of TGF-β in both cocultures, that is, with PD1/28- and NGFR-transduced cells (Fig. 4D). Still, PD1/28-transduced cells maintained a higher secretion compared with the control (143 pg/ml versus 70 pg/ml; p = 0.03). This suggests that PD1/28-transduced cells might better function in the vicinity of tumors.

To ascertain the influence of the genetic modification of T cells with PD1/28 on T cell cytotoxicity potential, we first measured the levels of surface expression of CD107a (LAMP-1), a degranulation marker, following coculture with SKMEL23 cells. As seen in Fig. 4E, we observed similar levels of CD107a expression by T cells transduced with PD1/28-F4 and NGFR-F4 (59.2% versus 56.1% of positive cells, respectively; p = 0.13). In both cases, no significant CD107a expression was noted in cocultures with the negative control melanoma line 888 (Fig. 4E). These observations were further corroborated by cell-mediated cytotoxicity assays using these cells; following coculture of transduced CD8+ T cells with CFSE-labeled melanoma cells, we did not observe a statistically significant difference in antitumor cytotoxicity, as exemplified by the propidium iodide–positive population (Fig. 4F: 52.1% for PD1/28-F4 versus 45.3% for NGFR-F4 using the SK-MEL23 target cell line; p = 0.23). No significant cytotoxicity was noted in cocultures with control HLA-A2888 melanoma cells (Fig. 4F). Therefore, compared with the NGFR-transduced population, the PD1/28-transduced cells did not exhibit a loss in cytotoxic potential.

In conclusion, upon stimulation by human melanoma lines and compared with the NGFR control, PD1/28-expressing T cells demonstrated an improved cytokine production capability.

Activation marker upregulation and increased proliferation of PD1/28-transduced T lymphocytes

In addition, we analyzed PD1/28-F4– and NGFR-F4–transduced T cells for surface expression of the T cell activation markers CD25, CD69, and CD137 following coculture with melanoma cells. Compared with the NGFR cell population, PD1/28-engineered cells demonstrated a statistically significant superior expression of these markers, as follows: for instance, for CD25, we detected 43.4% of positive cells for PD1/28 versus 28.9% for the control NGFR (Fig. 5A; p < 0.05). Similarly, we noted a proportion of 73.6% of positive cells for CD69 in the PD1/28 sample compared with 57.3% in the NGFR one (Fig. 5A).

The CD28 pathway has been shown to enhance the growth of T cells (36). We also show in this study that PD1/28-transduced T cells were stimulated with plate-bound OKT3 (0.1 mg/ml) in the presence of soluble PD1/Fc (4 μg/ml) and 30 IU/ml IL-2. Three days after stimulation, the cells were analyzed for CFSE dilution. These results are representative of three independent experiments, and the difference between the two groups was found statistically significant (p < 0.05, calculated using a Student paired t test). (C) Transduced PBLs with either PD1/28-F4 or NGFR-F4 cells were incubated with melanoma lines (as indicated) for 15 min and analyzed for intracellular expression of pERK. These results are representative of three independent experiments, and the difference between the two groups was found statistically significant (p < 0.04, calculated using a Student paired t test).

![FIGURE 5](http://www.jimmunol.org/)
cells can secrete higher levels of IL-2 (Fig. 4A) and upregulate CD25 (Fig. 5A) upon TCR engagement. Thus, we assessed PD1/28-engineered T cell proliferation by labeling them with CFSE, followed by stimulation with plate-bound OKT3 in the presence of soluble PDL1. Three days after stimulation, the cells were analyzed for CFSE dilution. As seen in Fig. 5B, PD1/28-expressing cells proliferated more than the control, as indicated by the lower MFI measured (18 for PD1/28 versus 41 for NGFR; *p < 0.05). PD1 has been shown to reduce the levels of ERK phosphorylation (37). Thus, we wanted to assess how PD1/28 expression would impact on pERK expression. PD1/28- or NGFR-transduced T cells expressing the F4 TCR were incubated with target melanoma cells and analyzed for intracellular pERK expression following intracellular staining with an appropriate Ab. As seen in Fig. 5C, PD1/28 expressed higher levels of pERK compared with the NGFR control (e.g., 41.1% versus 29.2% of pERK-positive cells, respectively; *p < 0.05). No significant pERK expression was observed in control cocultures with the melanoma line 888. Thus, T cells expressing the chimeric costimulatory converter PD1/28 display an improved activation and proliferative profile.

**PD1/28 mediates superior antitumor cytotoxicity in xenograft models**

PD1/28-transduced cells demonstrated heightened proliferation, cytokine secretion, and upregulation of activation markers, but we did not observe a significant increase in cell-mediated cytotoxicity (Fig. 4F). We assumed that a classical 4-h cytotoxicity assay may not necessarily fully demonstrate the antitumor activity of PD1/28-transduced cells. Thus, we decided to assess the antitumor function of PD1/28-transduced T cells in an ovo cytotoxicity assay we developed (38) based on a xenograft system using the chick embryo CAM model (39). The CAM is a highly vascularized membrane that enables gas exchange and that can be easily accessed by cutting a small window in the egg shell. A total of 5 \times 10^6 tumor cells (either SK-MEL23 or 526) was grafted on the CAM, as described in Materials and Methods. In general, between 70 and 90% of the treated eggs successfully developed melanoma tumors. The latter were treated by adoptively transferring 5 \times 10^6 T lymphocytes engineered to express either PD1/28-F4 or NGFR-F4 (control). Five to six days after the transfer, the tumors were excised, measured, and weighed in a blinded manner. Following treatment with PD1/28-transduced T cells, we observed striking regressions of tumors compared with control groups treated with NGFR-F4 cells (Fig. 6A). The average weight for tumors treated was reduced by ∼75% in the PD1/28-F4 T cell–treated tumors compared with the control treatment with NGFR-F4 cells (4.7 mg versus 18.4 mg, respectively), and the difference was found to be statistically significant (n = 6; *p = 0.01, calculated using a Student paired t test). Moreover, we produced single-cell suspensions from these tumors and analyzed them for the presence of CD3+/CD8+ human T cells by flow cytometry (Fig. 6C). Although we and the difference between the PD1/28 and the NGFR (control) groups was found statistically significant (p = 0.02, calculated using a Student paired t test), (D) Winn assay. Athymic nude mice were inoculated with SK-MEL23 and transduced lymphocytes (either PD1/28 or NGFR control, as indicated) in the flank. Tumor growth was measured in a blinded fashion using a caliper and calculated using the following formula: [D \times d (2)] / 6, where D is the largest tumor diameter and d its perpendicular one. Results are shown for the different time points as mean ± SEM (n = 5), and the difference between the PD1/28- and NGFR-treated groups was found statistically significant (p = 0.018). These results are representative of two independent experiments.

**FIGURE 6.** Human tumor regression in the CAM system. (A and B) Human melanoma tumors (derived from 526 or SK-MEL23, as indicated) that grew for 6 d on the CAM of chick embryo were treated with adoptively transferred T lymphocytes engineered to express either PD1/28-F4 or NGFR-F4 (control). At days 5–6 after treatment, the tumors were excised (A) and weighed (B) in a blinded manner. These results were obtained with three different donors, and the difference between the PD1/28 and the NGFR (control) groups was found statistically significant (n = 6; *p = 0.01, calculated using a Student paired t test). (C) We produced single-cell suspension from treated 526 tumors and analyzed them for the presence of CD3+/CD8+ human T cells by flow cytometry 1 d following T cell injection. These results are representative of three independent experiments, and the difference between the PD1/28 and the NGFR (control) groups was found statistically significant (p = 0.02, calculated using a Student paired t test). (D) Winn assay. Athymic nude mice were inoculated with SKMEL23 and transduced lymphocytes (either PD1/28 or NGFR control, as indicated) in the flank. Tumor growth was measured in a blinded fashion using a caliper and calculated using the following formula: [D \times d (2)] / 6, where D is the largest tumor diameter and d its perpendicular one. Results are shown for the different time points as mean ± SEM (n = 5), and the difference between the PD1/28- and NGFR-treated groups was found statistically significant (p = 0.018). These results are representative of two independent experiments.
detected the presence of human CD3<sup>+</sup>/CD8<sup>+</sup> T cells in both groups, we observed a statistically significant higher proportion of these in the PD1/28-treated group compared with the NGFR-control group (1.09% versus 0.12%, respectively; p = 0.02). This suggests that human T cells are able to traffic to and infiltrate human melanoma tumors grown on the CAM and that PD1/28 could endow T cells with improved persistence.

Finally, we performed a Winn assay to test the ability of PD1/28-transduced T cells to mediate enhanced antitumor activity in a xenograft model. Nude mice were inoculated with a mixture of SK-MEL23 cells and PD1/28-F4- or NGFR-F4-transduced cells (at E:T of 2:1), and we followed tumor development in the subxenograft model. In conclusion, PD1/28-expressing T cells demonstrated a higher cytotoxic potential compared with the NGFR control when used to treat melanoma tumors grown in the CAM model and in vivo.

**Discussion**

The PD1 pathway is a central negative regulator of T cell function and has been shown to be highly relevant in cancer (40). Several recent reports indeed demonstrate that its neutralization either using Abs (11, 41) or genetically engineered T cells [using RNA interference (42, 43)] may significantly improve T cell antitumor function. In this study, we show that it is possible to divert the negative signals PD1 induced into positive ones using a costimulatory converter in the form of chimeric PD1/28 receptor. Our present report confirms and extends a recent study describing a similar concept using a differently designed chimeric molecule (44). Unlike in the latter, we have described in this study the enhanced antitumor function of PD1/28-transduced cells in an Ag-specific setting using two different TCRs (Fig. 2). We have also studied different signaling moieties and were able to reach high levels of transgene expression without selection using a clinically approved retroviral platform, which was further optimized to drive the expression of three genes (the PD1/28 molecule and both F4 TCR chains). More importantly, we have demonstrated that primary human lymphocytes endowed with the PD1/28 molecule exhibited improved cytotoxicity in two xenograft models of human melanoma tumors.

As the induction of negative costimulation provides tumor cells with an immune escape mechanism (2, 6), it is possible to extend this approach to other negative costimulatory molecules such as CTLA-4, as recently shown in a mouse model (45). In addition, as reported in several studies and seen in this study (Figs. 1, 2), the composition of the chimeric signaling moiety can substantially impact on the receptor expression and function (46). Still, given the enhancing functional role of 4-1BB in chimeric receptor (47, 48), further optimization of costimulatory converters that include an additional signaling moiety derived from 4-1BB (in particular) or using other TNFR members such as OX40 (49) or CD27 (50) (in general) is warranted. The use of costimulatory converters could be also extended to other Ag-specific receptors such as chimeric Ag receptors or combined with small interfering RNA targeting the endogenous PD1 receptor, provided viral vectors can be efficiently designed to drive the expression of all these components. In turn, this approach could also be applied to chronic viral disease such as AIDS, as PD1 is highly expressed in T cells from HIV-infected patients (9).

Unlike Abs used to block inhibitory costimulatory pathways, the approach we describe in this study can endow T cells with a means, not only to counteract inhibitory costimulation, but also to activate positive costimulatory pathways, which could facilitate differentiation into memory cells, persistence over time, and enhanced function in the patients. Also, one could surmise that because tumors may escape from an immune response and be selected over time in vivo based on their high levels of PD1 expression, the use of costimulatory retargeting molecules may help revert this situation, lowering immunosuppression and thus enabling a broader T cell antitumor response.

The CAM model has been widely used for grafting xenograft tumors, facilitating studies of tumor development and angiogenesis in the past century (39). Nevertheless, to our knowledge, this study represents its first evaluation relative to a tumor xenograft mouse model in the context of adoptive T cell transfer treatment. As we obtained similar results in both models (Fig. 6), we trust that the use of the CAM-xenograft system could accelerate the evaluation process of cell-based treatments of cancer, as a preliminary model toward more elaborate systems.

In conclusion, we have shown that the PD1/28 costimulatory converter improves the antitumor activity of transduced T cells in an Ag-dependent setting, leading to tumor regression. We trust that this approach aimed at the diversion of costimulatory pathways bears important implications for the improvement of T cell–based treatments using gene-transfer approaches.

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**Disclosures**

The authors have no financial conflicts of interest.

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