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Transfer of TCR Genes into Mature T Cells Is Accompanied by the Maintenance of Parental T Cell Avidity

Mark P. Rubinstein,* Andre N. Kadima,* Mohamed L. Salem,* Christophe L. Nguyen,* William E. Gillanders,* Michael I. Nishimura, † and David J. Cole†*

The adoptive transfer of tumor-specific T cells expanded in vitro can be of significant therapeutic value in select cancer patients. This strategy is limited though, as it is often difficult, if not impossible, to obtain T cells of clinical value. The transfer of TCR genes to mature T cells to generate tumor-reactive T cells provides a potential mechanism to overcome these limitations. To evaluate the feasibility of such an approach and the quality of the resulting T cells, we generated replication-deficient retroviral vectors using the well-characterized OT-1 TCR genes. After transducing murine T cells, we were able to expand large numbers of Ag-specific T cells that were functionally active against tumor cells expressing the relevant Ag. Furthermore, we found that T cells expressing retrovirally encoded TCR had avidity that was similar to that of the parental clone. This maintenance of avidity was despite variable expression of the retrovirally encoded TCR and the presence of potentially competing endogenous TCRs. These results suggest that the inherent qualities of the TCR, as dictated by the coding sequence, are the most critical parameters in the generation of high-avidity T cells. The Journal of Immunology, 2003, 170: 1209–1217.

The adoptive transfer of Ag-specific T cells can be of therapeutic value in a wide range of human diseases. This was first demonstrated with a class of tumor-localized T cells (known as tumor-infiltrating lymphocytes), which, upon adoptive transfer concomitant with IL-2, can mediate tumor regression in up to one-third of patients with metastatic melanoma (1, 2). Adoptive T cell therapy has since been shown to be efficacious in the treatment of other cancers and viral diseases (3–8). However, there are significant limitations. In cancer patients, with the exception of a subset of tumors, there is an inability to isolate and expand T cells of therapeutic value (9). This may represent either a failure of the tumor to stimulate the generation of a tumor-specific T cell response (ignorance) or a suppression of an active immune response (tolerance or deletion) (10–15). Of additional complexity, the avidity of responding T cells may be significantly compromised in cancer patients, because the tumor, or in many cases closely related self-Ags, may be capable of selectively deleting the T cells of highest tumor avidity (16, 17). Consequently, even in those patients with expandable tumor-specific T cells, the resulting T cells may be of poor therapeutic ability. The recent identification of tumor-associated Ags and an increasing number of tumor Ag and viral-specific TCR genes (15, 18–23) now provide the opportunity of generating high-avidity T cells.

T cell specificity is dictated by gene products encoded for from two TCR genes, which in the majority of T cells are denoted as α and β TCR subunits. These membrane-bound proteins, which are covalently linked by a disulfide bond, are encoded for by a product of gene rearrangement (24). The surface expression of these two polymorphic TCR subunits is dependent on the coexpression of numerous nonpolymorphic TCR subunits, thus restricting the feasibility of αβ TCR gene transfer to the targeting of T cells and/or their progenitors. Because T cells are easier to culture and expand, they currently represent a more attractive gene transfer target as opposed to their progenitors.

Mature T cells express endogenous αβ TCR subunits which may compete with exogenous TCR subunits for cell surface expression and impede the generation of high-avidity T cells. Although there has been extensive research demonstrating redirection of T cell specificity via TCR gene transfer (25–30), the issue of heterodimer formation between endogenous and exogenous TCRs and the resulting T cell avidity has received limited attention. This may be partially a reflection of the widespread use of TCR-transgenic mice where allelic exclusion lessens the formation of such heterodimers (31, 32). Because available evidence suggests successful immunotherapy via adoptive T cell transfer is dependent on the generation of high-avidity T cells (17, 33–35), delineating the relationship between TCR gene transfer and avidity is of paramount importance.

Using retroviral vectors, we have developed a TCR gene transfer model using the well-characterized OT-1 TCR (36–38). Both the OT-1 α and β subunit genes, along with a drug-resistant gene, were placed into a retroviral vector. Consistent with other recent reports (29, 39–41), we show that T cells transduced with a TCR vector recognize and kill target cells with relevant Ag. We have further characterized TCR gene-modified T cells for both TCR expression and in vitro functional ability. Importantly, we demonstrate for the first time that despite the presence of endogenous TCRs and variable TCR expression, T cells modified by our OT-1 TCR gene transfer vector are of a high avidity similar to that of their parental clone and are able to recognize low levels of Ag.

Materials and Methods

Animals

All mice used were 6–14 wk old, purchased from The Jackson Laboratory (Bar Harbor, Maine), and maintained in specific pathogen-free conditions at the Medical University of South Carolina (Charleston, SC) in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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with National Institutes of Health guidelines. C57BL/6-Ly5.1 (B6.SJL-Ptprc<sup> Pep3</sup>/BoyJ) are congenic to wild-type C57BL/6-Ly5.2 mice at the Ly5.5 (CD45) allele (42), use of which allowed us to distinguish effector cells from APCs in functional assays listed below. OT-1 TCR-transgenic mice contain the OT-1 TCR (Vα2/NJβ5) transgene, encoding a TCR specific for the OVA epitope (SIINFEKL-H-2K<sup>B</sup>) (37). OT-1 TCR-transgenic mice were maintained on a C57BL/6-Ly5.1 background and assessed for transgene status by flow cytometric analysis of the peripheral blood with an Ab against the Vα2 subunit.

**Abs and other reagents**

The following mAbs were used for this study: B20.1, anti-Vα2; MR9-4, anti-Vβ5; 145-2C11, anti-CD3; 37.51, anti-CD28; A20, anti-ly5.1 (CD45.1); 53-6.7, anti-CD8; XMG1.2, anti-IFN-γ/H9253; 11B11, anti-IL-4; and 2.4G2, FC Block (all from BD Pharmingen, San Diego, CA). Murine IL-2 was reconstituted in 0.1% BSA/PBS and stored at −20°C according to the manufacturer’s recommendations (R&D Systems, Minneapolis, MN). The H-2K<sup>B</sup>-SIINFEKL tetramer was kindly provided by the MHC Tetramer Core Facility (National Institute of Allergy and Infectious Diseases, Allentown, PA). The ovASBN construct was transfected into GPE86 cells kindly provided by A. Bank (Columbia University, New York, NY) (47). Packaging cell clones were isolated by limiting dilution and screened for those producing high-titer vector. Twenty-four hours before harvesting supernatant, medium was changed. Retroviral supernatant was then harvested, filtered (0.2 μm polyethersulfone), and stored at −80°C.

**Transduction of murine T cells**

Mouse splenocytes were cultured using modifications of previously described protocols (48–51). Splenocytes were harvested from C57BL/6-Ly5.1 mice and activated with plate-bound anti-CD3 and anti-CD28 at 1 x 10<sup>6</sup> cells/ml in 96-well round-bottom plates (Costar plate 3799; Costar, Corning, NY). Forty-eight hours after stimulation, cells were transferred to 24-well plates (Costar plate 3526), murine IL-2 was added at 20 ng/ml, and a 25% final dilution of viral supernatant was also added. During the next week of culture, cells were maintained at ~1–2 million cells/ml. IL-2 was added every 2 days at 20 ng/ml, and a 25% final dilution of viral supernatant was added on days 5 and 7. On day 10, cells were restimulated with 96-well round-bottom plates precoated with anti-CD3 and anti-CD28 in medium with 500 μg/ml G418 without murine IL-2. On day 12, cells were transferred to 24-well plates in medium with 20 ng/ml murine IL-2 and 500 μg/ml G418. At this point, G418-resistant cells expanded and were used for experiments on days 15–17 of culture.

**Flow cytometry**

Cells were analyzed by flow cytometric analysis as previously described (52). Briefly, cells were washed in FACS buffer consisting of 0.5% BSA and 0.02% sodium azide in HBSS. Cells were stained with PE-labeled anti-Vα2 or anti-Vβ5 (BD Biosciences) and with FITC-labeled anti-Ly5.1 (BD Biosciences). Cells were analyzed by flow cytometry as previously described (52). Briefly, 51Cr-labeled target cells, either EL-4 cells, EL-4 cells pulsed with SIINFEKL peptide, or E.G7-OVA cells, were incubated with T cells in a 96-well U-bottom plate for 4 h at 37°C with 5% CO<sub>2</sub>. Culture supernatant was then harvested and radioactivity was determined. The percentage of specific 51Cr release was calculated according to the equation: percentage of specific lysis = ((experimental release – spontaneous release)/(maximum release – spontaneous release)) x 100. Spontaneous release was determined by incubation of targets in the absence of effectors, whereas maximum target release was determined by treatment of cells with 9% Triton X-100 solution. Similar results were obtained for all experiments showing both the CFSE-propidium iodide cytotoxicity assay (data not shown), a modification of a previously described protocol (53, 54).

**Intracellular cytokine assay and peptide titration assay**

Intracellular cytokine expression was detected using the CytoFix/Cytoperm Plus kit with GolgiStop (BD Pharmingen). Before analysis, T cells were stimulated in a 96-well round-bottom plate by coculture of 100,000 T cells with 100,000 target cells for 7 h. Target cells consisted of EL-4 cells, E.G7-OVA cells, or EL-4 cells pulsed with 6 h 31P SIINFEKL peptide. The concentration of peptide used to label EL-4 cells was either 5 μg/ml in Fig. 6 or 1.7 μg/ml with 3-fold dilutions in Fig. 7. Following coculture of effectors and targets, cells were stained with anti-Ly5.1 Ab to distinguish T cells from target cells. Subsequently, intracellular cytokine expression was determined using the CytoFix/Cytperm kit with GolgiStop (BD Pharmingen).

**Results**

Creation of functional TCR retroviral vectors

The ovASBN retroviral vector (Fig. 1) contains both the OT-1 α and β TCR subunits as well as the drug-resistant gene neomycin. The ability of this vector to express functional TCRs was first analyzed in the T cell hybridoma, 58α β /CD8, which is deficient in both the α and β TCR subunits. These cells contain a full complement of TCR CD3 subunits, which can be expressed on the cell surface only in the presence of both the exogenous α and β TCR subunits (45, 55). Transduction of 58α β /CD8 cells with the ovASBN vector followed by drug selection resulted in a dramatic increase in the expression of the CD3ε subunit (Fig. 2, A and B). This result importantly demonstrates that our ovASBN vector was capable of directing the simultaneous expression of three separate genes in a single cell. Of even greater importance, when ovASBN-transduced cells were presented with the relevant peptide, these cells specifically released IL-2 (Fig. 2C). This indicates that not only is the OT-1 TCR expressed on the surface of these cells, but that it is also functional. These results provided us a model to study exogenous TCR expression in the more complex environment of mature T cells.

Expression of retrovirally encoded TCR in murine T cells

Having demonstrated the ability of the ovASBN retroviral construct to direct expression of a functional TCR in the simple environment of a T cell hybridoma, our next goal was to assess this exogenous TCR expression in the more complex environment of a mature T cell. As a first step, we developed a gene transfer protocol for
transducing murine T cells. Specifically, murine splenocytes, which contain a high percentage of T cells, were stimulated via plate-bound anti-CD3/anti-CD28 and IL-2, which allows for the selective outgrowth of CD8<sup>+</sup>/H<sub>9251</sub>T cells (48). During this culture period, cells were transduced, with either the ovASBN vector or a control vector, and then selected for drug resistance. Using this protocol, we were routinely able to generate large numbers of transduced T cells after 2–3 wk of culture. For simplicity, we limit our discussion to three populations of such T cells, all generated via retroviral transduction and drug selection. These include 1) OT1control-vector (positive control) cells generated via transduction of T cells from OT-1 TCR-transgenic mice or the parental clone; 2) BL6control-vector (negative control) cells generated via transduction of T cells from nontransgenic C57BL/6 mice; and 3) BL6ovASBN-vector (experimental condition) T cells generated via transduction using the ovASBN vector of T cells from nontransgenic C57BL/6 mice.

Having generated retrovirally transduced T cells, the next step was to assess whether the retrovirally encoded TCR was being expressed on the surface. Using Abs specific for the TCR variable regions (the OT-1 TCR α subunit utilizes Vα2 and the β subunit utilizes Vβ5), we show that a high percentage of BL6ovASBN-vector cells expressed Vα2 (60%) or Vβ5 (56%) relative to the percentage of T cells expressing endogenous Vα2 (9%) or Vβ5 (20%), as indicated by staining BL6control-vector cells (Fig. 3, A and B). The expression of retrovirally encoded TCR in BL6ovASBN-vector cells was also manifest in an increase in the percentage of cells double positive for both Vα2 and Vβ5 relative to control cells (27% vs 1.7%, Fig. 3, C and D). On a per cell basis, the level of exogenous TCR was significantly more heterogeneous than that of endogenous TCR. We also demonstrated, using single OT-1 TCR subunit retroviral vectors, the expression of either the OT-1 α or β at levels of 54 and 47%, respectively (data not shown). Since TCRs require complementary α and β subunits to achieve surface expression (55, 56), these results demonstrate that the exogenous OT-1 TCR could pair with endogenous TCR subunits, also consistent with previous reports (36, 57, 58). To evaluate whether this heterogeneous expression or potential formation of TCR heterodimers compromised the ability of the retrovirally encoded TCR expression to recognize Ag, we stained cells with the (SIINFEKL/H-2K<sup>b</sup>)

FIGURE 1. The ovASBN retroviral construct: cDNA encoding the OT-1 TCR were cloned into this modified “pg1 SAMEN SRα” Moloney murine leukemia virus backbone. The 5’ long terminal repeat promoter drives expression of the α TCR gene while the SRα promoter drives the β TCR gene. Drug resistance is encoded by the neomycin resistance gene, which is immediately downstream of encephalomyocarditis virus internal ribosome entry site (IRES). SD, splice donor site; SA, splice acceptor site; ψ<sup>+</sup>, packaging signal; pA, polyadenylation signal; LTR, long terminal repeat. Arrows denote transcription start sites.

FIGURE 2. The ovASBN retroviral construct directs surface expression of the functional OT-1 TCR in a T cell hybridoma. A, FACS analysis shows control 58α-β/CD8<sup>+</sup> cells (not transduced) do not express CD3ε. B, In contrast, the majority of cells transduced with the ovASBN retroviral construct express CD3ε. Isotype control staining indicated by dotted line. C, Cells transduced with ovASBN construct specifically release IL-2 in response to relevant peptide. Data are representative of three independent experiments.
tetramer. Nearly 10% of BL6<sup>ovASBN-vector</sup> cells stained positive, indicating that individual cells are capable of simultaneously expressing both TCR subunits at levels high enough to confer Ag-specific binding (Fig. 4).

**Function of retrovirally encoded TCR genes on murine T cells**

The next step was to test the ability of retrovirally encoded TCR to bestow functional activity. We first evaluated this using cytotoxicity assays. Both OT1<sup>control-vector</sup> and BL6<sup>ovASBN-vector</sup> cells specifically recognized and lysed EL-4 target cells pulsed with the SIINFEKL peptide (Fig. 5). In addition to being cytotoxic, retrovirally transduced cells were evaluated for the ability to produce cytokine in response to relevant Ag. We found that both BL6<sup>ovASBN-vector</sup> and OT1<sup>control-vector</sup> cells specifically produced IFN-γ, as measured by intracellular flow cytometry, following coculture of T cells and target cells with relevant Ag (Fig. 6). We were unable to detect IL-4 production, suggesting the generation of a Th1 (59, 60)-biased population of T cells. Using IFN-γ production as a readout, we were able to quantify that ~15% of BL6<sup>ovASBN-vector</sup> cells were Ag specific in contrast to ~40% of OT1<sup>control-vector</sup> cells. Of those BL6<sup>ovASBN-vector</sup> cells staining positive, more than two-thirds recognized E.G7-OVA tumor cells. As it has been estimated that E.G7-OVA cells only express ~100 specific peptide-MHC complexes per cell (61), these results suggest that the majority of BL6<sup>ovASBN-vector</sup> cells able to recognize Ag were of high avidity (Fig. 6).

It should be noted that BL6<sup>ovASBN-vector</sup> cells were also tested for cytotoxicity against E.G7-OVA tumor cells. In contrast to the OT1<sup>control-vector</sup>-positive control cells, however, we were unable to convincingly show that BL6<sup>ovASBN-vector</sup> cells mediated the lysis of E.G7-OVA tumor cells in an Ag-specific manner (data not shown). Although this might reflect a loss of avidity or sensitivity to Ag recognition, we believe it more likely reflects other factors. First, we observed a fair amount of nonspecific lysis in our cytotoxicity assay against Ag-negative EL-4 cells. This may reflect the development of NK cell activity, which has been associated with culture conditions similar to those we used (62–67). It is also possible that expression of high levels of exogenous TCR might predispose or enhance the susceptibility of retrovirally modified T cells developing NK cell activity, although we have no direct evidence supporting this. Another significant factor that likely limits the sensitivity of our cytotoxicity assay is that the percentage of Ag-specific T cells among the bulk BL6<sup>ovASBN-vector</sup> cells was three to four times lower than among the bulk OT1<sup>control-vector</sup>-positive control cells (Fig. 6 and data not shown). Thus, this lower percentage of Ag-specific T cells combined with a potential increase in NK cell activity in the large percentage of Ag-nonspecific T cells among bulk BL6<sup>ovASBN-vector</sup> cells may have contributed to our inability to demonstrate cytotoxicity of E.G7-OVA tumor cells by BL6<sup>ovASBN-vector</sup> cells.

Given these potential limitations, to further examine the avidity issue, we used a peptide titration assay to directly compare the avidity of BL6<sup>ovASBN-vector</sup> and OT1<sup>control-vector</sup> cells and to assess the minimum amount of peptide necessary to achieve a half-maximal response in IFN-γ production. (The advantages of this IFN-γ-based peptide titration assay are multifold, including that 1) IFN-γ production is very tightly linked to Ag recognition (68, 69) and 2) this assay is not detrimentally affected by differences in the frequency of Ag-specific T cells.) We anticipated that OT1<sup>control-vector</sup> cells, which are able to exclude competing TCR through allelic exclusion, would be able to recognize lower levels of Ag than BL6<sup>ovASBN-vector</sup> cells that express competing sets of TCR on their surface. Contrary to our expectations, the half-maximal dose of peptide was only, at best, marginally different (~5-fold) between BL6<sup>ovASBN-vector</sup> and OT1<sup>control-vector</sup> cells (Fig. 7). The results shown are representative of four experiments. Additionally, the peptide-titration experiment revealed that a significant portion of BL6<sup>ovASBN-vector</sup> cells responded to lower levels of Ag than that found on E.G7-OVA target cells, as indicated by comparison of the percentage of cells responding to E.G7-OVA with those responding to titrated doses of peptide (Figs.
subunits exhibited heterogeneous expression relative to that of the endogenous TCR subunits. This heterogeneous expression could result from either variable gene expression or competition between exogenous and endogenous TCR subunits. The opportunity for individual OT-1 TCR subunits to form heterodimers with endogenous TCRs was verified by the transduction of T cells with alternative vectors containing only individual \( \alpha \) or \( \beta \) TCR subunit genes. Despite the potential for this heterodimer formation and the variable gene expression observed, a significant portion of these cells stained positive with the relevant tetramer, demonstrating not only simultaneous gene expression of all retrovirally encoded TCR genes, but sufficient levels of TCR subunit surface expression to achieve Ag recognition.

In addition to characterizing the expression of retrovirally encoded TCR, we analyzed the ability of such expression to confer Ag-dependent functional ability to mature T cells. Importantly, we demonstrated, for the first time, that murine splenocytes retrovirally transduced with TCR genes can be endowed with the ability to kill peptide-pulsed target cells in vitro. Consistent with this result, we also determined that around 15% of BL6\(^{\text{ovASBN-vector}}\) cells produced IFN-\( \gamma \) specifically in response to relevant tumor. This was particularly impressive in that only about half of OT1\(^{\text{control-vector}}\) cells, which were almost all TCR and CD8 positive (data not shown), produced IFN-\( \gamma \), suggesting that it may not be feasible to successfully functionally modify every cell in the BL6\(^{\text{ovASBN-vector}}\) condition. It was unclear why more OT1\(^{\text{control-vector}}\) control cells did not stain positive. Although it was possible that assaying for other cytokines, such as TNF-\( \alpha \) could have identified a higher percentage of Ag-specific cells, this would probably only have represented a minor contribution, as the percentage of Ag-specific cells identified by tetramer staining and IFN-\( \gamma \) staining correlated well. One possible explanation for why roughly only half of OT1\(^{\text{control-vector}}\) cells stained positive may relate to their activation state, because it has been reported that even though Ag-specific T cells may retain both TCR and CD8 expression, they may not always stain tetramer positive or exhibit Ag-specific functional qualities (74, 75). If the activation state is a relevant factor, this may also explain why roughly 25% of BL6\(^{\text{ovASBN-vector}}\) cells stained positive for high levels of exogenous dual \( \alpha \beta \) TCR expression while only 10–15% of BL6\(^{\text{ovASBN-vector}}\) cells were positive by IFN-\( \gamma \)-staining or tetramer analysis.

Another relevant issue in this article was that although IFN-\( \gamma \) staining and cytotoxicity correlated well in the analysis of OT1\(^{\text{control-vector}}\) control cells, BL6\(^{\text{ovASBN-vector}}\) cells failed to show Ag-specific cytotoxicity against E.G7-OVA tumor cells despite producing IFN-\( \gamma \) after coculture with E.G7-OVA tumor cells. As previously discussed, this may reflect limitations in our cytotoxicity assay including both differences in the percentage of Ag-specific T cells among the bulk populations and the possible generation of NK cell activity. The relevance of these limitations could be directly tested by sorting the bulk BL6\(^{\text{ovASBN-vector}}\) cells based on tetramer staining and directly assessing their cytotoxicity against E.G7-OVA tumor cells. However, even if these limitations do not explain the discrepancy between the results of our cytotoxicity and IFN-\( \gamma \)-based assays, it is important to note that in contrast to in vitro cytotoxicity, in vitro cytokine production following Ag exposure may be a much stronger predictor of in vivo therapeutic efficacy (76–79). As it has been previously shown that T cell clones isolated from TCR gene-modified human PBL can be segregated based on either cytotoxicity, cytokine expression, or both of these properties (29), the isolation of T cell clones in our murine system might prove useful in formally evaluating whether cytotoxicity or cytokine production is more useful as a predictor for in vivo therapeutic efficacy.

A key indicator of T cell therapeutic potential is T cell avidity, which refers to the overall binding affinity between a T cell and endogenous Ag. This avidity could be similar to that of their parental clone. However, it was recently demonstrated that T cells expressing a retroviral vector directed against the homolog of endogenous Ag levels of much lower than 100 peptide-MHC complexes per cell.

### Discussion

It was first demonstrated in 1998 that a complete retrovirally encoded TCR could be expressed in the peripheral blood of a mouse (70). Subsequently, it was shown that the retrovirally mediated transfer of TCR genes could also lead to the functional recognition of both tumor and virally infected cells in vitro (29, 30). It has now just recently been demonstrated that T cells expressing a retrovirally encoded TCR can also function in a therapeutic manner (40). Our data extend upon these reports (71–73) and characterize the avidity of T cells expressing an exogenous TCR. We show for the first time that despite variable TCR gene expression and the presence of endogenous TCRs, the avidity of the resulting T cells could be similar to that of their parental clone.

In addition to analyzing the Ag sensitivity of T cells following TCR gene transfer, we have examined parameters not previously characterized. Using Abs against both TCR subunits, we have uniquely characterized the expression of a complete TCR encoded from a single retroviral vector. Our results show that both TCR subunits exhibited heterogeneous expression relative to that of the endogenous TCR subunits. This heterogeneous expression could result from either variable gene expression or competition between exogenous and endogenous TCR subunits. The opportunity for individual OT-1 TCR subunits to form heterodimers with endogenous TCRs was verified by the transduction of T cells with alternative vectors containing only individual \( \alpha \) or \( \beta \) TCR subunit genes. Despite the potential for this heterodimer formation and the variable gene expression observed, a significant portion of these cells stained positive with the relevant tetramer, demonstrating not only simultaneous gene expression of all retrovirally encoded TCR genes, but sufficient levels of TCR subunit surface expression to achieve Ag recognition.

In addition to characterizing the expression of retrovirally encoded TCR, we analyzed the ability of such expression to confer Ag-dependent functional ability to mature T cells. Importantly, we demonstrated, for the first time, that murine splenocytes retrovirally transduced with TCR genes can be endowed with the ability to kill peptide-pulsed target cells in vitro. Consistent with this result, we also determined that around 15% of BL6\(^{\text{ovASBN-vector}}\) cells produced IFN-\( \gamma \) specifically in response to relevant tumor. This was particularly impressive in that only about half of OT1\(^{\text{control-vector}}\) cells, which were almost all TCR and CD8 positive (data not shown), produced IFN-\( \gamma \), suggesting that it may not be feasible to successfully functionally modify every cell in the BL6\(^{\text{ovASBN-vector}}\) condition. It was unclear why more OT1\(^{\text{control-vector}}\) control cells did not stain positive. Although it was possible that assaying for other cytokines, such as TNF-\( \alpha \) could have identified a higher percentage of Ag-specific cells, this would probably only have represented a minor contribution, as the percentage of Ag-specific cells identified by tetramer staining and IFN-\( \gamma \) staining correlated well. One possible explanation for why roughly only half of OT1\(^{\text{control-vector}}\) cells stained positive may relate to their activation state, because it has been reported that even though Ag-specific T cells may retain both TCR and CD8 expression, they may not always stain tetramer positive or exhibit Ag-specific functional qualities (74, 75). If the activation state is a relevant factor, this may also explain why roughly 25% of BL6\(^{\text{ovASBN-vector}}\) cells stained positive for high levels of exogenous dual \( \alpha \beta \) TCR expression while only 10–15% of BL6\(^{\text{ovASBN-vector}}\) cells were positive by IFN-\( \gamma \)-staining or tetramer analysis.

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APC that is capable of leading to a functional response (17, 33–35). T cell avidity can be influenced by a number of factors (34, 80, 81) including some not easily amenable by modifications in current TCR gene transfer approaches and others that may be directly influenced by the techniques used. Of the latter, the potential factors that might maximize the avidity of TCR gene-modified T cells include 1) the selection of a TCR with optimized affinity and 2) the appropriate surface expression of the TCR. In terms of TCR

FIGURE 5. The ovASBN retroviral vector confers Ag-specific cytotoxicity in primary T cells. Cytotoxicity was measured following a 4-h coculture of effector cells with target cells. Target cells included either EL-4 cells pulsed with SIINFEK (■) or EL-4 cells not pulsed (□). Effector cells included either OTI control-vector (A), BLG control-vector (B), or BLG ovASBN-vector (C) cells. Results are representative of four independent experiments.

FIGURE 6. The ovASBN retroviral vector bestows the ability of primary T cells to produce IFN-γ production following specific Ag engagement. Intracellular IFN-γ and IL-4 expression measured by FACS after an 8-h coculture of the indicated retrovirally transduced T cells with the following target cells: EL-4 cells (A–C), EL-4 cells pulsed with 5 μg/ml SIINFEKL peptide (D–F and J–L), or E.G7-OVA cells (G–I). Cells were gated by both FS/SS# and for expression of Ly5.1 (to exclude Ly5.2 target cells). BL6 control-vector cells did not produce significant levels of IFN-γ in any condition. In contrast, BLG ovASBN-vector and OTI control-vector cells produced IFN-γ in response to relevant Ag (E, F, H, and I). In contrast, IL-4 production was not detected in BLG ovASBN-vector and OTI control-vector cells with any target cell (J–L; data not shown). Number in upper right corner indicates the percentage of Ly5.1 T cells gating positive for cytokine production. Results are representative of three independent experiments.
affinity, this is probably more of a fixed variable dependent on the selection of an appropriate TCR. We chose the OT-1 TCR, which has been well characterized and is known to be of sufficient affinity to produce high-avidity T cells (82, 83). Although it appeared obvious that the selection of a good TCR for gene transfer would be critical, we also anticipated that the level of TCR expression would be a critical factor in influencing the avidity of the resulting T cell. Contrary to our expectations, despite the potential for heterodimer formation and variable gene expression, we were unable to detect a significant loss of T cell avidity in T cells expressing functional retrovirally encoded TCRs.

T cell avidity was evaluated in the context of titrated amounts of Ag to allow the identification of populations of T cells of increasing avidity, which respond to decreasing concentrations of Ag. Our baseline for comparison was OT1control-vector cells, which express a fairly uniform and high level of TCR expression and are known to be of high avidity (Fig. 4). With BL6ovASBN-vector cells, we expected to see a significant variation of avidity as a result of the variable expression of TCR observed. Additionally, since the OT-1 TCR is capable of pairing with endogenous TCRs, we anticipated that the formation of such heterodimers might negatively impact T cell avidity. Contrary to our expectations, of those OT1control-vector and BL6ovASBN-vector cells that were Ag specific, these cells showed only small differences (of <5-fold) in response to limiting concentrations of Ag. This difference appears extraordinarily small when compared with other publications showing at least 100- to 1000-fold differences in Ag sensitivity between low- and high-avidity T cells (17, 33–35, 84–86). Although we cannot exclude the possibility that there is some difference in avidity between BL6ovASBN-vector and OT1control-vector cells, we believe these results indicate T cell avidity can be most directly influenced by inherent properties of the TCR used. Although the level of TCR expression probably does have relevance, it may represent more of a threshold criterion, in that T cells either express appropriate levels of TCR or do not. In terms of heterodimer formation, unlike OT1control-vector cells, which were able to exclude the formation of most heterodimers by allelic exclusion, Ag-specific BL6ovASBN-vector cells were generated in the context of pre-existing endogenous TCRs. Thus, despite the presence of endogenous TCRs and variable gene expression, we were able to generate T cells of high avidity equal to the parental clone.

We should note some caution in interpretation of our results; they were obtained using the genes of a single TCR. The other report examining T cell avidity following TCR gene transfer to mature T cells found that the resulting T cells were of lower avidity (30). The results of this report in terms of T cell avidity are somewhat complicated though, as Ag-specific T cell clones were selected with high amounts of Ag, which might allow for the outgrowth of low-avidity T cells (17, 34). Nevertheless, it is quite possible that some special attributes of the OT-1 TCR allow it to function in a unique way at low levels of expression. In fact, the one report we are aware of that arrived at similar conclusions to our own in terms of the sensitivity of TCR function in the face of low expression also utilized the OT-1 TCR model (57). But, even if our findings are not universally true for all TCRs, they are of significant importance. With sufficient care, it should be possible to choose or even artificially generate TCRs that display optimized quality (18, 87, 88).

Regardless of the method used for obtaining viable TCR genes, the value of the TCR transfer approach is underscored by the many patients with either cancer, viral infection, or other life-threatening diseases, for whom it is not possible to expand T cells of therapeutic value. For such patients, the successful transfer of TCR genes would undoubtedly be a significant step in the development of improved T cell-based therapies. Our results support the utility of transferring TCR genes to create Ag-specific T cells for use in such a strategy.

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