Adoptively transferred effector cells derived from naïve rather than central memory CD8$^+$ T cells mediate superior antitumor immunity

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Effector cells derived from central memory CD8$^+$ T cells were reported to engraft and survive better than those derived from effector memory populations, suggesting that they are superior for use in adoptive immunotherapy studies. However, previous studies did not evaluate the relative efficacy of effector cells derived from naïve T cells. We sought to investigate the efficacy of tumor-specific effector cells derived from naïve or central memory T-cell subsets using transgenic or retrovirally transduced T cells engineered to express a tumor-specific T-cell receptor. We found that naïve, rather than central memory T cells, gave rise to an effector population that mediated superior antitumor immunity upon adoptive transfer. Effector cells developed from naïve T cells lost the expression of CD62L more rapidly than those derived from central memory T cells, but did not acquire the expression of KLRG-1, a marker for terminal differentiation and replicative senescence. Consistent with this KLRG-1$^-$ phenotype, naïve-derived cells were capable of a greater proliferative burst and had enhanced cytokine production after adoptive transfer. These results indicate that insertion of genes that confer antitumor specificity into naïve rather than central memory CD8$^+$ T cells may allow superior efficacy upon adoptive transfer.

Infusion of tumor-reactive T cells to treat cancer is transitioning from a promising possibility to a successful reality. Adoptive immunotherapy with T cells can effectively treat patients with EBV-associated malignancies and metastatic melanoma, and application of this treatment is broadening as our ability to generate T cells targeting diverse tumor antigens improves (1–10). Our expanding capacity to target novel antigens is driven, in part, by advances in genetic engineering that permit high efficiency transfer of genes encoding tumor specific T-cell receptors (TCR) into open repertoire mature T cells. These genetically modified T cells can specifically recognize tumor cells in vitro, and can induce objective tumor regression following infusion into patients (9).

The ability to engineer tumor recognition permits not only targeting of any antigen for which a specific TCR can be identified, but also selection of the CD8$^+$ T-cell subset from which the cells for therapy will be generated. Resting CD8$^+$ T cells exist as naïve (T_N), central memory (T_{CM}), and effector memory (T_{EM}) populations, each with distinct phenotypic and functional characteristics (11). In vitro stimulation of these subsets induces their proliferation and differentiation into the cytolytic effector cells (T_{EFF}) used for patient treatment. While the nature of CD8$^+$ T-cell subsets is well defined (12), the heritable influence of those populations on the traits of their effector cell progeny is not well studied (13, 14). Understanding this relationship might be important for generating optimal effector cells for patient treatment.

The characteristics of CD8$^+$ T-cell subsets have been elucidated primarily through study of viral infection (15–17). In this setting, memory cells are superior to naïve cells due to their increased precursor frequency (18), their rapid proliferation and their efficient acquisition of effector functions (12). However, these qualities might not be advantageous for adoptive immunotherapy where the precursor frequency is determined by the number of cells infused, and differentiation into effector cells occurs before cell infusion. Indeed, recent studies intimate this possibility; in nonhuman primates, induction of effector memory cells has been uniquely successful in protecting from simian immunodeficiency virus (19) yet, in another macaque study, adoptively transferred effector cells generated from effector memory cells rapidly perished (20).

Previous studies on the influence of CD8$^+$ T cell differentiation states have not focused on the relative efficacy of naïve T cells (20–23). With the emergence of TCR gene therapy, naïve cells, which represent the most common CD8$^+$ T-cell phenotype in many patients, have become an important potential source of effector cells. Herein we investigate the lineage relationship and therapeutic efficacy of effector cells of naïve or central memory origin, and we report the superior efficacy of effector cells derived directly from naïve T cells for adoptive immunotherapy of cancer.

Results

We used the pmel-1 TCR transgenic model of adoptive immunotherapy to study the development, function, and efficacy of tumor specific effector cells differentiated from naïve or central memory progenitors. This model reproduces the clinical challenge of breaking tolerance to a shared tumor/self antigen to induce regression of large, established tumors (24). Tumor-specific CD8$^+$ T-cell populations enriched for T_N or T_{CM} phenotype cells were isolated from pmel-1 splenocytes (Fig. S1). These cells displayed not only the phenotypic but also the functional qualities ascribed to naïve and central memory cells as, in response to antigenic stimulation, IFN-γ production and proliferation were more efficient in the T_{CM} subset (Fig. 1 *A* and *B*) (12). Emulating clinical protocol, effector CD8$^+$ T cells were generated from each population by two stimulations (Fig. 1*C*) (9). For simplicity, effector cells of naïve or central memory origin were termed T_{EFF}^N and T_{EFF}^{CM}, respectively.

Effector Cells Generated from Naïve or Central Memory Cells Acquire Cytolytic Effector Cell Phenotype and Function. Both T_{EFF}^N and T_{EFF}^{CM} demonstrated high levels of specific target killing consistent with effector CD8$^+$ T-cell function (Fig. 1*D*). They also expressed the cytolytic granule proteins that typify effector cells, perforin 1,

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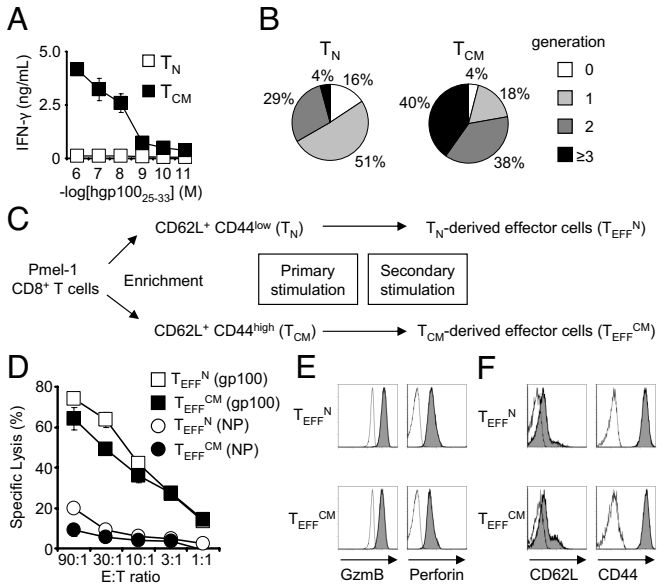

Fig. 1. Effector CD8$^+$ T cells derived from naïve or central memory cells acquire cytolytic phenotype and function. (*A*) Production of IFN-γ by freshly isolated cells in an overnight coculture assay. The error bars represent the standard error of the mean. (*B*) The number of cell divisions 2 days after peptide stimulation, as determined by CFSE dilution from a FACS histogram. (*C*) Schematic delineating generation of primary and secondary effector cells from naïve and central memory CD8$^+$ T cells. (*D*) Cytolytic function of T_{EFF}^N and T_{EFF}^{CM} as determined by ^{51}Cr release assay. The target peptide, $gp100_{25–33}$ or $NP_{366–374}$ is indicated in parenthesis. (*E*) Flow cytometric analysis indicating expression of granzyme B and perforin and (*F*) L-selectin and CD44. The open histograms indicate isotype antibody controls. All figures shown are representative of at least two independent experiments.

and granzyme (Fig. 1*E*). To characterize further their differentiation states, we determined expression of CD62L and CD44, the phenotypic markers by which the progenitor cell subsets were isolated. Both T_{EFF}^N and T_{EFF}^{CM} predominantly displayed the CD62L$^-$ CD44^{high} phenotype of effector cells (Fig. 1*F*) (25). Taken together, these data indicated that effector cells were generated from both the T_N and T_{CM} populations.

Effector CD8$^+$ T Cells of Naïve or Central Memory Origin Possess Distinct Gene Expression Signatures and Developmental Programs. We sought to determine if, despite acquisition of similar effector phenotype and function, T_{EFF}^N and T_{EFF}^{CM} retained distinct gene expression signatures. Oligonucleotide microarrays were used to study the two effector cell groups in the resting condition and 4 h after restimulation. A false discovery rate (FDR) of 2% and 2-fold or greater difference identified 1,020 and 86 probe sets that were differentially expressed between T_{EFF}^N and T_{EFF}^{CM} in rested and restimulated cells, respectively (Fig. 2 *A* and *B*). An annotated list of selected genes with reported function in T cells and greater than 5-fold differential expression is shown in Table S1. Real time RT-PCR validation of selected differentially expressed genes is displayed in Fig. S2. These microarray data revealed unique gene expression signatures of effector cells of naïve or central memory origin. Thus, T_N and T_{CM} appeared to confer distinct programs to their effector cell progeny.

To better understand the effector cell programs and lineage relationship of T_{EFF}^N and T_{EFF}^{CM} we studied changes in gene expression, phenotype, and function induced by antigen- and IL-2-driven differentiation. The T-box transcription factors T-bet and Eomesodermin (Eomes) control cytolytic development and function of CD8$^+$ T cells (26). T-bet (encoded by *Tbx21*) was less than 2-fold differentially expressed in T_{EFF}^N and T_{EFF}^{CM} on microarray and real time RT-PCR. However, *Eomes* was the most highly overexpressed transcription factor in T_{EFF}^{CM} on microarray for both resting and stimulated cells. We examined expression of *Eomes* as effector differentiation was induced by serial stimulation with antigen and IL-2. *Eomes* mRNA transcripts were approximately 100-fold greater in T_{EFF}^{CM} than T_{EFF}^N following primary stimulation (Fig. 2*C*). The relatively high *Eomes* mRNA in T_{EFF}^{CM} was maintained through secondary and tertiary stimulation. *Eomes* expression by T_{EFF}^N increased with each stimulation, but did not attain the level observed in T_{EFF}^{CM}. Thus, effector cells of T_N or T_{CM} origin acquired and maintained different levels of *Eomes* expression despite repeated stimulation with the same developmental cues. These patterns of *Eomes* expression suggested differences in the development and functional programs that were conferred by T_N or T_{CM}.

To further study the lineage relationship between T_{EFF}^N and T_{EFF}^{CM}, we examined expression of CD62L, the adhesion molecule that distinguishes the central memory from the effector memory lineage (Fig. 2*D*). Following primary stimulation, T_{EFF}^N displayed greater frequency of CD62L$^+$ CD44^{high} cells than T_{EFF}^{CM}. However, this phenotype was transient as secondary and tertiary stimulation induced loss of CD62L. In contrast, T_{EFF}^{CM} maintained a more stable, albeit diminishing, subset of CD62L$^+$ CD44^{high} cells suggesting that expression of CD62L is a T_{CM} lineage-specific trait that is not entirely lost with effector differentiation.

Because CD62L expression decreases with progressive differentiation of effector CD8$^+$ T cells (25, 27), we hypothesized that the CD62L$^+$ subpopulation of T_{EFF}^{CM} was less prone to terminal differentiation than the CD62L$^-$ subpopulation. To test for terminal differentiation of these subsets, we examined simultaneous expression of CD62L and killer cell lectin-like receptor subfamily G, member 1 (KLRG1), an inhibitory receptor that is expressed by senescent T cells (Fig. 2*E*) (28, 29). Serial stimulation did not induce KLRG1 expression in T_{EFF}^N. However, T_{EFF}^{CM} developed a subset of KLRG1^{high} cells, suggesting a greater propensity to terminal maturation. Remarkably, the frequency of KLRG1^{high} cells was equivalent in the CD62L$^-$ and CD62L$^+$ subsets indicating that the CD62L$^+$ subset was not protected from developing a senescent phenotype (Fig. 2*F*). This finding defies models of effector cell differentiation in which the CD62L$^+$ CD44^{high} T_{CM} phenotype is mutually exclusive with the KLRG1^{high} senescent T_{EFF} phenotype (3, 13). These data suggest, rather, that effector cells of T_{CM} origin might be a distinct lineage that can maintain the lineage-specific trait of CD62L expression even as they differentiate into senescent effector cells.

We further characterized T_{EFF}^N and T_{EFF}^{CM} by examining their secretion of cytokines. Effector CD8$^+$ T cells gain the ability to elaborate IFN-γ but lose the capacity to produce IL-2 as they progressively differentiate (3, 13, 25, 30, 31). Following primary stimulation, T_{EFF}^{CM} were capable of greater IFN-γ and lesser IL-2 production than T_{EFF}^N, which suggested that T_{EFF}^{CM} were more differentiated (Fig. 2*G*). Remarkably, following secondary stimulation T_{EFF}^N elaborated greater quantities of both IFN-γ and IL-2 (Fig. 2*G*). The reversal between primary and secondary stimulation in the effector cells that produced greater IFN-γ was confirmed by flow cytometry (Fig. 2*H*). The enhanced production of both IFN-γ and IL-2 by T_{EFF}^N was somewhat paradoxical, but it might be explained by relative cellular senescence of T_{EFF}^{CM}. Taken together with their tendency to develop a KLRG1^{high} phenotype, the data suggest that T_{EFF}^{CM} terminally differentiate and senesce more rapidly than T_{EFF}^N.

Effector CD8$^+$ T Cells Generated Directly from Naïve Cells Mediate More Potent Antitumor Activity. We tested the efficacy of effector cells derived from T_N or T_{CM} in tumor treatment. T_{EFF}^N and T_{EFF}^{CM} generated from a primary stimulation were adoptively transferred into nonlethally irradiated mice bearing established, vascularized tumors. Specific vaccination and IL-2 were coadmin-

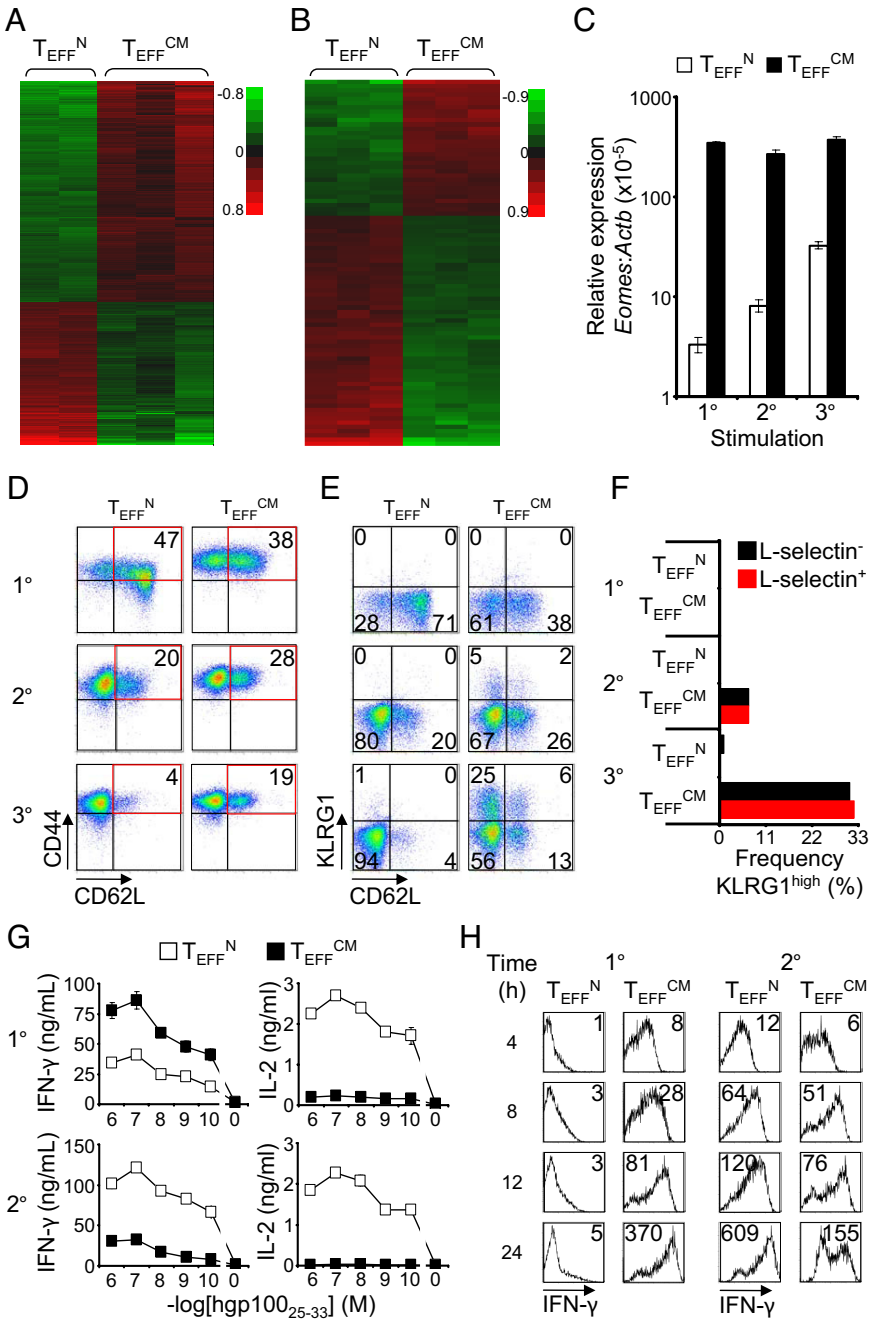

Fig. 2. Naïve and central memory cells confer distinct genetic signatures and developmental programs to their effector cell progeny. Microarray analysis of the gene expression of (*A*) rested and (*B*) stimulated T_{EFF}^{N} and T_{EFF}^{CM}. Probe sets with FDR ≤2% and ≥2-fold differences in expression are shown. Red and green colors indicate increased and decreased expression respectively. The scales are $\log_{10}$. (*C*) Relative expression of *Eomes* by T_{EFF}^{N} or T_{EFF}^{CM} as determined by real time RT-PCR. The *x* axis indicates the number of times the cells were stimulated with antigen and IL-2. Error bars represent the standard error of the mean. (*D*) Flow cytometric determination of the expression of CD62L and CD44 by T_{EFF}^{N} and T_{EFF}^{CM} through serial stimulations. The numbers on the dot plots indicate the frequency of cells with central memory ($CD62L^{+}$ $CD44^{high}$) phenotype. (*E*) Expression of CD62L and KLRG1. Quadrant frequencies are shown. (*F*) Graph derived from panel E. Frequencies of cells from the $CD62L^{+}$ or $CD62L^{-}$ fraction of T_{EFF}^{N} or T_{EFF}^{CM} with $KLRG1^{high}$ phenotype. (*G*) IFN-γ and IL-2 production, in coculture assays, by effector cells generated by primary or secondary stimulation of naïve or central memory cells. (*H*) Intracellular IFN-γ expression following peptide stimulation of T_{EFF}^{N} or T_{EFF}^{CM}. The mean fluorescence intensities are indicated. All figures shown are representative of at least two independent experiments.

istered to all treated mice. Both T_{EFF}^{N} and T_{EFF}^{CM} displayed significant antitumor activity. However, T_{EFF}^{N} were significantly more effective than T_{EFF}^{CM} (Fig. 3*A*). Greater efficacy of T_{EFF}^{N} was also observed with adoptive transfer of effector cells generated from a secondary stimulation (Fig. S3). However, it should be noted that higher numbers of secondary stimulation T_{EFF}^{N} were required to achieve antitumor activity similar to that seen with primary stimulation. These data are consistent with our previous findings indicating that T cells lose in vivo function with repeated in vitro stimulation (25).

We next studied the mechanisms underlying the differences in efficacy of T_{EFF}^{N} and T_{EFF}^{CM}. We reisolated cells from recipient mice 6 days following infusion and tested their capacity to elaborate IFN-γ and IL-2. T_{EFF}^{N} produced greater quantities of both cytokines (Fig. 3*B*). Furthermore, T_{EFF}^{CM} appeared to lose the ability to produce IL-2 following infusion, as they secreted no detectable IL-2 in this assay (Fig. 3*B*). Results were similar with adoptive transfer of cells generated from secondary stimulation (Fig. S4).

To test the importance of IFN-γ and IL-2 production by effector cells, we treated tumor bearing mice with cells deficient in these cytokines. IFN-$\gamma^{-/-}$ T_{EFF}^{N} but not IL-$2^{-/-}$ T_{EFF}^{N} displayed impaired antitumor responses (Fig. 3 *C* and *D*). Thus, IFN-γ was a crucial effector cytokine. However, IL-2 appeared to mark effective cells, rather than mediate the antitumor response. The finding that IL-2 deficiency did not impair the function of effector cells was counterintuitive considering the capacity of IL-2 to potentiate T cell-based adoptive immunotherapy. However, this result was consistent with other work showing no benefit to overexpression of IL-2 by adoptively transferred T cells (32).

We next tested the capacity of the effector cells to expand following adoptive transfer. T_{EFF}^{N} displayed greater clonal expansion in both the spleen and the tumor draining lymph nodes (Fig.

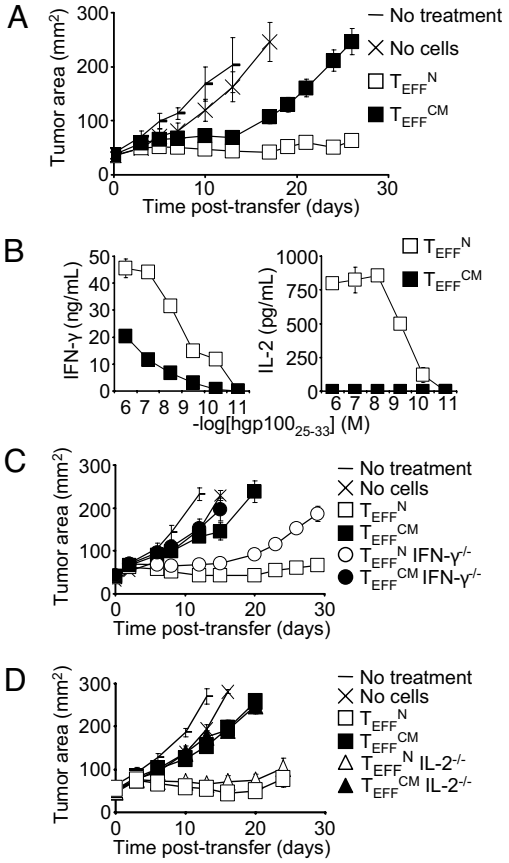

Fig. 3. Use of effector cells derived from naïve rather than central memory cells improves adoptive cell transfer therapy. (*A*) Tumor responses following treatment with 4×10^6 effector cells generated from naïve or central memory subsets. All groups except ''No treatment'' received adjuvant vaccine and IL-2. T_{EFF}^N versus T_{EFF}^{CM}, $P < 0.01$. (*B*) Production of IFN-γ and IL-2 by adoptively transferred cells reisolated from spleens 6 days following infusion. Cell numbers were equalized for the assay. (*C*) Tumor treatment with 7.65×10^5 wild-type or IFN-$\gamma^{-/-}$ effector cells. T_{EFF}^N versus IFN-$\gamma^{-/-}$ T_{EFF}^N, $P < 0.05$. (*D*) Tumor treatment with 1.05×10^6 wild-type or IL-$2^{-/-}$ effector cells. T_{EFF}^N versus IL-$2^{-/-}$ T_{EFF}^N, $P > 0.05$. All figures shown are representative of at least two independent experiments.

4 *A* and *B*). Consistent with this enhanced expansion, T_{EFF}^N less frequently exhibited the KLRG1^{high} phenotype of cells with limited proliferative potential (Fig. 4*C*). These findings were reproduced with adoptive transfer of effector cells generated from two simulations (Fig. S5). Taken together these data indicate the therapeutic superiority of effector cells generated from naïve cells, and they demonstrate greater capacity for cytokine production and expansion of these cells following infusion.

Application to Genetically Engineered Open Repertoire CD8$^+$ T Cells. Pmel-1 TCR-transgenic T cells develop in mice with a genetically restricted repertoire of self-specific precursors. The naturally occurring subsets of naïve and central memory cells in these mice might be artifactually induced by interactions with self-antigen or by differences in assembly and expression of the TCR. We sought to test if the principles elucidated in transgenic cells also applied to wild-type cells that developed with a full TCR repertoire. T_N or T_{CM} wild-type CD8$^+$ T cells were stimulated and transduced with a retroviral vector encoding the pmel-1 TCR. This transduction model emulates clinical therapies, and permits study of T-cell subsets that develop in physiological conditions. Paralleling results with transgenic cells, T_{EFF}^N produced less IFN-γ following the primary stimulation, but more IFN-γ following the second stimulation. Also consistent with the transgenic cells, T_{EFF}^N produced more IL-2 after both stimulations (Fig. 5*A*). These data supported the similarity of transgenic cells to transduced wild-type cells, and they again demonstrated that as T_{EFF}^N differentiated they exceeded T_{EFF}^{CM} in production of both IFN-γ and IL-2.

The efficacy of transduced T_{EFF}^N and T_{EFF}^{CM} was studied by infusion of cells into tumor bearing mice. Expression of the TCR is weaker with gene transduction than with transgenic animals, and tumor therapy is less robust when transduced cells are used. Nevertheless, consistent with findings from transgenic cells, antitumor immunity was greater with infusion of TCR tranduced T_{EFF}^N (Fig. 5*B*). This finding was reproduced by effector cells generated from secondary stimulation (Fig. 5*C*). These data validated work in the transgenic mouse model, and they again indicated that the distinct program conferred by naïve progenitors to their effector cell progeny directed development of more effective cells for adoptive immunotherapy.

Discussion

Studies of viral infection have illuminated the distinct traits and niche roles of CD8$^+$ T-cell subsets in physiological immunity. Adoptive immunotherapy is a nonphysiological setting in which lessons from the study of antiviral responses might not apply. In fact, the advantage of adoptive immunotherapy is its capacity to distort normal immunity by ablating the host immune system before cell infusion, administering massive numbers of antigen-specific T cells, and further activating transferred cells with immune adjuvants (1, 3, 13, 33). We found that the efficient physiological responses of central memory cells were not advantageous to their adoptively transferred effector cell progeny. Rather, effector cells derived from central memory cells rapidly differentiated, expediting senescence and resulting in diminished function following infusion. In contrast, effector cells of naïve origin displayed sustained effector development, with prolonged cytokine production, increased in

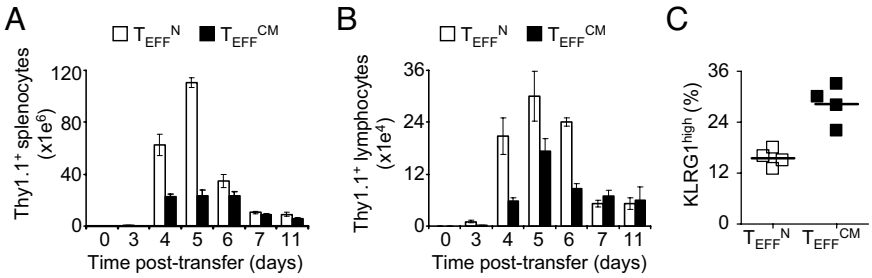

Fig. 4. Effector cells derived directly from naïve cells possess greater potential for clonal expansion following infusion. (*A* and *B*) The number of cells present in spleens and lymph nodes, respectively, following adoptive transfer of T_{EFF}^N or T_{EFF}^{CM}. Error bars indicate the standard error of the mean. ($n = 4$ for each group and each time point). (*C*) The frequency of transferred cells in recipient spleens displaying KLRG1^{high} phenotype 5 days following infusion. The transverse lines represent the means. $P = 0.0024$. All figures shown are representative of at least two independent experiments.

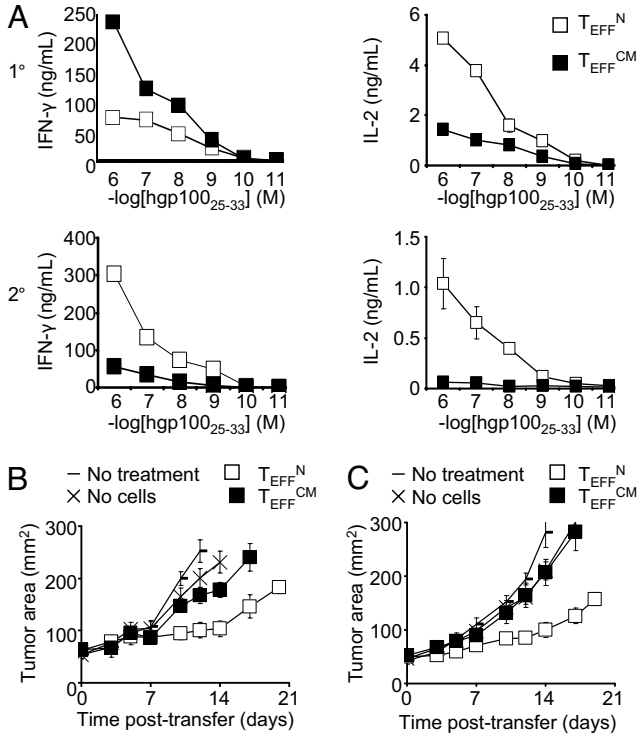

Fig. 5. Genetically engineered open repertoire cells reproduce the central findings of the transgenic mouse model. (*A*) Elaboration of IFN-γ and IL-2 by TCR transduced naïve- or central memory-derived effector cells. (*B*) Tumor growth following treatment with transduced effector cells generated by 4×10^6 primary or (*C*) 2×10^7 secondary stimulation of naïve or central memory cells. All figures shown are representative of at least two independent experiments.

vivo expansion, and, ultimately, more effective antitumor responses.

These data, showing effector cells with distinct traits determined by progenitor subsets, add to the increasing evidence that effector $CD8^+$ T cells are not a homogenous population but rather an amalgamation of subsets with diverse functions and fates. $CD8^+$ T cells can acquire Th1-, Th2-, Treg-, and Th17-type effector functions, which impact their abilities to combat infections and to mediate tumor regression (13, 34–36). Furthermore, effector cell subsets fated for either rapid extinction or for long-term survival as central memory or effector memory cells have been identified (37, 38). The effector cell subset destined for long-term survival tends to express IL-7Rα and lack expression of KLRG1 (15, 37). KLRG1, which identifies senescent effector cells with diminished replicative capacity, impairs proliferation through defective AKT (Ser-473) phosphorylation (28). In our studies, T_{EFF}^{CM} but not T_{EFF}^{N} acquired KLRG1 expression in vitro and in vivo reflecting a greater tendency of this effector cell lineage to develop phenotypic senescence and suggesting a mechanism for the diminished expansion of these cells that occurred following infusion. These findings further imply that the fate of short- or long-lived effector cells might be linked to their origin as naïve or memory T cells and underscore that the prevention of terminal differentiation, which can be accomplished using IL-21 or Wnt, is desirable in the generation of T-cell populations for adoptive immunotherapy (21, 31).

Naïve or memory progenitors developed into effector cells with distinct qualities, despite repeated stimulation with the same developmental cues. These differences could be due to development along separate pathways or to different positions along a common pathway. The tendency of both subsets to acquire *Eomes*, lose CD62L, and lose IL-2-production intimates that both subsets share a common developmental destination. However, the predilection of T_{EFF}^{CM} to retain a subset of CD62L positive cells indicates that effector cells could maintain certain lineage-specific traits. Furthermore, acquisition of KLRG1 by the $CD62L^+$ subset of T_{EFF}^{CM} suggests that this lineage-specific trait was preserved even in terminal differentiation. Although these findings were from in vitro rather than in vivo studies, they support a model of separate but converging differentiation pathways for development of effector cells derived from naïve or central memory T cells. Thus, the present manuscript expands the scope of previous studies that did not distinguish between effector cells of naïve or central memory origin (3, 12, 27).

A report from study of nonhuman primates demonstrated that effector cells of central, rather than effector memory origin were capable of prolonged survival following infusion (20). However, naïve-derived effector cells were not studied, and a therapeutic endpoint was not used. Consistent with that work, we found that central memory-derived effector cells could indeed persist following adoptive transfer (Fig. S6), but that effector cells generated directly from naïve T cells were superior upon adoptive transfer for proliferation, cytokine release, and tumor treatment efficacy.

The clinical implication of this work is that the efficacy of adoptive immunotherapy might be improved by generating effector cells for therapy from naïve cells. Naïve viral antigen-specific T cells in cord blood can be expanded for adoptive transfer (39). Furthermore, in preliminary studies of melanoma patients, naïve cells ($CD62L^+$ $CD45RO^-$) represent 20 to 60% of the $CD8^+$ T cells in leukapheresis samples and can be readily transduced with TCR-encoding vectors and expanded. The technique for isolating naïve cells for the treatment of patients with cancer in a Good Manufacturing Practices-compliant manner must be developed. However, this report lays the scientific groundwork for further study in human tissues, and it demonstrates the importance of selecting the optimal precursor cells for generation of effector cells for adoptive immunotherapy.

Methods

Mice and Cell Lines. Pmel-1 Thy1.1 (24), pmel-1 Thy1.1 IFN$\gamma^{-/-}$ (40), pmel-1 Thy1.1 IL-$2^{-/-}$, and C57BL/6 mice (The Jackson Laboratory) were bred and housed according to the guidelines of the Animal Care and Use Committee at the National Institutes of Health. Genotypes were confirmed by PCR analysis using The Jackson Laboratory protocols. B16 and MCA205 were obtained from the NCI Tumor Repository and grown in culture media.

$CD8^+$ T-Cell Culture. $CD8^+$ T cells were isolated from splenocytes by magnetic bead negative selection (Miltenyi Biotec or Stemcell Technologies). $CD44^{high}$ and $CD44^{low}$ cells were separated using biotinylated anti-CD44 antibodies (BD Biosciences) and anti-biotin magnetic beads (Miltenyi Biotec) or by fluorescence activated cell sorting (FACS) on the basis of CD62L and CD44 expression. For experiments using IL-$2^{-/-}$ or IFN-$\gamma^{-/-}$ cells, only FACS sorting was used. Primary stimulation was accomplished using plate-bound anti-CD3 (2 μg/mL) and soluble anti-CD28 (1 μg/mL) (BD Biosciences). Cells were expanded in culture media (24) containing 30 to 60 IU IL-2 (Novartis) for 6–7 days. Secondary and tertiary stimulations were with irradiated splenocytes pulsed with hgp100$_{25-33}$ (1 μM). Thus, the first ex vivo stimulation was always with CD3/CD28, and the second and third with peptide and IL-2.

$CD8^+$ T-Cell Transduction. Two days following initiation of stimulation as described above, cells were transduced with a MSGV1 retroviral vector with a codon optimized sequence encoding the pmel-1 TCR α- and β-chains linked by an internal ribosome entry site (41). Retrovirus was produced by transient transfection using the PlatE packaging line (Cell Biolabs Inc.) with cotransfection of the pCL-Eco (Imgenex) plasmid. A single transduction was performed using spinoculation on retronectin-coated plates.

In Vitro Assays. Flow cytometry was performed by labeling cells with fluorescent antibodies specific for Thy1.1, CD62L, CD44, CD8 (all from BD Biosciences), KLRG1, granzyme B, or perforin (all from eBioscience). Intracellular staining was performed per manufacturer protocol (BD Biosciences). Carboxyfluorescein succinimidyl ester (CFSE) labeling was per manufacturer protocol (Invitrogen). Cytokine quantities were determined by ELISA (R&D Systems). ^{51}Chromium release assays

were performed as described (25). Real-time PCR was conducted using commercially available primer/probe sets (Applied Biosystems).

Oligonucleotide Microarrays. Total RNA was isolated and processed using Expression 3′ amplification One-cycle Target Labeling and Control Reagents kit according to the manufacturer's directions (Affymetrix). Hybridization was performed according to the manufacturer's protocol. Laser detection (Affymetrix Scanner 3000 7G) was performed and signal intensities quantified (Affymetrix Genechip Command Console).

Microarray Analysis. Gene expression was quantitated with the Affymetrix Expression Console (Affymetrix). Signal values from the Expression Console software were normalized with an adaptive variance-stabilizing, quantile-normalizing transformation (P. J. Munson, GeneLogic Workshop of Low Level Analysis of Affymetrix GeneChip Data, 2001, software available at http://abs.cit.nih.gov/geneexpression.html). The transform, termed S10, is scaled to match the logarithm transform (base 10).

Differential gene expression was assessed by analysis of variance (ANOVA). A one-factor ANOVA of four treatment levels was performed, followed by post hoc tests comparing naïve to memory cell populations in the unstimulated and stimulated states. A fold change of at least 2 and a false discovery rate of 2% (42) was required to declare a probeset differentially expressed. The array data were deposited in Gene Expression Omnibus (GEO), accession number GSE16522.

Adoptive Cell Transfer. B16 melanoma tumors were initiated by s.c. injection of 5×10^5 cells. Cell transfer treatment occurred 7–9 days following tumor cell injection. Mice were pretreated with a conditioning regimen of 500 or 600 cGy total body irradiation the day of or the day before cell transfer. Cells were administered with 2×10^7 plaque forming units of recombinant vaccinia virus encoding gp100 and with 600,000 IU rhIL-2 (Novartis) given twice a day for a total of five or six doses (24). The number of cells administered ranged from 8×10^5 to 4×10^6 in primary stimulation experiments and from 1×10^7 to 4×10^7 in secondary stimulation experiments. Within any given experiment and for any head-to-head comparison, the number of T cells in each subset is normalized for the two groups. Greater numbers of secondary stimulation cells were required due to the loss of efficacy in vivo that accompanies repeated stimulation in vitro (25). Serial tumor measurements were performed by an investigator blinded to the treatment groups. Each treatment group consisted of five mice. Error bars reflect the standard error of the mean.

Statistics. Tumor curves were assessed by one-way Analysis of Variance (ANOVA) with a Bonferroni multiple comparisons posttest. Single-measurement comparisons between two groups were tested using unpaired *t*-tests. Prism GraphPad software (GraphPad Software Inc.) was used for these analyses.

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