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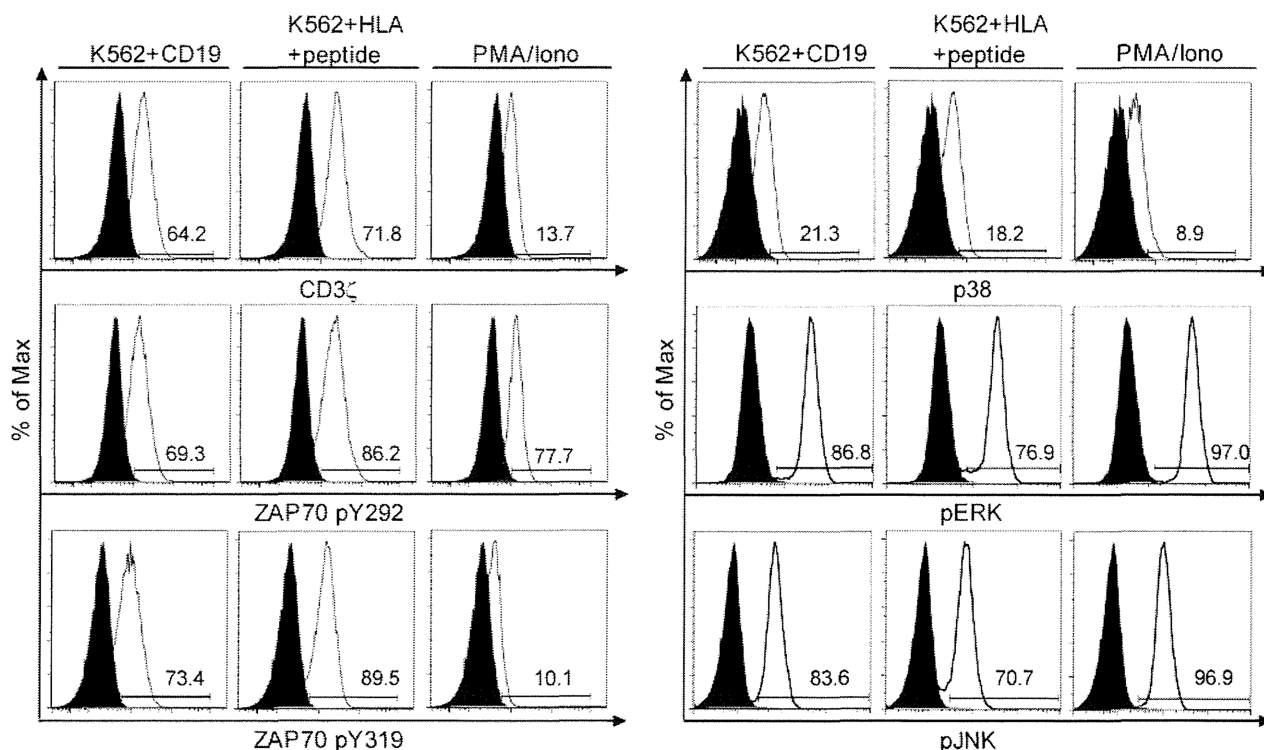

Figure 5. Analysis of intracellular signaling after stimulation through the virus-specific TCRs and CD19-CARs. Intracellular phospho-specific staining of CD3ζ, ZAP70 (pY292 and pY319), p38, ERK, and JNK. Bi-specific T cells were either stimulated with K562 (gray), the indicated K562 transfectants at a 1:5 ratio, or PMA/ionomycin for 2 minutes (CD3ζ and ZAP70), or 10 minutes (p38, ERK, and JNK), permeabilized and stained with phospho-specific mAbs, and analyzed by flow cytometry. The flow plots for CD3ζ, ZAP70, and p38 were performed on a FACSCanto II (BD Biosciences), and for ERK and JNK on a FACSCalibur (BD Biosciences). Data are representative of $B^*0702_{CMVpp65}$ bi-specific T cells and representative of 4 independent experiments with bi-specific T cells from 4 donors.

produce IFN-γ, TNF-α and IL-2 by intracellular cytokine staining than K562/CD19 stimulation, but this difference was not statistically significant (Figure 4B-C). We analyzed the rate at which bi-specific T cells became IFN-γ^+ by stimulating the cells with each APC for various times before fixation and intracellular staining. In the time-course analysis, ~ 10% more cells secreted IFN-γ after TCR stimulation at time points after 4 hours (Figure 4C). However, IFN-γ, TNF-α, and IL-2 secretion into the culture supernatant was equivalent after CD19 and viral Ag stimulation (Figure 4D). Taken together, these data demonstrate only slight differences in induction of effector functions whether the T cell is signaled through the endogenous TCR or the introduced CAR.

We next examined phosphorylation of proximal and distal signaling molecules including CD3ζ, ZAP70, p38, ERK, and JNK after Ag binding using flow-based intracellular staining with phospho-specific mAb. A short 2- to 10-minute stimulation with $CD19^+$ K562 or K562/B*0702 pulsed with an optimal concentration of $B^*0702_{CMVpp65}$ peptide induced phosphorylation of CD3ζ and ZAP70 at tyrosine 292 and 319 in the majority of T cells. In some but not all experiments, the mean fluorescence intensity of phosphoprotein staining was lower after stimulation through the CAR than through the TCR. However, both the proportion of T cells that exhibited phosphorylation of distal signaling molecules including ERK, JNK, and p38, and the mean fluorescence intensity of staining were not significantly different after stimulation through the CAR or the TCR (Figure 5).

The release of cytolytic granules and cytokines after ligand engagement and signaling through a CAR does not ensure that the CAR-modified T cell will undergo productive cell division after recognition of target cells. We observed similar up-regulation of CD25 on bi-specific T cells after stimulation through the TCR or the CAR, suggesting the cells would be similarly responsive to endogenous or exogenous IL-2 (Figure 6A). Indeed, CFSE labeling of T cells before stimulation with K562/CD19 or K562/viral Ag in cultures that were not supplemented with IL-2 showed that a comparable proportion of the bi-specific T_{CM}-derived T_E cells divided over 72 and 96 hours (Figure 6B and data not shown). Cell division was more robust after stimulation through the TCR or the CAR than after stimulation with CD3/28 beads (Figure 6B). T-cell proliferation induced by stimulation through either the CD19-CAR or the TCR also resulted in an equivalent increase in absolute cell number in the cultures over 4 days, even in the absence of supplemental IL-2, indicating that ligand engagement through the CAR did not induce greater activation-induced cell death than through the TCR, and that sufficient endogenous IL-2 was produced by T_{CM}-derived T_E cells to promote cell proliferation (Figure 6C). Thus, stimulation of T_{CM}-derived T_E cells through an introduced CD19-CAR that contains a CD28 costimulatory domain induces similar phosphorylation of signaling molecules, production of effector cytokines, and T-cell division as stimulation through an endogenous virus-specific T cell receptor on the same cell.

Discussion

Gene transfer to redirect the specificity of any human T lymphocyte to recognize cancer cells through the expression of a TCR specific for a tumor-associated Ag or a CAR specific for a tumor cell-surface molecule, is a developing area in cancer therapy. Several issues remain to be resolved including the type of T cell

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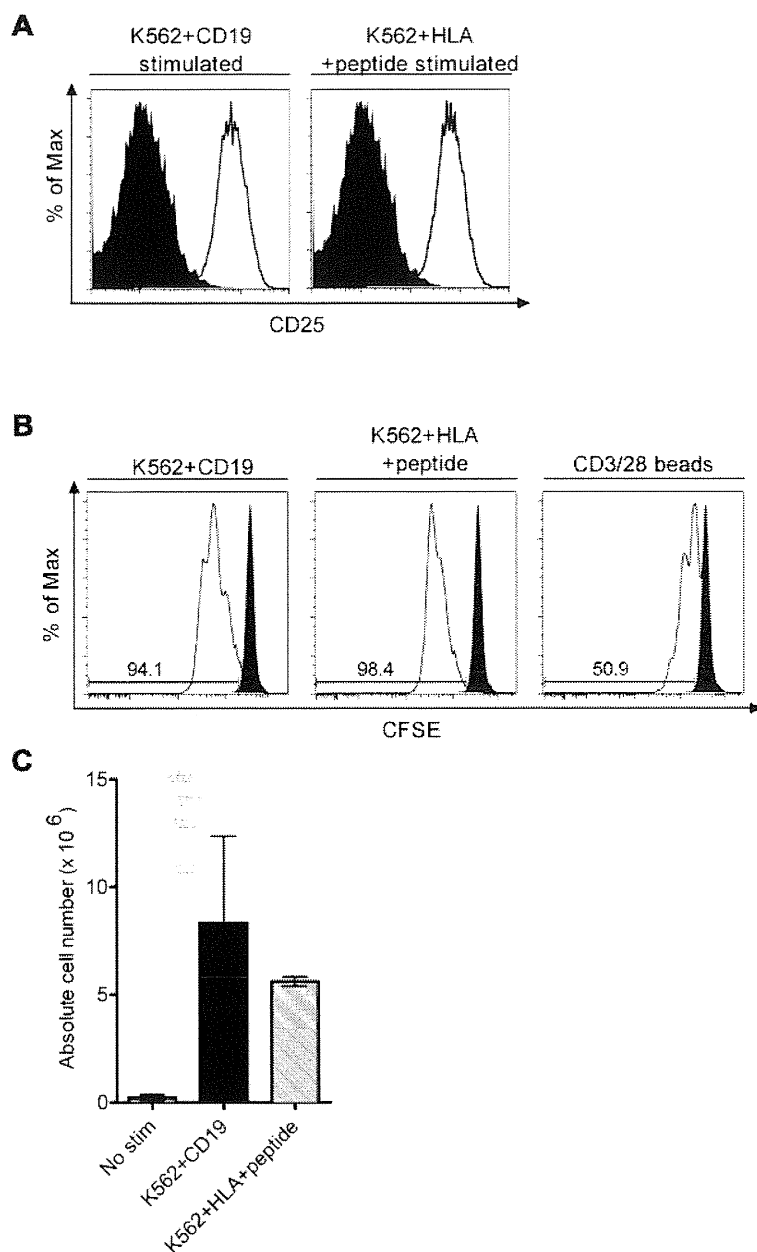

Figure 6. T-cell activation and division after stimulation through the virus-specific TCRs and CD19-CARs. (A) Up-regulation of CD25 after CD19 or TCR stimulation. Bi-specific T cells were stimulated with an equal number of either K562/CD19 or K562/HLA-B*0702 cells pulsed with CMVpp65 peptide or left unstimulated and examined for CD25 expression 24 hours later. Expression of CD25 on unstimulated cells is shown in the gray histograms and expression of CD25 on stimulated T cells is shown in the white histograms. (B) CFSE dilution assay after CD19 or TCR stimulation. Bi-specific T cells were labeled with CFSE, stimulated with an equal number of the indicated K562 transfectants or with CD3/28 beads, and CFSE staining intensity was analyzed 72 hours after stimulation. CFSE staining of unstimulated T cells is shown in the gray histograms and those of stimulated in the white histograms. Data are representative of 3 independent experiments. (C) Cell growth of bi-specific T cells after CD19 or TCR stimulation without exogenous IL-2. A total of 2×10^6 bi-specific T cells were stimulated with either K562/CD19 transfectants or K562/HLA transfectants pulsed with CMV peptide, and the absolute cell count was determined by the standard trypan blue dye exclusion method 4 days after stimulation. Data are pooled from 4 independent experiments with bi-specific T cells from 4 donors. The mean and SD of the absolute cell number is shown.

(naive, central memory, or effector memory) to modify, and the design of receptor constructs that mimic physiologic signals in the engineered T cells. A potential use of gene-modified donor T cells is to induce a GVL effect after allo-HSCT to reduce the unacceptably high rates of relapse that are currently experienced by patients with advanced malignancies.[1-5,8] The use of T cells from a normal donor circumvents the potential difficulty deriving autologous T-cell products from lymphopenic patients who have had extensive prior chemotherapy, but the application of T-cell therapy after allo-HSCT requires knowledge of the specificity of the endogenous TCR(s) to avoid causing GVHD. Unselected autologous CAR-modified T-cell therapy has recently been reported to exhibit profound antitumor activity in CLL.[34,35] The methodology described here would enable the application of this approach to more aggressive B-cell malignancies where allo-HSCT is the standard of care and where engineering and infusing unselected donor T cells would carry a risk of GVHD. We show that bi-specific (CD19-CAR, virus-specific) $CD8^+$ T_E cells can be rapidly derived from T_{CM} precursors of normal donors. We selected T_{CM} cells for genetic modification because they exhibit superior proliferation to Ag compared with T_{EM} cells,[22] and we have shown that gene-modified virus-specific T_E cells derived from T_{CM} cells, but not from T_{EM} cells, persist long-term after adoptive transfer in animal models.[21,23] To select $CD8^+$ T_{CM}, we used 3 mAbs (CD4, CD45RA, and CD14) for depletion of undesired cells and a CD62L mAb for positive enrichment. This 2-step depletion and enrichment was highly effective in removing and selecting the desired subsets, and enriched for $CD8^+CD62L^+$ memory cells, although a $CD62L^+$ $CD13^+$ myeloid population that phenotypically and morphologically resembled basophils remained after enrichment. The proportion of contaminating $CD13^+$ cells varied between donors; however, these cells did not persist in culture and did not significantly alter T-cell proliferation or transduction efficiency.

We focused on deriving CMV- and EBV-specific T cells from T_{CM} for genetic modification because a majority of donors retain virus-specific $CD8^+$ memory T cells for these pathogens, and the adoptive transfer of virus-specific T cells for even a few epitopes

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after allo-HSCT confers antiviral immunity to immunodeficient HSCT recipients without causing GVHD.[17-19,36] It has been suggested that CAR-modified virus-specific T_E cells may exhibit superior persistence in vivo as a consequence of signals delivered through the virus-specific TCR.[37,38] The frequency and proportion of the memory CD8$^+$ T-cell response to CMV and EBV that resides in the CD62L$^+$ T_{CM} subset varied for each virus and among individuals, but we could reliably derive T_E cells for multiple HLA class I–restricted epitopes from both viruses from a majority of donors. In addition, the addition of lentivirus shortly after Ag stimulation led to efficient and preferential transduction of the virus-specific T cells, which could then be expanded in short-term culture by stimulation through the CAR. We show that selection with reversible MHC streptamers as a final enrichment provides highly pure populations of bi-specific T cells, and avoids transferring T cells that could cause GVHD. It is possible that an additional benefit of this therapy is that the virus-specific CTL would provide antiviral protection in the post-allo-HSCT setting, although the consequences of inserting a CAR on TCR signaling in vivo are uncertain, and will require careful analysis in the clinical application of this approach.

The derivation of highly pure human T_E cells that expressed both a virus-specific TCR and a tumor-targeting CAR enabled comparative analysis of signaling through the naturally expressed TCR and the introduced CAR on the same T cells. For these studies, we used K562 cells that could present CD19 to the CAR or HLA/peptide complexes to the TCR as APC to control for potential differences in adhesion or other molecules that might be differentially expressed if tumor cells and virus-infected cells were used as APCs. We found that ligation of the TCR and CAR evoked uniform phosphorylation of CD3ζ, ZAP70, ERK, JNK, and p38 and induced similar secretion of IFN-γ, TNF-α, and IL-2. Moreover, signaling through either the TCR or the CAR resulted in target cell lysis, and induced proliferation and expansion of the T cells in the absence of exogenous IL-2. The CD19-specific CAR used in our study has a CD28 signaling domain in tandem with CD3ζ, based on prior work showing that addition of CD28 improves proliferation and IL-2 and IFN-γ secretion.[14] The incorporation of additional signaling domains from CD134 or CD137 has been shown to further increase cytokine secretion and lytic activity, and to improve cell viability in vitro and in a xenogeneic mouse engraftment model.[39-42] It is likely that the cellular response to signaling through a CAR will depend on many factors including the specificity and affinity of the scFv component of the CAR, incorporation of costimulatory signaling domains, and ligand density and location on the target cell. The strategy of deriving highly pure bi-specific T cells allows comparative analysis of signaling events induced by ligation of different CAR constructs with those after endogenous TCR signaling, and may prove useful in selecting the features for individual CARs that more closely approximate physiologic signaling, and preserve T-cell function and survival in vivo.

Our results also have implications for clinical applications of CAR-modified T cells beyond allo-HSCT. The T-cell pool in humans contains a diversity of phenotypic and functionally distinct T cells that have different effector functions and migratory capacity that are imprinted by earlier Ag experience.[43] Moreover, the frequency of these subsets of T cells varies substantially from person to person depending on age and pathogen exposure, and may be altered in cancer patients by the type and extent of prior chemotherapy.[44,45] Thus, transduction of bulk populations of autologous T cells may not provide cell products with uniform capacity to survive, mediate desired antitumor effector function, and migrate in vivo. The strategy of purifying defined subsets of T cells, including those with a defined TCR specificity before genetic modification may provide cell products that have more predictable activity after adoptive transfer.

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Authorship

Contribution: S.T. designed and performed experiments, analyzed the data, and wrote the manuscript; T.N.Y. designed and performed experiments, and analyzed data; R.A.G. performed experiments and analyzed data; C.J.T. performed experiments and helped write the manuscript; M.C.J. contributed reagents and helped write the manuscript; and S.R.R. designed experiments, analyzed the data, and wrote the manuscript.

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