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Natural Expression of the CD19 Antigen Impacts the Long-Term Engraftment but Not Antitumor Activity of CD19-Specific Engineered T Cells

Eleanor J. Cheadle,*1 Robert E. Hawkins,* Hayley Batha,† Allison L. O’Neill,* Simon J. Dovedi,*1 and David E. Gilham*

T cells gene-modified to express chimeric Ag receptors (CARs) have shown potent antitumor activity in vivo and are in clinical trials at locations worldwide. However, CAR activity has been investigated in mouse models in which Ag expression is restricted to the tumor. To explore the impact of normal tissue expression of the target Ag, we developed a mouse CD19-specific CAR to investigate antitumor efficacy against a syngeneic B cell lymphoma cell line within a background of normal CD19+ host B cells. Mouse T cells engrafted with the amCD19/CD3ζ CAR specifically lysed A20 lymphoma targets and B cells in vitro. These T cells also eradicated a 12-d established disseminated A20 lymphoma in mice preconditioned with 6 Gy total body irradiation. In the short-term (7 d after adoptive transfer), amCD19z T cells underwent Ag-dependent proliferation in vivo with a concomitant depletion in host B cell levels. However, the levels of amCD19z CAR+ T cells decreased significantly at later time points, at which point host B cells returned, eventually reaching normal levels. In contrast, CAR+ T cells lacking a signaling domain or specificity for mCD19 persisted over extended periods in blood and spleen. Importantly, no overt clinical signs of autotoxicity were observed in tumor-free or tumor-bearing mice treated with amCD19z T cells over an extended period of time. These observations highlight the importance of studying the activity of CAR+ T cells in autologous models that have the normal range of tissue expression of Ag. The Journal of Immunology, 2010, 184: 1885–1896.

Adoptive cellular therapy is of increasing interest and is being actively investigated for the treatment of many malignancies. B cell malignancies seem to be particularly amenable to immunotherapy, with monoclonal Abs and bone marrow transplant/donor lymphocyte infusions commonly used in treatment schedules (1, 2). More recently, mouse models demonstrated the efficacy of gene-modified T cells targeting B cell malignancies through chimeric Ag receptors (CARs). Murine and human T cells expressing CAR consisting of an Ab-type recognition domain (scFv) giving CD19 specificity linked to the CD3ζ signaling molecule have been shown to eradicate established human CD19+ lymphoma when given in conjunction with lymphodepleting conditioning regimens or to immunodeficient mice (3–7). These receptors are now in early-stage clinical trials at several sites worldwide. However, murine models of CAR+ T cells targeting CD19 have not fully mimicked the human setting because, unlike in patients, the CAR+ T cells tested have not been tested for function against a background of normal CD19 Ag on B cells because the receptors have been targeted against human CD19. To address the question of whether CD19 expression on normal B cells affects the function and safety of gene-modified T cells, we generated a CAR targeting murine CD19. In a fully syngeneic mouse model, we demonstrate that T cells targeting CD19 expressed in its natural form on tumor and normal B cells can eradicate systemic B cell lymphoma; however, T cells bearing the anti-mouse CD19/CD3ζ CAR do not persist long-term compared with control, nonsignaling CAR.

Materials and Methods

General reagents and cell culture

Hamster anti-mouse CD3ε (clone145-2C11) and hamster anti-mouse CD28 (clone 37.51) were obtained from BD Pharmingen (Cowley, U.K.). Human IL-2 (Proleukin) was obtained from Novartis (Camberley, U.K.), and recombinant murine IL-7 was purchased from R&D Systems (Abingdon, U.K.). FITC-, PE-, PE-Cy5-, PE-Cy7-, and APC-conjugated mAbs to human CD19, human CD34, and mouse CD4, CD8, CD19, CD44, CD62L, and CD107a were obtained from BD Pharmingen; unless otherwise stated, all chemical reagents were purchased from Sigma-Aldrich (Poole, U.K.). Matched Ab pairs to IFN-γ and IL-2 were obtained from BD Pharmingen (R4-6A2 and JES6-1A12/5H4, respectively).

Cell culture

Unless otherwise stated, all cell culture media were obtained from Invitrogen (Paisley, U.K.). 293T (ATCC CRL-11268) and GP+e86 (8) cells were cultured in DMEM glutamax media supplemented with 10% FCS and 2% tryptophan. The A20 BALB/c B cell lymphoma line (ATCC TIB-208) and 1D3 hybridoma [a gift from Prof. D.T. Fearon (9)] were grown in RPMI 1640 (BioWhittaker, Wokingham, U.K.) supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 50 mM 2-ME, and streptomycin (complete RPMI).

Generation of A20 cell line expressing luciferase

Firefly luciferase (luc2) was digested from the pGL4.14[luc2/Hygro] vector (Promega, Southampton, U.K.) KpnI, XbaI, cloned into the pCR2.1 TOPO vector (KpnI, SpeI), and subcloned into the retroviral vector rKat.IRES.GFP.
as an EcoRI, NotI fragment (10). Amphotrophic retroviral supernatant was generated by transient transfection of 293T (T) and (A20) cells, and (A20) cells were infected by centrifugation of cells in viral supernatant at 1200 × g for 3 h. Following cell sorting for GFP, A20 luc cells were generated by four rounds of limiting dilution and testing for luciferase expression using the Bright-Glo Luciferase reagent. The limit of detection was 31.25 pg/ml. At the end of the culture period, cells were enzymed in PBS/1% FCS containing a 1:1000 dilution of anti-human IgG (γ-chain specific) (Sigma-Aldrich) for 30 min on ice. Following an additional washing step, samples were acquired on a FACScalibur. The number of remaining CD19+ or CD34+ cells was calculated using the formula: (number of Ag-positive cell events/number of bead events) × number of beads added. Degranulation was measured during a 4-h coculture of 10⁶ tumor cells and 10⁶ T cells by the addition of GolgiStop (BD Biosciences, Oxford, U.K.), at the manufacturer’s recommended dilution, and 0.5 μg rat IgG2A PE (eBioscience, Hatfield, U.K.) or CD107a PE (BD Biosciences) during the culture period. Cells were stained for CD34 (PE-Cy5) and analyzed on a FACScan. T cells were also cultured with BALB/c B cells isolated using the untouched B cell isolation kit (Miltenyi Biotec), as described above.

**Intracellular cytokine staining**

A total of 1 × 10⁶ T cells were cultured with 5 × 10⁵ A20/CT26 cells for 16 h in the presence of GolgiPlug (BD Biosciences) in 1 ml volume in 24-well plates. Cells were stained with anti-CD4 FITC, CD8 PE-Cy7, and CD34 APC for 30 min at 4°C. Cells were fixed and permeabilized for 45 min in fix/perm buffer (eBioscience) and stained with rat IgG1-PE or anti-IFN-γ-PE for 30 min at 4°C, washed with permeabilization buffer (eBioscience), and acquired on a FACScalibur.

**In vitro CFSE proliferation assay**

amCD19mtm/amCD19z T cells were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (Invitrogen), and 10⁶ T cells were cultured with media, 5 × 10⁵ untouched B cells, or A20 luc cells in 1 ml complete RPMI containing 10 IU/ml IL-2. After 3 days, IL-2 in 24-well plates in triplicate. After 3 days, IL-2 was removed, and cells were stained with CD4 or CD8 PE-Cy7, CD34 APC, or CD19 PE. CountBright counting beads were added, and samples were acquired on a FACScalibur (BD Biosciences).

**In vivo function of amCD19z T cells**

All in vivo studies were carried out under the auspices of the Animals (Scientific Procedures) Act 1986 and under U.K. Coordinating Committee for Cancer Research guidelines; animals were housed under specific pathogen-free conditions. Six- to eight-week-old BALB/c mice were purchased from Harlan (Loughborough, U.K.) and received varying combinations of i.p. injections of saline or 150–200 mg/kg cyclophosphamide (Cytoxan, Baxter, Germany), 6 Gy low-dose irradiation, i.v. injections of 5 × 10⁶ A20 luc cells, and i.v. injections of saline, control T cells, amCD19mtm, amCD19z, or ahCD19z T cells at varying doses and time points. Heparinized blood from the tail vein was collected at various time points after cell infusion and was analyzed for the presence of CD4⁺, CD8⁺, CD19⁺ T cells and CD19⁺ blood by flow cytometry. The number of cells/ml blood was enumerated by the addition of CountBright counting beads during the acquisition process and the assumption that 1 g blood is equivalent to 1 ml blood. Mice were assessed for tumor growth by in vivo bioluminescence imaging at various time points. Briefly, mice received an i.p. injection of 150 mg/kg luciferin (Caliper Life Sciences, Runcorn, U.K.), anesthetized with isoflurane and were imaged for bioluminescence using an IVIS Lumina II in vivo imaging system (Caliper Life Sciences). Mice were imaged for up to 2 min on the dorsal and ventral sides using a 12.5-cm field of view, with medium binning and F-stop 1. Bioluminescence is displayed as photons per second. Mice were culled at the onset of hind leg paralysis, ill health, >20% loss in body weight, or at specified time points. Splenocytes and bone marrow were isolated and cryopreserved in 90% FCS, 10% DMSO in liquid nitrogen for later analysis by flow cytometry. Where appropriate, T cells were labeled with 4 μM carboxyfluorescein diacetate succinimidyl ester and were washed extensively immediately prior to i.v. infusion.

**Serum ELISA**

Serum was isolated from clotted blood obtained by cardiac puncture or from the tail vein. Total IgG was quantified by a sandwich-based ELISA using 10 ng/ml goat anti-mouse IgG (Sigma-Aldrich) and HRP goat anti-mouse IgG (Sigma-Aldrich) at known concentrations of murine IgG Sigma-Aldrich).

**Results**

Mouse T cells bearing a chimeric receptor targeting murine CD19 are functional in vitro

An scFv targeting murine CD19 was generated from the 1D3 hybridoma (9) by PCR, as previously described (12). To confirm specificity for target Ag expression on normal mouse B cells, the scFv (termed amCD19) was cloned as a fusion with human IgG1 (hFc) and expressed in 293T cells. The secreted anti-mouse CD19 hFc fusion protein efficiently stained the mouse B cell lymphoma
cell line A20 to levels comparable with that of the parental 1D3 Ab (Fig. 1A). Cell lines lacking mouse CD19 did not stain with the fusion protein (data not shown).

To test the scFv in the context of a CAR, the amCD19 scFv was cloned in place of the anti-human CD19 scFv present within the pMP71.tCD34.2A.ahCD19z retroviral vector (3, 4). The chimeric receptor consisted of the scFv fused to the CD3ζ sequence (amCD19z) and was expressed downstream of the truncated CD3ζ marker gene (tCD3ζ). A control nonsignaling receptor was also generated, whereby the amCD19 scFv was expressed as a fusion with the mouse H2-Kb class I transmembrane domain (amCD19mtm) and expressed using the pMP71 vector with tCD3ζ marker gene (15).

Immunoblotting confirmed that cells transduced with the amCD19z chimeric receptor vector expressed an immunoreactive protein of the predicted molecular mass (Fig. 1B). Mouse splenic T cells engrafted with the amCD19z vector demonstrated a mean 1.3-fold higher ($n = 5$) transduction level in CD4+ subsets than in CD8+ cell subsets (data not shown), which was similar to that seen with the amCD19mtm CAR (1.6-fold). In our study, CD4+ T cells from BALB/c mice preferentially expanded after in vitro activation, which we believe is the likely explanation for this observation. All transduced T cells expressed TCR-β as shown by double staining of TCR-β and CD4 in Fig. 1C (CD8 costaining was also seen; data not shown). amCD19z T cells specifically lysed A20 B cell lymphoma target cells in short-term killing assays (Fig. 1D), with no killing observed with control ahCD19z T cells or on CD19+ CT26 cells. amCD19z T cells also degranulated during a 4-h coculture with A20 cells, as determined by a relative increase in CD107a expression in the CAR+ T cell population (59.0% ± 3.9% of CD3ζ+ T cells; Fig. 2A) compared with that observed in amCD19mtm T cells (11.4% ± 0.5%) or upon culture with CT26 cells (data not shown). amCD19z T cells were equally effective in longer-term cytotoxicity assays against A20 target cells (Fig. 2B) and normal B cells (Fig. 2C). During the 24-h coculture, there was no increase in the number of amCD19z T cells compared with amCD19mtm T cells (Fig. 2B, right panel).

**FIGURE 1.** Generation of murine T cells expressing the amCD19z CAR. A, A20 cells were stained with supernatant from 293T cells transfected with ahCD19scFv.hFc or amCD19scFv.hFc, followed by PE-anti human hFc or PE-amCD19 Ab and analyzed on a flow cytometer. B, Western blot probed for CD3ζ of murine T cells bearing an amCD19mtm or amCD19z CAR. C, amCD19mtm/amCD19z T cells were analyzed by flow cytometry 5 d after transduction for the expression of TCR-β. Cells were gated on CD3ζ- murine T cells bearing an amCD19mtm or amCD19z CAR. B, amCD19mtm/amCD19z T cells were analyzed by flow cytometry 5 d after transduction for the expression of TCR-β. Cells were gated on CD3ζ- murine T cells bearing an amCD19mtm or amCD19z CAR. C, amCD19mtm/amCD19z T cells were analyzed by flow cytometry 5 d after transduction for the expression of TCR-β. Cells were gated on CD3ζ- murine T cells bearing an amCD19mtm or amCD19z CAR. D, Four-hour cytotoxicity of amCD19z T cells or ahCD19z T cells on [35S]labeled A20 or CT26 cells at increasing E:T ratios.

**FIGURE 2.** Murine T cells bearing the amCD19z CAR kill CD19+ targets. A, A total of $10^5$ A20 cells were cultured with $5 \times 10^4$ (45% transduced) amCD19mtm (top row) or amCD19z T cells (bottom row) for 4 h in the presence of GolgiStop and rat IgG2aPE or CD107aPE, stained for CD3ζ and analyzed on a flow cytometer. Cells were gated on the live lymphocyte to exclude tumor cells. B, A total of $10^5$ A20 cells were cocultured for 24 h with $5 \times 10^4$ (45% transduced) mock, amCD19mtm, or amCD19z T cells. The number of tumor cells and CD3ζ+ (transduced T cell marker) T cells were enumerated by the addition of CountBright beads and the acquisition of mCD19+ and CD3ζ+ cells on a flow cytometer. Data are representative of at least two independent experiments. C, A total of $5 \times 10^4$ A20/B cells were cocultured for 24 h with $10^6$ amCD19mtm/amCD19z T cells (15% transduced). The number of CD19+ cells was enumerated by flow cytometry, as described above.
**FIGURE 3.** Murine T cells bearing the amCD19z CAR secrete cytokines and proliferate in response to CD19 Ag. A. A total of $10^5$ A20 cells were cocultured for 24 h with $2 \times 10^5$ (15% transduced) amCD19mtm or amCD19z T cells in 96-well plates. Supernatants were analyzed by ELISA for IFN-γ and IL-2. Data are presented as cytokine/10^5 transduced cells and are representative of at least three independent experiments. B. A total of $5 \times 10^5$ A20 or B cells were cocultured for 24 h with $10^6$ (15% transduced) amCD19mtm or amCD19z T cells in 24-well plates. Supernatants were analyzed by ELISA for IFN-γ and IL-2. A total of $5 \times 10^5$ A20 cells (C) or CT26 cells (D) were cultured with $10^6$ amCD19mtm/amCD19z T cells (30% transduced) for 16 h in the presence of Brefeldin A and analyzed for IFN-γ–producing cells by flow cytometry. Quadrants were set using the rat IgG1 PE isotype control, which gave <0.1% background (data not shown). E. A total of $10^5$ CFSE-labeled amCD19mtm/amCD19z T cells (15% CD34+) were cultured for 4 d with $5 \times 10^5$ A20 cells/untouched B cells or media in triplicate. Representative plots for CFSE labeling and CD34 staining on CD4- and CD8-gated lymphocytes are shown after 4 d coculture with A20 cells. F. The number of CD34+ cells that were CFSE bright (undivided) or CFSE dim (divided) cells was quantified in CD4 and CD8 lymphocytes 7 d after culture with A20 cells and 10 IU/ml IL-2 by the addition of CountBright beads prior to acquisition on a flow cytometer. Data are presented as mean ± SEM of triplicates and are representative of two independent experiments.
In vitro Ag-specific proliferation of amCD19z T cells

Given the observation that amCD19z T cells could produce IL-2 during antigenic challenge, the in vitro proliferative potential of these cells was investigated after coculture with CD19-expressing A20 cells or B cells using a CFSE-based flow cytometry assay. No significant proliferation of CD4+ or CD8+ CAR-expressing T cells was found after 1 d of culture (data not shown); however, at days 4 (Fig. 3E) and 7 (Fig. 3F), CD8+ amCD19z T cells had clearly undergone several rounds of cell division upon culture with A20 cells, whereas CD4+ amCD19z T cells had undergone only one round or no division. No proliferation of amCD19mntm T cells was seen upon culture with the CD19+ cell line CT26 (Fig. 3E, 3F). CD8+CAR+ T cell proliferation was not driven by low levels (10 IU/ml) of IL-2 alone (Fig. 3F), suggesting that ligation of CD19 Ag by CAR was able to induce proliferation in CD8 T cells in vitro when the CAR contains a CD3ζ signaling domain but little or no proliferation in CD4 T cells. Indeed, a similar pattern of proliferation was observed when amCD19z T cells were cultured in the complete absence of exogenous IL-2.

Specific proliferation of amCD19z T cells in preconditioned mice

T cells adoptively transferred into mice and humans are thought to undergo homeostatic expansion driven by the common γ-chain cytokines, including IL-7 and -15 (16). We wanted to determine whether the presence of accessible Ag on tumor and host B cells further influenced CAR+ T cell proliferation upon adoptive transfer into conditioned mice. CFSE-labeled amCD19z T cells or control nonsignaling amCD19mntm T cells were adoptively transferred into A20 tumor-bearing or nontumor-bearing specific-pathogen-free BALB/c mice that had been preconditioned with 6 Gy total body irradiation (TBI) (Fig. 4). Although preconditioning with 6 Gy irradiation significantly decreased the numbers of B cells in spleen, bone marrow, and blood, CD19+ cells were still present at significant levels 24 h postconditioning at the time when the amCD19z T cells are infused (3.8 ± 2.2 × 10^6 per spleen compared with 1.45 ± 0.4 × 10^6 in unconditioned animals). Splenocytes were examined 6 d after adoptive transfer for the relative frequency of CFSE-labeled CD34+ (CAR marker gene), CD4+, and CD8+ T cells. In the tumor-free animals, the majority of amCD19mntm T cells (CD4+ and CD8+) had retained the CFSE label (72% ± 3% CD4+CD34+CFSE and 79% ± 3% CD8+CD34+CFSE; Fig. 4), indicative of a lack of in vivo proliferation. In contrast, the majority of CD4+ and CD8+ amCD19z T cells had undergone a greater number of cell divisions and no longer expressed the CFSE label (90% ± 1% CD4+CD34+CFSE and 82% ± 2% CD8+CD34+CFSE; Fig. 4). Moreover, no significant difference in the CAR+ T cell proliferative response was seen in tumor bearing versus nontumor bearing mice suggesting that CD19 expression on B cells alone was sufficient to induce proliferation of amCD19z T cells (Fig. 4B).

This specific proliferation translated to a relative increase (2- to 3-fold) in the number of amCD19z T cells in the peripheral blood 7 d after adoptive transfer into conditioned mice compared with amCD19mntm T cells. Engraftment appeared unrelated to the CAR T cell dose given (Supplemental Fig. 1); therefore, engraftment data were pooled from multiple experiments. This observation held true when cyclophosphamide (1.8 ± 0.3 × 10^6 amCD19z versus 7.1 ± 1.6 × 10^6 amCD19mntm) or 6 Gy TBI was used for preconditioning (6.4 ± 1.2 × 10^6 amCD19z versus 2.2 ± 0.3 × 10^5 amCD19mntm). At this day 7 time point, there was a significant increase in the number of engrafted CD8+ amCD19z T cells compared with CD8+ amCD19mntm T cells in cyclophosphamide- (p < 0.0001) and TBI-conditioned animals (p = 0.019; Fig. 5A). Although there was also a trend to increased numbers of CD4+ amCD19z T cells compared with CD4+ amCD19mntm T cells, these differences were only statistically significant when TBI was used (p = 0.007). In cyclophosphamide-pre-treated mice, the relative engraftment of amCD19z T cells was also compared with T cells bearing an irrelevant CD3ζ-containing CAR (ahCD19z); once again, there was a statistically significant increase in the number of CD8+ (p = 0.003) and CD4+ (p = 0.047) amCD19z T cells compared with ahCD19z control T cells (Fig. 5A). Taken together, these data indicate that amCD19z T cells engraft to higher levels in vivo than control T cells in conditioned mice.

amCD19z T cells fail to persist in vivo for extended periods after adoptive transfer to preconditioned mice

To determine the duration of amCD19z T cell persistence in conditioned mice, animals received 1.25 × 10^6 CAR+ T cells following TBI preconditioning and were serially bled to monitor for CAR+ T cells over a 62-d period. Although the number of CD4+ amCD19mntm T cells in the peripheral blood remained fairly constant over the 62-d time period, CD4+ amCD19z T cells decreased below the limit of detection (~2.500 cells/ml) by day 62 (Fig. 5B). A similar pattern was seen in the CD8+ T cell population; CD8+ amCD19z T cells decreased below the limit of detection by day 43 (Fig. 4B). In contrast, CD8+ amCD19mntm T cell levels...
remained constant over the study period and were still detectable at day 62. Mice were culled at day 81, and their spleens were analyzed for CD4+ and CD8+ amCD19mtm/amCD19z T cells (Fig. 6A). A scenario similar to that in the periphery was seen, with significantly fewer CD4+ and CD8+ amCD19z T cells detected compared with amCD19mtm T cells (CD4: 0.09% ± 0.015% versus 1.4% ± 0.12%; p = 0.007; CD8: 0% versus 0.99% ± 0.05; p = 0.01). This was in contrast to the percentage of total CD4 and CD8 T cells, which did not vary between the two groups. Furthermore, although B cell depletion was seen at early time points following amCD19z T cell transfer, there was a gradual resurgence in B cells, with B cells reappearing around day 43 and approaching levels seen in amCD19mtm T cell-treated mice by day 62 in the peripheral blood (Fig. 5C) and by day 81 in the spleen (Fig. 6B). The loss of amCD19z T cells from the circulation and resurgence of B cells was even more pronounced in cyclophosphamide-preconditioned mice (data not shown); amCD19z T cells had decreased below the limit of detection by day 43, and B cells had fully recovered to control levels by day 81 in the spleen. The loss of amCD19z T cells was not dependent on T cell dose, because cyclophosphamide-preconditioned mice that received a 6-fold higher dose of amCD19z T cells showed B cell resurgence to normal levels within 28 d of T cell transfer, with a similar loss of amCD19z T cells (data not shown). To confirm that the loss of amCD19z T cells in the spleen was not simply due to an increase in the number of non-CAR+ cells, we repeated the experiment detailed in Fig. 6A and 6B and quantified the numbers of amCD19mtm and amCD19z T cells at day 119 in the spleen. As detailed in previous experiments, a gradual decrease in the number of amCD19z T cells over time in the peripheral blood was seen compared with amCD19mtm T cells, with a concomitant resurgence in B cells to normal levels by day 84 (data not shown). At day 119, there were 8-fold fewer CD4+amCD19z+ T cells than CD4+amCD19mtm+ T cells in the spleen (2.3 × 10^4 ± 9.5 × 10^3 cells/ml)
amCD19z [0.18% of CD4 T cells] versus 1.9 \times 10^5 \pm 6 \times 10^3 \text{amCD19mtm} [2\% of CD4 T cells)]. A similar scenario was seen with the CD8 T cell population (7.4 \times 10^3 \pm 6 \times 10^3 \text{amCD19z} [0.2\% of CD8 T cells] versus 4.2 \times 10^5 \pm 6.7 \times 10^3 \text{amCD19mtm} [1.9\% of CD8 T cells]).

To confirm that the reduction in amCD19z T cells, compared with amCD19mtm T cells, was not due to some kind of selective survival advantage of the nonsignaling CAR targeting CD19, cyclophosphamide-preconditioned mice were adoptively transferred with 12 \times 10^6 \text{amCD19z} or control ahCD19z T cells, and the relative increase in amCD19z T cells, compared with controls, was confirmed after 7 d in tail bleeds (Fig. 6C). Two hundred and fifty-six days later, analysis of the spleens of these mice confirmed that the amCD19z T cells remained at a very low level that was...
close to the level of detection (0.05% ± 0.01 CD4+; 0.018% ± 0.02 CD8+), whereas the ahCD19z control T cells formed a significantly greater proportion of total splenocytes (0.45 ± 0.18 CD4+; 0.6 ± 0.24 CD8+; p < 0.01 against amCD19z T cell frequency) (Fig. 6D). Consistent with this lack of amCD19z T cells, the number of CD19 B cells in this group had returned to normal levels (Fig. 6E). Bone marrow was also analyzed to determine whether amCD19z T cells had trafficked to and remained at the source of newly emerging B cells; however, neither amCD19z nor ahCD19z T cells were detected in the bone marrow (data not shown).

We wanted to investigate whether amCD19z T cells differed phenotypically from amCD19mtm T cells prior to their appearance in vivo. To this end, we compared the phenotype of CAR+ T cells 21 d after engraftment, when B cells were still depleted in amCD19z-treated mice, and amCD19z T cells were still detectable. amCD19z T cells displayed a small increase in CD44+CD62L+ effector memory markers compared with amCD19mtm T cells, with 93% ± 0.77% of amCD19z T cells being effector memory cells (CD44+CD62L+) compared with 88% ± 1% of amCD19mtm T cells (data not shown). There were no differences in the proportion of central memory cells (4.8% ± 0.9% versus 6.3% ± 0.5%, respectively). amCD19z T cells expressed slightly higher levels of CD28 and lower levels of ICOS than amCD19mtm T cells, but the inhibitory ligand programmed death-1 (PD-1) was expressed in only a small percentage of CAR+ T cells (Fig. 6F). amCD19z T cells were still functional at this time, as measured by the production of the effector cytokines IFN-γ and IL-2 when splenocytes taken from mice 21 d after T cell transfer were cultured for 24 h with the A20 CD19+ B cell lymphoma cell line (Fig. 6G) at a CAR E:T ratio of 0.04:1.

Taken together, these experiments indicate that amCD19z T cells proliferate and engraft in preconditioned immune-competent mice. However, over time, amCD19z T cells decrease in numbers until they are barely detectable, whereas control nonsignaling/irrelevant CAR remain detectable at consistently higher levels. The reduction in amCD19z T cells coincides with the return of circulating B cells in the animal.

**Murine T cells bearing an amCD19z CAR are highly effective at eradicating established, disseminated B cell lymphoma**

Our previous studies demonstrated that mouse T cells bearing a CD3ζ-containing CAR targeting hCD19 are able to eradicate a 13-d established A20 B cell lymphoma engineered to express human CD19 in immunodeficient mice (4). To assess the efficacy of amCD19z T cells, we established a metastatic model of B cell lymphoma, and used bioluminescent in vivo imaging to assess tumor development over time. BALB/c mice received a systemic injection of A20 B cell lymphoma cells expressing luciferase (A20Luc) via the tail vein 11 d before preconditioning and 12 d prior to adoptive transfer of T cells. Because of the cyclophosphamide sensitivity of the A20 tumor, 6 Gy TBI was used to precondition tumor-bearing recipient mice 1 d prior to T cell infusion. As in previous experiments, there was a statistically significant increase in the number of CD8+ amCD19z T cells in the peripheral blood at day 7 compared with amCD19mtm T cells, but there was no overall difference in the levels of engraftment in mice that received 10^6 or 4 × 10^6 amCD19z T cells (data not shown). Mice were imaged on day 8; those that received either dose of amCD19z T cells showed no discernable evidence of tumor (Fig. 7A). This was in contrast to mice that received saline at day 0; all surviving mice (five of six) had a positive bioluminescence signal (the remaining mouse had been culled on day 7 because of the onset of hind leg paralysis as a result of tumor). Moreover, all mice that received 4 × 10^6 amCD19mtm T cells had a positive signal for bioluminescence on the dorsal side, ventral side, or both (four of six mice) or had been culled as the result of the onset of hind leg paralysis on day 5 or 7. Mice that received amCD19z T cells had no detectable tumor through day 71, whereas all saline- and amCD19mtm control-treated mice succumbed to tumor within 30 d. There was a clear survival advantage for the mice treated with amCD19z T cells compared with amCD19mtm (p < 0.001, log-rank [Peto] test), whereas there was no significant difference between either control group (amCD19mtm versus saline; p = 0.7843). However, as in nontumor-bearing mice, no amCD19z T cells could be detected in the spleen at later time points (120 d), even after rejection of an A20 rechallenge on day 76 and B cells had returned to normal levels. In a separate experiment, the ability of amCD19z T cells to deplete B cells was compared in mice that were tumor-free or tumor bearing at the time of T cell infusion. In keeping with the experiments shown in Fig. 7, tumor regressed in all amCD19z-treated mice by day 8, and no differences in the nadir of B cell counts was seen in tumor-bearing versus tumor-free mice, with B cells beginning to return in three of five mice by day 42 (~5000/ml) and increasing to ~5 × 10^7/ml at day 63 (data not shown).

To determine tumor burden in these animals prior to treatment, in a subsequent experiment, mice were imaged immediately prior to adoptive T cell transfer; they demonstrated strong bioluminescent signals correlating with widespread disseminated B cell lymphoma that resolved within 6 d of amCD19z T cell transfer (Fig. 7C).

When survival analysis was compared from four independent experiments, it became apparent that there seemed to be a threshold dose of amCD19z T cells below which no therapeutic activity was observed (Fig. 7D). At a dose <1 × 10^6 amCD19z T cells per animal, only 1 of 10 animals survived for a period of 38 d, whereas 20 of 21 mice treated with a minimum of 10^6 amCD19z T cells survived for the same period, with 18 of these followed out to 120 d with no detectable tumor. No control-treated animal survived until the 38-d time point.

Although treated mice remained tumor-free for >100 d, the gradual resurgence in B cells over time in amCD19z-treated mice suggests that tumor may reoccur in patients at later time points when aCD19z T cells are no longer detectable. If tumor was to reoccur, it may be beneficial to give additional infusions of aCD19z T cells. To address this, we used B cell depletion in mice as a surrogate readout for CART T cell activity. BALB/c mice were pretreated with 200 mg/kg cyclophosphamide, followed by an infusion of amCD19mtm or amCD19z T cells. Following the first T cell infusion, B cells were depleted in amCD19z-treated mice for 28–35 d, compared with 7–14 d in amCD19mtm-treated mice (Supplemental Fig. 2). At day 42, mice received an additional 200 mg/kg cyclophosphamide, followed by a second infusion of T cells. As before, specific B cell depletion was seen in the amCD19z-treated mice. B cells returned to normal levels in amCD19mtm-treated mice within 16 d, whereas they remained undetectable in amCD19z-treated mice. Even 40 d after the second infusion of amCD19z T cells, B cells numbers were still greatly reduced in three of five mice, suggesting that repeated infusions of aCD19z T cells may have further therapeutic effect if tumor recurrence was to occur.

**No apparent signs of toxicity are observed in preconditioned mice engrafted with amCD19z T cells**

Finally, mice that had received amCD19z T cells after cyclophosphamide preconditioning (but no tumor) were followed long-term (up to 196 d, n = 20; up to 256 d, n = 7) for evidence of toxicity (Fig. 8). No significant differences in survival were observed between mice that received amCD19z T cells and ahCD19z T cells (p = 0.36) or saline (p = 0.72) or mice that received no treatment at all (p = 0.86), suggesting that observed deaths/culling under Home Office Project License conditions were due to natural
causes related to the life span of the laboratory mice. Importantly, there was no evidence of T cell-related toxicity in mice that had to be culled, and no phenotypic differences were observed between mice that received amCD19z T cells and other treatments. However, it must be noted that these mice were housed under specific pathogen-free conditions. We examined whether circulating Ab levels were affected by amCD19z therapy and found that total IgG remained significantly depleted 21 d after T cell transfer. By day 91, total IgG levels were beginning to return to normal levels, which correlated with the return of B cells in amCD19z-treated mice by this time point (Fig. 8B, 8C). The number of plasma cells (B220+CD138+) was also significantly reduced in the spleens of amCD19z-treated mice 21 d after T cell transfer (0.1% ± 0.009% versus 0.7% ± 0.04%; data not shown).

FIGURE 7. amCD19z T cells eradicate systemically established B cell lymphoma in vivo. A, BALB/c mice (six per group) received 150 mg/kg cyclophosphamide i.p. on day −13, a systemic injection of $5 \times 10^5$ A20luc cells via the tail vein on day −12, 6 Gy irradiation on day −1, and $10^6$ (data not shown) or $4 \times 10^6$ amCD19z T cells or $4 \times 10^6$ amCD19mtm T cells or saline via the tail vein on day 0. On days 8, 21, and 71, mice received an i.p. injection of 150 mg/kg vi-Luciferin, were anesthetized with isoflurane, and were imaged for up to 2 min on the dorsal and ventral sides for bioluminescence. Bioluminescence was converted to photons per second, and ventral images are shown from days 8 and 21. (Mouse #2 with amCD19mtm T cells was positive for bioluminescence when imaged from the dorsal side.) Data are representative of three independent experiments. *Mice culled because of hind leg paralysis prior to day 8. B, Mice were culled at the onset of hind leg paralysis, and a pseudosurvival Kaplan–Meier survival curve is shown. C, BALB/c mice (three per group) received 150 mg/kg cyclophosphamide i.p. on day −13, a systemic injection of $5 \times 10^5$ A20luc cells via the tail vein on day −12, 6 Gy irradiation on day −1, and $8.7 \times 10^6$ amCD19z T cells (48% CD34) via the tail vein on day 0. Bioluminescence imaging following an i.p. injection of 150 mg/kg vi-Luciferin was performed on day 0 prior to T cell infusion and on days 6, 13, 20, and 27. Ventral images are shown, but similar results were found when mice were imaged on the dorsal side (data not shown). D, Survival of mice at day 38 from four independent experiments with varying doses of amCD19mtm and amCD19z T cells is shown. All mice that were alive at day 38 were also tumor-free, as assessed by in vivo imaging.
The provision of CARs with more potent signaling domains, such as CD28, may prove to be important, because these were shown to prevent activation-induced cell death and improve T cell persistence in vivo (4, 16, 18–22). In this study, chemotherapy (cyclophosphamide) and irradiation (6 Gy TBI) were used to study amCD19z T cell engrafment. Within 1 wk of adoptive transfer, CD4+ and CD8+ amCD19z T cells proliferated in vivo. However, only CD8+ amCD19z T cells underwent Ag-driven proliferation in vitro, suggesting that other factors, perhaps homeostatic cytokines (23), were playing a role in the costimulation of CD4+ T cells in vivo. Attempts were made to replicate this in vitro with the addition of IL-7 and -15 to cultures, but this did not enhance CD4 T cell proliferation (data not shown).

Interestingly, amCD19mtm T cells did not seem to undergo significant proliferation upon transfer into lymphodepleted animals, and we hypothesize that this is due to the use of mice maintained under strict specific pathogen-free conditions. Correspondingly, significant increases in the number of engrafted CAR+ T cells were seen in the peripheral blood 7 d after transfer compared with T cells bearing the nonsignaling amCD19mtm or the irrelevant targeted ahCD19z CARs, indicating that the increase in amCD19z T cells required Ag binding and signaling from the CAR.

Although the engineered T cells persisted at early time points within the peripheral circulation, it became clear that amCD19z T cells were effectively lost by 2–3 mo, which correlated with a resurgence in the numbers of circulating B cells. The suggestion from this observation was that the amCD19z T cells were targeting normal B cells and that, after a period of time, these T cells most likely die out through excessive Ag stimulation as a result of the regenerating pool of B cells. Preconditioning with irradiation or cyclophosphamide reduced the B cell compartment to ~1% of its original size, which, in vivo, may prove to be of a sufficient size to promote the proliferation of the amCD19z T cells; however, as the lymphodepleting effects of the conditioning end, the flood back of B cells seems to be too much for the engrafted amCD19z T cells.

We previously showed that CD4+ and CD8+ amCD19z T cells are able to eradicate tumor in vivo (4), and the lack of a clear correlation between the disappearance of CD4+ and CD8+ amCD19z T cells and B cell resurgence suggests that both subsets play a key role in initial B cell depletion.

The provision of CARs with more potent signaling domains, such as CD28, may prove to be important, because these were shown to prevent activation-induced cell death and improve T cell persistence in vivo (7, 24–26); however, our in vitro studies indicated that amCD19z T cells were receiving at least some costimulation by interaction with CD19+ target cells (17) and indicated that amCD19z T cells were receiving at least some costimulation in vitro, suggesting that other factors, perhaps homeostatic cytokines (23), were playing a role in the costimulation of CD4+ T cells in vivo. Attempts were made to replicate this in vitro with the addition of IL-7 and -15 to cultures, but this did not enhance CD4 T cell proliferation (data not shown). Interestingly, amCD19mtm T cells did not seem to undergo significant proliferation upon transfer into lymphodepleted animals, and we hypothesize that this is due to the use of mice maintained under strict specific pathogen-free conditions. Correspondingly, significant increases in the number of engrafted CAR+ T cells were seen in the peripheral blood 7 d after transfer compared with T cells bearing the nonsignaling amCD19mtm or the irrelevant targeted ahCD19z CARs, indicating that the increase in amCD19z T cells required Ag binding and signaling from the CAR.

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cell depletion due to potentially greater enhanced homeostatic proliferation of T cells as a result of increased availability of bacterial Ags. Indeed, microbial translocation was shown to increase the effectiveness of adoptively transferred T cells following TBI as a result of signaling through TLR4 (28).

Critically, amCD19z T cells were highly effective at eradicating 12-d established, disseminated A20 B cell lymphoma, although there seemed to be a minimum dose of amCD19z T cells (10⁶ cells) that was required for optimal therapy. Similar to the scenario seen in the tumor-free mice, amCD19z T cells do not persist long-term after adoptive transfer, and B cells returned to normal levels. Because amCD19z T cells were able to eradicate tumor within 6 d of transfer, during which time amCD19z T cells are still undergoing rapid proliferation and expansion, this models suggests that a CD3ζ-alone CAR is sufficient to eradicate tumor. Because cyclophosphamide and TBI can deplete T regulatory cells in vivo (29), this may have increased the potency of amCD19z T cells but was not sufficient to induce tumor regression alone, as evidenced by the continued growth of tumor in the amCD19zmtm-treated mice. Although second-generation CARs containing costimulatory domains may have a long-term survival advantage over amCD19z T cells, the ability of a CD3ζ-alone CAR to eradicate tumor, in conjunction with only the short-term persistence and resurgence of B cells, may be clinically advantageous. Prolonged B cell depletion, as may be seen with more potent second-generation receptors, could lead to secondary complications, such as lethal infections, as a result of the lack of humoral immunity (30, 31).

The key observation in this model was that there were no clinically manifest signs of toxicity driven by the CD3ζ CAR T cells, similar to other studies treating murine leukemia with cyclophosphamide and immune effector cells (20). As discussed above, B cell depletion for periods $\geq$ 1 mo was observed; however, there was no apparent long-term effect of CAR T cell activity because the levels of B cells returned to normal, suggesting that there was no permanent damage caused to the hemopoietic system as a result of therapy. No toxicity was directly apparent as a result of B cell depletion, although the animals were maintained under highly controlled specific pathogen-free conditions; the full implications of B cell depletion may not become apparent within this clean environment. Indeed, circulating Ab levels, as measured by total IgG, were significantly reduced, even after the B cells had returned. However, it is important to note that rituximab treatment in patients with non-Hodgkin’s lymphoma (NHL) leads to a significant decrease in serum IgG and IgM levels out to 11 mo (5). The lack of observed toxicity in this model using a CD3ζ CAR was encouraging and provides support for our current phase I trial testing the efficacy of T cells bearing an anti-human CD19.CD3ζ CAR in patients with NHL. The observations from this model system suggest that accessibility to Ag alone is insufficient to cause toxicity, because the CD19 Ag is available at high levels on normal cells present within the anatomical locations that CAR T cells will rapidly populate after systemic infusion (i.e., the peripheral circulation and within secondary lymphoid tissues). This model suggests that the choice of Ag is likely to be as critical as target accessibility for on-target toxicity.

We have developed a novel model based upon targeting gene-modified T cells against the mouse CD19 Ag; using a first-generation CAR, we observed efficient antitumor therapy with no apparent adverse effects. The field of engineered T cells is rapidly evolving, and this model will be used to test the efficacy and on-target toxicity of more powerful receptor combinations (33, 34) to provide information that may prove pertinent to the design of future CAR-based clinical trials.

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