A novel chimeric antigen receptor containing a JAK–STAT signaling domain mediates superior antitumor effects

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The adoptive transfer of T cells engineered with a chimeric antigen receptor (CAR) (hereafter referred to as CAR-T cells) specific for the B lymphocyte antigen CD19 has shown impressive clinical responses in patients with refractory B cell malignancies1–7. However, the therapeutic effects of CAR-T cells that target other malignancies have not yet resulted in significant clinical benefit8–11. Although inefficient tumor trafficking and various immunosuppressive mechanisms can impede CAR-T cell effector responses, the signals delivered by the current CAR constructs may still be insufficient to fully activate antitumor T cell functions. Optimal T cell activation and proliferation requires multiple signals, including T cell receptor (TCR) engagement (signal 1), co-stimulation (signal 2) and cytokine engagement (signal 3)12. However, CAR constructs currently being tested in the clinic contain a CD3ζ (TCR signaling) domain and co-stimulatory domain(s) but not a domain that transmits signal 3 (refs. 13–18). Here we have developed a novel CAR construct capable of inducing cytokine signaling after antigen stimulation. This new-generation CD19 CAR encodes a truncated cytoplasmic domain from the interleukin (IL)-2 receptor β-chain (IL-2Rβ) and a STAT3-binding tyrosine-X-X-glutamine (YXXQ) motif, together with the TCR signaling (CD3ζ) and co-stimulatory (CD28) domains (hereafter referred to as 28-ΔIL2RB-z(YXXQ)). The 28-ΔIL2RB-z(YXXQ) CAR-T cells showed antigen-dependent activation of the JAK kinase and of the STAT3 and STAT5 transcription factors signaling pathways, which promoted their proliferation and prevented terminal differentiation in vitro. The 28-ΔIL2RB-z(YXXQ) CAR-T cells demonstrated superior in vivo persistence and antitumor effects in models of liquid and solid tumors as compared with CAR-T cells expressing a CD28 or 4-1BB co-stimulatory domain alone. Taken together, these results suggest that our new-generation CAR has the potential to demonstrate superior antitumor effects with minimal toxicity in the clinic and that clinical translation of this novel CAR is warranted.

Cytokines that share common γ-chains in their receptors have a fundamental effect on T cell immunity, mainly through activation of the JAK–STAT signaling pathway19. Whereas IL-2, IL-7 and IL-15 predominantly induce STAT5 activation through tyrosine residues within the common γ-chain, the IL-2R β-chain (for IL-2 and IL-15) or the IL-7R α-chain (for IL-7), IL-21 preferentially activates STAT3 through its association motif YXXQ within the IL-21 receptor20. In addition to its critical role in memory cell formation and effector cell differentiation, IL-21 functions synergistically with other cytokines to promote T cell proliferation21–23. Indeed, the forced expression of cytokine-encoding genes in CAR-T cells improves their persistence and antitumor effects in vivo, highlighting the importance of signal 3 for CAR-T cell functions24–26. However, constitutive cytokine expression poses a risk for autonomous T cell growth, potentially causing cancers, as well as for serious adverse events such as cytokine release syndrome (CRS) and neurotoxicity by CAR-T cells with excessive growth potential27,28. Here we aimed to determine whether delivery of cytokine signaling only after antigen engagement could enhance antitumor effects of CAR-T cells.

To induce JAK–STAT pathway activation in CAR-T cells in an antigen-dependent manner, we first incorporated a full-length or truncated cytoplasmic domain of IL-2Rβ between the cytoplasmic domains of CD28 and CD3ζ (Fig. 1a and Supplementary Table 1). Second, we added a YXXQ motif at the C terminus of CD3ζ for STAT3 recruitment (to obtain 28-IL2RB-z(YXXQ) or 28-ΔIL2RB-z(YXXQ))29. We also generated CAR constructs with only a CD28 and CD3ζ cytoplasmic domain (construct 28-z) or a 4-1BB and CD3ζ cytoplasmic domain (BB-z) for comparison. A CD19-specific monoclonal antibody (clone FMC63)-derived single-chain variable fragment (scFv) targeting CD19 was used for antigen recognition 30. All CAR constructs were N-terminally linked to a truncated version of nerve growth factor receptor (NGFR) via a 2A peptide sequence derived from porcine teschovirus 1 (P2A). The 28-ΔIL2RB-z(YXXQ), but not the 28-IL2RB-z(YXXQ), CAR was efficiently expressed on the cell surface, specifically in the NGFR+ T cell population, and it transmitted T cell activation signals comparable to those of the

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Figure 1 Generation of the CD19-specific chimeric antigen receptor constructs to induce JAK–STAT pathway activation. (a) A schematic diagram of the anti-CD19 CAR constructs used in this study. An FMC63-derived scFv was linked to CD28 and CD3ζ (to generate 28-z), to CD8α, 4-1BB and CD3ζ (to generate 28-ζ), to CD28-α, 4-1BB and CD3ζ (to generate IL2RB(FLSL)-ζ(YXXQ) CAR, IL2RBζ(YXXQ) CAR, respectively). All of the CARs were linked to truncated NGFR (ANGFR) with or without an internal deletion, and CD3ζ with the YXXQ motif (to generate 28-IL2RBζ(YXXQ) and 28-IL2RBζ(YXXQ), respectively). (b) Representative FACS plots (b) and mean fluorescence intensity (MFI) of phosphorylated STAT3 and STAT5 within the CD8+ population over time as assessed by flow cytometry (c) in T cells that were transduced with the indicated CAR-encoding construct and stimulated with IL-2 (300 IU/ml) or IL-21 (50 ng/ml) were used as control. (d) Schematic representation of CAR constructs with either the STAT3- or STAT5-binding domain alone. (e) Quantification of STAT3 and STAT5 phosphorylation in T cells that were transduced with constructs encoding the indicated CARs 120 min after stimulation with NALM-6 (n = 4 different donor samples). Values were determined by repeated measures analysis of variance (ANOVA) with Tukey’s multiple comparisons test; n.s., not significant; P = 75.44 for pSTAT3, F = 99.46 pSTAT5; degrees of freedom = 15. In c,e, horizontal lines denote mean values.
28-z and BB-z CARs after encountering antigen, as demonstrated by CD69 upregulation and phosphorylation of the kinases ERK and Akt (Supplementary Figs. 1a,b and 2a–c). The 28-ΔIL2RB-z(YXXQ) CAR-T cells showed a significant upregulation of phosphorylated STAT3 (pSTAT3) and pSTAT5 in an antigen-dependent manner (Fig. 1b,c and Supplementary Fig. 3a,b). Although STAT3 and STAT5 phosphorylation was also observed in CAR-negative T cells after antigen stimulation, expression of the 28-ΔIL2RB-z(YXXQ) CAR triggered higher levels of pSTAT3 and pSTAT5 in CAR-positive cells, suggesting that the phosphorylation event was likely induced by cell-intrinsic signaling (Supplementary Fig. 4a–d). Replacement of tyrosine by phenylalanine within the STAT3-association motif Tyr-Leu-Ser-Leu (YLSL) of the IL-2Rβ domain (28-ΔIL2RB(FLSL)-z(YXXQ)) significantly reduced pSTAT3 levels, and the deletion of the YXXQ motif at the C terminus (28-ΔIL2RB-z) decreased pSTAT3 levels (Fig. 1d,e and Supplementary Fig. 5a–c). These results suggested that the YLSL and YXXQ motifs mainly contributed to the enhanced pSTAT3 and pSTAT5 levels, respectively. On the basis of these data, we further analyzed the functional attributes of the 28-ΔIL2RB-z(YXXQ) CAR-T cells.

After in vitro antigen stimulation, the 28-ΔIL2RB-z(YXXQ) CAR-T cells achieved significantly greater proliferation than the 28-z and BB-z CAR-T cells, regardless of cytokine supplementation, which resulted from both more rapid cellular division and less activation-induced cell death (Fig. 2a–c and Supplementary Fig. 6a,b). Both the STAT3 interaction and STAT5 interaction domains were required to promote CAR-T cell proliferation (Fig. 2a and Supplementary Fig. 6a). Of note, the CAR-T cells with the STAT3-association motif maintained the less differentiated CD8+CD45RA+CD62L+CCR7+ T cell population significantly better than the other CAR-T cells (Fig. 2d and Supplementary Fig. 7). T cells within this population mostly expressed CD27, CD28 and CD95, which corresponded to a marker phenotype for stem-cell-like memory T cells31. Consistent with these results, cotreatment with the STAT3 inhibitor S3I-201 and the STAT5 inhibitor pimozide abrogated the proliferative advantage of the 28-ΔIL2RB-z(YXXQ) CAR-T cells, and inhibition of STAT3 signaling in the 28-ΔIL2RB-z(YXXQ) CAR-T cells decreased the CD45RA+CD62L+CCR7+ T cell population significantly better than the other CAR-T cells (Fig. 2e and Supplementary Fig. 8a–c). Although STAT3 activation can promote expression of programmed cell death ligand 1 (PD-L1) in several types of tumor cells, such as lymphoma cells and lung cancer cells32,33, JAK–STAT pathway activation did not have effects in addition to those seen after antigen stimulation for the upregulation of PD-L1 or other immunoinhibitory molecules in antigen-stimulated CAR-T cells (Supplementary Fig. 9). After repeated stimulations, all CAR-T cells showed reduced proliferation and cytokine production and upregulation of certain exhaustion markers (Supplementary Figs. 10 and 11). These results suggest that retrovirally transduced CAR-T cells undergo functional impairment accompanied by chronic antigen exposure, as reported previously34,35. 28-z CAR-T cells showed substantially decreased proliferation and increased expression of programmed cell death 1 (PD-1), lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin and mucin domain 3 (TIM-3), as compared with that of the BB-z and 28-ΔIL2RB-z(YXXQ) CAR-T cells. 28-ΔIL2RB-z(YXXQ) or 28-ΔIL2RB(FLSL)-z(YXXQ)-transduced CD8+ T cells maintained better proliferation, IL-2 secretion and cytokine polyfunctionality than other CAR-T cells. These attributes have been described in less differentiated memory T cells36,37. To compare CAR-T cell functions after exposure to antigen in vivo, CAR-T cells were transplanted into immunodeficient NOD-SCID IL-2Rγnull (NSG) mice bearing tumors derived from NALM6 human leukemia cells (Fig. 2f). Persisting CAR-T cells were isolated from the spleen and analyzed for proliferative capacity and cytokine secretion in vitro. Similar to the in vitro data, the 28-ΔIL2RB-z(YXXQ) CAR-T cells showed better proliferation and cytokine polyfunctionality than the 28-z and BB-z CAR-T cells (Fig. 2g,h). These results suggest a key role of STAT3 in suppressing terminal differentiation of T cells, which is consistent with recent human and mouse studies38,39.

To assess the genetic mechanisms underlying the functional properties of the 28-ΔIL2RB-z(YXXQ) CAR-T cells, we compared the gene expression profiles of CD8+ CAR-T cells at 4, 24 and 72 h following antigen stimulation in vitro. The number of differentially expressed genes among the 28-z, BB-z and 28-ΔIL2RB-z(YXXQ) CAR-T cells progressively increased over time (Supplementary Table 2). Unsupervised hierarchical clustering and principal component analysis of those genes mostly classified different CAR-T cells into independent subclusters (Fig. 3a,b). We further investigated whether the 28-ΔIL2RB-z(YXXQ) CAR induced gene expression profiles that reflected JAK–STAT pathway activation. Gene set enrichment analysis (GSEA) revealed significant enrichment of genes induced by IL-21 (but not by IL-2, IL-7 or IL-15) in the 28-ΔIL2RB-z(YXXQ) CAR-T cells at 24 h after stimulation (Fig. 3c and Supplementary Fig. 12a). Moreover, the 28-ΔIL2RB-z(YXXQ) CAR-T cells were enriched for expression of previously identified STAT3 target genes (Fig. 3d)40, which further corroborated that STAT3 pathway was preferentially activated by the 28-ΔIL2RB-z(YXXQ) CAR. These profiles were not evident at 4 h, and were maintained at 72 h, after activation (Supplementary Fig. 12b,c). Notably, the 28-ΔIL2RB-z(YXXQ) CAR-T cells also showed increased expression of multiple genes encoding effector molecules, such as GZMA, GZMB, GZMH, GZMK and PRF1, which we validated by qPCR analysis (Fig. 3e). We then evaluated the cytolytic efficacy of the 28-ΔIL2RB-z(YXXQ) CAR-T cells relative to that of the other CAR-T cells. All of the CAR-T cells demonstrated similarly potent in vitro cytotoxicity against CD19+ cells (Supplementary Fig. 13). However, the 28-ΔIL2RB-z(YXXQ) CAR-T cells showed superior cytolytic activity following repeated antigen exposures, treatments that progressively attenuated the effector functions of the other CAR-T cells as previously reported (Fig. 3f,g)35.

Next we studied the antileukemic potency of the 28-ΔIL2RB-z(YXXQ) CAR-T cells in vivo. NSG mice were treated with 5 × 106 CAR-T cells on day 14 after injection with 5 × 106 NALM6 cells that had been transduced with a construct expressing an EGFP–firefly luciferase fusion protein (hereafter referred to as NALM6-GL cells) (Fig. 4a). The 28-ΔIL2RB-z(YXXQ)-transduced T cells efficiently suppressed leukemia progression as compared to that with the 28-z and BB-z CAR-T cells, which resulted in significantly longer overall survival of the 28-ΔIL2RB-z(YXXQ) CAR-T cell–treated mice (Fig. 4b,c and Supplementary Fig. 14). Notably, the 28-ΔIL2RB-z(YXXQ) CAR-T cells showed significantly better persistence in the peripheral blood (Fig. 4d). Two infusions of CAR-T cells (5 × 106 cells per infusion, separated by 2 d) resulted in better control of leukemia and higher levels of secreted IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α with all of the tested CAR constructs, although the 28-ΔIL2RB-z(YXXQ) CAR-T cells showed better persistence than the other CAR-T cells (Supplementary Fig. 15a–g). IL-6 was below the limits of sensitivity and not detectable in this model (data not shown). Human T cells transplanted into NSG mice cause xenogeneic graft-versus-host disease (GVHD) by recognizing xenogeneic antigens through endogenous TCR engagement. The persisting CAR-positive and CAR-negative T cells induced GVHD in
Figure 2  The 28-ΔIL2RB-z(YXXQ) CAR-T cells show a superior proliferative capacity and maintain less differentiated memory T cell phenotypes after antigen stimulation. (a) Fold expansion of the CD4+ and CD8+ CAR-T cell populations 7 d after stimulation with the NALM-6 or K562 cell lines (n = 8 different donor samples). P values were determined by repeated-measures one-way ANOVA with Tukey’s multiple-comparisons test for the NALM-6 data (F = 20.12 for CD4+ T cells; F = 36.57 for CD8+ T cells; degrees of freedom = 39) or by paired t-test for comparison between the NALM-6 and K562 data in individual CAR-T cells (t = 10.34 (CD4+-28-z), t = 8.16 (CD4+-BB-z), t = 13.98 (CD4+-28-ΔIL2RB-z(YXXQ)), t = 10.26 (CD4+-28-ΔIL2RB-(FLSL)-z(YXXQ)), t = 6.33 (CD4+-28-BB-z), t = 10.57 (CD8+-BB-z), t = 9.88 (CD8+-28-ΔIL2RB-z(YXXQ)), t = 12.63 (CD8+-28-ΔIL2RB-(FLSL)-z(YXXQ)) and t = 7.44 (CD8+-28-ΔIL2RB-z); degrees of freedom = 7). (b) CAR-T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with NALM-6 cells. The mean fluorescence intensity of CFSE was analyzed 3 d following the stimulation (n = 4 different donor samples; repeated measures one-way ANOVA with Tukey’s multiple comparisons test; F = 36.84 for CD4+ T cells, F = 38.44 for CD8+ T cells; degrees of freedom = 19). (c) The frequency of dead cells within CD4+ or CD8+ CAR-T cell population, as analyzed by flow cytometry 24 h after stimulation with NALM-6 cells (n = 4 different donor samples). P values were determined by repeated-measures one-way ANOVA with Tukey’s multiple-comparisons test (F = 53.83 for CD4+ T cells; F = 114.41 for CD8+ T cells; degrees of freedom = 24). (d) The frequency of CD45RA+CD62L+CCR7+ cells in the CD8+ CAR-T cell population 7 d after stimulation with NALM-6 cells (n = 9 different donor samples). P values were determined by repeated-measures one-way ANOVA with Tukey’s multiple-comparisons test (F = 14.35; degrees of freedom = 44). (e) The frequency of dead cells within CD4+ or CD8+ CAR-T cell population, as analyzed by flow cytometry 24 h after stimulation with NALM-6 cells (n = 4 different donor samples). P values were determined by repeated-measures one-way ANOVA with Tukey’s multiple-comparisons test (F = 53.83 for CD4+ T cells; F = 114.41 for CD8+ T cells; degrees of freedom = 24). (d) The frequency of CD45RA+CD62L+CCR7+ cells in the CD8+ CAR-T cell population 7 d after stimulation with NALM-6 cells (n = 9 different donor samples). P values were determined by repeated-measures one-way ANOVA with Tukey’s multiple-comparisons test (F = 14.35; degrees of freedom = 44). (e) The fold expansion of CD8+ CAR-T cells on day 7 after stimulation with NALM-6 cells and culture with or without 25 μM of S3I-201 (STAT3 inhibitor) and/or 5 μM of pimozide (STAT5 inhibitor) for 3 d (n = 6 different donor samples). P values were determined by repeated-measures one-way ANOVA with Tukey’s multiple-comparisons test for each condition (F = 46.52 for DMSO; F = 15.67 for S3I-201; F = 19.17 for pimozide; F = 1.85 for S3I-201 + pimozide; degrees of freedom = 17). Horizontal lines indicate mean values; n.s., not significant. (f) Experimental design. NSG mice were intravenously (i.v.) infused with NALM-6 cells and subsequently transplanted with CAR-T cells. Spleen cells were then isolated from the mice and re-stimulated with NALM-6 or K562, and proliferation and cytokine production were measured. (g,h) Fold expansion after 7 d of culture (g) and cytokine production (h) of CD8+ CAR-T cells treated as in show in f. The data shown are the sum of two independent experiments (n = 6 mice per group) and are presented as mean ± s.d. P values were determined by Student’s T test (F = 10.85 for fold expansion; F = 12.42 for IL-2; F = 2.17 for IFN-γ; F = 0.81 for TNF-α; F = 7.51 for IL-2+IFN-γ+TNF-α) cells; degrees of freedom = 29); n.s., not significant.
Log2-transformed values were compared by repeated-measures one-way ANOVA with Tukey’s multiple comparisons test (analyzed by qPCR and relative to ubiquitin C (UBC)). False discovery rates (FDRs) to adjust for multiple-hypothesis testing are presented.

In the time course analysis, the 28-IL2RB-z(YXXQ) CAR-T cells have unique gene expression profiles and show potent cytotoxic activity after repetitive antigen stimulations. The 28-z, BB-z and 28-IL2RB-z(YXXQ) CAR-T cells were stimulated with NALM-6 cells, and their gene expression profiles were compared by microarray analysis at 4, 24 and 72 h after stimulation (n = 4 different donor samples). The data shown are unsupervised hierarchical clustering (a) and principal component analysis (b) of differentially expressed genes at 4 h (left), 24 h (middle) and 72 h (right) after stimulation (raw P < 0.01 by repeated measures one-way ANOVA). Var., variance. Gene set enrichment analysis (GSEA) for the expression profiles of the 28-IL2RB-z(YXXQ) CAR-T cells as compared to those for the 28-z or BB-z CAR-T cells using genes induced by the IL-21 treatment (c) or the STAT3 target genes (d) as gene sets (n = 4 samples for each CAR). Nominal P values, as determined by an empirical phenotype-based permutation test, and false discovery rates (FDRs) to adjust for multiple-hypothesis testing are presented. (e) Expression of the genes associated with cytolytic activity, as analyzed by qPCR and relative to ubiquitin C (UBC) expression levels, in CAR-T cells that were stimulated for 24 h (n = 4 different donor samples). Log2-transformed values were compared by repeated-measures one-way ANOVA with Tukey’s multiple comparisons test (F = 24.52 for GZMA; F = 43.68 for GZMB; F = 32.5 for GZMH; F = 43.43 for GZMK; F = 32.54 for PRF1; degrees of freedom = 11). Horizontal lines denote the mean values. (f,g) Experimental design to test effect of repeated stimulations of CAR-T cells with NALM-6 cells at an E:T cell ratio of 1:3 (f) and cytotoxic activity of CAR-T cells against NALM-6 cells on days 0, 3 and 6, as evaluated by flow cytometry (n = 4 technical replicates for individual CAR-T cells). Data are mean ± s.d. P values were determined by ordinary one-way ANOVA with Tukey’s multiple-comparisons test for each round of stimulation (F = 0.41 for day 0; F = 44.94 for day 3; F = 46.75 for day 6; degrees of freedom = 19). Similar results were obtained in an independent experiment. In e.g., *P < 0.001; n.s., not significant.

Figure 3 The 28-IL2RB-z(YXXQ) CAR-T cells have unique gene expression profiles and show potent cytotoxic activity after repetitive antigen stimulations. (a,b) The 28-z, BB-z and 28-IL2RB-z(YXXQ) CAR-T cells were stimulated with NALM-6 cells, and their gene expression profiles were compared by microarray analysis at 4, 24 and 72 h after stimulation (n = 4 different donor samples). The data shown are unsupervised hierarchical clustering (a) and principal component analysis (b) of differentially expressed genes at 4 h (left), 24 h (middle) and 72 h (right) after stimulation (raw P < 0.01 by repeated measures one-way ANOVA). Var., variance. (c,d) Gene set enrichment analysis (GSEA) for the expression profiles of the 28-IL2RB-z(YXXQ) CAR-T cells as compared to those for the 28-z or BB-z CAR-T cells using genes induced by the IL-21 treatment (c) or the STAT3 target genes (d) as gene sets (n = 4 samples for each CAR). Nominal P values, as determined by an empirical phenotype-based permutation test, and false discovery rates (FDRs) to adjust for multiple-hypothesis testing are presented. (e) Expression of the genes associated with cytolytic activity, as analyzed by qPCR and relative to ubiquitin C (UBC) expression levels, in CAR-T cells that were stimulated for 24 h (n = 4 different donor samples). Log2-transformed values were compared by repeated-measures one-way ANOVA with Tukey’s multiple comparisons test (F = 24.52 for GZMA; F = 43.68 for GZMB; F = 32.5 for GZMH; F = 43.43 for GZMK; F = 32.54 for PRF1; degrees of freedom = 11). Horizontal lines denote the mean values. (f,g) Experimental design to test effect of repeated stimulations of CAR-T cells with NALM-6 cells at an E:T cell ratio of 1:3 (f) and cytotoxic activity of CAR-T cells against NALM-6 cells on days 0, 3 and 6, as evaluated by flow cytometry (n = 4 technical replicates for individual CAR-T cells). Data are mean ± s.d. P values were determined by ordinary one-way ANOVA with Tukey’s multiple-comparisons test for each round of stimulation (F = 0.41 for day 0; F = 44.94 for day 3; F = 46.75 for day 6; degrees of freedom = 19). Similar results were obtained in an independent experiment. In e.g., *P < 0.001; n.s., not significant.
NSG mice were subcutaneously (s.c.) injected with the CD19-expressing A375 melanoma cells (A375-CD19) (day –21) and then treated with $5 \times 10^5$ CAR-T cells on days 0 and 4. (c) Kaplan–Meier curve for the overall survival of the mice (n = 6 mice per group) and the frequency of CD8$^+$ CAR-T cells in the peripheral blood (n = 4 mice for 28-z CAR-T cells on day 21; n = 6 for the other groups). (d) Representative data of two experiments are shown. In c, P values were determined by log-rank test and adjusted with Bonferroni correction; in d, P values were determined by ordinary one-way ANOVA with Tukey’s multiple-comparisons test (F = 17.19, degrees of freedom = 17 for day 7; F = 7.42, degrees of freedom = 15 for day 21). (e-g) NSG mice were intravenously infused with CD19$^+$ primary B-ALL cells (day –35) and adoptively transferred with $5 \times 10^5$ BB-z or 28-z CAR-T cells (day 0). The serial monitoring of the CD45$^+$CD19$^+$ B-ALL cells in the peripheral blood (%)(n = 5 mice per group) (e), the Kaplan–Meier curve for the event-free survival of the mice (n = 5 mice group) (f) and the frequency of the CD8$^+$ CAR-T cells in the peripheral blood on days 7, 14 and 28 (n = 5 mice per group) (g) are shown. In f, P values were determined by log-rank test, adjusted with Bonferroni correction. In h, P values were determined by t-test (t = 2.58 for day 7; t = 4.28 for day 14; t = 2.46 for day 21; degrees of freedom = 8). The data are representative of two experiments using different B-ALL samples. (h) Experimental design. NSG mice were subcutaneously (s.c.) injected with the CD19-expressing A375 melanoma cells (A375-CD19) (day –21) and then treated with $5 \times 10^5$ CAR-T cells on days 0 and 4. (i-k) Frequency of CD8$^+$ CAR-T cells in the peripheral blood and subcutaneous tumors on day 7, tumor progression (j) and overall survival (k) of mice treated as described in h (n = 7 mice per group). In i, P values were determined by ordinary one-way ANOVA with Tukey’s multiple comparisons test (F = 12.61 for peripheral blood; F = 27.46 for tumor; degrees of freedom = 20). In k, P values were determined by log-rank test, adjusted with Bonferroni correction. Representative results of two experiments are shown. In d, g, i, horizontal lines indicate mean values ± s.d. *P < 0.001 by the statistical test indicated in the respective legend.
some mice after leukemia eradication (Supplementary Fig. 16a,b and Supplementary Table 3). The 28-AIL2RB-z(XXYQ) CAR-T cells did not increase the development of GVHD as compared to that in mice treated with the 28-z and BB-z CAR-T cells. Indeed, all CAR-T cells induced similar progressive weight loss with comparable latency when infused into tumor-free mice (Supplementary Fig. 17a). There was no significant difference in the development of lethal GVHD among induced similar progressive weight loss with comparable latency when compared to 28-z and BB-z CAR-T cells. Improved expansion of the CAR-T cells showed better persistence in peripheral blood and prevented leukemia progression for longer periods than the BB-z CAR-T cells (Fig. 4f–g). Finally, we tested whether the JAK–STAT pathway activation in the CAR-T cells could improve their therapeutic effects against solid tumors. We adoptively transferred various CAR-engineered T cells into NSG mice that had subcutaneously been inoculated with A375 melanoma cells transduced with CD19 (A375-CD19) (Fig. 4h). As seen in the mouse models of leukemia, the 28-AIL2RB-z(XXQ) CAR-T cells showed superior expansion in the peripheral blood and tumor mass, which resulted in better control of the tumor cells (Fig. 4i–k). At the time of the death of the mice, the tumor cells maintained CD19 expression, and the CAR-T cells had almost disappeared within the tumor mass, suggesting that the sustained persistence of CAR-T cells is required to suppress tumor progression (Supplementary Fig. 18a–c).

In summary, we have developed a novel CAR construct that activates the JAK–STAT pathway. The 28-AIL2RB-z(XXQ) CAR triggered gene expression profiles analogous to those triggered by IL-21 treatment, which provided the T cells with distinct functional properties, including superior proliferative capacity and effector functions as compared to 28-z and BB-z CAR-T cells. Improved expansion of the 28-AIL2RB-z(XXQ) CAR-T cells in vivo could potentially increase the risk for CRS, which should be cautiously addressed when this construct is clinically translated. Our novel CAR design can be used in any CAR-T cell, independent of antigen specificity, to enhance their antitumor efficacy.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Y.K. and N.H. designed the project; Y.K., S.T.T., M.A., C.-H.W. and K.S. performed the experiments; M.D.M. and M.O.B. provided critical human samples and contributed to the writing of the manuscript; and Y.K. and N.H. analyzed the results and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Construction of CAR-expressing genes. The 28-z CAR-encoding gene was generated by linking the sequences encoding the FMC63-derived scFv to those of the extracellular, transmembrane and cytoplasmic domain of CD28 (amino acids 114–220) and the cytoplasmic domain of CD3z. The BB-z-encoding CAR was generated by inserting the sequences encoding the hinge and transmembrane domain of CD8α and the 4-1BB cytoplasmic domain between the sequences encoding the scFv and cytoplasmic domain of CD3z. To induce JAK–STAT signaling in the CAR, the cytoplasmic domain of full-length (amino acids 266–551) or truncated (amino acids 266–337 and 530–551) IL-2Rβ was linked between CD28 and cytoplasmic domain of CD3z. The YYXX motif was created by site-directed mutagenesis at the C terminus region of CD3z domain. For the 28-ΔIL2RB (FSL)-zYYXX) CAR, tyrosine within the YLNL motif was substituted with phenylalanine to abrogate STAT5 recruitment. All CAR-expressing genes were linked to the sequence encoding ΔNGFR using a furin–Ser-Gly-Ser-Gly–P2A sequence and cloned into the pMX vector. The amino acid sequences of the CAR signaling domains are provided in Supplementary Table 1.

In vitro culture of human T cells. Peripheral blood mononuclear cells obtained from healthy donors were prepared by Ficoll–Paque PLUS density gradient centrifugation (GE Healthcare). The CD3+ and CD8+ cells were purified through negative magnetic selection using the Pan T Cell Isolation Kit and the CD8+ T Cell Isolation Kit (Miltenyi Biotec), respectively. The purified T cells were stimulated with irradiated artificial antigen-presenting cells that expressed a membrane-bound form of anti-CD3 (clone OKT3) and the co-stimulatory molecules CD80 and CD83 (aAPC/mOKT3 cells) at an E:T cell ratio of 5:1. On the following day, 100 IU/ml IL-2 and 10 ng/ml IL-15 (PeproTech) were added to the cultures. The culture medium was replenished every 2–3 d. CAR-transduced T cells were restimulated with NALM-6 or K562 at an E:T cell ratio of 1:1 7 d after the initial stimulation. The number of CAR-T cells was determined by calculating the product of the total number of T cells and the percentage of ΔNGFR+ CD4+ or CD8+ T cells. The fold expansion was calculated by dividing the number of expanded CAR-T cells after 7 d of culture by the respective number on day 0.

Cell lines. Both aAPC/mOKT3 and K562-CD19 are derived from the human erythroleukemic cell line K562 (American Type Culture Collection; ATCC) and the CD8+ T cell line was obtained from DSMZ (Braunschweig, Germany). The A375 melanoma cell line was obtained from ATCC. All cell lines were routinely cultured by the respective number on day 0.

Cytokine production analysis. For intracellular cytokine staining, CAR-T cells were stimulated with NALM-6 cells at an E:T cell ratio of 1:1. Brefeldin A (BioLegend) was added to the culture medium after 2 h to inhibit extracellular secretion of cytokines, and the cells were further cultured for 4 h. After staining for the surface markers, the cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and then stained with PE–anti-IL-2 (clone SFCI21-Thy2D3; Beckman Coulter) and FITC–anti-HLA-ABC (clone O323; BioLegend), APC–anti-CD45 (clone HI30; BioLegend), FITC–anti-HLA-A2 (clone BB7.2; BioLegend) and PE–anti-CD19 (clone HIB19, BioLegend). The CAR-transduced T cells were stained with biotin-labeled protein L (GenScript), followed by streptavidin–PE (Thermo Fisher Scientific). For the CFSE dilution assay, T cells were labeled with 5 μM CFSE (Thermo Fisher Scientific) before culture. Dead cells were discriminated with the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific). The stained cells were analyzed with a FACScanto II instrument (BD Biosciences). The data analysis was performed using the FlowJo software (Tree Star).

Analysis of phosphoproteins by intracellular flow cytometry. Phosphorylated proteins in the CAR-T cells were analyzed by intracellular flow cytometry following coculture with NALM-6 or K562 cells at an E:T cell ratio of 1:1. The cells were fixed with 1.6% formaldehyde, followed by permeabilization by ice-cold methanol. The following antibodies were used: Alexa-Fluor-647-conjugated anti-phospho-STAT3(Tyr705) (clone 4/P/Stat3, BD Biosciences), Alexa-Fluor-647-conjugated anti-phospho-STAT5(Tyr694) (clone 47/Stat5, BD Biosciences), Alexa-Fluor-647-conjugated anti-phospho-p44/p42 MAPK (Erk1/Erk2) (Th202/Tyr204) (clone 20A, BD Biosciences) and anti-phospho-AktThr308 (clone D25E6, Cell Signaling Technology). Alexa-Fluor-647-conjugated anti-rabbit IgG (H+L) (Jackson ImmunoResearch) was used as the secondary antibody following the staining with the anti-phospho-Akt antibody. The STAT3 inhibitor S3I-201 (Selleck Chemicals) and the STAT5 inhibitor pimozone (Cayman Chemical) were used at concentrations of 25 μM and 5 μM, respectively.

Immunoblotting analysis. Equal amounts of protein were separated on 8% gels by SDS–PAGE and transferred to Immobilon-P PVDF membranes (Millipore). The membranes were probed with the primary antibodies at 4 °C overnight and then washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse-IgG (H+L) or anti-rabbit-IgG (H+L) secondary antibody (Promega). The following primary and secondary antibodies were used: anti-STAT3 (clone D32Z2G, Cell Signaling Technology; 1:1,000 dilution), anti-phospho-STAT3(Tyr705) (clone D3A7, Cell Signaling Technology; 1:1,000 dilution), anti-STAT5 (Cell Signaling Technology; 1:1,000 dilution), anti-phospho-STAT5(Tyr694) (clone D47E7, Cell Signaling Technology; 1:1,000 dilution), anti-β-actin (clone C4, Santa Cruz Biotechnology; sc-77778; 1:1,000 dilution), HRP-conjugated anti-mouse-IgG(H+L) (Promega, W4021; 1:1,000 dilution) and HRP-conjugated anti-rabbit-IgG(H+L) (Promega, W4011; 1:1,000 dilution). All images were acquired with ChemiDoc MP system (Bio-Rad) and Image Lab software (Bio-Rad). Protein levels for each blot were quantified with ImageJ software.

Flow cytometry. The following antibodies were used for the flow cytometry analysis: allopseudocyanin (APC)- and Cy7-conjugated anti-CD4 (clone RPA-T4; BioLegend), phycoerythrin (PE)- and Cy7-conjugated anti-CD8 (RPA-T4; BioLegend), PE–Cy7–anti-CD8 (clone SFC121Thy2D3; Beckman Coulter), PE–anti-CD8 (clone RPA-T8; BioLegend), Pacific Blue–conjugated anti-CD8 (clone B9.11; Beckman Coulter), PE–anti-CD69 (clone FN50; BioLegend), fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (clone MEM-56; Thermo Fisher Scientific), PE–anti-CD62L (clone DREG-36; BioLegend), Pacific Blue–anti-CCR7 (clone G043H7; BioLegend), APC–Cy7–anti-CD27 (clone O323; BioLegend), APC–anti-CD28 (clone CD28.2; BioLegend), PerCP–Cy5.5–conjugated anti-CD95 (clone DX2; BD Biosciences), Alexa-Fluor-488-conjugated anti-CD279 (clone EH12.2H7; BioLegend), PE–anti-CD274 (clone 29E.2A3; BioLegend), APC–Cy7–anti-CD66 (clone F38-2E2; BioLegend), PerCP–Cy5.5–anti-CD223 (clone C987W; BioLegend), FITC–anti-CD271 (clone ME20.4; BioLegend), PerCP–Cy5.5–anti-CD271 (clone ME20.4; BioLegend), V450–conjugated anti-CD271 (clone C40-1457; BD Biosciences), APC–anti-CD45 (clone HJ30; BioLegend), FITC–anti-HLA-A2 (clone BB7.2; BioLegend) and PE–anti-CD19 (clone HIB19, BioLegend). The CAR-transduced T cells were stained with biotin-labeled protein L (GenScript), followed by streptavidin–PE (Thermo Fisher Scientific). For the CFSE dilution assay, T cells were labeled with 5 μM CFSE (Thermo Fisher Scientific) before culture. Dead cells were discriminated with the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific). The stained cells were analyzed with a FACSaria II instrument (BD Biosciences). The data analysis was performed using the FlowJo software (Tree Star).

Microarray analysis. The CAR-transduced CD8+ T cells were cocultured with NALM-6 cells and purified at 4, 24 and 72 h after stimulation using a FACSaria cell sorter (BD Biosciences). RNA was extracted from the sorted cells using the

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RNeasy Micro Kit (Qiagen). The gene expression profiles were analyzed using the Affymetrix Human Gene 2.0 ST Array by the Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, Ontario, Canada). The raw data were normalized and annotated with the Affymetrix Expression Console version 1.4.1 (Affymetrix). The differentially expressed genes among the three types of CAR-T cells were extracted by repeated-measures one-way ANOVA using the Affymetrix Transcriptome Analysis Console (Affymetrix) (P < 0.01 with repeated-measures one-way ANOVA). An unsupervised hierarchical clustering was performed, and a heat map was generated using the HeatPlus software package from Bioconductor. A principal component analysis was performed using the function ‘pcomp’ in the R package ‘stats’, and the data were shown on a two-dimension plot using the ‘ggplot2’ package from GitHub. A GSEA was performed using the GSEA v2 software (Broad Institute). The genes induced by the cytokine treatment in the CD8+ T cells were analyzed using published gene expression data (GSE58262). Significantly upregulated genes were identified in individual cytokine-treated T cells relative to those in control T cells by unpaired t-test (P < 0.01). STAT3 target genes were extracted according to a previous study analyzing gene expression profiles and chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq)\(^8\). The genes that were significantly downregulated in STAT3-deficient T cells, as compared to those in wild-type T cells (GSE21670; P < 0.05 by unpaired t-test), and had STAT3-binding sites in the promoter regions were extracted.

**Analysis of serum cytokine concentrations.** The serum concentrations of human IL-2, IFN-γ, TNF-α, and IL-6 in NALM-6-bearing mice were measured using an enzyme-linked immunosorbent assay (ELISA). The following kits from R&D Systems were used for quantification of the cytokines: Human IL-2 ELISA Max Deluxe (BioLegend), Human IFN-γ Quantikine ELISA Kit (R&D Systems), Human TNF-α ELISA Max Deluxe (BioLegend) and Human IL-6 Quantikine ELISA Kit (R&D Systems). The results were normalized to the levels of UBC, and the relative expression levels were calculated using the 2^-ΔΔCT method. The average log₂-transformed expression values of all samples was normalized to 0 in each gene. The following primers were used for the real-time PCR experiments: GZMA forward, CCTGTGATTTGGAATGAATGTTG, and reverse, AGGGCTTCCAGAATCTCCAT; GZMB forward, GGCCCC ACAATATCAAAGAA, and reverse, GATGGGCTTTTTTCACAGGGATA; GZMH forward, CCATTCTCTCCTCTGTTGG, and reverse, TGAAACA AGGCCATGTTG; GZMK forward, TTCCTTTTCTTTCAGACCT; GZMB reverse, CGACCGTTCAGACGCTTCA; PRF1 forward, CCTCTCTCCTACGGAGTGCT; and reverse, TCTGTGCTCTCCTCAGAGTGC; and UBC forward, ATTTGGGTCCGCCGTTCCTTG, and reverse, TGCCCTGACATTCTGATGTT.

**Mouse experiments.** In the mouse experiments, male NSG mice that were bred at the Princess Margaret Cancer Centre animal facility were used. The model for leukemia treatment, animals were intravenously injected with 5 million CD19⁺ NALM-6 leukemia cells that had been transduced with PMX-EGFP-fluorescent luciferase (NALM6-GL cells) or 10 million primary CD19⁺ B-ALL cells. Mice were irradiated with 1.5 Gy using X-RAD 320 (Precision X-Ray, Inc.) before the transplantation of the primary ALL cells to enhance engraftment. The CD8⁺ T cells were retrovirally transduced with each anti-CD19-CAR-encoding gene and expanded for 2 weeks. One or two infusions of 5 million CAR-T cells were administered to the mice 14 d after the transplantation of the NALM6-GL cells, and 500,000 CAR-T cells were infused 35 d after transplantation of the primary B-ALL cells. In vivo imaging of the NALM6-GL within the mice was performed with Xenogen IVIS Spectrum and analyzed with Living Image software (Perkin Elmer). The engraftment of the primary B-ALL cells was regularly monitored by analyzing the peripheral blood. Mice were monitored at least once daily and euthanized by CO₂ inhalation after they became moribund due to the leukemia progression or had more than 20% weight loss. In the model for solid tumor treatment, the NSG mice were subcutaneously injected with 5 × 10⁶ A375 melanoma cells that had been transduced with a construct expressing CD19 (at day -21 before T cell infusion) and intravenously infused with 5 × 10⁵ CD19⁺ T cells on days 0 and 4. The volume of the inoculated tumors was monitored every 2–3 d until they reached >300 mm³ or an ulcer had formed in the tumor. In the tumor-free model, NSG mice were irradiated with 1.5 Gy and adoptively transferred with 5 million or 10 million CAR-T cells the next day. The development of xenogeneic GVHD was monitored daily in each experiment. The mice were euthanized when they exhibited one of the following symptoms: more than 20% loss of initial body weight, pronounced lethargy, hunching posture, severe diarrhea or severe dermatitis. The mice were randomly assigned to treatment groups in each experiment. No statistical methods were used to predetermined sample size. The investigators were not blinded to allocation during experiments and outcome assessment.

**Statistical analysis.** Statistically significant differences between two groups were assessed using a two-tailed paired or unpaired t-test. Comparisons between more than two groups were carried out by ANOVA with Tukey's multiple-comparisons test. Significance was defined as P < 0.05. In the mouse experiments, the overall survival and event-free survival of the mice that were treated with the T cells were depicted by a Kaplan–Meier curve, and the survival difference between the groups was compared using the log-rank test. When more than two groups were compared, the P value was adjusted according to the number of comparisons with the Bonferroni method. All statistical analyses were performed using GraphPad Prism 6 software. No statistical method was used to predetermined the sample size.

**Study approval.** This study was performed in accordance with the Helsinki Declaration and approved by the Research Ethics Board of the University Health Network, Toronto, Canada. Written informed consent was obtained from all healthy donors who provided the peripheral blood samples. All animal experiments were approved by the Ontario Cancer Institute–Princess Margaret Cancer Centre Animal Care Committee at the University Health Network and performed in accordance with Canadian Council on Animal Care guidelines.

**Life Sciences Reporting Summary.** Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

**Data availability.** The microarray data has been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE103906.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

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Please do not complete any field with "not applicable" or n/a. Refer to the help text for what text to use if an item is not relevant to your study. For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

Experimental design

1. Sample size
   Describe how sample size was determined.
   Samples sizes were estimated based on preliminary experiments. We did not use a statistical method to predetermine sample size.

2. Data exclusions
   Describe any data exclusions.
   No data were excluded throughout the studies.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.
   The in vitro data were reproduced in multiple (at least three) samples. For in vivo studies to see antitumor effects of CAR-T cells, we repeated experiments twice and obtained similar results.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   For in vivo studies, mice were randomly assigned to treatment groups in each experiment.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   The investigators were not blinded to group allocation during data collection or analysis. All of our data analysis both in vitro and in vivo is based on the objectively measurable data: absolute cell counts, frequency, fluorescence intensity, body weight, tumor size, and survival. Blinding does not affect these data values.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☒   | ☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☐   | ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐   | ☒ A statement indicating how many times each experiment was replicated |
| ☐   | ☒ The statistical test(s) used and whether they are one- or two-sided |
| ☒   | ☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☐   | ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☐   | ☒ Test values indicating whether an effect is present |
| ☒   | ☐ Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted. |
| ☒   | ☐ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☒   | ☐ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation) |

See the web collection on statistics for biologists for further resources and guidance.
# Software

**Policy information about availability of computer code**

7. **Software**

Describe the software used to analyze the data in this study.

- GraphPad Prism 6; Flowjo (version 9.7.6); Image Lab (version 5.2.1); Imagej (version 1.48);
- Affymetrix Expression Console (version 1.4.1); Affymetrix Transcriptome Analysis Console (version 3.1.0.5); R (version 3.1.0); Bioconductor (version 2.14); HeatPlus (version 2.10.0);
- stats (version 3.1.0); ggplot2 (version 2.1.0); GSEA (version 2); Living Image (version 4.3.1).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

# Materials and reagents

**Policy information about availability of materials**

8. **Materials availability**

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

- Artificial antigen presenting cells and CAR constructs will be available upon reasonable request under MTA.

9. **Antibodies**

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The following antibodies were used for the flow cytometry analysis: APC-Cy7-anti-CD4 (clone RPA-T4; BioLegend; #300518; lot number B244839), PE-Cy7-anti-CD4 (RPA-T4; BioLegend; #300512; lot number B159354), PE-Cy7-anti-CD8 (clone SFCI21Thy2D3; Beckman Coulter; #603861; lot number 7617042), PE-anti-CD8 (clone RPA-T8; BioLegend; #301051; lot number 162300), Pacific-Blue-anti-CD8 (clone B9.11; Beckman Coulter; #A82791; lot number 32), PE-anti-CD69 (clone FN50; BioLegend; #310906; lot number B224547), FITC-anti-CD45RA (clone MEM-56; Thermo Fisher Scientific; #MHC45RA01; lot number 795595F), PE-anti-CD62L (clone DREG-56; BioLegend; #304806; lot number B238376), Pacific Blue-anti-CCR7 (clone G043H7; BioLegend; #353210; lot number B224088), APC-Cy7-anti-CD27 (clone O323; BioLegend; #302815; lot number B213295), APC-anti-CD28 (clone CD28.2; BioLegend; #302911; lot number B208224), PerCP/Cy5.5-anti-CD95 (clone DX2; BD Biosciences; #561655; lot number 4290805), Alexa Fluor 488-anti-CD279 (clone EH12.2H7; BioLegend; #329936; lot number B231939), PE-anti-CD274 (clone 29E.2A3; BioLegend; #329706; lot number B236176), APC/Cy7-anti-CD366 (clone F38-2E2; BioLegend; #345026; lot number B234886), PerCP/Cy5.5-anti-CD223 (clone C987W; BioLegend; #125212; lot number B231176), FITC-anti-CD271 (clone ME20.4; BioLegend; #345104; lot number B223717), PerCP/Cy5.5-anti-CD271 (clone ME20.4; BioLegend; #345112; lot number B218745), V450-anti-CD271 (clone C40-1457; BD Biosciences; #562123; lot number 7138758), APC-anti-CD45 (clone H130; BioLegend; #304012; lot number B190802), FITC-anti-CD3 (clone B8B2; BioLegend; #343304; lot number B150785), PE-anti-CD19 (clone HIB19, BioLegend; #302028; lot number B231046), biotin-labeled protein L (GenScript; #M00097; lot number 16F001065), streptavidin-PE (Thermo Fisher Scientific; #S866; lot number 1865801), Alexa Fluor 647-anti-phospho-STAT3 (Tyr705) (clone 7D6; Cell Signaling Technology; #302028; lot number 7129683), Alexa Fluor 647-anti-phospho-STAT5 (Tyr694) (clone 47Stat/5Stat; BD Biosciences; #612599; lot number 7082834), Alexa Fluor 647-anti-phospho-p44/42 MAPK (Erk1/2) (Thermo Fisher Scientific; #S866; lot number 1865801), Alexa Fluor 647-anti-phospho-STAT3 (Tyr705) (clone 4/P-Stat3; BD Biosciences; #557815; lot number 7129683), anti-phospho-STAT5 (clone D3A7, Cell Signaling Technology; #302028; lot number 7108853), PE-anti-IFN-γ (clone 4S.B3; BioLegend; #502909; lot number B224364). All the antibodies are validated for use in flow cytometry. Data are available on manufacturer's website.

The following antibodies were used for immunoblotting: anti-STAT3 (clone D3Z2G, Cell Signaling Technology; #13038; lot number 3), Alexa Fluor 647-anti-rabbit IgG (H+L) (Jackson ImmunoResearch; #711-605-152), FITC-anti-IL-2 (clone 5344.111; BD Biosciences; #340448; lot number 7108853), PE-anti-IFN-γ (clone 4S.B3; BioLegend; #502528; lot number B182253), and PE anti-TNF-α (clone MAb11; BioLegend; #502909; lot number B224364). All the antibodies are validated for use in immunoblotting. Data are available on manufacturer’s website.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.
      aAPC/mOKT3 and K562-CD19: derived from the human erythroleukemic cell line K562 directly obtained from American Type Culture Collection (ATCC)
      NALM-6: directly obtained from DSMZ (Braunschweig, Germany)
      A375 melanoma cell line: directly obtained from ATCC
   b. Describe the method of cell line authentication used.
      Authentication of cells was carried out by short-tandem repeat (STR) analysis.
   c. Report whether the cell lines were tested for mycoplasma contamination.
      All cell lines were routinely assessed for the presence of mycoplasma contamination using a PCR-based technology.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
      None of the cell lines used are listed in the ICLAC.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
    Provide all relevant details on animals and/or animal-derived materials used in the study.
    Eight- to twelve-week old male NOD-scid IL2rgnull (NSG) mice bred at the Princess Margaret Cancer Centre animal facility were used.

Policy information about studies involving human research participants

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.
    Peripheral blood mononuclear cells were obtained from healthy donors at the Princess Margaret Cancer Centre under approval of the Research Ethics Board of the University Health Network, Toronto, Canada. Population characteristics of individual donors are not available to researchers.
    Primary CD19+ B-ALL samples were obtained from newly diagnosed patients at the Princess Margaret Cancer Centre.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Peripheral blood mononuclear cells were obtained from healthy donors and T cells were purified as described in Methods. In mouse experiments, peripheral blood was collected from the tail. Red blood cells were lysed before analysis of the peripheral blood and spleen cells. Subcutaneous tumor cells were ground into a single-cell suspension.

6. Identify the instrument used for data collection. FACSCanto II (BD Biosciences)

7. Describe the software used to collect and analyze the flow cytometry data. FlowJo software (Tree Star)

8. Describe the abundance of the relevant cell populations within post-sort fractions. The purity was verified by flow cytometry analysis.

9. Describe the gating strategy used. The gating strategy for each analysis is provided in Supplementary Figures. After gating on forward scatter (FSC) vs. side scatter (SSC), doublets were excluded by FSC-H vs. FSC-W and SSC-H vs. SSC-W gating. For analysis of spleen cells and subcutaneous tumor cells within the mice, debris was excluded by FSC vs. SSC gating as shown in Supplementary Figures.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.