Supplementary Figure 1, K. Adachi
Supplementary Figure 1. Generation of CAR-T cells producing both IL-7 and CCL19. (a) Schemas of conventional (Conv.) and 7×19 CAR (7×19) gene constructs were shown. (b) Transduction with anti-hCD20 conventional (Conv.) or 7×19 (7×19) CAR construct was conducted by retroviral vectors. The expression levels of CAR were analyzed by flow cytometry. The numbers in histograms represent the percentages of positively stained cells. Similar results were obtained from ten independent experiments. (c) CAR-T cells were co-cultured with mitomycin C-treated P815-hCD20 (red bar) or immobilized anti-CD3 mAb (gray bar) for 3 or 5 days. As negative controls, CAR-T cells were co-cultured with mitomycin C-treated P815 (blue bar) or without any stimulations (white bar). The culture supernatants were examined for concentrations of IL-7 or CCL19 by ELISA. Data represent the mean ± SEM of triplicate wells. Open circle represents the values of individual well. N.D.: not detected. Similar results were obtained from two independent experiments.
**Supplementary Figure 2.** Continuous expansion of 7×19 CAR-T cells in response to the Ag. Conventional (black square) or 7×19 (red circle) anti-hCD20 CAR-T cells were cultured with or without mitomycin C-treated P815-hCD20 as stimulant, which was repeatedly supplemented once a week. The absolute numbers of viable cells were measured. Data represent the mean ± SEM of triplicate wells.
Supplementary Figure 3. Transduction with anti-FITC 7×19 CAR construct induced the generation of CAR-T cells producing both IL-7 and CCL19. Transduction with anti-FITC conventional (Conv.) or 7×19 (7×19) CAR construct was conducted by retroviral vectors. (a) The expression levels of CAR were examined by flow cytometry. The numbers indicate the percentages of positively stained cells. Similar results were obtained from ten independent experiments. (b) CAR-T cells were cultured for 3, 5, or 7 days in the absence (white bar) or presence of either immobilized FITC-conjugated trastuzumab (red bar) or anti-CD3 mAb (gray bar). The concentrations of IL-7 or CCL19 in the supernatants were measured by ELISA. Data represent the mean ± SEM of triplicate wells. Open circle represents the values of individual well. N.D.: not detected. Similar results were obtained from two independent experiments.
Supplementary Figure 4, K. Adachi
**Supplementary Figure 4.** *In vitro* assays with anti-FITC 7×19 CAR-T cells. (a, b) Anti-FITC CAR-T cells were labeled with CytoTell Blue, and stimulated with immobilized FITC-conjugated trastuzumab for the indicated periods. (a) The dilution of CytoTell Blue was analyzed by flow cytometry. The numbers of cell divisions are indicated in the histograms. The numbers in donut charts represent percentages of the each gated fraction in the cultured cells (0; gray, 1; black, 2; green, 3; red, and > 4; blue). Conv. and 7×19 indicate conventional CAR-T cells and 7×19 CAR-T cells, respectively. (b) The absolute number of viable cells (left panel) and viability (right panel) were measured. (c, d) Anti-FITC CAR-T cells were stimulated with immobilized FITC-conjugated trastuzumab (c, d) or anti-CD3 (c) in the lower chambers of the transwell plate. On day 3, T cells (c) or dendritic cells (d) were incubated in the upper chambers for 3 (c, d) or 5 hours (c). The cells migrated from the upper chamber to the lower chamber were enumerated by flow cytometry. (b-d) White and red columns indicate conventional and 7×19 CAR-T cells, respectively. Data represent the mean ± SD of triplicate wells. Open circle represents the values of individual well. *P* values; (b, left) day 3: 0.0054, day 5: 0.0019, day 7: 3.6 × 10⁻⁴. (b, right) day 5: 0.049, day 7: 0.0048. (c) anti-CD3, 3 hours: 0.0024, FITC, 3 hours: 0.028, anti-CD3, 5 hours: 0.0024, FITC, 3 hours: 0.010. (d) 6.0 × 10⁻⁵. *P* values were calculated by two-sided t test. *: *P* < 0.05, **: *P* < 0.01, ***: *P* < 0.005. (e) Cytotoxic potential of conventional (Conv.: circles) and 7×19 (squares) CAR-T cells against P815-hCD20 in the presence of unlabeled rituximab (Ab: open symbols) or FITC-conjugated rituximab (FITC-Ab: filled symbols) at the indicated effectors/targets (E/T) ratios was measured. Data represent the mean ± SD of triplicate wells. (a-e) One representative data from two independent experiments are shown.
Supplementary Figure 5. Expressions of CD127 and CCR7 on naïve T cells, conventional or 7×19 CAR-T cells, DC, P815-hCD20, or 3LL-hCD20. (a) The expression levels of CD127 on naïve spleen T cells, anti-FITC conventional or 7×19 CAR-T cells, P815-hCD20, and 3LL-hCD20 were analyzed by flow cytometry. Naïve T cells were identified as CD3⁺ CD19⁻ population. CAR⁻ and CAR⁺ populations were identified as FITC-conjugated dextran beads-negative and -positive cells, respectively. P815-hCD20 or 3LL-hCD20 were subcutaneously (s.c.) inoculated on DBA/2 or C57BL/6 mice, respectively. The masses of the tumor were resected and enzymatically digested to single cells. Tumor cells were identified as hCD20⁺ population. (b) The expression levels of CCR7 were analyzed on the same populations in (a) as well as DC identified as CD11c⁺ population in spleen cells. (a, b) Red and gray histograms indicate stained and unstained samples, respectively. Excess amount of non-labeled anti-CD16/CD32 Ab was added into the staining reactions so as to prevent non-specific binding of anti-CD127 and anti-CCR7 Abs with Fcγ receptor. The numbers in histograms represent the percentages of positively stained cells. Conv.: T cells containing conventional CAR-T cells. 7×19: T cell containing 7×19 CAR-T cells. Except for the data of the tumor cells, one representative data from two independent experiments are shown. As for the tumor cells, one representative data from three mice are exhibited.
Supplementary Figure 6. Prolongation of the survival of tumor-bearing mice by the treatment with 7×19 CAR-T cells. DBA/2 mice were inoculated s.c. with P815-hCD20 on day 0, and subsequently treated with i.v. injection of 1 × 10^6 conventional or 7×19 anti-hCD20 CAR-T cells on day 3. As a control, the mice were left untreated after the tumor inoculation (no treat.). Median survival time (days) and 95 % confidence interval (CI) were as follows; no treatment: 31.5 (28-35), Conv.: 35 (28-35), 7×19: 42 (38.5-52.5). P values were calculated by two-sided t test. N = 5 mice/group.
Supplementary Figure 7. Dose-escalation experiment with conventional CAR-T cells. DBA/2 mice were inoculated s.c. with P815-hCD20. The mice were treated with i.p. injection of CPA followed by i.v. injection of $1 \times 10^6$, $2 \times 10^6$, or $4 \times 10^6$ conventional anti-hCD20 CAR-T cells. As a control, the mice were left untreated after the tumor inoculation. N = 4 mice/group.
Supplementary Figure 8. Expression levels of immune checkpoint molecules on conventional and 7×19 CAR-T cells. (a) DBA/2 mice were inoculated s.c. with P815-hCD20. The mice were treated with i.p. injection of CPA, and then given i.v. injection of anti-hCD20 7×19 CAR-T cells. One day after CAR-T cell injection, tumor mass was resected and enzymatically digested to single cells. Expression levels of Programmed cell death ligand 1 (PD-L1) on the tumor cells were analyzed by flow cytometry. Tumor cells were identified as hCD20⁺ population. Red and gray histograms indicate stained and unstained samples, respectively. The numbers in histograms represent the percentages of positively stained cells. One representative data from three mice were shown. (b) Conventional (Conv., upper panels) or 7×19 (7×19, lower panels) anti-hCD20 CAR-T cells were co-cultured with P815-hCD20. Expression levels of Programmed cell death 1 (PD-1), Lymphocyte-activation gene 3 (LAG3), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) on the CAR-T cells were analyzed by flow cytometry on day 4. The numbers in dot plots represent the percentages of cells in each quadrant, and those in parentheses represent the percentages of CAR-T cells expressing immune checkpoint molecule in entire CAR-T cells. (a, b) Excess amount of non-labeled anti-CD16/CD32 Ab was added into the staining reactions so as to prevent non-specific binding of Abs with Fcγ receptor. Data shown is derived from single experiment.
Supplementary Figure 9. Improved anti-tumor effects of 2nd generation 7×19 CAR-T cells. (a, b) Inoculation with P815-hCD20 and pretreatment with CPA on DBA/2 mice were conducted as described in Supplementary Figure 7, followed by the injection of 2nd generation conventional or 7×19 anti-hCD20 CAR-T cells. As a control, the mice were left untreated after the tumor inoculation (no treat.). Survival of the mice (a) and the volume of the tumors (b) were assessed. Tumor volumes during the initial 14 days are shown in the insets. 2nd 28 Conv.: the mice treated with CD28-based 2nd generation conventional CAR-T cells. 2nd BB Conv.: the mice treated with 4-1BB-based 2nd generation conventional CAR-T cells. 2nd BB 7×19: the mice treated with 4-1BB-based 2nd generation 7×19 CAR-T cells. (a) Median survival time (days) and 95 % CI were as follows; no treatment: 38.5 (24.5-42), 2nd 28 Conv.: 42 (28-52.5), 2nd BB Conv.: 45.5 (35-not estimated, NE), 2nd 28 7×19: not reached, NR (NE-NE), 2nd BB 7×19: NR (NE-NE). P values calculated by two-sided t test between 7×19 CAR-T cell-treated group and conventional CAR-T cell-treated group are shown. N = 5 mice/group.
Supplementary Figure 10. Anti-tumor effects of 7×19 CAR-T cells in lung tumor model. (a-c) C57BL/6 mice were inoculated s.c. with 3LL-hCD20. (a) The mice were treated with i.p. injection of CPA, and then given i.v. administration of anti-hCD20 CAR-T cells. (b, c) Anti-hCD20 CAR-T cells were injected i.v. without CPA pretreatment. (a-c) As control, the mice were left untreated after the tumor inoculation (no treat.). Survival of the mice (a, b) and the volume of tumors (c) were assessed. Tumor volumes during the initial 14 days are shown in the insets. (a, b) Median survival time (days) and 95 % CI were as follows; (a) no treatment: 56 (24.5-59.5), Conv.: 52.5 (49-56), 7×19: NR (NE-NE). (b) no treatment: 49 (38.5-59.5), Conv.: 38.5 (28-49), 7×19: NR (NE-NE). P values calculated by two-sided t test among the groups are shown. N = 5 mice/group.
Supplementary Figure 11. Anti-tumor effects of 7×19 CAR-T cells targeting endogenous cancer antigen. (a, b) C57BL/6 mice were inoculated s.c. with PAN02. The mice were treated with i.p. injection of CPA, and then given i.v. administration of anti-mesothelin CAR-T cells. As a control, the mice were left untreated after the tumor inoculation (no treat.). Survival of the mice (a) and the volume of the tumors (b) were assessed. Tumor volumes during the initial 14 days are shown in the insets. (a) Median survival time (days) and 95 % CI were as follows; no treatment: 56 (52.5-56), Conv.: 52.5 (38.5-56), 7×19: NR (NE-NE). P values calculated by two-sided t test among the groups are shown. N = 5 mice/group.

Supplementary Figure 11, K. Adachi
Supplementary Figure 12, K. Adachi
Supplementary Figure 12. Generation of CAR-T cells producing either IL-7 or CCL19. (a) Schemas of IL-7-expressing CAR (7 CAR) and CCL19-expressing CAR (19 CAR) constructs were shown. (b) The transduction into T cells with anti-hCD20 conventional (Conv.), 7×19, 7, or 19 CAR constructs were conducted by retroviral vectors. The expression levels of CAR were analyzed by flow cytometry. The numbers in histograms represent the percentages of positively stained cells. Similar results were obtained from more than five independent experiments. (c) CAR-T cells were co-cultured with mitomycin C-treated P815-hCD20 (red bar) or immobilized anti-CD3 mAb (gray bar). As controls, CAR-T cells were co-cultured with mitomycin C-treated P815 (blue bar) or without any stimulations (white bar). The culture supernatants were examined for concentrations of IL-7 or CCL19 by ELISA. Data represent the mean ± SEM of triplicate wells. Open circle represents the values of individual well. Asterisks represent the samples under the detection levels. Similar results were obtained from two independent experiments. (d) Inoculation with P815-hCD20 and pretreatment with CPA on DBA/2 mice were conducted as described in Supplementary Figure 7. The mice were given i.v. administration of $1 \times 10^6$ Conv., 7×19, 7, 19 anti-hCD20 CAR-T cells or a mixture of $0.5 \times 10^6$ each of 7 and 19 anti-hCD20 CAR-T cells (7 + 19). As a control, the mice were left untreated after the tumor inoculation (no treat.). Survival of the mice was assessed. Median survival time (days) and 95 % CI were as follows; no treatment: 35 (17.5-49), Conv.: 40.25 (35-49), 7 CAR: 45.5 (42-52.5), 19 CAR: 38.5 (35-42), 7×19: NR (NE-NE), 7+19: 45.5 (42-NE). $P$ values calculated by two-sided t test among the groups are shown. N = 5 mice/group (no treat., 7×19, 7 CAR, 7+19) or 4 mice/group (Conv., 19 CAR).
Supplementary Figure 13. Requirement of concomitant signaling of IL-7 and CCL19 for the anti-tumor effects of 7×19 CAR-T cells. (a) Inoculation with P815-hCD20 and pretreatment with CPA on DBA/2 mice were conducted as described in Supplementary Figure 7. The mice were given i.v. administration of Conv. or 7×19 anti-hCD20 CAR-T cell. A cohort of the mice treated with 7×19 CAR-T cells were further treated with i.p. injection of anti-CD127 Ab twice a week. As a control, the mice were left untreated after the tumor inoculation (no treat.). Survival of the mice was assessed. Median survival time (days) and 95 % CI were as follows; no treat.: 38.5 (28-42), Conv.: 56 (38.5-59.5), 7×19: NR (NE-NE), 7×19 + anti-CD127: 45.5 (42-49). P values calculated by two-sided t test among the groups are shown. N = 5 mice/group (Conv., 7×19, 7×19 + anti-CD127) or 4 mice/group (no treat.). (b) Inoculation of 3LL-hCD20 and pretreatment with CPA on C57BL/6 mice were conducted as described in Supplementary Figure 10a. Anti-hCD20 7×19 CAR-T cells were generated from T cells of wild-type (WT) or CCR7-deficient (CCR7 KO) mice, and were injected i.v. into the mice. The mean ± SD of tumor volume on day 17 in each group is shown. Open circle represents the values of individual mice. P value shown in the graph was calculated by two-sided t test. N = 5 mice/group.
Supplementary Figure 14. Infiltration of both T cells and DC into the tumor tissues by the treatment with 7×19 CAR-T cells. The areas positive for colors of each fluorescent dye in Figure 3b or 3c were calculated by Hybrid Cell Count program (KEYENCE). Co-localization of T cells and DC in (b) indicates the area positive for both red and green (i.e. detected as yellow) in Figure 3c. no treat.: untreated group. Conv.: mice treated with conventional CAR-T cells. 7×19: mice treated with 7×19 CAR-T cells.
Supplementary Figure 15, K. Adachi
Supplementary Figure 15. Accumulation of 7x19 CAR-T cells in tumor tissue but not in normal organs. (a, b) C57BL/6 mice were inoculated s.c. with 3LL-hCD20, and given i.v. injection of conventional or 7x19 anti-hCD20 CAR-T cells, or control non-transduced T cells without CPA treatment. The injected cells were prepared from congenic C57BL/6 mice showing CD90.1⁺, CD90.2⁻, and luciferase⁺ phenotype. Survival of the mice (a) and the volume of the tumors (b) were assessed. (b) Tumor volumes during the initial 14 days are shown in the insets. The mice treated with non-transduced activated T cells are shown as Non-transduced. Conv. indicates the mice treated with conventional CAR-T cells. 7x19 indicates the mice treated with 7x19 CAR-T cells. (a) Median survival time (days) and 95% CI were as follows; no treatment: 49 (28-59.5), Conv.: 56 (45.5-63), 7x19: NR (NE-NE). P values calculated by two-sided t test among the groups are shown. N = 5 mice/group. (c) C57BL/6 mice were inoculated s.c. with 3LL-hCD20 on day 0, followed by i.p. injection of CPA. Then, conventional or 7x19 CAR-T cells, or control non-transduced T cells generated from the luciferase⁺ congenic mice were injected i.v. into the mice. In vivo bioluminescence imaging analysis to detect transferred donor T cells was conducted on day 18 after the tumor inoculation. Similar results were obtained from two independent experiments.
Supplementary Figure 16. Infiltration of host-derived endogenous T cells as well as transferred donor T cells in the tumor tissue of the mice treated with 7×19 CAR-T cells. (a) C57BL/6 mice were inoculated s.c. with 3LL-hCD20. The mice were treated with i.p. injection of CPA, and then given i.v. injection of conventional or 7×19 anti-hCD20 CAR-T cells, or non-transduced T cells. The injected cells were derived from the CD90.1\(^+\), CD90.2\(^-\) congenic C57BL/6 mice. On day 19, tumor tissues were resected from the mice, and were stained for CD90.1 (green) and CD3 (red). Microscopic examination was conducted at × 400 magnification. Similar results were obtained from two independent experiments. (b) C57BL/6 mice were inoculated s.c. with 3LL-hCD20 and given i.v. injection of conventional or 7×19 anti-hCD20 CAR-T cells, or non-transduced T cells generated from the CD90.1\(^+\) CD90.2\(^-\) congenic mice without CPA treatment. A cohort of the mice treated with 7×19 CAR-T cells were further treated with i.p. injection of anti-CD90.2 Ab twice a week. The mean ± SD of tumor volume on day 14 in each group is shown. Open circle represents the values of individual mice. N = 5 mice/group. *: All mice exhibited complete regression, resulting in zero value of tumor volume.
Supplementary Figure 17, K. Adachi
Supplementary Figure 17. Development of memory phenotype in donor CAR-T cells and endogenous T cells by the treatment with 7×19 CAR-T cells. C57BL/6 mice were inoculated s.c. with 3LL-hCD20 and given i.v. injection of conventional or 7×19 anti-hCD20 CAR-T cells generated from the CD90.1+, CD90.2− congenic mice. On day 28, spleen cells were harvested and used for following assays. (a) The recipient and donor T cells were identified as CD90.2+ and CD90.1+ cells, respectively. The expression of memory T cell markers (CD44 and CD62L) and CAR on CD4+ and CD8+ T cells were analyzed by flow cytometry. The numbers in dot plots or histograms indicate the percentages of cells in each gate. (b, c) The spleen cells were co-cultured with mitomycin C-treated 3LL-hCD20 (b) or the parental 3LL tumor (c). The production of IFN-γ was examined by intracellular cytokine staining. (b) The numbers in histograms indicate the percentages of IFN-γ-positive cells in CD90.1+ donor T cells. (c) The cells positive for IFN-γ in CD90.2+ endogenous CD8+ T cells were detected by flow cytometry. The numbers in dot plots indicate the percentages of cells in each quadrant. Conv.: mice treated with conventional CAR-T cells. 7×19: mice treated with 7×19 CAR-T cells. (a-c) Similar results were obtained from two independent experiments.
Supplementary Figure 18, K. Adachi
**Supplementary Figure 18.** The diversity of TCR repertoire in T cells before and after the treatment with 7×19 CAR-T cells. Inoculation with P815-hCD20, pretreatment with CPA, and administration of anti-hCD20 CAR-T cells were conducted as described in *Supplementary Figure 7*. One hundred forty days after tumor inoculation, spleen cells were harvested from the tumor-rejected mice, and the CAR-positive and CAR-negative T cells were expanded by *in vitro* stimulation with P815-hCD20 and parental hCD20-negative P815. CD8⁺ CAR⁺ or CD8⁺ CAR⁻ populations were sorted by flow cytometer for TCR repertoire analysis. As control, the same populations were sorted from the T cells transduced with anti-hCD20 7×19 CAR, prior to injection into mice. TCR repertoire was analyzed by the next generation sequencer, and the frequency of V and J region usage in α and β chains were displayed as 3-D graphs. The numbers in the graphs represent the diversity index calculated as 1 - Pielou's evenness (a higher number indicates a less diversity). N = 2 samples /group. Representative graphs are shown, and the diversity indices are expressed as the average of the two.
Supplementary Figure 19. Tumor-specific memory responses in the mice treated with 7×19 CAR-T cells. (a) Inoculation with P815-hCD20, pretreatment with CPA on DBA/2 mice, and administration of anti-hCD20 CAR-T cells were conducted as described in Supplementary Figure 7. One hundred forty days after tumor inoculation, the tumor-rejected mice were re-challenged with P815-hCD20 and the parental P815 on the left and right flanks, respectively. (b) C57BL/6 mice were inoculated s.c. with 3LL-hCD20 and given i.v. injection of anti-hCD20 7×19 CAR-T cells without CPA treatment. One hundred twenty days after tumor inoculation, the tumor-rejected mice were re-challenged with 3LL-hCD20 and the parental 3LL on the left and right flanks, respectively. (a, b) As control, naïve mice were inoculated with the tumors in the same fashion. The volumes of the tumors were assessed.

Supplementary Figure 19, K. Adachi
Supplementary Figure 20. Serum levels of IL-7 and CCL19 in the mice treated with 7×19 CAR-T cells. C57BL/6 mice were inoculated s.c. with 3LL-hCD20. The mice were treated with i.p. injection of CPA, and then given i.v. injection of conventional or 7×19 anti-hCD20 CAR-T cells. The sera were collected on the indicated days and examined for the concentrations of IL-7 or CCL19 by ELISA. Data represent the mean + SD. Sample sizes (N) were as follows; Pre-treatment: 15 mice. Non-treated, day 6: 4 mice, day 13 and 21: 3 mice. Conv., all time points: 5 mice. 7×19, day 6 and 13: 5 mice, day 21: 4 mice. Asterisks represent the samples under the detection levels.
**Supplementary Figure 21.** Undetectable oncogenic changes of immune cells in the mice treated with 7x19 CAR-T cells. (a) C57BL/6 mice were inoculated s.c. with 3LL-hCD20 and given i.v. injection of luciferase-positive 7x19 anti-hCD20 CAR-T cells. *In vivo* bioluminescence images of the mice in dorsal and ventral positions were examined on day 75 and 76, respectively. Data shown is derived from single experiment. (b) Inoculation with P815-hCD20, pretreatment with CPA on DBA/2 mice, and administration of anti-hCD20 CAR-T cells were conducted as described in *Supplementary Figure 7*. On day 110, spleen cells were harvested from the tumor-rejected mice and cultured without any stimulants. As control, spleen cells harvested from naïve mice were cultured in the same fashion. The absolute numbers of viable cells were measured by using trypan blue at the indicated time points from three mice per group (red: tumor-rejected mice, black: naïve mice). Data are shown as mean ± SEM of triplicate wells per mouse.