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Supplementary Materials for

Single-cell antigen-specific landscape of CAR T infusion product identifies determinants of CD19-positive relapse in patients with ALL

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The PDF file includes:

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Figs. S1 to S19

Other Supplementary Material for this manuscript includes the following:

Table S2

Supplementary Table 1. Patient demographics and response documentation of discovery cohort

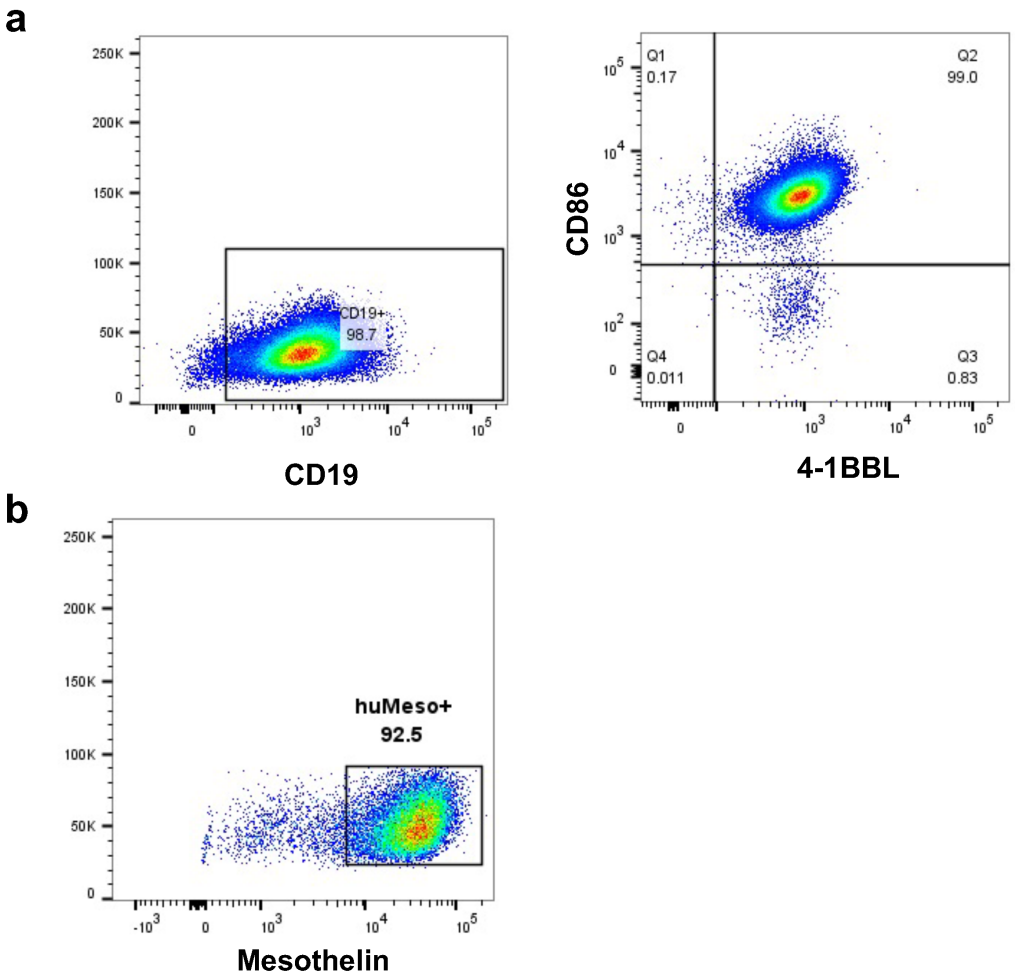
ID	Infusion date	Age	Sex	Response to CART19	B cell aplasia (BCA)	Relapse	Time to relapse (days)	Relapse type	Last contact days
CHP112	4/16/13	9.6	F	MRD neg CR	Yes	No	N/A	N/A	2171
CHP139	6/11/14	15.2	F	MRD neg CR	Yes	Yes	1807	CD19 negative	2000
CHP151	9/9/14	15.5	F	MRD neg CR	Yes	No	N/A	N/A	1833
CHP158	2/25/15	4.9	F	MRD neg CR	Yes	No	N/A	N/A	1696
CHP165	6/23/15	14.5	M	MRD neg CR	Yes	No	N/A	N/A	1637
CHP157	2/10/15	12.3	M	MRD neg CR	Yes	Yes	632	CD19 positive	1135
CHP161	4/7/15	9.7	F	MRD neg CR	Yes	Yes	287	CD19 positive	602
CHP171	9/8/15	9.8	M	MRD neg CR	Yes	Yes	595	CD19 positive	944
CHP110	3/19/13	10.0	F	MRD neg CR	Yes	Yes	79	CD19 positive	221
CHP111	4/1/13	9.9	F	MRD positive	Yes	Yes	44	CD19 positive	69
CHP117	5/9/13	16.2	M	No response	N/A	N/A	N/A	N/A	79
CHP167	7/9/15	21.5	M	No response	N/A	N/A	N/A	N/A	31

Supplementary Table 2. Differentially expressed genes between proliferating, partially active, and fully active cell populations across the 4 experimental conditions. **Attached as separate files**.

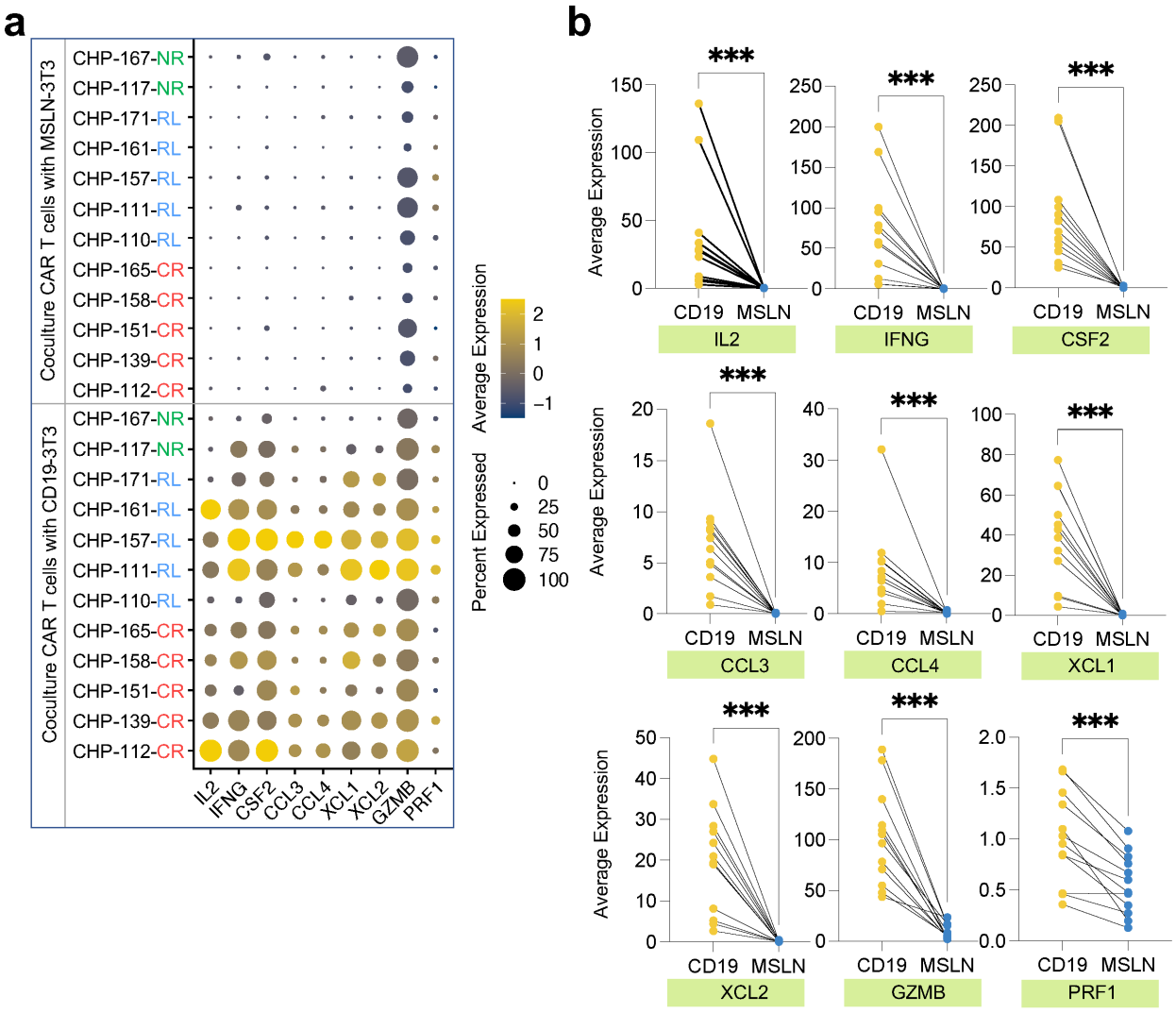
Supplementary Table 3. Patient demographics and response documentation of validation cohort

ID	Infusion date	Age	Response to CART19	B cell aplasia (BCA)	Relapse	Time to relapse (days)	Relapse type	Last contact days
CHP959-112	4/16/13	9.6	MRD neg CR	Yes	No	N/A	N/A	2171
CHP959-115	5/21/13	15.0	MRD neg CR	Yes	No	N/A	N/A	2395
CHP959-118	7/16/13	9.3	MRD neg CR	Yes	No	N/A	N/A	2134
CHP959-145	7/22/14	11.1	MRD neg CR	Yes	No	N/A	N/A	1828
CHP959-154	2/17/15	16.2	MRD neg CR	Yes	No	N/A	N/A	1764
CHP959-158	2/25/15	4.9	MRD neg CR	Yes	No	N/A	N/A	1696
CHP959-165	6/23/15	14.5	MRD neg CR	Yes	No	N/A	N/A	1637
16CT022-02	10/18/16	13.7	MRD neg CR	Yes	No	N/A	N/A	1098
16CT022-04	11/15/16	20.4	MRD neg CR	Yes	No	N/A	N/A	1072
16CT022-06	11/22/16	6.0	MRD neg CR	Yes	No	N/A	N/A	1105
16CT022-08	12/13/16	20.3	MRD neg CR	Yes	No	N/A	N/A	906
16CT022-09	12/20/16	8.5	MRD neg CR	Yes	No	N/A	N/A	902
16CT022-10	12/6/16	17.5	MRD neg CR	Yes	No	N/A	N/A	1029
16CT022-11	12/6/16	22.3	MRD neg CR	Yes	No	N/A	N/A	1094
16CT022-12	1/3/17	11.3	MRD neg CR	Yes	No	N/A	N/A	910
16CT022-13	12/27/16	12.6	MRD neg CR	Yes	No	N/A	N/A	994
16CT022-14	12/27/16	10.1	MRD neg CR	Yes	No	N/A	N/A	1091
16CT022-20	1/24/17	29.1	MRD neg CR	Yes	No	N/A	N/A	913
16CT022-27	4/11/17	13.3	MRD neg CR	Yes	No	N/A	N/A	932
16CT022-30	4/18/17	11.1	MRD neg CR	Yes	No	N/A	N/A	916
16CT022-32	3/28/17	19.1	MRD neg CR	Yes	No	N/A	N/A	986
16CT022-36	6/6/17	3.7	MRD neg CR	Yes	No	N/A	N/A	916
16CT022-38	6/27/17	8.7	MRD neg CR	Yes	No	N/A	N/A	741
16CT022-39	7/25/17	2.8	MRD neg CR	Yes	No	N/A	N/A	737
16CT022-40	7/18/17	14.8	MRD neg CR	Yes	No	N/A	N/A	755
16CT022-41	8/1/17	13.3	MRD neg CR	Yes	No	N/A	N/A	724
CHP959-104	1/8/13	6.1	MRD neg CR	Yes	Yes	184	CD19 positive	1148
CHP959-105	1/29/13	7.8	MRD neg CR	Yes	Yes	244	CD19 positive	952
CHP959-110	3/19/13	10.0	MRD neg CR	Yes	Yes	79	CD19 positive	221
CHP959-111	4/1/13	9.9	MRD neg CR	Yes	Yes	44	CD19 positive	69
CHP959-124	9/17/13	9.2	MRD neg CR	Yes	Yes	185	CD19 positive	617

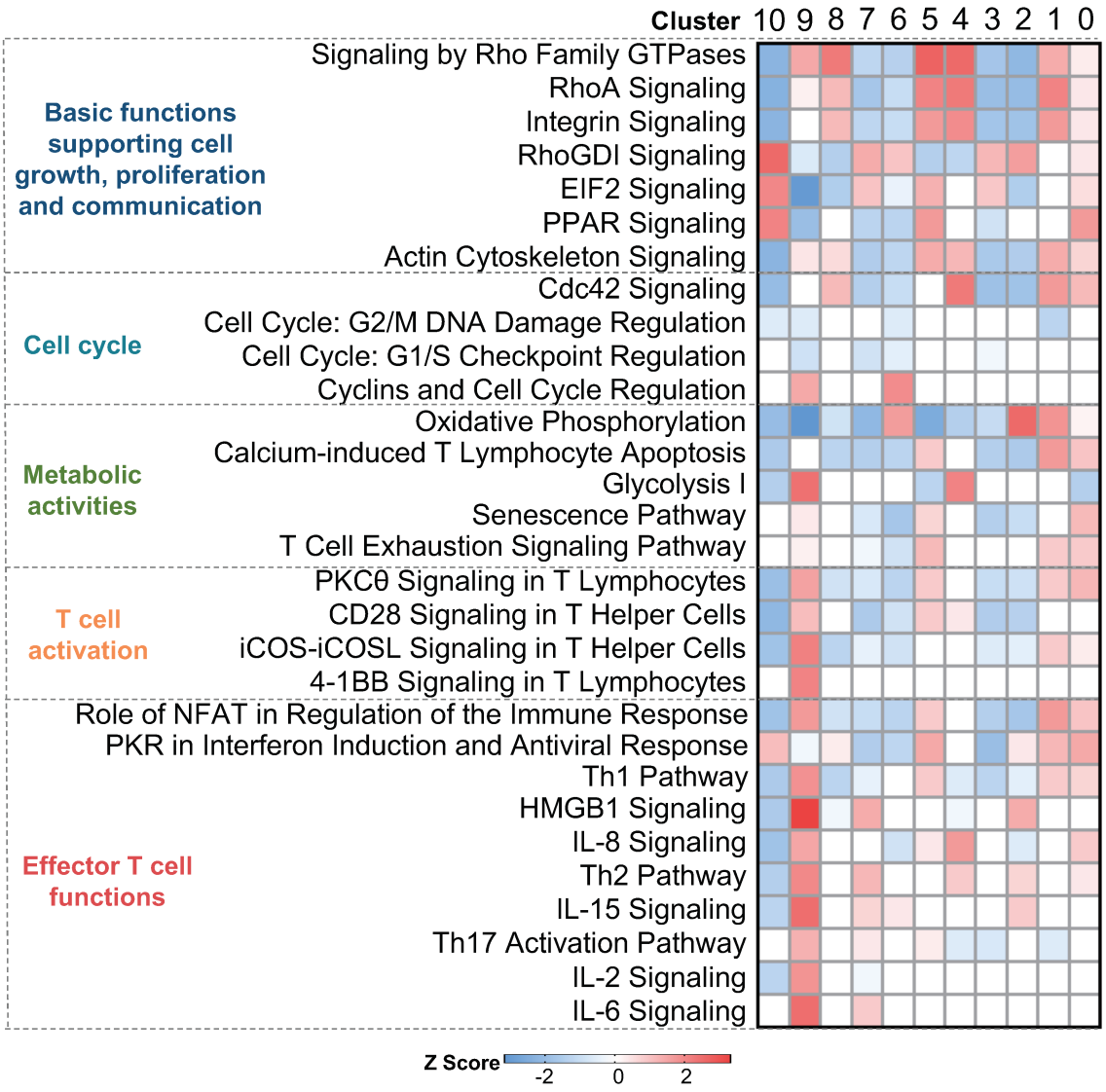
CHP959-144	8/5/14	15.2	MRD neg CR	Yes	Yes	289	CD19 positive	1928
CHP959-150	9/30/14	17.3	MRD neg CR	Yes	Yes	272	CD19 positive	1855
CHP959-152	10/28/14	9.9	MRD neg CR	Yes	Yes	1367	CD19 positive	1581
CHP959-153	12/2/14	15.8	MRD neg CR	Yes	Yes	510	CD19 positive	534
CHP959-157	2/10/15	12.3	MRD neg CR	Yes	Yes	632	CD19 positive	1135
CHP959-161	4/7/15	9.7	MRD neg CR	Yes	Yes	287	CD19 positive	602
CHP959-163	5/5/15	13.3	MRD neg CR	Yes	Yes	757	CD19 positive	892
CHP959-169	7/28/15	1.7	MRD neg CR	Yes	Yes	104	CD19 positive	859
CHP959-171	9/8/15	9.8	MRD neg CR	Yes	Yes	595	CD19 positive	944
CHP959-172	9/22/15	13.3	MRD neg CR	Yes	Yes	518	CD19 positive	1456
16CT022-18	1/10/17	15.3	MRD neg CR	Yes	Yes	230	CD19 positive	505
16CT022-29	3/14/17	8.3	MRD neg CR	Yes	Yes	372	CD19 positive	980
16CT022-37	6/6/17	13.7	MRD neg CR	Yes	Yes	156	CD19 positive	176
16CT022-51	10/3/17	12.4	MRD neg CR	Yes	Yes	279	CD19 positive	723
16CT022-52	10/10/17	23.0	MRD neg CR	Yes	Yes	668	CD19 positive	783
16CT022-55	9/26/17	5.0	MRD neg CR	Yes	Yes	428	CD19 positive	769
16CT022-69	2/13/18	6.5	MRD neg CR	Yes	Yes	146	CD19 positive	216
16CT022-77	2/5/19	11.7	MRD neg CR	Yes	Yes	272	CD19 positive	319



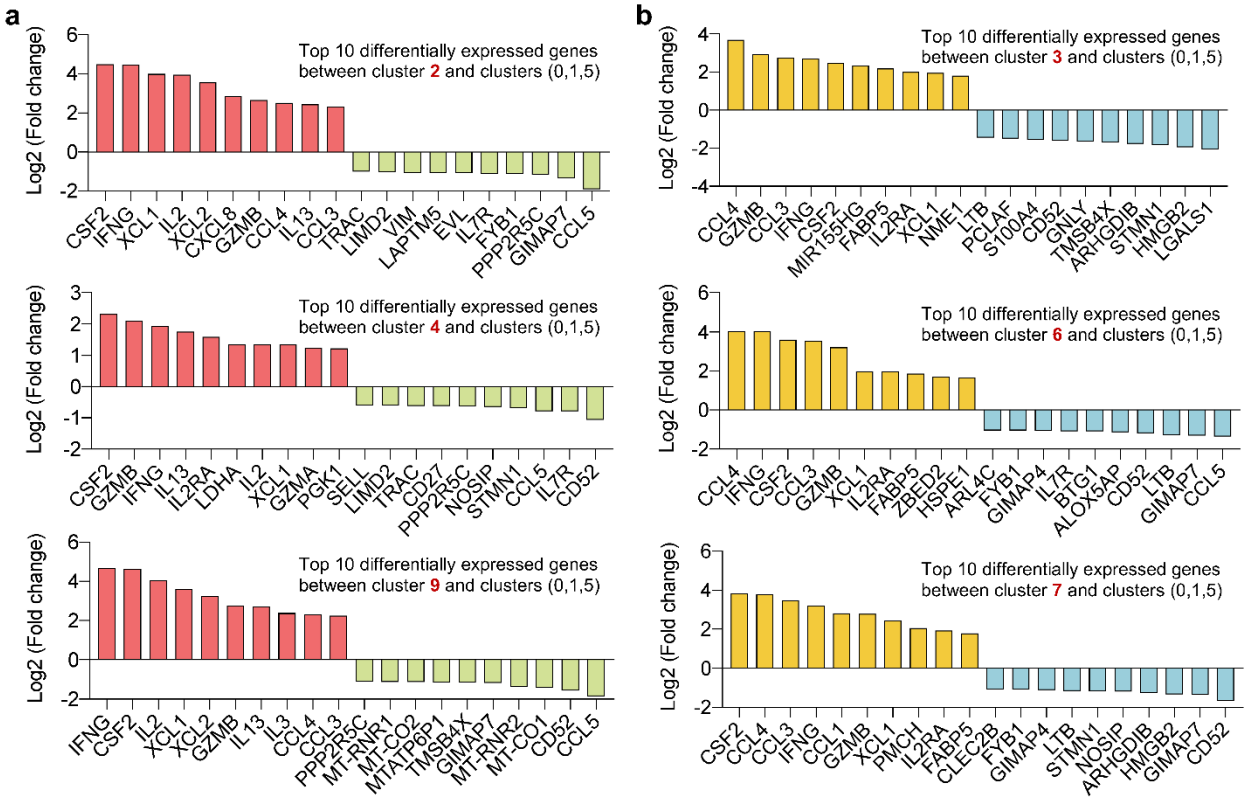
Supplementary Figure 1. Flow cytometry evaluation of engineered 3T3 cells. (**a, b**) Representative flow cytometry plots showing stable expression of human CD19, CD86, 4-1BB ligand (**a**) and mesothelin (**b**) in 3T3 cells.



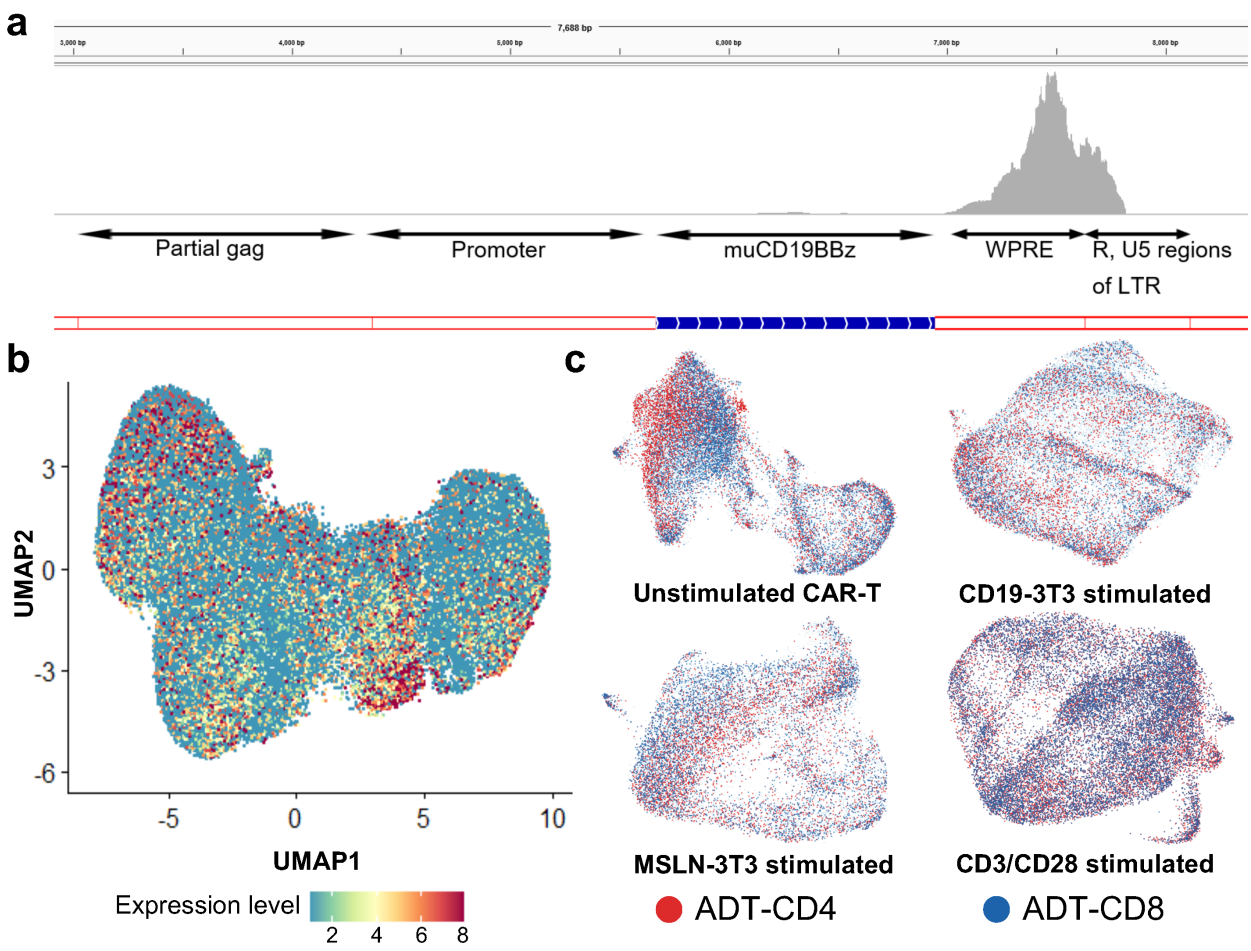
Supplementary Figure 2. Comparison of functional gene expression between CD19-3T3 and MSLN-3T3 (control) conditions. (**a**) Dotplot of T cell functional gene expression of each patient in CD19-3T3 and MSLN-3T3 coculture conditions. The size of circle represents proportion of single cells expressing the gene, and the color shade indicates normalized expression level. (**b**) The average expression level of functional genes across all single cells in each patient and their comparison between CD19-3T3 and MSLN-3T3 conditions. The *P* values were calculated with Mann-Whitney test. *** P <0.001.



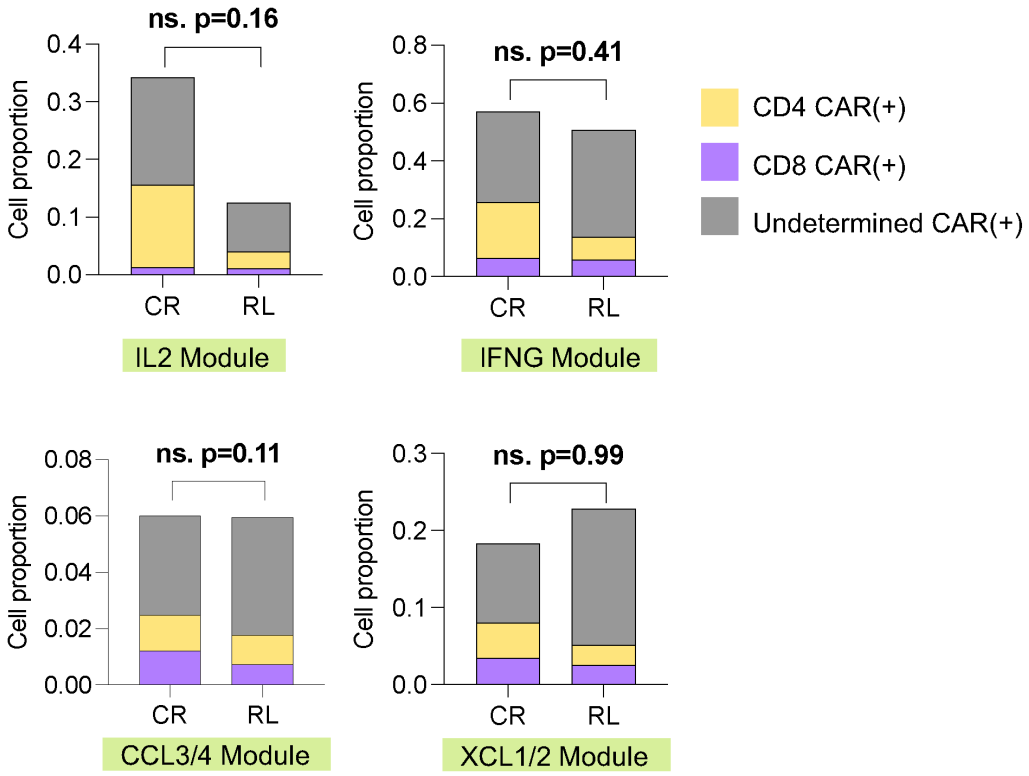
Supplementary Figure 3. Canonical signaling pathways of each cluster identified in the global clustering analysis of all the CAR T cells profiled in our project. Differentially expressed genes of each cluster are used to identify the biological pathways. A statistical quantity, called z score, is computed and used to characterize the activation level. z score reflects the predicted activation level ($z < 0$, inhibited; $z > 0$, activated; $z \geq 2$ or $z \leq -2$ can be considered significant).



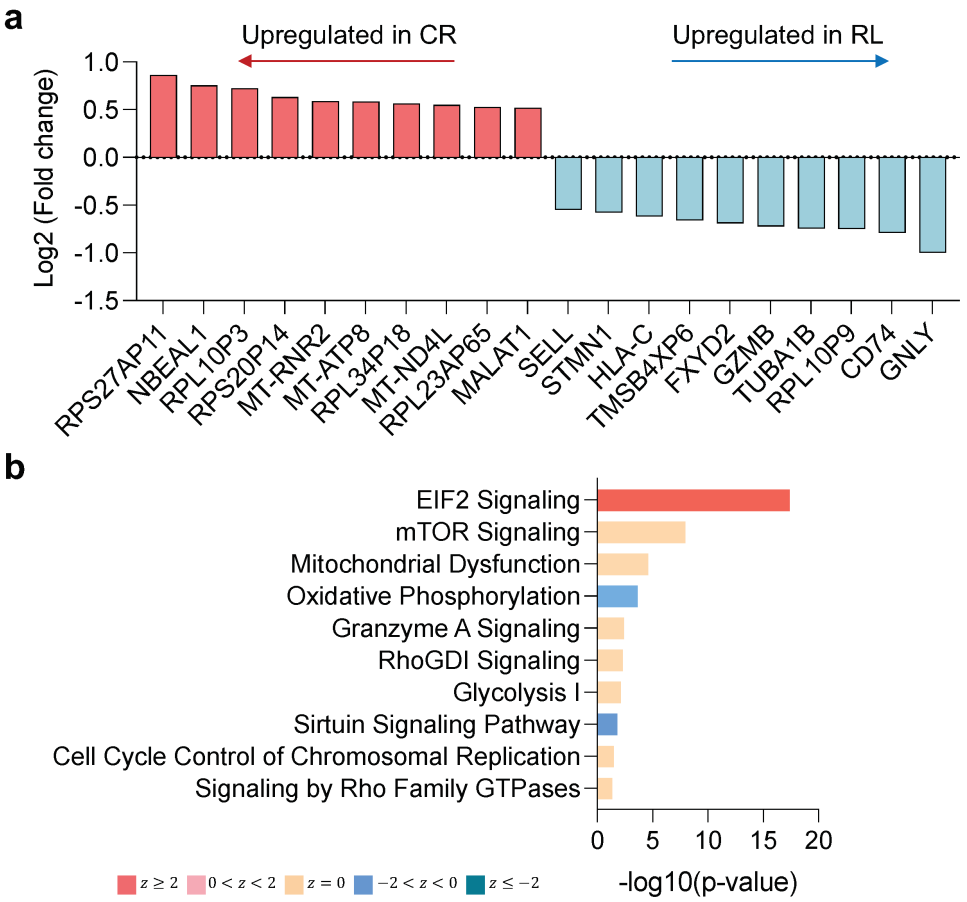
Supplementary Figure 4. Differentially expressed gene analysis of stimulation condition-related clusters. (**a**) Top 10 differentially expressed genes between clusters enriched in CD19-3T3 stimulated CAR T cells vs. MSLN-3T3 stimulated cells (**b**) Top 10 differentially expressed genes between clusters enriched in anti-CD3/CD28 beads stimulated CAR T cells vs. unstimulated cells.



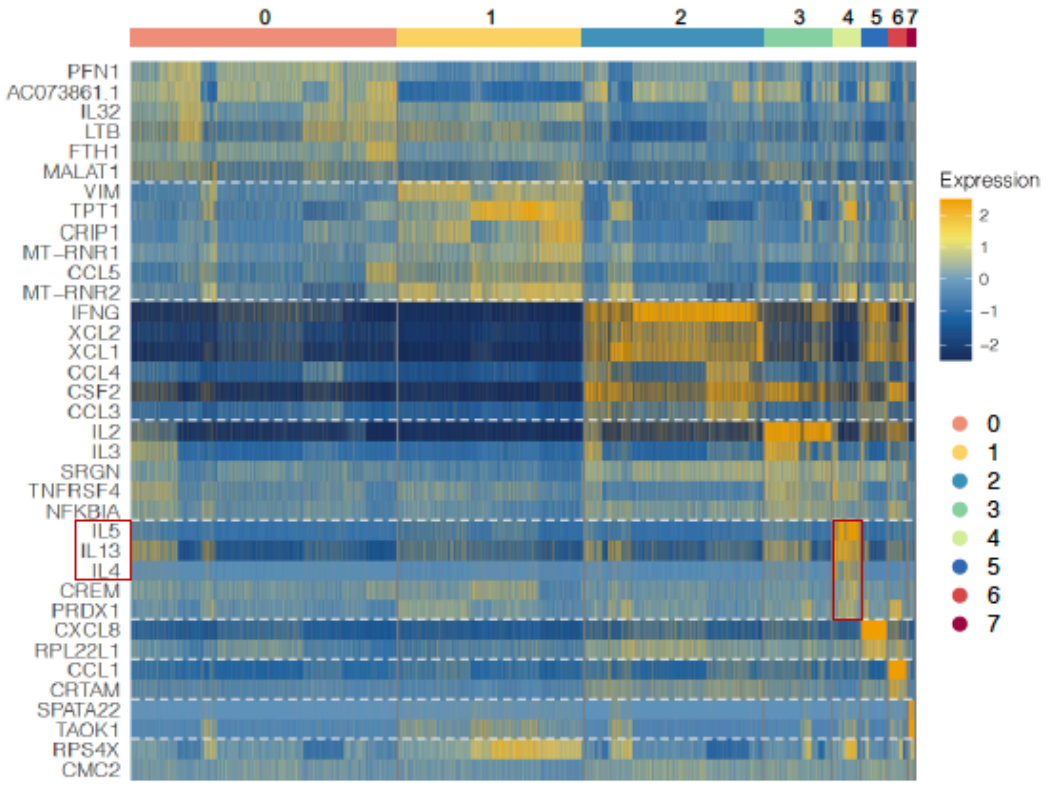
Supplementary Figure 5. Detection of CAR structure, CAR gene expression and CD4/CD8 cellular molecule expression. (**a**) Identify the expression of the lentiviral vector elements in the CAR construct. (**b**) The distribution of CAR gene expression among all profiled single cells. (**c**) ADT-CD4 and ADT-CD8 expression determined by CITE-seq data in four stimulation conditions.



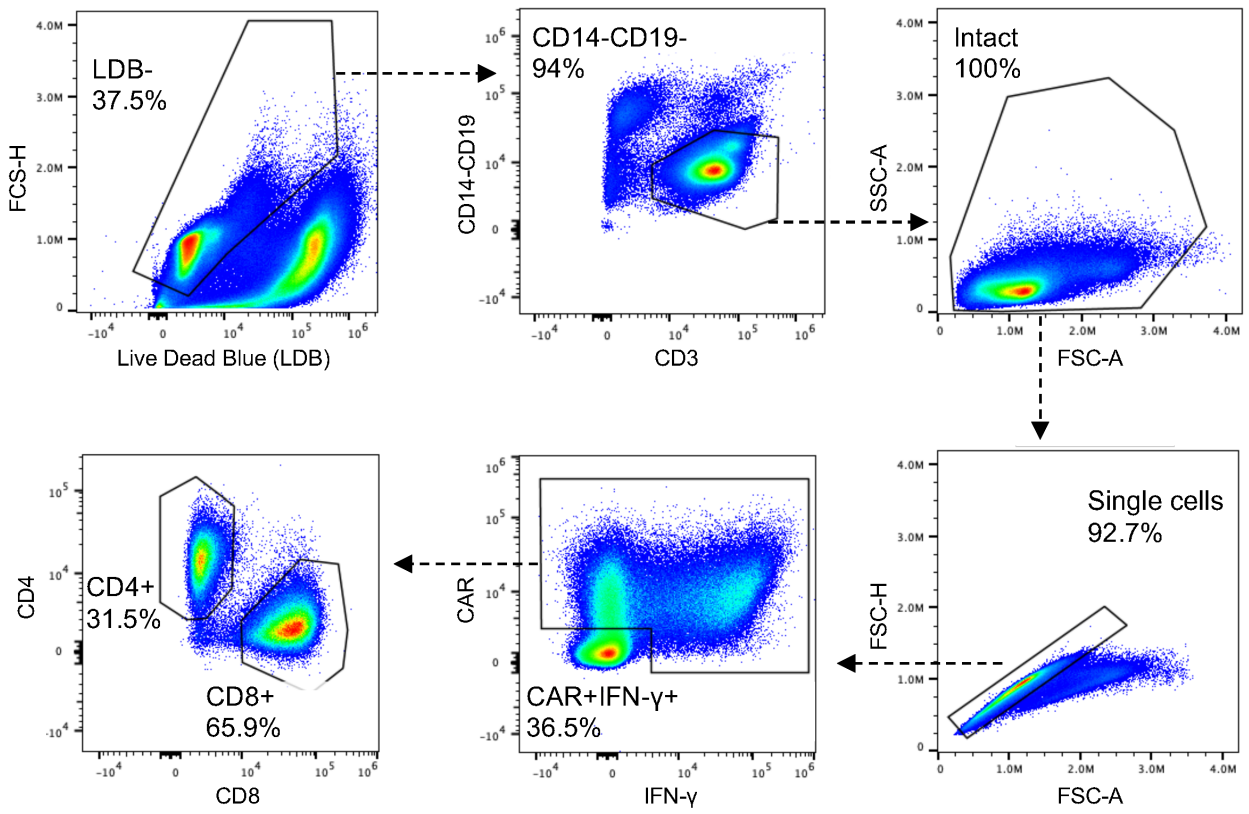
Supplementary Figure 6. Comparison of identified modules (except for Th2 module) between CR and RL patients. The *P* values were calculated with Mann-Whitney test. ns. Not significant.



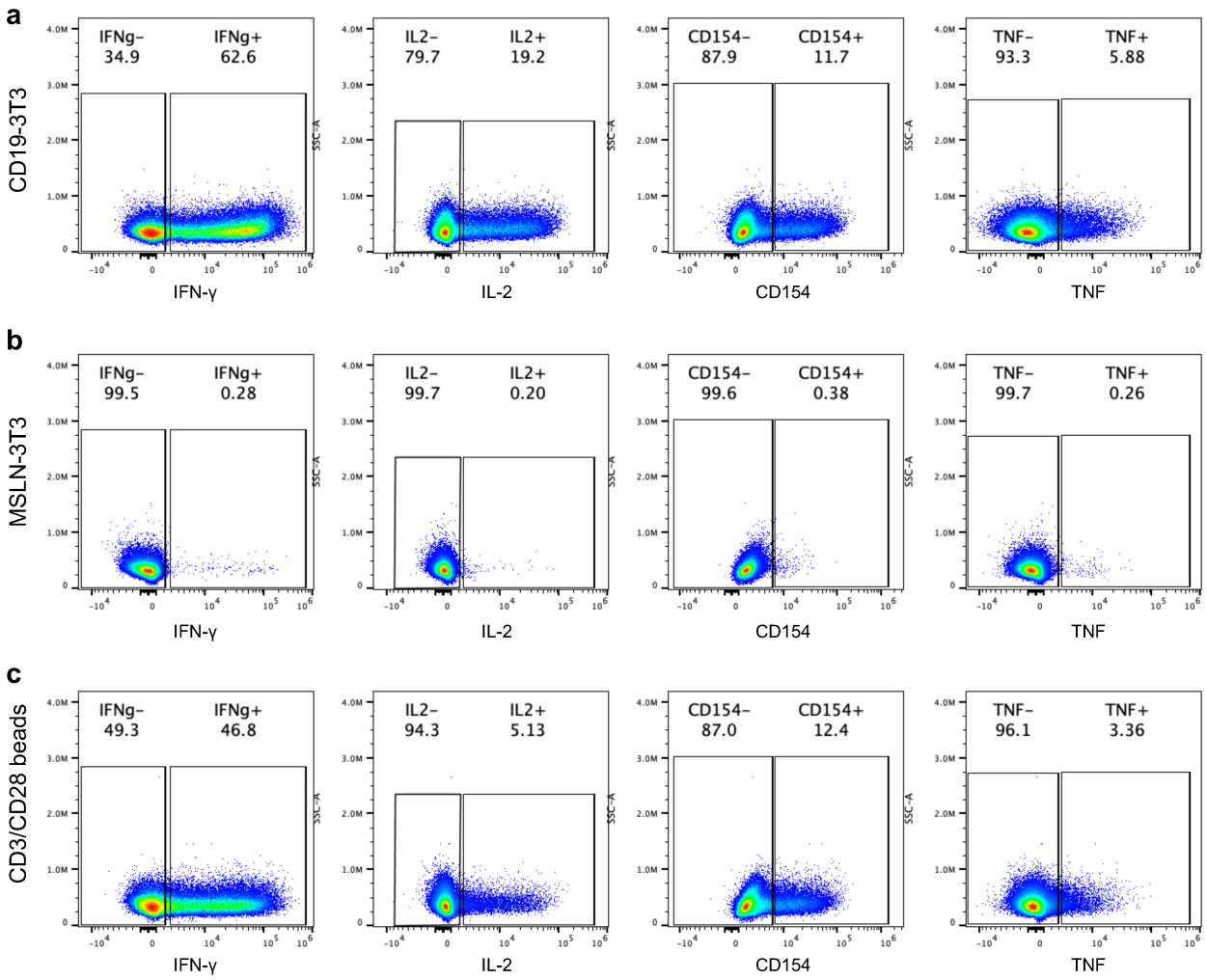
Supplementary Figure 7. Differentially expressed genes between unstimulated baseline CAR T cells from CR and RL patients and the corresponding pathways. (**a**) Top 10 DEGs upregulated in CR or RL group. (**b**) Corresponding canonical pathways regulated by the highly differential genes identified in (**a**). z score reflects the predicted activation level ($z < 0$, inhibited; $z > 0$, activated; $z \geq 2$ or $z \leq -2$ can be considered significant).



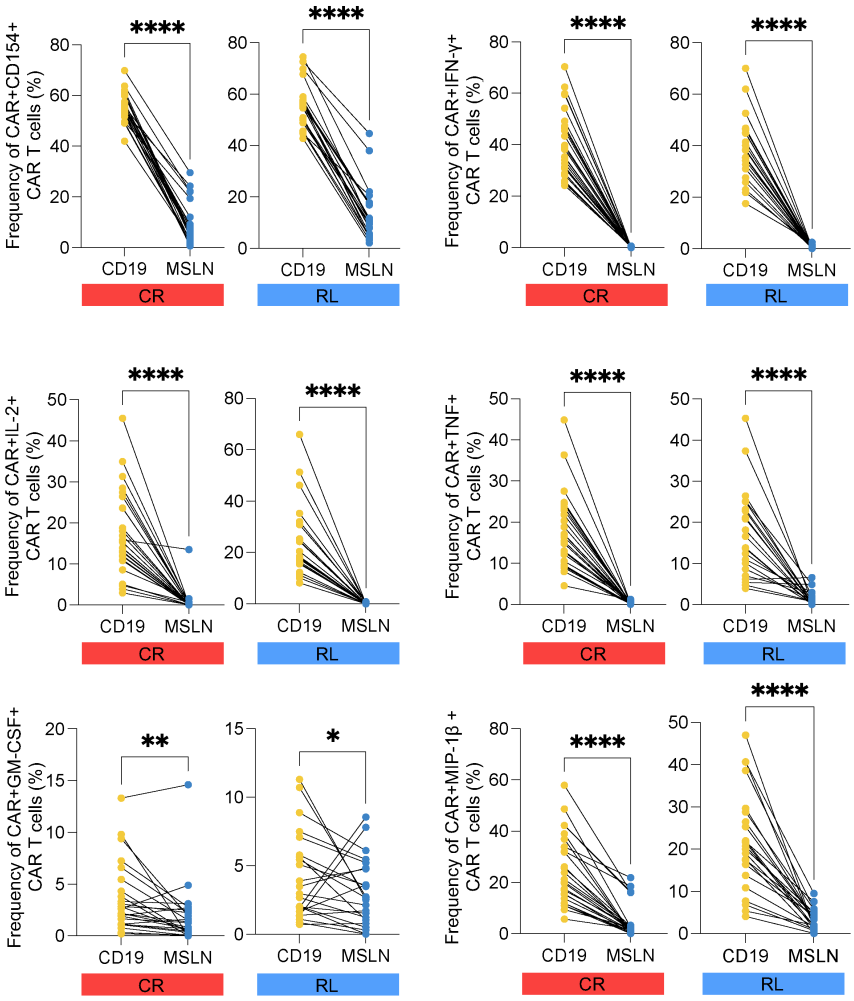
Supplementary Figure 8. Differentially expressed genes of each cluster identified in isolated CAR+ cells from CR and RL patients. Heatmap of top 6 DEGs of each cluster was shown, among which the highly expression of Th2-related genes *IL13*, *IL5* and *IL4* in cluster 4 was indicated.



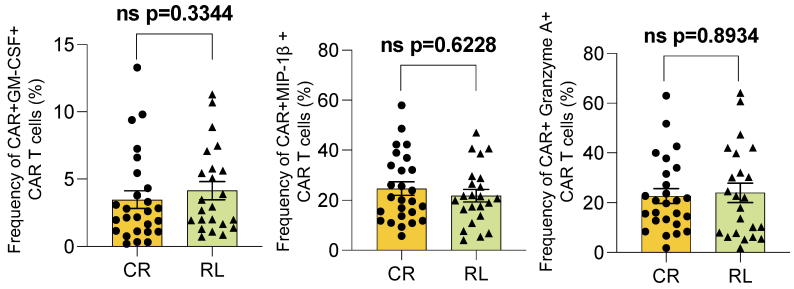
Supplementary Figure 9. Gating strategy of flow cytometry data analysis. The Live Dead Blue (LDB) was used to select live cells, followed by CD14-CD19−/CD3+ subset, intact and single cell selection. CAR+IFN-γ+ were applied to select fully activated CAR+ CAR T cells, and CD4+ or CD8+ subpopulation was analyzed separately.



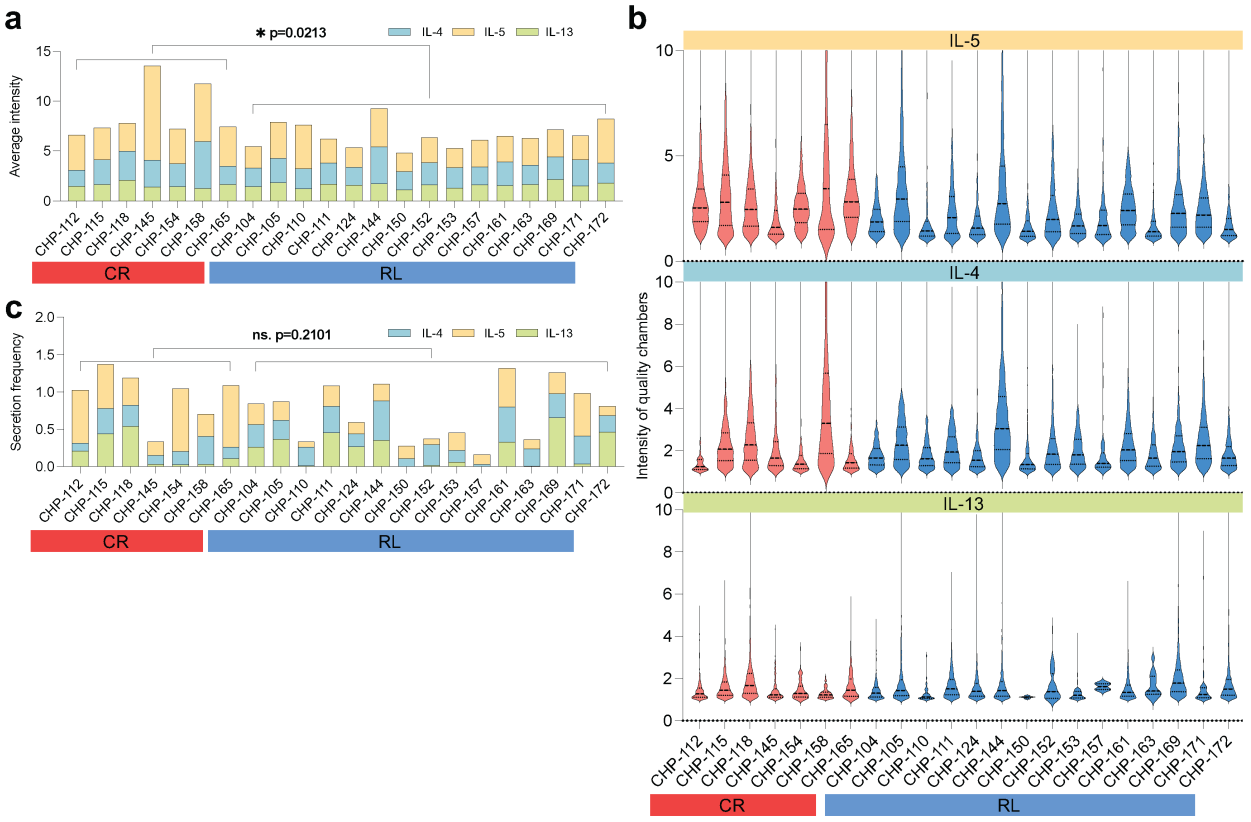
Supplementary Figure 10. Representative flow plot showing activation-related signature's frequency in different coculture conditions. (**a**) CAR T cells cocultured with CD19-3T3 cells produce high level of IFN-γ, IL-2, CD154 and TNF. (**b**) CAR T cells cocultured with MSLN-3T3 have rare IFN-γ, IL-2, CD154 and TNF production. (**C**) Stimulation with CD3/CD28 beads induces comparable IFN-γ, IL-2, CD154 and TNF expression as compared with CD19-3T3 coculture.



Supplementary Figure 11. Frequency comparison of CD154+, IFN-γ+, IL-2+, TNF+, GM-CSF+ and MIP-1β+ CAR T cells between CD19-3T3 and MSLN-3T3 coculture conditions. Results of CR and RL patients are presented separately. Each dot represents the frequency of each patient in specific response group. All the *P* values were calculated with Mann-Whitney test. * *P* <0.05, ** *P* <0.01, **** *P* <0.0001.



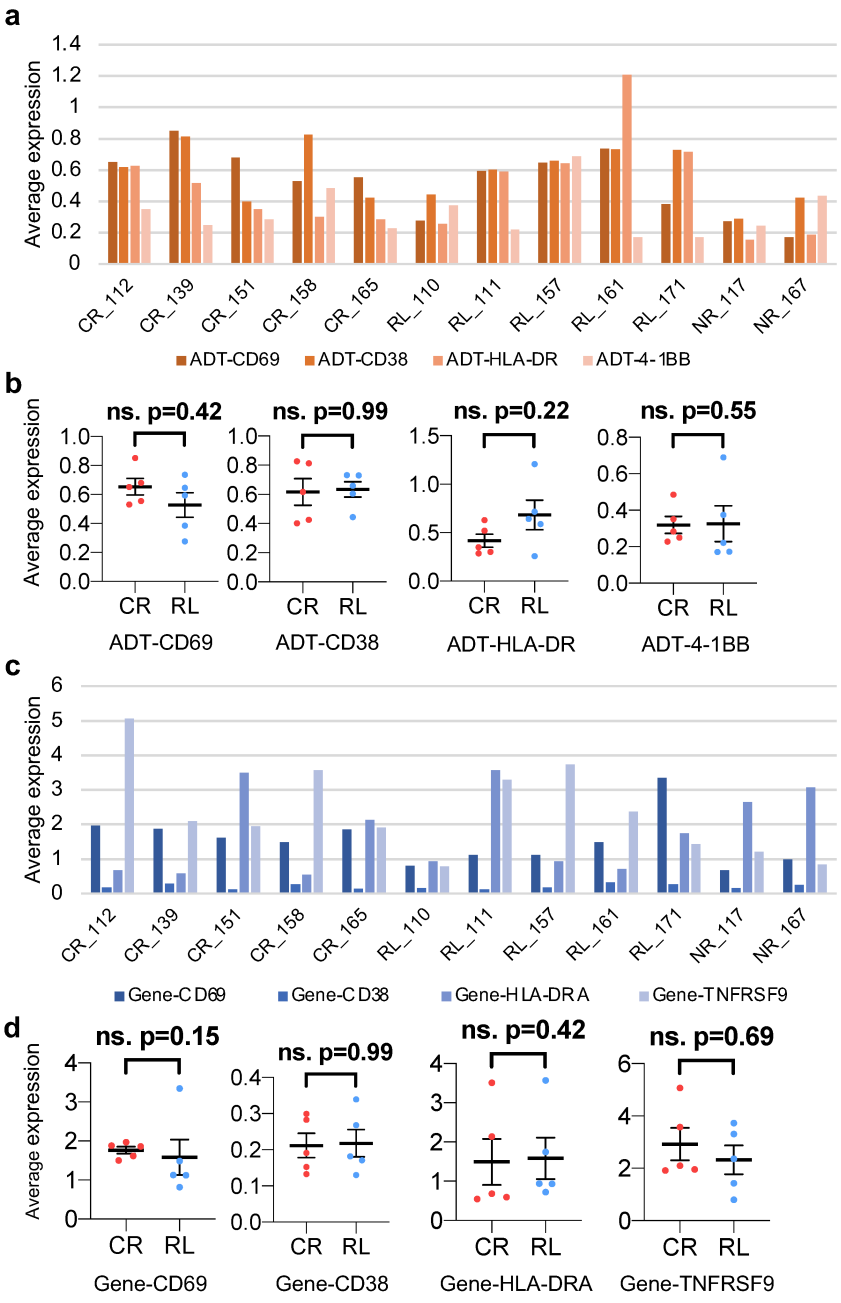
Supplementary Figure 12. Frequency comparison of GM-CSF+, CCL4+, and Granzyme A+ CAR T cells between CR and RL response groups. Results CAR T cells are cocultured with CD19-3T3 cells. All the *P* values were calculated with Mann-Whitney test. ns. Not significant. Scatter plots show mean±s.e.m.



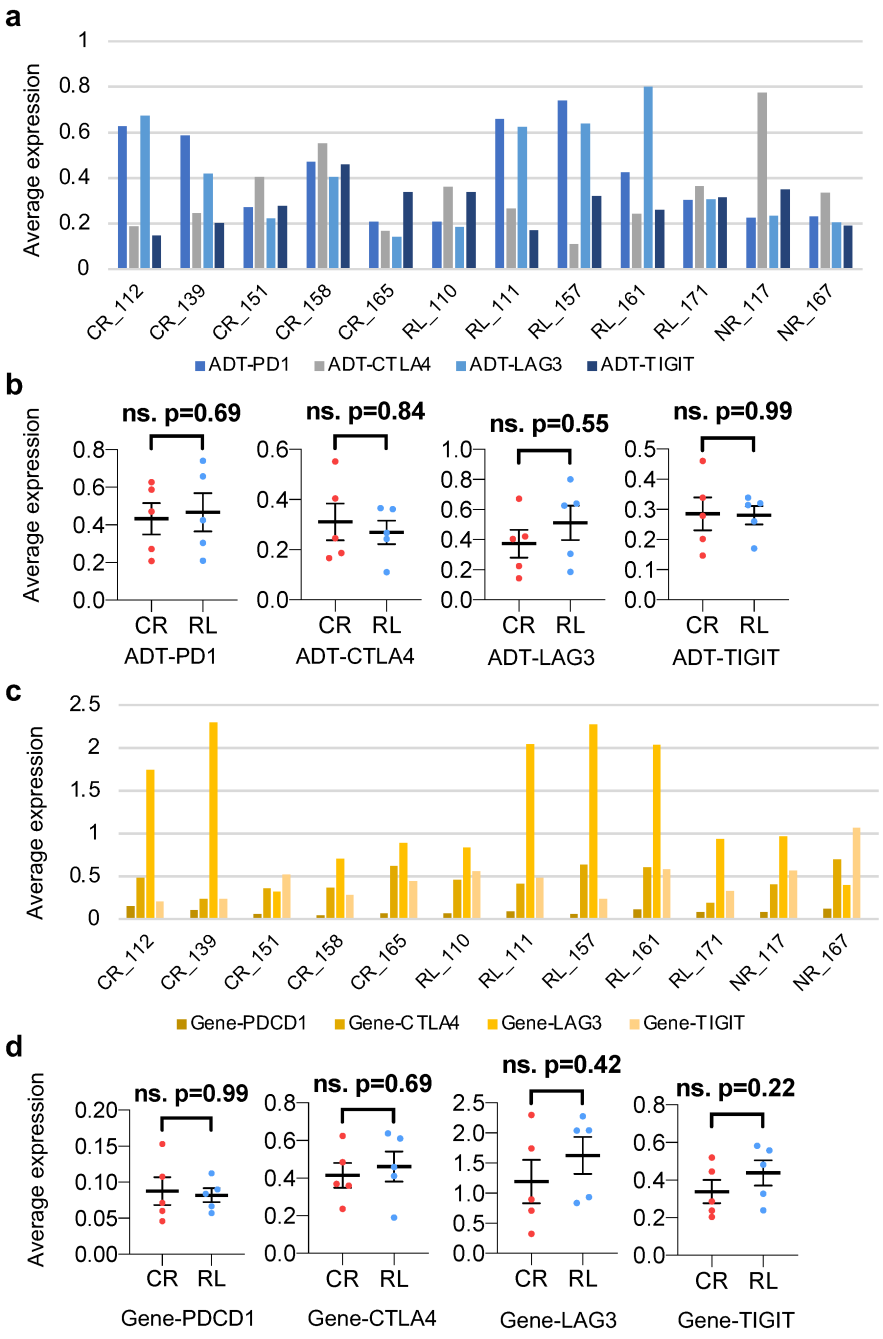
Supplementary Figure 13. Comparison of the average intensity and secretion frequency of Th2 cytokines between CR and RL patients. (**a**) The average signal intensity of quality chambers. (**b**) The intensity distribution of all the quality chambers. (**C**) The secretion frequency of Th2 cytokines. The *P* value was calculated with Mann-Whitney test. ns. Not significant.

	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{EF}
CD62L	+	+	+	−	−
CCR7	+	+	+	−	−
CD45RA	+	+	−	−	+
CD45RO	−	+	+	+	−

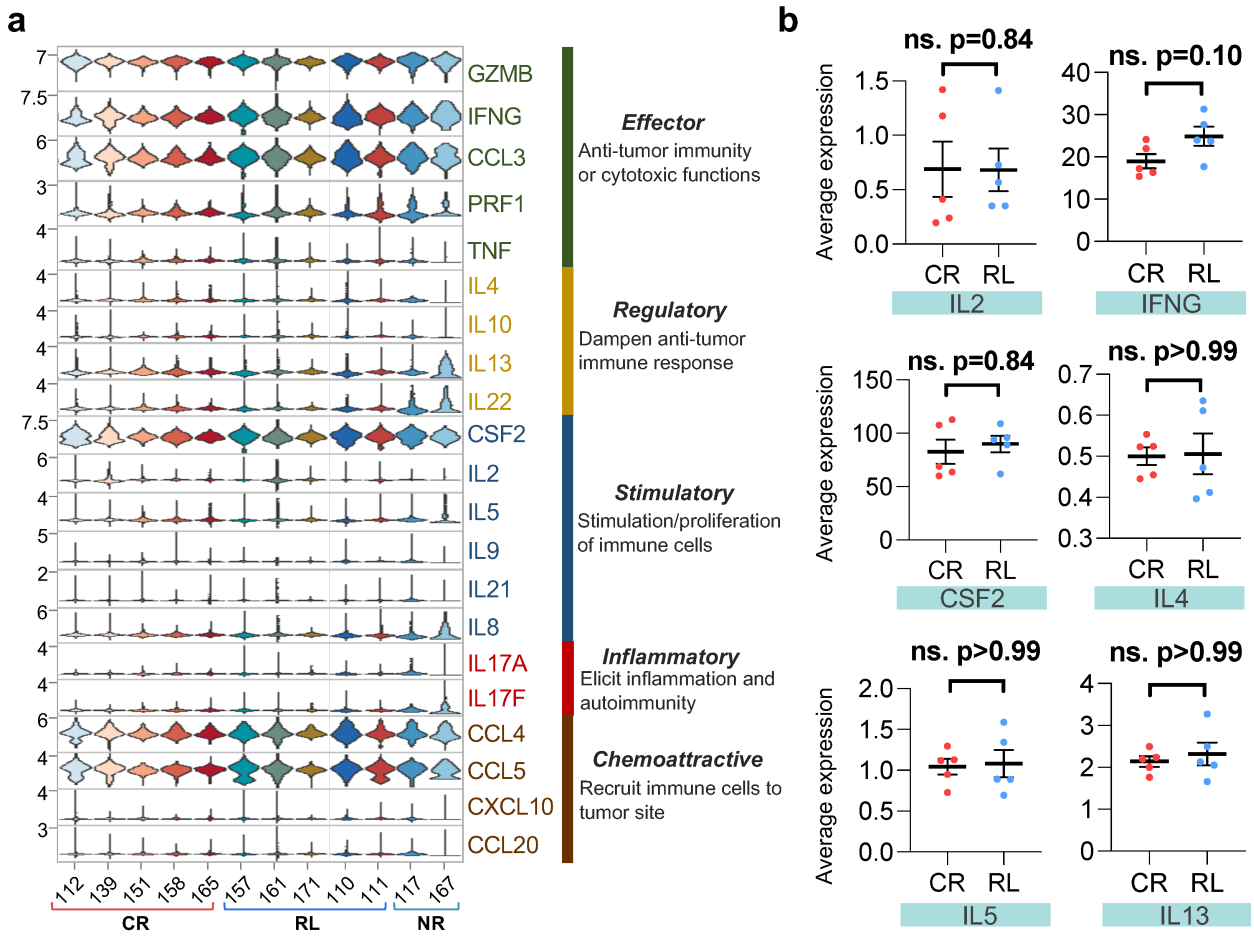
Supplementary Figure 14. A four-marker scheme to determine T cell differentiation states. T_N, naïve; T_{SCM}, stem cell-like memory; T_{CM}, central memory; T_{EM}, effector memory; T_{EF}, effector T cells.



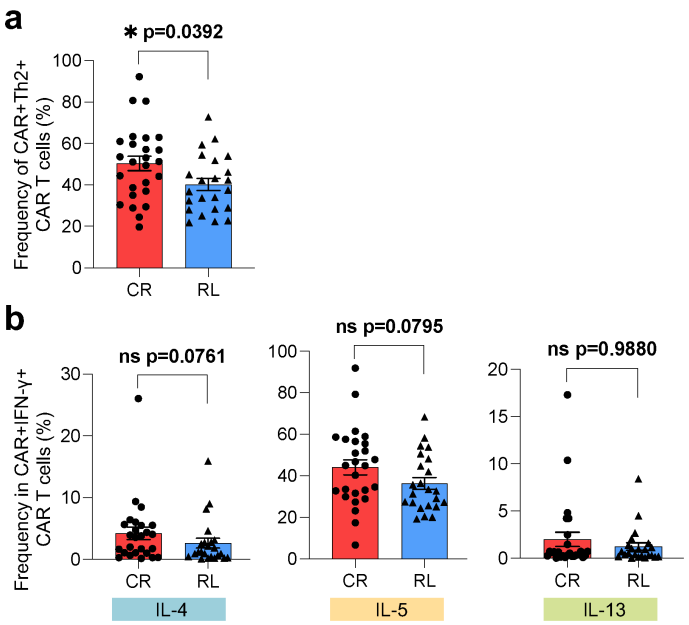
Supplementary Figure 15. Expression of activation-related surface protein ADTs and encoding genes in CD19 stimulated CAR T cells and their comparison between response groups. (**a**) The expression level of ADT-CD69, ADT-CD38, ADT-HLA-DR, ADT-4-1BB in each patient. (**b**) Comparison of the four protein markers expression between CR and RL patients. (**c**) The expression level of genes *CD69*, *CD38*, *HLA-DRA*, *TNFRSF9* in each patient. (**d**) Comparison of the four encoding genes expression between CR and RL patients. Each scatter point represents the average expression value of all single cells of specific patient. The P values were calculated with Mann-Whitney test. ns. Not significant. Scatter plots show mean±s.e.m.



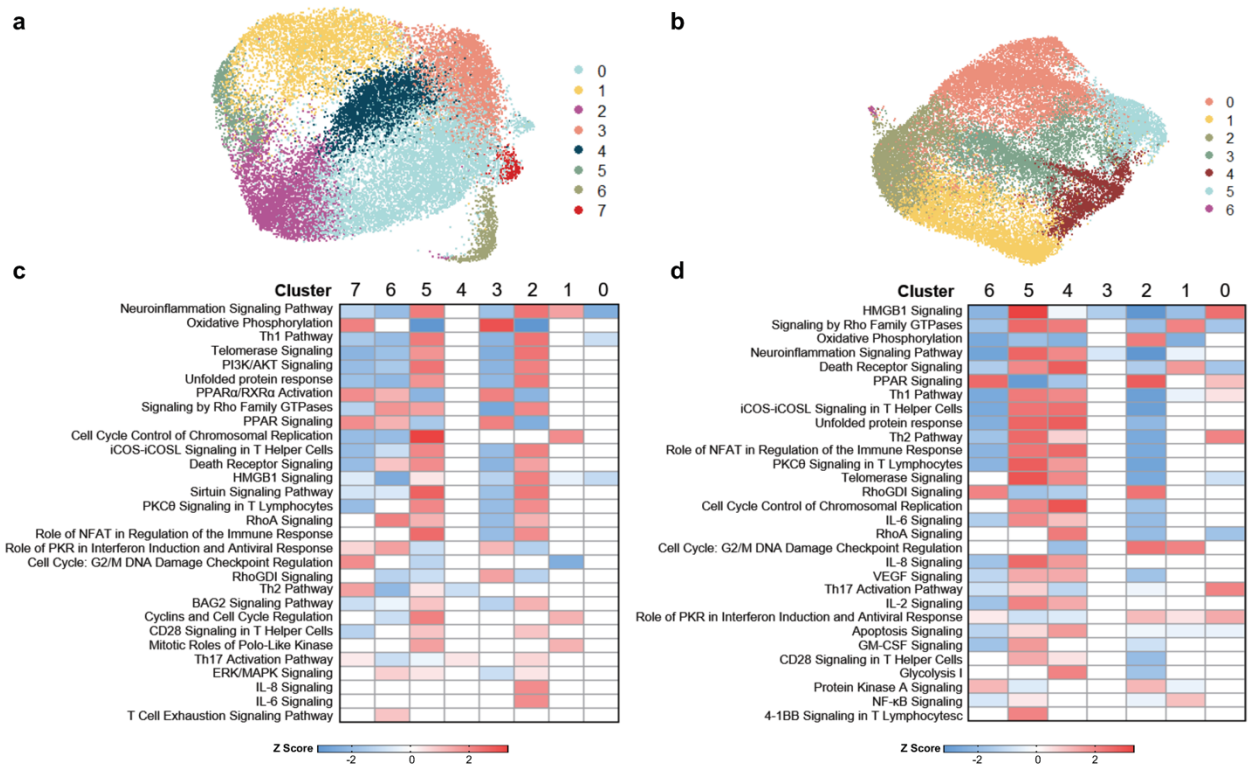
Supplementary Figure 16. Expression of co-inhibitory surface protein ADTs and encoding genes in CD19 stimulated CAR T cells and their comparison between response groups. (**a**) The expression level of ADT-PD1, ADT-CTLA4, ADT-LAG3, ADT-TIGIT in each patient. (**b**) Comparison of co-inhibitory protein markers expression between CR and RL patients. (**c**) The expression level of genes *PDCD1*, *CTLA4*, *LAG3*, *TIGIT* in each patient. (**d**) Comparison of co-inhibitory genes expression between CR and RL patients. Each scatter point represents the average expression value of all single cells of specific patient. The *P* values were calculated with Mann-Whitney test. ns. Not significant. Scatter plots show mean±s.e.m.



Supplementary Figure 17. Cytokine gene expression pattern of TCR-stimulated CAR-T cells. (**a**) Single cell expression level violin plot of all key immunologically relevant cytokine genes from CAR+ cells. (**b**) Quantification of expression level of selected cytokine genes and comparisons between CR and RL group. Each scatter point represents the average expression value of all single cells of specific patient. The P values were calculated with Mann-Whitney test. ns. Not significant. Scatter plots show mean±s.e.m.



Supplementary Figure 18. Frequency Th2+ CAR T cells in CD3/CD28 beads stimulation condition. (**a**) The comparison of CAR+Th2+ CAR T cell frequency between CR and RL patients. Th2 measures the total frequency of IL-4+, IL-5+, and IL-13+ cells. (**b**) The frequency comparison of each Th2-related cytokine+ CAR T cells between CR and RL patients. All the *P* values were calculated with Mann-Whitney test. ns. Not significant. Scatter plots show mean±s.e.m.



Supplementary Figure 19. Global pathway analysis and comparison of CD19-specific or anti-CD3/CD28-beads stimulated pre-infusion CAR T cells. (**a**) UMAP clustering of anti-CD3/CD28 beads stimulated cells. (**b**) UMAP clustering of CD19-3T3 stimulated cells. (**c**) Canonical pathway analysis and comparison of clusters in (**a**). (**d**) Canonical pathway analysis and comparison of clusters in (**b**). Differentially expressed genes of each cluster are used to identify the biological pathways. z score reflects the predicted activation level ($z < 0$, inhibited; $z > 0$, activated; $z \geq 2$ or $z \leq -2$ can be considered significant).