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NEWS & COMMENTARY

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CD4⁺ CAR T cells in for the long journey

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Graphical Abstract text (to accompany Figure 1 on the ToC listing):

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23 In a recently published article, Melenhorst *et al.* performed longitudinal analysis on CAR T
24 cells isolated from patients over 10 years post therapy, revealing expansion of a long-lived
25 CD4⁺ CAR T cell population with a cytotoxic phenotype.

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30 *Main text*

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32 Since the first clinical trials about a decade ago ^{1, 2}, chimeric antigen receptor (CAR) T cell
33 therapy has generated much interest in the clinic due to its remarkable capacity to induce
34 complete responses in a subset of patients. CAR T cell therapy involves genetic modification
35 of autologous T cells to express a synthetic receptor. These target a specific tumour antigen to
36 re-direct T cell killing towards cancer cells. Specifically, CAR T cells targeting the B cell
37 antigen, CD19, have demonstrated the ability to induce durable remissions in the treatment of
38 CD19⁺ B cell malignancies. Clinical responses vary between cancer types, ranging from as
39 high as 90% complete remission rates in acute lymphoblastic leukaemia (ALL) to 30–50% in
40 chronic lymphoblastic leukaemia (CLL) and non-Hodgkin's lymphoma (NHL) ^{3,4}.
41 Remarkably, a significant proportion of patients remain in relapse-free remission for years
42 following treatment. Elucidating the characteristics of CAR T cells in these patients may help
43 in further improvement of CAR T cell therapy. Whilst phenotypic markers of CAR T cells that
44 are predictive of persistence during the acute phase of the response have previously been
45 determined ⁵, a recent study by Melenhorst *et al.* performed the first longitudinal analysis of
46 CTL019 CAR T cells at >10 years clinical remission in 2 CLL patients, revealing fascinating
47 insights into CAR T cell persistence and phenotype over time ⁶.

48 Melenhorst *et al.* demonstrated that CTL019 cells were detectable 10 and 9 years post-
49 treatment in patients 1 and 2, respectively, which correlated with sustained B cell aplasia.
50 Through cytometry by time-of-flight (CyTOF) and multiomic single-cell analysis, the CAR T
51 cell phenotypes were assessed over time. Both patients received a 'bulk' CAR T cell product
52 derived from PBMCs, such that it contained a mixture of CD4⁺, CD8⁺ and non-conventional

53 CAR T cells. Whilst CD4⁺ CAR T cells constituted 70% and 5% of all CAR T cells in the acute
54 phase of the response in patients 1 and 2 respectively, both patients exhibited a marked
55 expansion of CD4⁺ CAR T cells over time, which constituted >95% of the CAR T cell
56 population from 3.4 years onwards for patient 1 and 7.2 years for patient 2. Characterisation of
57 these CD4⁺CAR⁺ T cells indicates that they exhibited a high proliferative potential with high
58 Ki-67 expression and about 30% of CD4⁺ CAR T cells in S, G2 or M phase of the cell cycle.
59 Further analysis of these long-lived CD4⁺CAR⁺ T cells demonstrated the expression of PD-1,
60 LAG-3, TIGIT and TIM-3 as well as transcription factors TOX, BATF and EOMES, but low
61 expression of TCF7 and FOS.

62 Interestingly, CD4⁺ CAR T cells expressed genes indicative of cytolytic potential including
63 perforin, granzyme A and K (but not granzyme B) and, importantly, had the capacity to
64 degranulate upon co-incubation with antigen-positive tumour cells. In this respect, these cells
65 resemble previously reported Perforin⁺ Granzyme A⁺ Granzyme B⁻ cytolytic CD4⁺ T cells
66 found to be important for immune-mediated control of melanoma and bladder cancer ^{7, 8}.
67 However, as both granzyme A and granzyme K are minimally cytotoxic relative to granzyme
68 B ^{9, 10}, the true cytotoxic potential of the CD4⁺ CAR T cells described here is unclear.
69 Consistent with previous work indicating a critical role for CD8⁺ CAR T cells in the acute
70 phase of the response, 25% of the CAR T cells in each patient at the earliest time point of ~2
71 months were granzyme B⁺ CD8⁺ CAR T cells. Thus, from this data, Melenhorst *et al.* propose
72 that CAR T cell therapy is governed by two major phases, an initial response phase involving
73 CD8⁺ T cells, followed by long-term remission dominated by Ki67^{hi} CD4⁺ CAR T cells
74 (**Figure 1**).

75 More broadly, these observations further highlight the importance of CD4⁺ CAR T cells in
76 maintaining durable anti-tumour responses. Previous studies have demonstrated that a defined
77 ratio of CD8⁺ and CD4⁺ CAR T cells can lead to greater tumour control ^{11–13}, an observation
78 that has led to one FDA approved product, Breyanzi (lisocabtagene maraleucel), having a pre-
79 defined ratio of CD8⁺ and CD4⁺ CAR T cells. Although CD4⁺ CAR T cells have been shown
80 to elicit cytotoxic function *in vitro* ¹⁴, the primary mechanism by which CD4⁺ CAR T cells
81 enhance therapeutic effects in the early elimination of tumour cells *in vivo* was identified to be
82 through IL-2 mediated support of CD8⁺ CAR T cell responses, rather than a direct cytotoxic
83 role of CD4⁺ CAR T cells ¹². While the relative role of CD4⁺ and CD8⁺ CAR T cells in the
84 early elimination of tumour cells remains unclear from the present study, these data raise the

85 possibility that whilst CD8⁺ CAR T cells play a critical role in early disease elimination, it is
86 cytotoxic CD4⁺ CAR T cells that are critical for long-term disease control.

87 In a second aspect of the study, Melenhorst *et al.* performed TCR sequencing and lentiviral
88 vector integration site (LVIS) analysis to investigate the clonal evolution of CAR T cells over
89 time. The lentiviral integration site for the CAR is random for each T cell and so provides a
90 barcode that acts as a proxy measure of CAR T cell clonality. Moreover, theoretically if a CAR
91 inserts into the genome at a point that disrupts the expression of a negative regulator of T cell
92 function this may in itself improve efficacy. Thus, identifying the lentiviral integration site and
93 its impact on clonal evolution is of significant interest to the field, largely sparked by the
94 observation that a TET2 integration event was responsible for the clonal expansion of CAR T
95 cells in one CLL patient¹⁵. However, in the current study, assessment of CAR integration sites
96 in the dominant clones revealed no common integration sites suggesting this was not a major
97 factor driving CAR T cell persistence. Consistent with this observation, TCR-seq analysis
98 demonstrated little clonality in persisting CAR T cells, suggesting this may be more stochastic
99 than previously anticipated.

100 Lastly, it is notable that from approximately 5 years post CAR T cell delivery, both patients
101 displayed a near absence of B cells, indicating that anti-CD19 CAR T cells remained active in
102 the depletion of both healthy and malignant B cells populations. This raises the question as to
103 whether the constant antigen stimulation provided by healthy B cells (a form of antigen
104 presenting cell) is critically required for the long-term persistence and maintenance of CD19
105 targeting T cells. This is of particular importance when considering how to improve CAR T
106 cell persistence in the solid tumours where CAR T cells do not naturally engage with antigen
107 presenting cells. This notion again signifies the importance of strategies that boost CAR T cell
108 persistence and function *in vivo* through vaccine-like approaches¹⁶ and/or utilise the initial
109 CAR T cell response to drive long-lasting endogenous immune responses against the tumour
110¹⁷.

111 In summary, these results provide novel insight into the dynamics of long-term CAR T cell
112 persistence, adding further support to the importance of CD4⁺ CAR T cells in driving clinical
113 efficacy. These data also suggest that lentiviral integration site is not a major determinant of
114 CAR T cell clonality, a finding that corroborates other recent analyses¹⁸. Further investigations
115 are required to confirm these findings in a larger cohort of patients and determine whether
116 CD4⁺ CAR T cells play an active role in disease suppression, or are more simply a by-product

117 of continued targeting of healthy CD19⁺ B cells. Moreover, this study highlights the probable
118 role that healthy CD19⁺ cells have in promoting the persistence of anti-CD19 CAR T cells.
119 Given this mechanism is not applicable to CAR T cell therapies targeting solid tumour antigens,
120 where CAR T cell persistence is inferior, an interesting possibility raised by this study is that
121 targeting of endogenous CD19 is actually critical for achieving long-term efficacy. Overall,
122 these findings could help to inform future development of strategies to enhance CAR T cell
123 therapy.

124

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130

131 **Conflicts of interest**

132 PAB declares the following conflicts: research funding from AstraZeneca, Bioartis, Bristol-Myers-
133 Squibb and Gilead Sciences.

134

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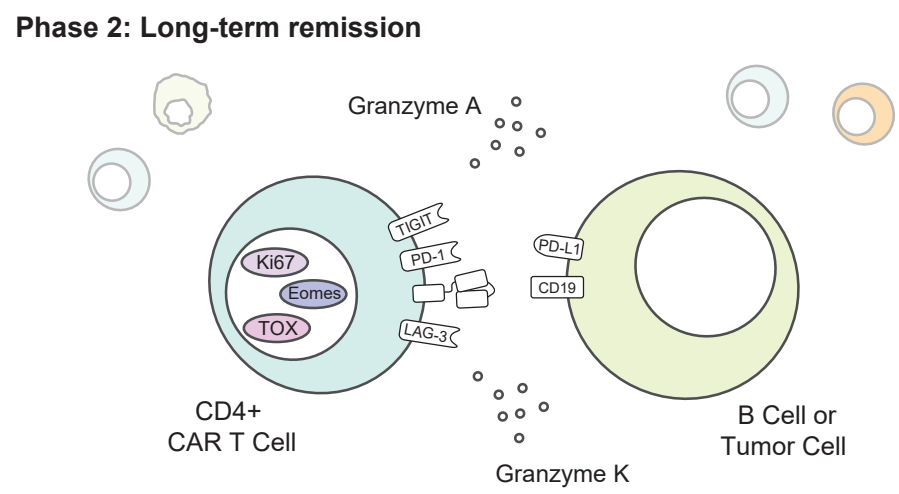
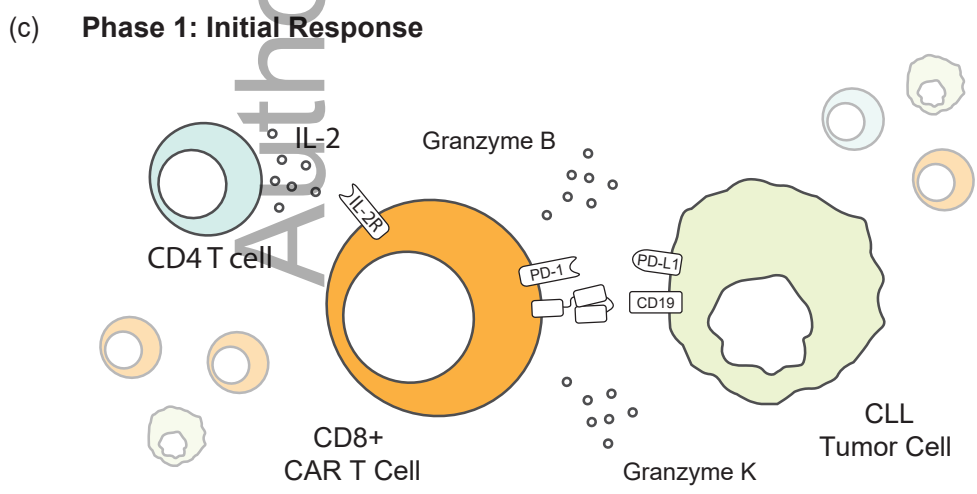
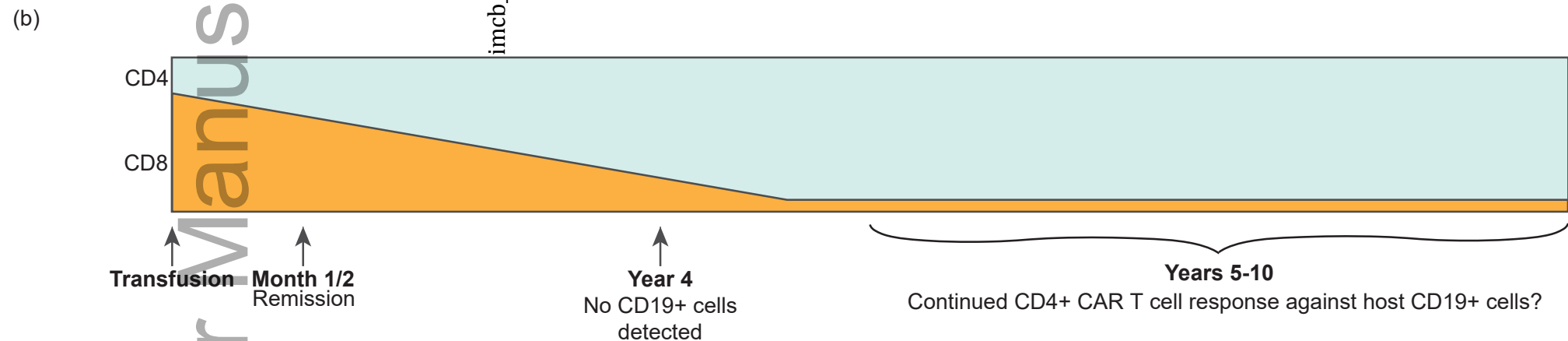
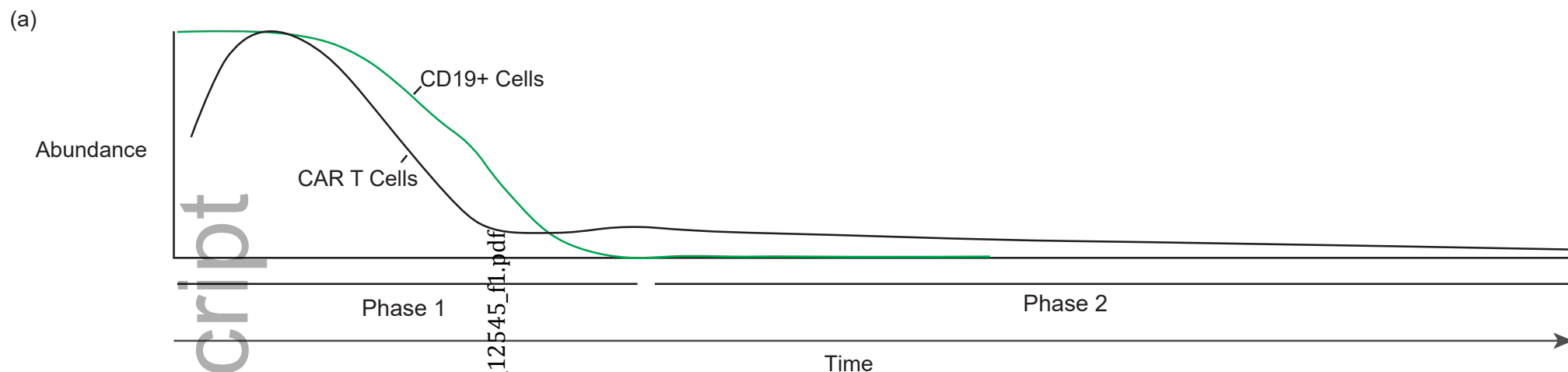
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179

180 **Figure 1.** Dynamics of CAR T cell response over 10 years. **(a)** The anti-CD19 CAR T cell
181 response can be sub-divided into the acute phase (Phase I) where CAR T cells initially expand
182 and subsequently contract in line with reduced numbers of CD19⁺ cells (either tumour cells or
183 healthy B cells). This is followed by a longer phase where CAR T cells persist at low levels
184 and the numbers of CD19⁺ cells remain low. **(b, c)** In the initial stages, the CAR T cell
185 population is constituted of a mixture of CD8⁺ T cells with a classical cytotoxic phenotype and
186 CD4⁺ CAR T cells that provide IL-2 support to this response. In the long-term remission phase,
187 CD4⁺ T cells make up the majority of CAR T cells and their persistence may in part be driven
188 by healthy B cells.

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