

Patients with gastrointestinal irritability after TGN1412-induced cytokine 2 storm displayed selective expansion of gut-homing $\alpha\beta$ and $\gamma\delta$ T-cells

Item Type	Journal article
Authors	McCarthy, Neil;Stagg, Andrew;Price, Claire;Mann, Elizabeth;Gellatly, Nichola;Al-Hassi, Hafid Omar;Knight, Stella;Panoskaltsis, Nicki
Citation	McCarthy, N.E., Stagg, A.J., Price, C.L. et al. (2021) Patients with gastrointestinal irritability after TGN1412-induced cytokine 2 storm displayed selective expansion of gut-homing $\alpha\beta$ and $\gamma\delta$ T-cells, <i>Cancer Immunology Immunotherapy</i> . 70, pp.1143–1153. https://doi.org/10.1007/s00262-020-02723-4
DOI	10.1007/s00262-020-02723-4
Publisher	Springer Nature
Journal	<i>Cancer Immunology Immunotherapy</i>
Download date	2026-05-12 06:51:04
License	https://creativecommons.org/licenses/by-nc-nd/4.0/
Link to Item	http://hdl.handle.net/2436/623628

1 **Patients with gastrointestinal irritability after TGN1412-induced cytokine**
2 **storm displayed selective expansion of gut-homing $\alpha\beta$ and $\gamma\delta$ T-cells**
3

4 Neil E McCarthy^{1,3}, Andrew J Stagg^{1,4}, Claire L Price^{1,5}, Elizabeth R Mann^{1,6},
5 Nichola L Gellatly¹, Hafid O Al-Hassi^{1,7}, Stella C Knight^{1,8} & Nicki
6 Panoskaltsis^{1,2,9}

7
8 **This work was undertaken at:** ¹Antigen Presentation Research Group,
9 Imperial College London, Northwick Park & St. Mark's campus, London, UK;
10 ²Department of Haematology, Imperial College London, Northwick Park & St.
11 Mark's campus, London, UK.

12
13 **Author Affiliations:**

14
15 ³MRC Career Development Fellow, Centre for Immunobiology, The Blizard
16 Institute, Bart's and the London School of Medicine and Dentistry, Queen Mary
17 University of London, London, UK. n.e.mccarthy@qmul.ac.uk
18

19 ⁴Reader, Centre for Immunobiology, The Blizard Institute, Bart's and the
20 London School of Medicine and Dentistry, Queen Mary University of London,
21 London, UK.

22
23 ⁵Scientific Director, Lucid Group Communications, Buckinghamshire, UK.

24
25 ⁶Wellcome Trust and Royal Society Sir Henry Dale Fellow, Lydia Becker
26 Institute of Immunology and Inflammation, University of Manchester
27

28 ⁷Senior Lecturer in Cancer Research, Research Institute in Healthcare Science,
29 Faculty of Science and Engineering, University of Wolverhampton, UK.
30

31 ⁸Professor of Immunopathology, Imperial College London, and Consultant in
32 Immunopathology, London North West University Healthcare NHS Trust,
33 Antigen Presentation Research Group, Northwick Park and St. Mark's Campus,
34 London, UK.

35
36 ⁹Associate Professor, Department of Hematology and Medical Oncology,
37 Winship Cancer Institute, Emory University School of Medicine; BioMedical
38 Systems Engineering Laboratory, Wallace H. Coulter Department of
39 Biomedical Engineering, Georgia Institute of Technology, Atlanta, USA.
40
41
42
43
44

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33

34
35

Corresponding Authors:

Nicki Panoskaltsis, MD PhD FRCP
Department of Hematology and Medical Oncology
Winship Cancer Institute
Emory University School of Medicine
Atlanta, GA 30322
USA
nicki.panoskaltsis@emory.edu

Neil McCarthy, PhD
Centre for Immunobiology, The Blizard Institute
Barts and The London School of Medicine and Dentistry
Queen Mary University of London
4 Newark Street, London, E1 2AT
UK
n.e.mccarthy@qmul.ac.uk

Abstract: 99 words **Manuscript:** 3,462 words

Figures: 3

Supplementary Tables / Figures: 1 / 3

References: 47

Keywords:

Cytokine storm
Cytokine Release Syndrome
TGN1412
V δ 2⁺ $\gamma\delta$ T-cells
Immunotherapy
Immune-related adverse events (irAEs)

1 **Declarations**

2
3 **Funding:** The North West London Hospitals NHS Trust; Cancer Research UK;
4 The Northwick Park Hospital Leukemia Research Trust Fund.

5
6 **Conflicts of Interest:** None of the authors declare a financial conflict of
7 interest. NP, SCK, CLP, HOA, ERM and NG declare no conflicts of interest.
8 NEM is supported by a Career Development Award from The Medical Research
9 Council (Grant Ref: MR/R008302/1) and is in receipt of a project grant from
10 Bart's and The London Charity (MGU0465). He has also received consultancy
11 fees and funding for research from ImCheck Therapeutics SAS. AJS research
12 is supported by grants from Gilead Sciences, AbbVie, The Medical College of
13 St Bartholomew's Hospital Trust, Bowel & Cancer Research, and Bart's Charity.
14 SCK, NEM and AJS have done contract work for Parexel pre-dating the work
15 described in this report. At the time of this work and report, Parexel Clinical
16 Trials Unit had a short-term contract with the Antigen Presentation Research
17 Group (APRG) to use a Class II cabinet within the laboratory. The APRG has
18 also been contracted to perform immunological studies by a pharmaceutical
19 company, the tissue specimens for which were supplied on behalf of that
20 company via Parexel which is located adjacent to the APRG department. There
21 is no conflict of interest involved.

22
23 **Ethics Approval:** Ethics approval had been obtained for the TGN1412 trial (by
24 the investigators – none of the authors of this report were involved in the clinical
25 trial). At the time of the trial-related serious adverse event, clinical and immune
26 monitoring ensued as a matter of standard clinical care; no studies were done
27 outside what was required for clinical care of the patients. Discussions between
28 the Ethics Committee, MHRA and Expert Scientific Group set-up by the Minister
29 of Health (UK) at the time in order to investigate the trial outcome unanimously
30 concluded that the monitoring (as outlined in this report) should continue for
31 standard of care, and that specific ethics approval was not required due to the
32 extraordinary circumstances.

33
34 **Consent to Participate:** Patients consented to clinical follow-up and immune
35 monitoring. None of the authors of this work were involved with the conduct of
36 the clinical trial or any of the pre-clinical testing of TGN1412. The patient cohort
37 had consented to the TGN1412 first-in-man clinical trial that resulted in the
38 cytokine storm serious adverse event. At the time of the start of sample
39 collection for the current report, the patients had been removed from the trial
40 and were being treated based on clinical need, rather than trial protocol.

41
42 **Consent for Publication:** Patients have provided written informed consent to
43 the publication of the clinical follow-up and immune monitoring data.

44
45 **Availability of Data and Material:** As this is a clinical cohort follow-up, and not
46 data provided on a clinical trial, the data are unavailable due to personal privacy
47 protections.

48
49 **Code Availability:** Not applicable.

50

1 **Authors' Contributions:** NEM and AJS were involved in the planning and
2 execution of all experiments, interpretation of data, and in preparation of the
3 manuscript. CLP, ERM, NLG and HOA contributed to a number of experiments.
4 NP had overall responsibility for the patients and clinical follow-up, and SCK
5 and NP supervised the project, interpreted data, and prepared the manuscript.

6

7

8 **ACKNOWLEDGEMENTS**

9 Financial support for some of the work was provided by The North West London
10 Hospitals NHS Trust incorporating Northwick Park Hospital, Cancer Research
11 UK, and The Northwick Park Hospital Leukaemia Research Trust Fund. We are
12 also grateful to Sarah Clarson of Beckman Coulter for providing the TCR-V β
13 repertoire kit used in these studies. Above all, we thank the six patients who
14 have given consent for presentation of their personal data.

15

16

17

1 **ABSTRACT**

2 Following infusion of the anti-CD28 superagonist monoclonal antibody
3 TGN1412, three of six previously healthy, young male recipients developed
4 gastrointestinal irritability associated with increased expression of
5 'gut-homing' integrin $\beta 7$ on peripheral blood $\alpha\beta$ T-cells. This subset of patients
6 with intestinal symptoms also displayed a striking and persistent expansion of
7 putative $V\delta 2^+$ $\gamma\delta$ T-cells in the circulation which declined over a two-year period
8 following drug infusion, concordant with subsiding gut symptoms. These data
9 demonstrate that TGN1412-induced gastrointestinal symptoms were
10 associated with dysregulation of the 'gut-homing' pool of blood $\alpha\beta$ and $\gamma\delta$ T-
11 cells, induced directly by the antibody and/or arising from the subsequent
12 cytokine storm.

13

14 **SIGNIFICANCE**

15 Following TGN1412-induced cytokine storm, 3 of 6 patients developed
16 gastrointestinal irritability associated with expansion of gut-homing $\alpha\beta$ and $\gamma\delta$ T-
17 cells. These findings may elucidate the pathology of immune-related adverse
18 events affecting the gut.

19

20

21

22

23

1 **Introduction**

2 In higher primates, the blood T-cell pool contains diverse $\alpha\beta$ T-cells and
3 semi-invariant 'unconventional' T-cells that recognize either microbial peptides
4 or metabolites, respectively (1). In both cases, antigen activation can stimulate
5 these cells to upregulate the gut-homing integrin $\alpha4\beta7$ and traffic to the intestine
6 (2-4). Gut microbes, and the metabolic activities these perform, vary between
7 host species. Consequently, the compounds generated and their conditioning
8 effects on peripheral blood T-cell responses are likely to differ between mice
9 and humans (5, 6). The influence of these microbial products may also diverge
10 between individual recipients of agonist/antagonist immunotherapies; gut
11 bacteria from patients with melanoma who respond to immune checkpoint
12 blockade are enriched for anabolic functions proposed to stimulate host
13 immunity (7). However, the extent to which peripheral blood T-cell responses
14 contribute to these modulatory effects *in vivo* remains unclear.

15 Some of the most common antigen-specific lymphocytes in human blood
16 are gut-tropic T-cells specialized to detect various bacterial metabolites (1, 8).
17 However, the frequencies and phenotypes adopted by these cells can differ
18 between individuals and age groups (9, 10), and their impact on
19 immunotherapeutic outcomes in treated patients is not well understood. It is
20 now well-recognized that checkpoint inhibitors can be associated with immune-
21 related adverse events (irAEs) affecting the gut, most notably symptoms of
22 diarrhea and colitis following blockade of cytotoxic T-lymphocyte antigen-4
23 (CTLA-4) or programmed cell death 1 (PD-1) in patients with melanoma (7, 11).
24 However, it is still unclear to what extent gastrointestinal irAEs are caused by

1 disruption of local mucosal immunoregulation, versus systemic drug effects on
2 gut-homing lymphocytes (12).

3 In March 2006, six healthy volunteers suffered from cytokine release
4 syndrome (CRS) during a phase 1 first-in-man clinical trial of the monoclonal
5 antibody TGN1412 (13). In pre-clinical studies, this anti-CD28 super-agonist
6 induced preferential lymphocytosis of regulatory T-cells in the absence of
7 systemic inflammation (14, 15); the immunological basis for antibody-induced
8 CRS and resultant lymphopenia in the human trial has remained unclear.
9 In addition to acute symptoms of CRS from which all six patients recovered
10 (13), three patients suffered from prolonged gastrointestinal irritability of
11 unknown etiology, suggesting unexpected TGN1412 antibody and/or CRS
12 effects on gastrointestinal immunity. We therefore undertook a detailed
13 investigation of peripheral blood distribution and expression levels of integrin
14 $\beta 7$ aiming to understand the immunological basis for these symptoms. These
15 analyses revealed that blood $\alpha\beta$ T-cells from patients who suffered from gut
16 irritability displayed significantly enhanced levels of $\beta 7$ expression that were not
17 observed in either asymptomatic patients or healthy controls. In addition,
18 TGN1412-induced gut symptoms were associated with a striking expansion of
19 circulating $\gamma\delta$ T-cells (putative phosphoantigen metabolite-responsive V γ 9V δ 2+
20 lineage) that was still evident two years after drug infusion. Together, these
21 data suggest that in three of six recipients, the TGN1412 antibody or
22 subsequent cytokine storm caused sustained dysregulation of the gut-homing
23 T-cell pool, which gradually normalized over the two-year period following
24 antibody infusion, concordant with subsiding gastrointestinal symptoms.

25

1 **METHODS**

2 ***Clinical trial***

3 Details of the first 30 days of clinical follow-up of the serious adverse event
4 (SAE) have been reported previously. The patients presented herein
5 correspond with those previously identified as follows (13): 1-B, 2-A, 3-F, 4-E,
6 5-C, and 6-D. The TGN1412 antibody was produced by TeGenero AG
7 (Würzburg, Germany), manufactured by Boehringer Ingelheim (Germany), and
8 the clinical trial was conducted by contract research organization PAREXEL
9 International (Waltham, MA, USA) on leased premises at Northwick Park
10 Hospital, London, UK. The authors of this report were not involved in either pre-
11 clinical or clinical testing of TGN1412.

12

13 ***Patients and data sources***

14 Patients were clinically followed, off trial, and assessed as a cohort following
15 the SAE (13). Based on clinical need and requirements for SAE follow-up, the
16 lead clinician (NP) requested immunological monitoring, including analysis of
17 peripheral blood T-cell subsets alongside intracellular and serum cytokine
18 levels. Monitoring commenced 10 days after infusion of TGN1412 and the
19 patients were evaluated at 21 time-points over the subsequent two years. All
20 patient blood samples were anonymized and the scientists performing the
21 immunological tests were not aware of patient symptoms, signs, or clinical
22 laboratory data. Patients were assessed by the lead clinician at the same
23 intervals wherein blood was procured for monitoring. Control blood samples
24 from healthy male volunteers (n=24) were obtained in parallel with the patient
25 samples after written informed consent. The six volunteers who received

1 TGN1412 were male and had a median age of 29.5 years (range 19-34) at the
2 time of recruitment into the first-in-man trial. Healthy control volunteers were
3 male and had a median age of 30 years (range 19-42). All patients were well
4 during the two-week period preceding the clinical trial and were without
5 significant medical history. Patients B and C were lost to immunological follow-
6 up after 15 and 22 months, respectively. Following development of
7 gastrointestinal symptoms in three of the six patients, additional assessment of
8 $\beta 7$ integrin expression on peripheral blood T-cells was introduced for all patients
9 at four separate time-points over the two-year follow-up. All clinical information
10 was withheld from the scientists who performed these analyses (NEM, AJS,
11 CLP, ERM, NLG, HOA, SCK) until laboratory investigations were complete. All
12 six patients consented to immunological monitoring and have given written
13 informed consent to the publication of data presented in this report.

14

15 ***Immune monitoring***

16 Specific leukocyte subset monitoring began on Day+10 following TGN1412
17 infusion and was repeated every three or four days for the first two weeks, then
18 weekly for four weeks, then every four weeks for three months, then every six
19 weeks for the remainder of eight months (time-points 1-17). In year two of
20 monitoring, patients were evaluated every three months (time-points 18-21). In
21 the first six months, whole blood was assessed for T-cell subsets, numbers,
22 phenotypes and intracellular cytokine expression. After six months, the tests
23 were rationalized to those that were most informative. Additional correlates of
24 immune function included assessment of T-cell receptor V β repertoire (kit kindly
25 donated by Beckman Coulter), and T-cell homing markers for the

1 gastrointestinal tract and skin based on expression of $\beta 7$ integrin and
2 cutaneous leukocyte antigen (CLA), respectively. The bulk of these data are
3 presented elsewhere – this report focuses on the gut-homing subsets. These
4 studies were conducted in a laboratory that operates under Good Laboratory
5 Practice (GLP) principles, undertakes exploratory research and were
6 performed using established laboratory protocols that were Minimal Information
7 About T-cell Assays (MIATA) compliant (Supplementary MIATA information).
8 The assays and reagents employed were previously validated and tested for
9 assay performance during the course of standard general investigative
10 research.

11

12 ***Flow Cytometry***

13 Peripheral whole blood was obtained by venipuncture into sodium-heparin
14 Vacutainer™ tubes (Becton-Dickinson) and then directly labeled with
15 monoclonal antibody (mAb; Supplementary Table 1) for 15min at room
16 temperature. After mAb labeling, Optilyse C reagent (Immunotech, Marseilles)
17 was used to lyse erythrocytes for 15min before washing the cells twice in cold
18 FACS buffer (2% FCS, 0.02% sodium azide, and 1mM EDTA in PBS) for 5min
19 at 300G. Cell pellets were fixed in 0.4mL paraformaldehyde (1%) and stored at
20 4°C in the dark until acquired on a FACSCalibur flow cytometer using CellQuest
21 software (Becton-Dickinson). All analyses were performed using WinList
22 software (Verity Software House, Maine, USA). Absolute cell counts were
23 determined using Flow-Count™ Fluorospheres (Beckman Coulter) added to
24 the cells immediately prior to acquisition.

25 Viable cells were gated according to their characteristic light-scatter
26 properties, and individual leukocyte subsets identified based on expression of

1 subset-specific surface antigens. Major CD3⁺ T-cell subsets were identified
2 based on differential expression of CD8 (CD8⁺ T-cells were CD3⁺/CD8⁺ and
3 putative CD4⁺ T-cells were CD3⁺/CD8⁻), since CD8 is less susceptible than CD4
4 to down-regulation during T-cell stimulation used in the intracellular cytokine
5 determination protocol. In each subset, naïve and memory populations were
6 enumerated by further double staining; naïve cells were CD45RA⁺/CD45RO⁻
7 and memory cells were CD45RA⁻/CD45RO⁺. Expression of CD69 in CD4⁺ and
8 CD8⁺ T-cell subsets was used to identify activated T-cells.

9

10 ***Intracellular cytokine staining***

11 Peripheral whole blood cells were cultured in complete medium (Dutch-
12 modified RPMI-1640 medium, 10% FCS, 20mM L-glutamine, 100u/mL
13 penicillin, 100µg/mL streptomycin) with or without monensin (3µM), PMA
14 (10ng/mL), and ionomycin (2µM) for 4h at 37°C, 5%CO₂. Cells were then
15 surface-labeled with anti-CD3 and anti-CD8 mAb for 15min at room
16 temperature. Optilyse C reagent (Immunotech, Marseilles) was used for lysis
17 of erythrocytes (0.5mL per 100µL aliquot of blood) and the samples were
18 incubated for 15min at room temperature. The remaining cells were twice
19 washed in cold FACS buffer for 5min at 300G, re-suspended in 100µL
20 Leucoperm A (Serotec, Oxford), and then incubated for 15min at room
21 temperature. The partially fixed cells were next washed twice in FACS buffer
22 and re-suspended in 100µL Leucoperm B (Serotec, Oxford). For intracellular
23 staining, the cells were labeled with 5µL anti-cytokine mAb for 30min on ice
24 then washed twice in FACS buffer and fixed in 0.4mL paraformaldehyde (1%)

1 prior to storage in the dark at 4°C (acquisition by flow-cytometry was performed
2 within 24 hours).

3

4 **Statistics**

5 Statistical analyses were conducted using SigmaStat™3.5 or SigmaPlot™11.0
6 software (Systat Software UK Ltd, London). The TCR-Vβ repertoire data were
7 compared using Kruskal-Wallis One-Way Analysis of Variance on Ranks. One-
8 Way Analysis of Variance with All-Pairwise Multiple Comparison Procedures
9 (Holm-Sidak method) was used to compare β7 integrin expression over time
10 between patient subsets and 10 healthy controls. Differences in expression of
11 γδ-TCR between patients and controls were evaluated by Student's t-test.

12

13

1 **RESULTS**

2 Three of six patients (A, B and E) who received TGN1412 suffered from
3 gastrointestinal symptoms (Common Terminology Criteria for Adverse Events
4 grade 1-2 irAE), manifesting as loose and frequent bowel motions or diarrhea
5 (primarily after consuming spicy foods; hereafter described as 'gut irritability'),
6 not present prior to drug exposure. These symptoms started within one month
7 of TGN1412 infusion and subsequently decreased in intensity over the two-year
8 follow-up period. Symptoms persisted in patients B and E at two years. Patient
9 B displayed the most pronounced gut symptoms and in the first year of follow-
10 up underwent a full gastrointestinal work-up including duodenal biopsies (which
11 were normal), and removal of a colonic polyp which exhibited non-specific
12 inflammation.

13

14 **Gut irritability in TGN1412 recipients was associated with increased β 7** 15 **expression by circulating $\alpha\beta$ T-cells**

16 Integrin $\alpha4\beta7$ binding to MAdCAM-1 facilitates leukocyte recruitment into
17 intestinal tissues (16). Accordingly, T-cell expression of $\alpha4\beta7$ is significantly
18 modulated during active gut inflammation, and inhibition of the $\alpha4\beta7$:MAdCAM-
19 1 axis has been an effective therapeutic strategy in patients with inflammatory
20 bowel disease (IBD) (17, 18). Development of gut symptoms in three of the six
21 TGN1412 recipients prompted us to assess T-cell expression of $\beta7$ integrin at
22 four separate time-points over the two-year follow-up period. CD45RA⁺
23 (predominately naïve) T-cells in the blood of both patients and healthy controls
24 uniformly expressed an intermediate level of $\beta7$, whereas CD45RA⁻ (antigen-
25 experienced effector/memory) T-cells included both $\beta7^+$ and $\beta7^-$ subsets,

1 representing putative gut-homing and non-intestinal populations, respectively
2 (Fig. 1a). In healthy volunteers, memory T-cells were evenly distributed
3 between $\beta 7^+$ and $\beta 7^-$ subsets (median ratio 0.98, interquartile range 0.80-1.19;
4 Fig. 1b). Patients C, D, and F, who did not exhibit gastrointestinal symptoms,
5 were indistinguishable from control subjects at all time points analyzed. In
6 contrast, patients with gut irritability (A, B, and E) displayed increased $\beta 7^+$
7 memory T-cells at 8.6 months ($p < 0.001$) and 10.2 months ($p = 0.003$) post-
8 TGN1412 infusion (Fig. 1b). At 8.6 months, both $CD45RA^+$ and $CD45RA^-$ T-
9 cells from patients A, B, and E also exhibited higher levels of $\beta 7$ integrin
10 expression per cell (mean fluorescence) compared with T-cells from healthy
11 controls, although this had normalized by one year post-infusion
12 (Supplementary Fig. 1a and b). Sustained changes in both $CD8^+$ and $CD8^-$
13 (presumed $CD4^+$) memory T-cells contributed to the elevated $\beta 7$ expression
14 detected in patients with gut irritability (Supplementary Fig. 1c and d). Fewer
15 than 8% of $\beta 7^+$ memory T-cells from either patients or controls expressed
16 $CD103/\alpha E$ integrin, the alternative binding partner for $\beta 7$ (Fig. 1c), consistent
17 with reports that $\beta 7$ primarily forms complexes with the $\alpha 4$ subunit on blood T-
18 cells (16), and confirming that the data presented here reflected changes in the
19 patients' $\alpha 4\beta 7^+$ compartment. Together, these findings indicated that gut
20 irritability in three of six patients infused with TGN1412 was associated with a
21 sustained increase in gut-homing potential among both naïve and memory $\alpha\beta T$ -
22 cells.
23

1 **TGN1412-induced gut irritability correlated with peripheral blood**
2 **expansion of putative V δ 2⁺ $\gamma\delta$ T-cells**

3 In addition to the features outlined above, the blood of patients who developed
4 gut irritability after TGN1412 infusion contained a distinct subset of CD3^{hi} T-
5 cells that was not present in the circulation of either asymptomatic patients or
6 healthy controls (Fig. 2a). These cells displayed a CD4⁻CD8⁻ 'double negative'
7 phenotype characteristic of unconventional lymphocytes (data not shown) and
8 expressed uniformly high levels of CD45RO (Fig. 2b) and β 7 integrin (Fig. 2e),
9 but lacked CD103 (Fig. 2f) and did not express any of the common V β -TCR
10 repertoire variants assayed at 8.6 and 12.1 months following TGN1412 infusion
11 (Supplementary Fig. 2). These features strongly implicated an expansion of
12 'unconventional' V γ 9V δ 2⁺T-cells (hereafter V δ 2⁺T-cells) which express high
13 levels of α 4 β 7 in human blood (3, 19) and are rapidly recruited to mucosal
14 tissues in higher primates *in vivo* (20, 21). Further support for this lineage
15 identity was later provided by the absence of $\alpha\beta$ -TCR (Fig. 2g) and lack of
16 markers for natural killer cells (CD56) or invariant natural killer T-cells (antibody
17 6B11; data not shown), but high expression levels of $\gamma\delta$ -TCR (22) (Fig. 2h) as
18 well as NKG2D, and CD161 (23, 24) (data not shown).

19 Analysis at 15 months post-infusion confirmed that typical low numbers
20 of $\gamma\delta$ T-cells were present in peripheral blood from unaffected patients C, D, and
21 F, as well as in six healthy controls analyzed in tandem, but these cells were
22 not CD3^{hi} (data not shown). In contrast, total $\gamma\delta$ T-cells (including both CD3⁺ and
23 CD3^{hi} subsets) remained significantly increased in patients with gut irritability
24 (A, B, E; 7.92-8.59%) compared with healthy controls (2.5-6.5%; p=0.002) even
25 at this late time point (more than one year post-infusion). In the blood of patients

1 A, B, and E, the $\gamma\delta$ T-cell pool reached peak numbers (16% of total T-
2 lymphocytes) approximately 1.5 months post-TGN1412 exposure, followed by
3 a gradual decline coincident with improvement in gastrointestinal symptoms
4 (Fig. 2i and j). Up to 25% of these $\gamma\delta$ T-cells displayed an activated/CD69⁺
5 phenotype, and expression of this marker decreased steadily over the following
6 seven months (Fig. 2k). No other clinical or laboratory features correlated with
7 $\gamma\delta$ T-cell expansion as observed in the three symptomatic patients. Importantly,
8 expansion of gut-homing lymphocytes in the patients with gut irritability was not
9 restricted to $\gamma\delta$ T-cells alone, because higher numbers of $\beta 7^+$ T-cells were still
10 detected in these individuals (Fig. 1) when the $\gamma\delta$ T-cell (CD3^{hi}) population was
11 excluded from this analysis (Supplementary Fig. 3).

12 The IFN γ -producing subset of blood V $\delta 2^+$ T-cells declines naturally with
13 age and is lost more rapidly in men after the age of 30 (25), with both ethnic
14 and environmental variables further impacting on the dynamics of this
15 compartment (26). To determine whether the expanded $\gamma\delta$ T-cells detected in
16 TGN1412 recipients remained functionally competent, and also to understand
17 how these cells might be contributing to gastrointestinal irritability, we next
18 assessed cytokine expression using a standard intracellular staining approach.
19 The $\gamma\delta$ T-cell population produced low-levels of IL-10 *in vitro* in the absence of
20 exogenous stimulation (Fig. 3), but did not appear to spontaneously produce
21 either IFN γ or IL-4. However, $\gamma\delta$ T-cell reactivation with phorbol myristate
22 acetate and ionomycin resulted in substantial production of IFN γ across a wide
23 range of time points analyzed, starting from one month post-infusion, when
24 symptoms of gut-irritability were first identified. Together, these data suggest
25 that development of intestinal symptoms in patients infused with TGN1412 was

- 1 associated with sustained expansion of circulating V δ 2⁺T-cells with IFN γ -
- 2 producing capacity, as well as increased gut-homing potential within the blood
- 3 $\alpha\beta$ T-cell pool.
- 4

1 **DISCUSSION**

2 This report provides unique evidence that TGN1412 antibody, or the
3 subsequent CRS, dysregulated intestinal immunity in three of six drug
4 recipients, outside of an identified infectious etiology; gut irritability was
5 associated with sustained enhancement of mucosal trafficking in both
6 conventional and unconventional T-cell subsets.

7 While the immunological response to TGN1412 infusion was surprisingly
8 uniform in many respects (13), the long-term impact of the drug on mucosal
9 immunity varied markedly between patients. Typical populations of $\beta 7^+ \alpha \beta T$ -
10 cells (16) were present as expected in all trial patients, but enhanced $\beta 7$
11 expression levels and the surprising expansion of $\gamma \delta T$ -cells were unique to
12 patients with symptoms of gut irritability. This variability of response may reflect
13 patient-specific differences in homeostatic T-cell reconstitution after TGN1412-
14 induced lymphopenia (13) and/or differential $\gamma \delta T$ -cell responses to high levels
15 of cytokines such as $TNF\alpha$ (27) following infusion of TGN1412. The peripheral
16 blood location, kinetics, magnitude, and duration of these $\gamma \delta T$ -cell expansions,
17 together with uniform expression of CD45RO (28) and high surface levels of
18 $\beta 7$, strongly implicate the $V\gamma 9V\delta 2^+$ T-cell lineage which responds to non-peptide
19 'phosphoantigens' (pAg) derived from microbes and stressed/transformed host
20 cells (29, 30). $V\gamma 9V\delta 2^+$ T-cells undergo rapid polyclonal expansion in the first
21 few weeks of human life, likely driven by pAg-producing bacteria within the gut
22 microbiome; thereafter, the repertoire displays progressive selection of shared
23 or 'public' pAg-reactive clones (defined by characteristic $V\gamma 9JP$ and $V\delta 2$
24 chains) (31). With advancing age, the $V\delta 2^+$ T-cell compartment becomes
25 increasingly oligoclonal, but different individuals may still display diverse or

1 'private' V γ 9V δ 2⁺ clonal expansions with distinct effector phenotypes (32),
2 potentially including variable expression of gut-homing markers. It is therefore
3 possible that the TGN1412 recipients with gut symptoms (A, B, and E) featured
4 V δ 2⁺ clonotypes that were absent from the blood of those without (C, D and F).
5 These cells may have exhibited different thresholds for pAg activation and
6 intestinal recruitment in the context of cytokine storm. Rapid expansion of
7 V γ 9JP⁺ $\gamma\delta$ T-cells has also been observed during immune reconstitution of
8 patients undergoing allogeneic hematopoietic stem cell transplantation, but the
9 clonotypes generated following donor cell infusion were substantially different
10 from those present either in the donor or the recipient pre-transplant (33).
11 Indeed, while V δ 2⁺T-cells constitute only a minor fraction of total circulating
12 lymphocytes in healthy individuals, their number and activation state in
13 peripheral blood and body tissues has been correlated with therapeutic and
14 clinical outcomes (34-36). Work from our own laboratory has also demonstrated
15 that activated blood V δ 2⁺T-cells rapidly up-regulate β 7 and can populate
16 human gut lamina propria where they induce substantial mucosal production of
17 IFN γ (3). In patients with Crohn's disease, gut-homing potential and pro-
18 inflammatory properties of V δ 2⁺T-cells are enhanced (36), suggesting that they
19 play a key role in human gut immunity and inflammation. In future, the
20 microbiome composition of pAg-producing bacteria and blood $\gamma\delta$ T-cell
21 repertoire may also prove to be important determinants of clinical outcome in
22 patients undergoing immunotherapy.

23 The V δ 2⁺ lineage is absent in rodents and does not recognize antigen in
24 the context of MHC (37). Instead, V δ 2⁺T-cells respond to butyrophilin (BTN)
25 proteins, considered as part of the B7 family of costimulatory receptors (37),

1 with critical roles recently identified for both BTN3A1 and BTN2A1 (38). V δ 2⁺T-
2 cells lack alloreactivity while displaying potent anti-tumor and anti-microbial
3 functions, such that reconstitution of this lineage after chemotherapy-induced
4 lymphopenia may reduce infection rates in patients receiving hematopoietic
5 stem cell transplantation, without increased incidence of graft versus host-
6 disease (33, 39). Expansion of blood V δ 2⁺T-cells has been observed in CRS,
7 most notably in healthcare workers exposed to SARS-CoV-1 in the 2003
8 outbreak; these individuals displayed strikingly similar features including
9 relatively stable $\alpha\beta$ T-cell numbers and TCR-V β repertoire, whereas marked
10 expansions of V δ 2⁺T-cells with IFN γ -producing capacity were still evident three
11 months after disease onset (40). These findings resemble data from nonhuman
12 primate models in which pAg injection stimulates blood V δ 2⁺T-cell expansion
13 *in vivo* (41), leading to accumulation of an IFN γ -producing subset both in lungs
14 and intestinal mucosa (20), accompanied by robust Th1 immune protection
15 against a range of different pathogens.

16 V δ 2⁺T-cells undergo expansion in response to a variety of microbial
17 infections and can dominate the blood lymphocyte pool for extended periods
18 (10). It is also now widely recognized that V δ 2⁺T-cells display tissue-tropic
19 phenotypes consistent with trafficking to barrier sites where pAg-producing
20 microbes and tumors frequently originate (29, 30). In particular, V δ 2⁺T-cells are
21 associated with effective host immunity to pAg-producing mycobacteria and
22 robust responses to bacillus Calmette-Guérin (BCG) vaccination (42, 43),
23 which induces population expansion and upregulation of CD69 and IFN γ
24 expression *in vitro*. Notably, these responses are enhanced in BCG-responders
25 compared with non-sensitized controls (44, 45), and the pool of V δ 2⁺T-cells

1 generated lacks lymph node homing receptors while displaying homogenous
2 expression of CD28 (45). Therefore, TGN1412 may have directly stimulated
3 $V\delta 2^+$ T-cells in the trial patients, and prior microbial exposures such as BCG
4 may have influenced subsequent responses to mucosal pathogens and/or
5 cytokine storm (as also postulated in the context of COVID-19 (46)). Indeed,
6 while previous studies have primarily linked $V\delta 2^+$ T-cell expansion with host
7 protection against bacterial pathogens, these lymphocytes can also lyse
8 stressed host cells infected with viruses including influenza (47) and SARS-
9 CoV-1 (40). Together, these data suggest that monitoring of gut-homing $\alpha\beta$ and
10 $\gamma\delta$ T-cell populations is likely to shed important new light on the initiation,
11 propagation, monitoring, and resolution of mucosal symptoms in human
12 subjects with irAEs or suffering CRS as a result of immunotherapy or severe
13 infections such as COVID-19.

14

1 **References**

- 2
3 1. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB (2015) The
4 burgeoning family of unconventional T cells. *Nat. Immunol.* 16: 1114-23. doi:
5 10.1038/ni.3298
- 6 2. Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B,
7 Weissman IL, Hamann A, Butcher EC (1993) Alpha 4 beta 7 integrin mediates
8 lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell.* 74:
9 185-95.
- 10 3. McCarthy NE, Bashir Z, Vossenkamper A et al. (2013) Proinflammatory
11 Vdelta2+ T Cells Populate the Human Intestinal Mucosa and Enhance IFN-
12 gamma Production by Colonic alphabeta T Cells. *J. Immunol.* 191: 2752-63.
13 doi: 10.4049/jimmunol.1202959
- 14 4. Juno JA, Wragg KM, Amarasena T et al. (2019) MAIT Cells Upregulate
15 alpha4beta7 in Response to Acute Simian Immunodeficiency Virus/Simian HIV
16 Infection but Are Resistant to Peripheral Depletion in Pigtail Macaques. *J.*
17 *Immunol.* 202: 2105-20. doi: 10.4049/jimmunol.1801405
- 18 5. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak
19 G (2009) Metabolomics analysis reveals large effects of gut microflora on
20 mammalian blood metabolites. *Proc. Natl. Acad. Sci. U. S. A.* 106: 3698-703.
21 doi: 10.1073/pnas.0812874106
- 22 6. Schroeder BO, Backhed F (2016) Signals from the gut microbiota to
23 distant organs in physiology and disease. *Nat. Med.* 22: 1079-89. doi:
24 10.1038/nm.4185
- 25 7. Gopalakrishnan V, Spencer CN, Nezi L et al. (2018) Gut microbiome
26 modulates response to anti-PD-1 immunotherapy in melanoma patients.
27 *Science.* 359: 97-103. doi: 10.1126/science.aan4236
- 28 8. McCarthy NE, Eberl M (2018) Human gammadelta T-Cell Control of
29 Mucosal Immunity and Inflammation. *Frontiers in immunology.* 9: 985. doi:
30 10.3389/fimmu.2018.00985
- 31 9. Ryan PL, Sumaria N, Holland CJ, Bradford CM, Izotova N, Grandjean
32 CL, Jawad AS, Bergmeier LA, Pennington DJ (2016) Heterogeneous yet stable
33 Vdelta2(+) T-cell profiles define distinct cytotoxic effector potentials in healthy
34 human individuals. *Proc. Natl. Acad. Sci. U. S. A.* 113: 14378-83. doi:
35 10.1073/pnas.1611098113
- 36 10. Morita CT, Jin C, Sarikonda G, Wang H (2007) Nonpeptide antigens,
37 presentation mechanisms, and immunological memory of human

- 1 Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition
2 of prenyl pyrophosphate antigens. *Immunol. Rev.* 215: 59-76. doi:
3 10.1111/j.1600-065X.2006.00479.x
- 4 11. Berman D, Parker SM, Siegel J, Chasalow SD, Weber J, Galbraith S,
5 Targan SR, Wang HL (2010) Blockade of cytotoxic T-lymphocyte antigen-4 by
6 ipilimumab results in dysregulation of gastrointestinal immunity in patients with
7 advanced melanoma. *Cancer Immun.* 10: 11.
- 8 12. Abu-Sbeih H, Ali FS, Alsaadi D, Jennings J, Luo W, Gong Z, Richards
9 DM, Charabaty A, Wang Y (2018) Outcomes of vedolizumab therapy in patients
10 with immune checkpoint inhibitor-induced colitis: a multi-center study. *J*
11 *Immunother Cancer.* 6: 142. doi: 10.1186/s40425-018-0461-4
- 12 13. Suntharalingam G, Perry M, Ward S, Brett S, Castello-Cortes A, Brunner
13 M, Panoskaltzis N (2006) Cytokine Storm in a phase 1 trial of the anti-CD28
14 monoclonal antibody TGN1412. *The New England Journal of Medicine.* 355:
15 1018-28.
- 16 14. Beyersdorf N, Gaupp S, Balbach K et al. (2005) Selective targeting of
17 regulatory T cells with CD28 superagonists allows effective therapy of
18 experimental autoimmune encephalomyelitis. *J Exp Med.* 202: 445-55.
- 19 15. TGN1412 Investigator's Brochure. TeGenero Immunotherapeutics.
20 http://www.mhra.gov.uk/home/idcplg?IdcService=GET_FILE&dDocName=CO
21 [N2023518&RevisionSelectionMethod=LatestReleased](http://www.mhra.gov.uk/home/idcplg?IdcService=GET_FILE&dDocName=CO). Accessed 5 May 2006
- 22 16. Rott LS, Briskin MJ, Andrew DP, Berg EL, Butcher EC (1996) A
23 fundamental subdivision of circulating lymphocytes defined by adhesion to
24 mucosal addressin cell adhesion molecule-1. Comparison with vascular cell
25 adhesion molecule-1 and correlation with β 7 integrins and memory
26 differentiation. *J Immunol.* 156: 3727-36.
- 27 17. Sandborn WJ, Feagan BG, Rutgeerts P et al. (2013) Vedolizumab as
28 induction and maintenance therapy for Crohn's disease. *N. Engl. J. Med.* 369:
29 711-21. doi: 10.1056/NEJMoa1215739
- 30 18. Feagan BG, Rutgeerts P, Sands BE et al. (2013) Vedolizumab as
31 induction and maintenance therapy for ulcerative colitis. *N. Engl. J. Med.* 369:
32 699-710. doi: 10.1056/NEJMoa1215734
- 33 19. Brandes M, Willimann K, Lang AB, Nam KH, Jin C, Brenner MB, Morita
34 CT, Moser B (2003) Flexible migration program regulates gamma delta T-cell
35 involvement in humoral immunity. *Blood.* 102: 3693-701. doi: 10.1182/blood-
36 2003-04-1016
37 2003-04-1016 [pii]

- 1 20. Ali Z, Shao L, Halliday L, Reichenberg A, Hintz M, Jomaa H, Chen ZW
2 (2007) Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate-driven
3 antimicrobial and cytotoxic responses of pulmonary and systemic
4 Vgamma2Vdelta2 T cells in macaques. *J. Immunol.* 179: 8287-96. doi:
5 179/12/8287 [pii]
- 6 21. Ryan-Payseur B, Frencher J, Shen L, Chen CY, Huang D, Chen ZW
7 (2012) Multieffector-Functional Immune Responses of HMBPP-Specific
8 Vgamma2Vdelta2 T Cells in Nonhuman Primates Inoculated with *Listeria*
9 *monocytogenes* $\{\Delta\}$ actA prfA*. *J. Immunol.* 189: 1285-93. doi:
10 10.4049/jimmunol.1200641
- 11 22. Thibault G, Bardos P (1995) Compared TCR and CD3 ϵ expression of $\alpha\beta$
12 and $\gamma\delta$ T cells. Evidence for the association of two TCR heterodimers with three
13 CD3 ϵ chains in the TCR/CD3 complex. *J Immunol.* 154: 3814-20.
- 14 23. Provine NM, Binder B, FitzPatrick MEB et al. (2018) Unique and
15 Common Features of Innate-Like Human V δ 2+ $\gamma\delta$ T Cells and Mucosal-
16 Associated Invariant T Cells. *Frontiers in immunology.* 9. doi:
17 10.3389/fimmu.2018.00756
- 18 24. Kong Y, Cao W, Xi X, Ma C, Cui L, He W (2009) The NKG2D ligand
19 ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells
20 through both TCRgammadelta and NKG2D. *Blood.* 114: 310-7. doi:
21 10.1182/blood-2008-12-196287
- 22 25. Caccamo N, Dieli F, Wesch D, Jomaa H, Eberl M (2006) Sex-specific
23 phenotypical and functional differences in peripheral human Vgamma9/Vdelta2
24 T cells. *J. Leukoc. Biol.* 79: 663-6. doi: 10.1189/jlb.1105640
- 25 26. Esin S, Shigematsu M, Nagai S, Eklund A, Wigzell H, Grunewald J
26 (1996) Different percentages of peripheral blood gamma delta + T cells in
27 healthy individuals from different areas of the world. *Scand. J. Immunol.* 43:
28 593-6.
- 29 27. Li H, Luo K, Pauza CD (2008) TNF- α is a positive regulatory factor for
30 human V γ 2V δ 2 T cells. *J Immunol.* 181: 7131-7.
- 31 28. Miyawaki T, Kasahara Y, Taga K, Yachie A, Taniguchi N (1990)
32 Differential expression of CD45RO (UCHL1) and its functional relevance in two
33 subpopulations of circulating TCR-gamma/delta+ lymphocytes. *Journal of*
34 *Experimental Medicine.* 171: 1833-8.
- 35 29. Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, Jomaa H (2003)
36 Microbial isoprenoid biosynthesis and human gammadelta T cell activation.
37 *FEBS Lett.* 544: 4-10. doi: S0014579303004836 [pii]

- 1 30. Scheper W, Sebestyén Z, Kuball J (2014) Cancer Immunotherapy Using
2 gammadelta T Cells: Dealing with Diversity. *Frontiers in immunology*. 5: 601.
3 doi: 10.3389/fimmu.2014.00601
- 4 31. Papadopoulou M, Dimova T, Shey M et al. (2020) Fetal public
5 Vgamma9Vdelta2 T cells expand and gain potent cytotoxic functions early after
6 birth. *Proc. Natl. Acad. Sci. U. S. A.* 117: 18638-48. doi:
7 10.1073/pnas.1922595117
- 8 32. Willcox CR, Davey MS, Willcox BE (2018) Development and Selection
9 of the Human Vgamma9Vdelta2(+) T-Cell Repertoire. *Frontiers in immunology*.
10 9: 1501. doi: 10.3389/fimmu.2018.01501
- 11 33. Ravens S, Schultze-Florey C, Raha S et al. (2017) Human gammadelta
12 T cells are quickly reconstituted after stem-cell transplantation and show
13 adaptive clonal expansion in response to viral infection. *Nat. Immunol.* 18: 393-
14 401. doi: 10.1038/ni.3686
- 15 34. Davey MS, Lin CY, Roberts GW et al. (2011) Human neutrophil
16 clearance of bacterial pathogens triggers anti-microbial gammadelta T cell
17 responses in early infection. *PLoS Pathog.* 7: e1002040. doi:
18 10.1371/journal.ppat.1002040
- 19 35. Laggner U, Di Meglio P, Perera GK et al. (2011) Identification of a novel
20 proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a
21 potential role in psoriasis. *J. Immunol.* 187: 2783-93. doi:
22 10.4049/jimmunol.1100804
- 23 36. McCarthy NE, Hedin CR, Sanders TJ et al. (2015) Azathioprine therapy
24 selectively ablates human Vdelta2(+) T cells in Crohn's disease. *J. Clin. Invest.*
25 125: 3215-25. doi: 10.1172/JCI80840
- 26 37. Rhodes DA, Reith W, Trowsdale J (2016) Regulation of Immunity by
27 Butyrophilins. *Annu. Rev. Immunol.* 34: 151-72. doi: 10.1146/annurev-immunol-
28 041015-055435
- 29 38. Eberl M (2020) Antigen recognition by human gammadelta T cells: one
30 step closer to knowing. *Immunol. Cell Biol.* doi: 10.1111/imcb.12334
- 31 39. Perko R, Kang G, Sunkara A, Leung W, Thomas PG, Dallas MH (2015)
32 Gamma delta T cell reconstitution is associated with fewer infections and
33 improved event-free survival after hematopoietic stem cell transplantation for
34 pediatric leukemia. *Biol. Blood Marrow Transplant.* 21: 130-6. doi:
35 10.1016/j.bbmt.2014.09.027

- 1 40. Poccia F, Agrati C, Castilletti C et al. (2006) Anti-severe acute respiratory
2 syndrome coronavirus immune responses: the role played by V gamma 9V
3 delta 2 T cells. *J. Infect. Dis.* 193: 1244-9. doi: 10.1086/502975
- 4 41. Sicard H, Ingoure S, Luciani B, Serraz C, Fournie JJ, Bonneville M,
5 Tiollier J, Romagne F (2005) In vivo immunomanipulation of V gamma 9V delta
6 2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate
7 model. *J. Immunol.* 175: 5471-80. doi: 175/8/5471 [pii]
- 8 42. Shen L, Frencher J, Huang D et al. (2019) Immunization of
9 Vgamma2Vdelta2 T cells programs sustained effector memory responses that
10 control tuberculosis in nonhuman primates. *Proc. Natl. Acad. Sci. U. S. A.* 116:
11 6371-8. doi: 10.1073/pnas.1811380116
- 12 43. Dantzer KW, de la Parte L, Jagannathan P (2019) Emerging role of
13 gammadelta T cells in vaccine-mediated protection from infectious diseases.
14 *Clin Transl Immunology.* 8: e1072. doi: 10.1002/cti2.1072
- 15 44. Hoft DF, Brown RM, Roodman ST (1998) Bacille Calmette-Guerin
16 vaccination enhances human gamma delta T cell responsiveness to
17 mycobacteria suggestive of a memory-like phenotype. *J. Immunol.* 161: 1045-
18 54.
- 19 45. Martino A, Casetti R, Sacchi A, Poccia F (2007) Central memory
20 Vgamma9Vdelta2 T lymphocytes primed and expanded by bacillus Calmette-
21 Guerin-infected dendritic cells kill mycobacterial-infected monocytes. *J.*
22 *Immunol.* 179: 3057-64. doi: 179/5/3057 [pii]
- 23 46. Netea MG, Giamarellos-Bourboulis EJ, Domínguez-Andrés J, Curtis N,
24 van Crevel R, van de Veerdonk FL, Bonten M (2020) Trained immunity: a tool
25 for reducing susceptibility and severity of SARS-CoV-2 infection. *Cell.* doi:
26 <https://doi.org/10.1016/j.cell.2020.04.042>
- 27 47. Li H, Xiang Z, Feng T et al. (2013) Human Vgamma9Vdelta2-T cells
28 efficiently kill influenza virus-infected lung alveolar epithelial cells. *Cell. Mol.*
29 *Immunol.* 10: 159-64. doi: 10.1038/cmi.2012.70
30
31

1 **FIGURE LEGENDS**

2

3 **Fig. 1 Blood T-cell expression of $\beta 7$ integrin and CD103 following**4 **TGN1412-induced cytokine storm.** CD3⁺ T-cells were identified in whole5 blood and $\beta 7$ expression on memory (CD45RA⁻) and naïve (CD45RA⁺) subsets6 was assessed. **(a)** Representative data for a healthy control and two patients7 are shown, one in whom $\beta 7^+$ cells were prominent in the memory T-cell8 population (patient B) and one in whom $\beta 7^+$ memory T-cells appeared normal

9 (patient C). Staining with isotype-matched control antibodies was contained

10 within the boxed region in the lower left of the plots. **(b)** Summary data for11 memory T-cells showing the ratio of $\beta 7^+:\beta 7^-$ cells assessed at four separate

12 time-points over a period of seven months. In patients with gut irritability (A, B,

13 and E), the ratio of $\beta 7^+:\beta 7^-$ cells was significantly higher at 8.6 and 10.2 months14 than was observed in healthy controls. **(c)** Proportion of $\beta 7^+$ memory T-cells

15 expressing CD103 in the patients, assessed over four separate time-points.

16 Patient B, the most symptomatic, displayed the highest ratio of $\beta 7^+:\beta 7^-$ memory17 T-cells and lowest percentage of CD103⁺ cells, suggesting a selective18 expansion of $\alpha 4\beta 7^+$ 'gut-homing' memory T-cells. Data for Patient D were not

19 available at time points 10.2 and 12.1 months, nor for patient A at 12.1 months.

20 **Fig. 2 Prolonged expansion of circulating $\gamma \delta$ T-cells (putative V $\delta 2^+$) in**21 **patients with gut irritability after TGN1412-induced cytokine storm.**22 Peripheral blood CD3⁺ T-cells **(a – R1)** included a CD3^{hi} subset (black23 histogram; identified by $\gamma \delta$ TCR-specific mAb used in **2h**) which exhibited a24 CD4⁻CD8⁻ 'double negative' phenotype characteristic of unconventional

25 lymphocytes (data not shown). This discrete population uniformly expressed

1 CD45RO and was clearly identifiable in the blood of patients A, B, and E (**b –**
2 **R2**; representative example from patient A), but not in patient C, D, or F (**c –**
3 **R2**; representative example from patient C), or in 24 healthy controls analysed
4 in parallel (**d – R2**; representative example). Example analyses in (**b**), (**c**) and
5 (**d**) are taken from 10 days post-TGN1412 infusion. CD3^{hi}T-cells in the patients
6 with gut symptoms uniformly expressed $\beta 7$ but not CD103/ αE (**e and f**)
7 indicating that these cells displayed the $\alpha 4\beta 7$ heterodimer which mediates
8 homing to the intestine (representative example from patient B). Staining with
9 a specific mAb confirmed that the CD3^{hi} cells were $\alpha\beta$ -TCR⁻ (**g – R3**) but
10 strongly expressed $\gamma\delta$ -TCR (**h**; black histogram - unfilled trace indicates isotype
11 control) and mapped to the CD3^{hi} population observed previously (**a – R1**; black
12 histogram). While total T-cell numbers in the patients were comparable at early
13 time points (**i**) the CD3^{hi} $\gamma\delta$ T-cells subset was present only in patients A, B, and
14 E, and persisted for up to two years post-TGN1412 infusion, decreasing slowly
15 over time (**j**). In patient E, symptoms of gut irritability and diarrhea worsened at
16 month 21, accompanied by an increase in CD3^{hi} $\gamma\delta$ T-cells at month 24 at which
17 time symptoms had improved and he had more formed stool (although still not
18 normal). Peak population size of CD3^{hi} $\gamma\delta$ T-cells (16% of the total T-lymphocyte
19 pool) was reached approximately 1.5 months post-TGN1412 exposure.
20 Between 5-25% of this population displayed an activated (CD69⁺) phenotype
21 that decreased steadily over seven months (**k**). The median values and inter-
22 quartile ranges of data obtained from healthy subjects (n = 24) are provided for
23 reference (horizontal dashed lines).

24 **Fig. 3 The expanded $\gamma\delta$ T-cell population spontaneously produced IL-10**
25 **and expressed IFN γ upon reactivation.** To assess the functional potential of

1 CD3^{hi}β7⁺γδT-cells, whole blood cells were cultured with or without monensin
2 and exogenous stimuli (PMA and ionomycin) for four hours prior to surface
3 labelling and intracellular staining with anti-cytokine monoclonal antibodies for
4 analysis by flow-cytometry. CD3^{hi}CD45RO⁺γδT-cells spontaneously produced
5 low levels of IL-10 in the absence of exogenous stimulation. By 1.5 months
6 post-TGN1412 infusion, a substantial proportion of CD3^{hi}γδT-cells produced
7 IFNγ upon reactivation with PMA and ionomycin. Dotted lines represent median
8 and interquartile range of values obtained from conventional CD4⁺ αβT-cells in
9 all patients (no CD3^{hi} cells were identifiable in healthy volunteers to serve as
10 matched controls).

11

Fig. 1

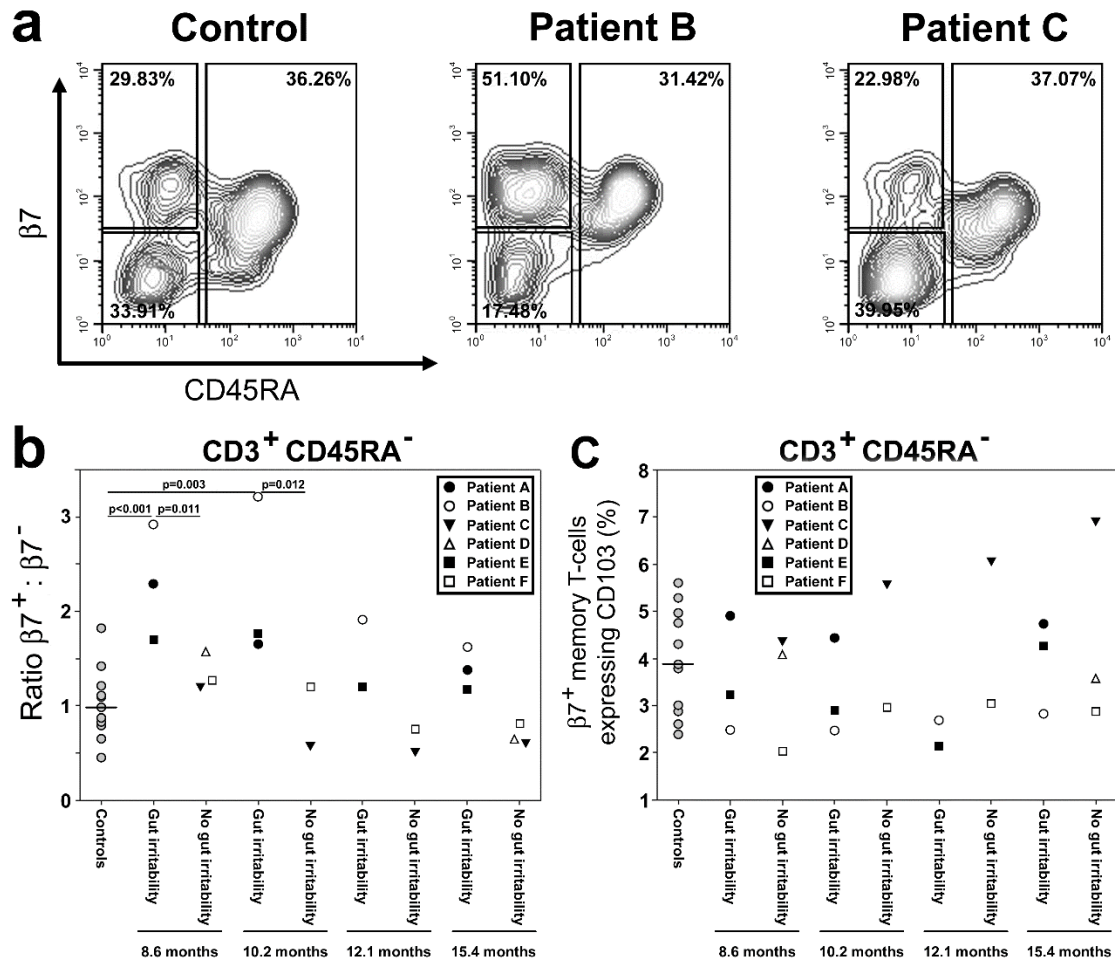


Fig. 2

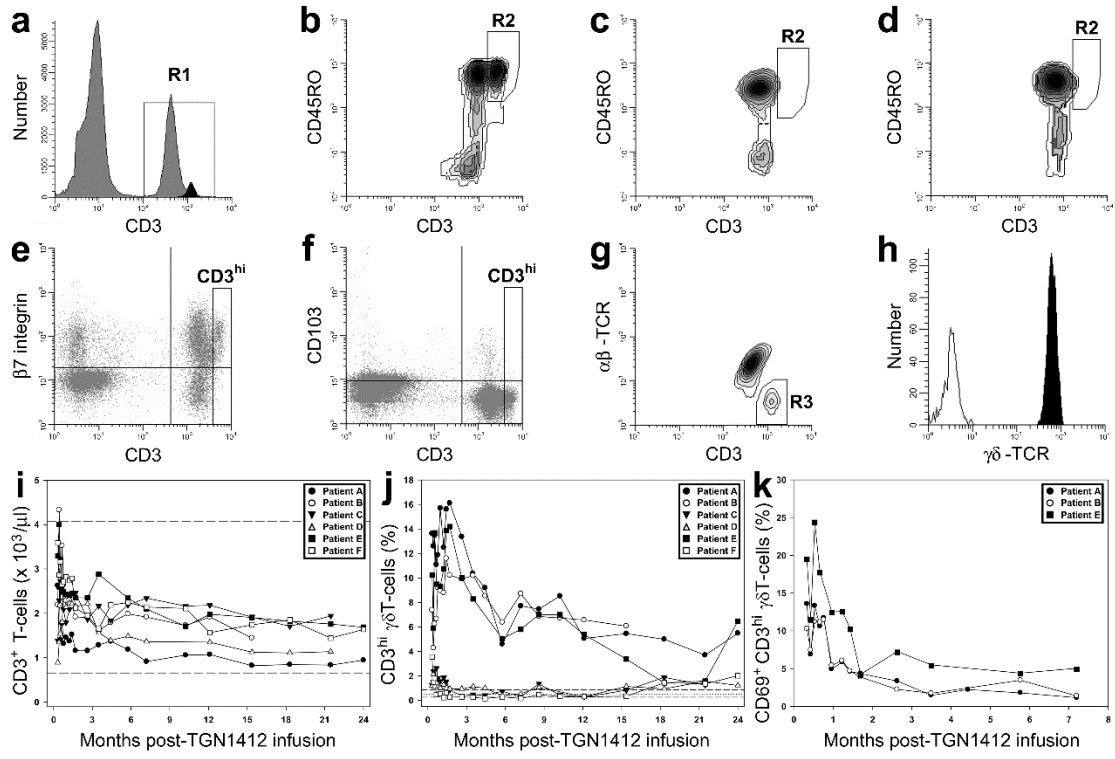
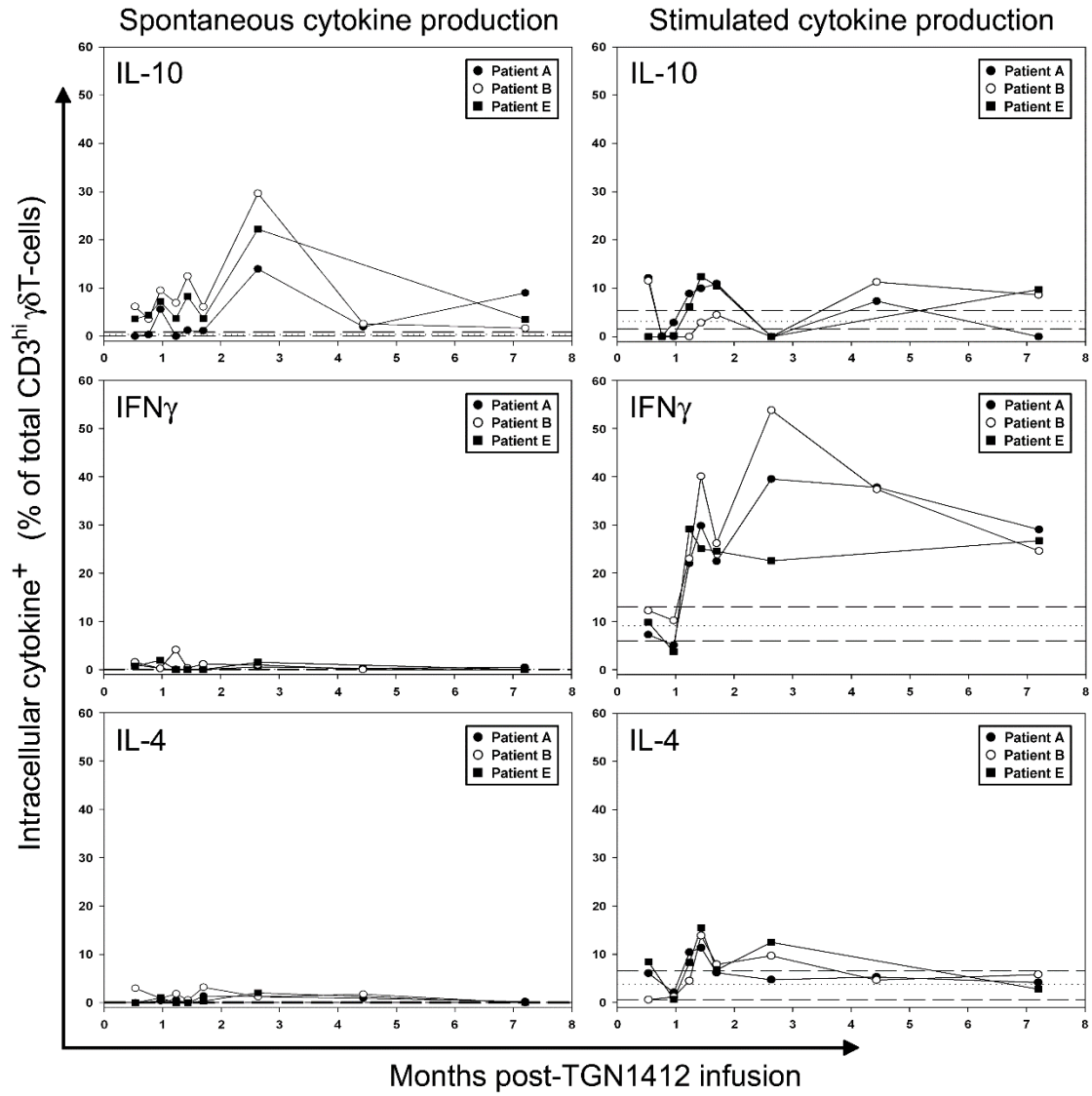


Fig. 3



Supplementary Table 1. Anti-human monoclonal antibodies used for flow cytometry^a.

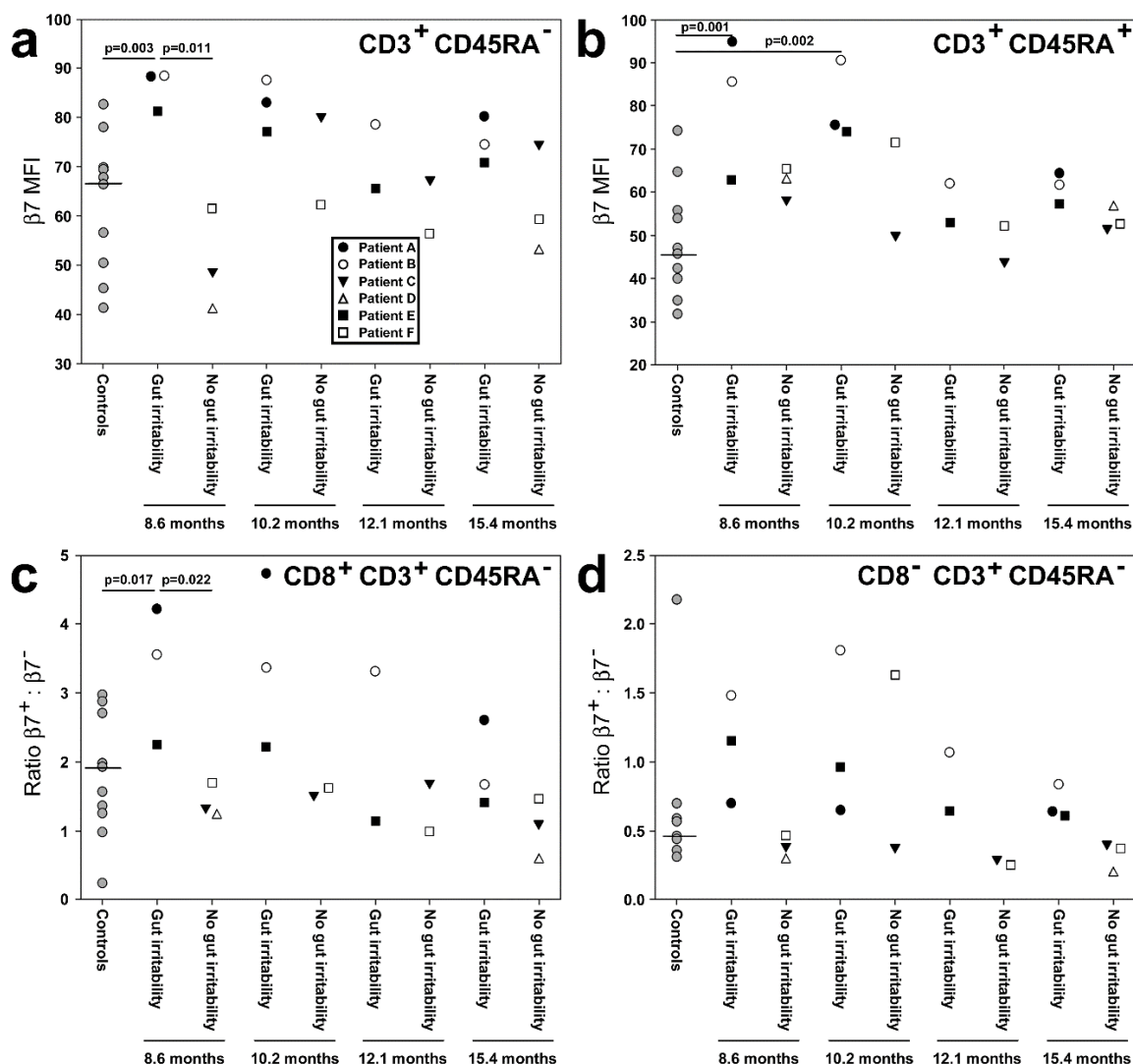
Anti-Human Monoclonal Antibody	Fluorochrome Conjugate Label^c	Clone or catalog number
$\gamma\delta$ -TCR ^b	FITC	11F2
$\alpha\beta$ -TCR ^b	FITC	WT31
CD4	FITC	SK3
CD8	FITC	SK1
CD11c	FITC	KB90
CD25	FITC	ACT-1
CD45	FITC	2D1
CD103	FITC	Ber-ACT8
CD161	FITC	DX12
invariant NKT-cells	FITC	6B11
NKG2D	FITC	1D11
murine IgG1	FITC	X40
Streptavidin	FITC	cat no.554060
CD3	PE	UCHT1
CD4	PE	SK3
CD8	PE	RPA-T8
CD28	PE	L293
CD45RO	PE	UCHL1
CD56	PE	MY31
CD69	PE	L78
CCR5/CD195	PE	2D7
β 7 integrin	PE	FIB504
murine IgG1	PE	X40
murine IgG2a	PE	G155-178
rat IgG2a	PE	R35-95
IFN- γ	PE	D9D10
IL-10	PE	JES3-9D7
IL-4	PE	MP4-25D2
CD3	PE-Cy5	UCHT1
CD8	PE-Cy5	RPA-T8
CD45RA	PE-Cy5	HI100
CD45RO	PE-Cy5	UCHL1
DC exclusion cocktail	PE-Cy5	cat no.MCA2248C
murine IgG2b	PE-Cy5	27-35
CD8	PC5	B9.11
CD4	PerCP	SK3
CD3 ^b	PerCP-Cy5.5	SK7
CD8	PerCP-Cy5.5	SK1
CD3	APC	UCHT1
CD4	APC	RPA-T4

CD8	APC	SK1
CCR9	APC	248621
HLA-DR	APC	L243
murine IgG2a	APC	20102
rat IgG2a	APC	17-4321
CLA	Biotin	HECA-452
IgM	Biotin	R4-22

^aAll mAb were purchased from BD Biosciences apart from CD11c-FITC and CD25-FITC (Dako), NKG2D-FITC (Abcam), IFN γ -PE, IL-10-PE and IL-4-PE, and dendritic cell exclusion cocktail PE-Cy5 (AbD Serotec), CD8-PC5 (Beckman Coulter), rat IgG2a-APC (eBioscience), CCR9-APC and murine IgG2a-APC (R&D Systems).

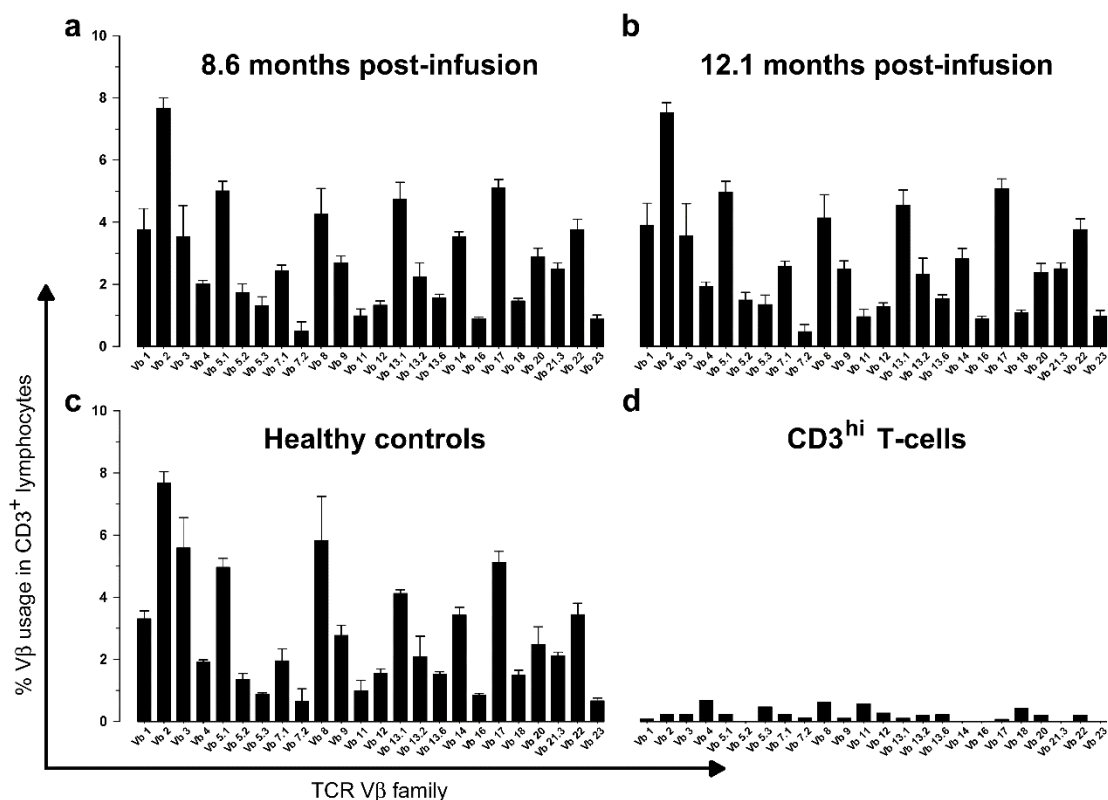
^bTCR $\alpha\beta/\gamma\delta$ /CD3 cocktail was comprised of WT31-FITC, 11F2-PE and SK7-PerCP-Cy5.5.

^cFITC: Fluorescein isothiocyanate; PE: Phycoerythrin; PE-Cy5: Phycoerythrin-Cyanine5.1; PC5: Phycoerythrin-Cyanine5.1; PerCP: Peridinin-chlorophyll-protein Complex Conjugate; PerCP-Cy5.5: Peridinin-chlorophyll-protein Complex CY5.5 Conjugate; APC: Allophycocyanin



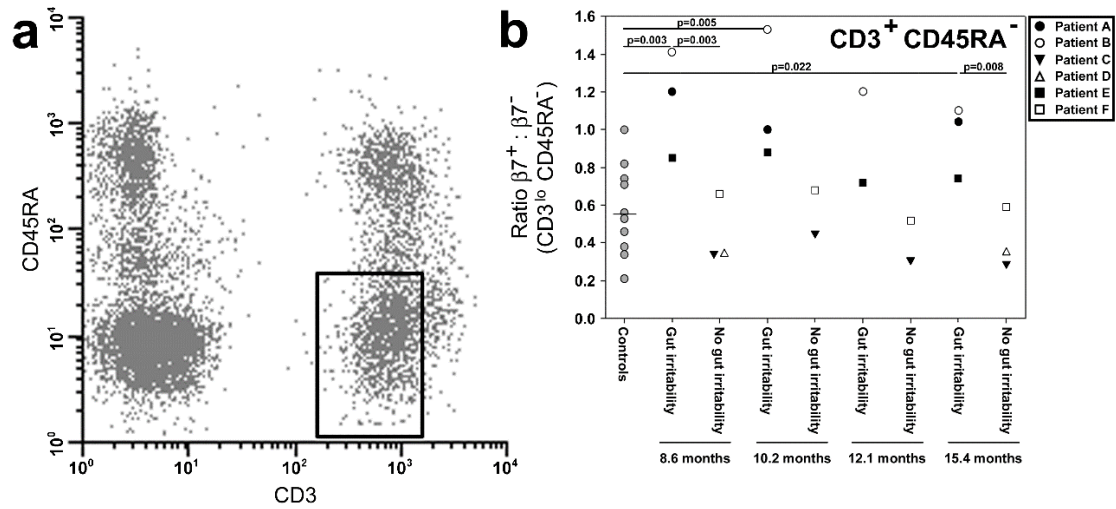
Supplementary Fig. 1 Enhanced integrin $\beta 7$ expression levels on blood T-cells from patients with gut irritability. The level of $\beta 7$ expression, measured as mean fluorescence intensity (MFI) of $\beta 7$ -expressing cells, was determined on $CD45RA^-$ memory T-cells (**a**) and on $CD45RA^+$ naïve T-cells (**b**). The level of $\beta 7$ was increased on both memory and naïve T-cells from patients with gut symptoms at 8.6 months post-infusion. Levels of $\beta 7$ on cells from patients with gut irritability (A, B, and E) remained elevated at 10.2 months following drug administration in the case of naïve T-cells (**b**). However, $\beta 7$ levels on patient T-cells were indistinguishable from those on T-cells from healthy controls by one year post infusion. The ratio of $\beta 7^+ : \beta 7^-$ expression was also

determined separately for CD8⁺ **(c)** and CD8⁻ (putative CD4⁺) **(d)** memory T-cell populations. Changes in both CD8⁺ and putative CD4⁺ memory T-cells contributed to the enhanced expression of β 7 in the affected patients. There was evidence of heterogeneity among the patients with gut symptoms; patient B, who developed the most pronounced and sustained gut symptoms, displayed enhanced β 7 expression in both CD8⁺ and CD8⁻ populations which normalised by 15 months, but such changes were confined to CD8⁺ T-cells only in patient A.



Supplementary Fig. 2 CD3^{hi} T-cells do not express common Vβ segments.

CD3⁺ T-cells in peripheral blood were labelled with monoclonal antibodies against 24 common Vβ segments to assess the TCR repertoire of the CD3^{hi} cells. T-cell Vβ repertoire was assessed at both **(a)** 8.6 months and **(b)** 12.1 months post-TGN1412 infusion. **(c)** The distribution of Vβ segments among patient T-cells was indistinguishable from the profile of six healthy age-matched volunteers analysed in parallel ($p=0.856$). In contrast, only ~5% of CD3^{hi} cells expressed any detectable Vβ segment, suggesting that these might be oligoclonal cells expressing uncommon Vβ chains, or alternatively represented a non- $\alpha\beta$ lineage **(d)**; a representative example from patient A is shown at 12.1 months post-TGN1412 infusion). Error bars indicate standard error of Vβ segment expression.



Supplementary Fig. 3 Expansion of CD3^{hi}γδT-cells does not account for increased β7 expression observed among total CD3⁺ T-cells from patients with gut symptoms. When CD3^{hi}T-cells were excluded from the analysis of CD45RA⁻ memory T-cells **(a)**, increased skewing towards β7 expression was still observed **(b)**.