# Patients with gastrointestinal irritability after TGN1412-induced cytokine storm displayed selective expansion of gut-homing αβ and γδ T-cells

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**Abstract:** 99 words **Manuscript:** 3,260 words

**Figures:** 3

**Supplementary Tables / Figures:** 1 / 3

**References:** 45

**Keywords:**
Cytokine storm
Cytokine Release Syndrome
TGN1412
Vδ2+ γδT-cells
Immunotherapy
Immune-related adverse events (irAEs)

## Declarations

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

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

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

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

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

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

**Code Availability:** Not applicable.

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-Vβ
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.
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16
17

## ABSTRACT

Following infusion of the anti-CD28 superagonist monoclonal antibody TGN1412, three of six previously healthy, young male recipients developed gastrointestinal irritability associated with increased expression of 'gut-homing' integrin β7 on peripheral blood αβT-cells. This subset of patients with intestinal symptoms also displayed a striking and persistent expansion of putative $V\delta2^{+}$ γδT-cells in the circulation which declined over a two-year period following drug infusion, concordant with subsiding gut symptoms. These data demonstrate that TGN1412-induced gastrointestinal symptoms were associated with dysregulation of the 'gut-homing' pool of blood αβ and γδT-cells, induced directly by the antibody and/or arising from the subsequent cytokine storm.

## SIGNIFICANCE

Following TGN1412-induced cytokine storm, 3 of 6 patients developed gastrointestinal irritability associated with expansion of gut-homing αβ and γδT-cells. These findings may elucidate the pathology of immune-related adverse events affecting the gut.

1 **Introduction**

2 In higher primates, the blood T-cell pool contains diverse αβT-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 α4β7 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 β7 aiming to understand the immunological basis for these symptoms. These
15 analyses revealed that blood αβT-cells from patients who suffered from gut
16 irritability displayed significantly enhanced levels of β7 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 γδT-cells (putative phosphoantigen metabolite-responsive Vγ9Vδ2+
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 (Würzberg, 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 β7 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 Vβ repertoire (kit kindly
25 donated by Beckman Coulter), and T-cell homing markers for the

gastrointestinal tract and skin based on expression of β7 integrin and cutaneous leukocyte antigen (CLA), respectively. The bulk of these data are presented elsewhere – this report focuses on the gut-homing subsets. These studies were conducted in a laboratory that operates under Good Laboratory Practice (GLP) principles, undertakes exploratory research and were performed using established laboratory protocols that were Minimal Information About T-cell Assays (MIATA) compliant (Supplementary MIATA information). The assays and reagents employed were previously validated and tested for assay performance during the course of standard general investigative research.

***Flow Cytometry***

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

Viable cells were gated according to their characteristic light-scatter 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 37ºC, 5%$CO_2$. 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%)

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

### *Statistics*

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

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 β7**
15 **expression by circulating αβT-cells**

16 Integrin α4β7 binding to MAdCAM-1 facilitates leukocyte recruitment into
17 intestinal tissues (16). Accordingly, T-cell expression of α4β7 is significantly
18 modulated during active gut inflammation, and inhibition of the α4β7: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 β7 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 β7, 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 $\beta7^+$ and $\beta7^-$ 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 $\beta7^+$
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 β7 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 β7 expression
14 detected in patients with gut irritability (Supplementary Fig. 1c and d). Fewer
15 than 8% of $\beta7^+$ memory T-cells from either patients or controls expressed
16 CD103/$\alpha_E$ integrin, the alternative binding partner for β7 (Fig. 1c), consistent
17 with reports that β7 primarily forms complexes with the α4 subunit on blood T-
18 cells (16), and confirming that the data presented here reflected changes in the
19 patients' $\alpha4\beta7^+$ 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 αβT-
22 cells.
23

**TGN1412-induced gut irritability correlated with peripheral blood expansion of putative Vδ2$^+$ γδT-cells**

In addition to the features outlined above, the blood of patients who developed gut irritability after TGN1412 infusion contained a distinct subset of CD3$^{hi}$ T-cells that was not present in the circulation of either asymptomatic patients or healthy controls (Fig. 2a). These cells displayed a CD4$^-$CD8$^-$ 'double negative' phenotype characteristic of unconventional lymphocytes (data not shown) and expressed uniformly high levels of CD45RO (Fig. 2b) and β7 integrin (Fig. 2e), but lacked CD103 (Fig. 2f) and did not express any of the common Vβ-TCR repertoire variants assayed at 8.6 and 12.1 months following TGN1412 infusion (Supplementary Fig. 2). These features strongly implicated an expansion of 'unconventional' Vγ9Vδ2$^+$T-cells (hereafter Vδ2$^+$T-cells) which express high levels of α4β7 in human blood (3, 19) and are rapidly recruited to mucosal tissues in higher primates *in vivo* (20, 21). Further support for this lineage identity was later provided by the absence of αβ-TCR (Fig. 2g) and lack of markers for natural killer cells (CD56) or invariant natural killer T-cells (antibody 6B11; data not shown), but high expression levels of γδ-TCR (22) (Fig. 2h) as well as NKG2D, and CD161 (23, 24) (data not shown).

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

A, B, and E, the γδT-cell pool reached peak numbers (16% of total T-lymphocytes) approximately 1.5 months post-TGN1412 exposure, followed by a gradual decline coincident with improvement in gastrointestinal symptoms (Fig. 2i and j). Up to 25% of these γδT-cells displayed an activated/CD69$^{+}$ phenotype, and expression of this marker decreased steadily over the following seven months (Fig. 2k). No other clinical or laboratory features correlated with γδT-cell expansion as observed in the three symptomatic patients. Importantly, expansion of gut-homing lymphocytes in the patients with gut irritability was not restricted to γδT-cells alone, because higher numbers of β7$^{+}$T-cells were still detected in these individuals (Fig. 1) when the γδT-cell (CD3$^{hi}$) population was excluded from this analysis (Supplementary Fig. 3).

The IFNγ-producing subset of blood Vδ2$^{+}$T-cells declines naturally with age and is lost more rapidly in men after the age of 30 (25), with both ethnic and environmental variables further impacting on the dynamics of this compartment (26). To determine whether the expanded γδT-cells detected in TGN1412 recipients remained functionally competent, and also to understand how these cells might be contributing to gastrointestinal irritability, we next assessed cytokine expression using a standard intracellular staining approach. The γδT-cell population produced low-levels of IL-10 *in vitro* in the absence of exogenous stimulation (Fig. 3), but did not appear to spontaneously produce either IFNγ or IL-4. However, γδT-cell reactivation with phorbol myristate acetate and ionomycin resulted in substantial production of IFNγ across a wide range of time points analyzed, starting from one month post-infusion, when symptoms of gut-irritability were first identified. Together, these data suggest that development of intestinal symptoms in patients infused with TGN1412 was

1 associated with sustained expansion of circulating Vδ2$^+$T-cells with IFNγ-
2 producing capacity, as well as increased gut-homing potential within the blood
3 αβT-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 $\beta7^{+}\alpha\beta$T-
10 cells (16) were present as expected in all trial patients, but enhanced β7
11 expression levels and the surprising expansion of γδT-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 γδT-cell responses to high levels
15 of cytokines such as TNFα (27) following infusion of TGN1412. The peripheral
16 blood location, kinetics, magnitude, and duration of these γδT-cell expansions,
17 together with uniform expression of CD45RO (28) and high surface levels of
18 β7, strongly implicate the $V\gamma9V\delta2^{+}$T-cell lineage which responds to non-peptide
19 'phosphoantigens' (pAg) derived from microbes and stressed/transformed host
20 cells (29, 30). Indeed, Vγ9Vδ2+ T-cells are already known to undergo rapid
21 polyclonal expansion in the first few weeks of human life, likely driven by pAg-
22 producing bacteria within the gut microbiome, after which the repertoire
23 displays progressive selection of shared or 'public' pAg-reactive clones (defined
24 by characteristic Vγ9JP and Vδ2 chains) (31). With advancing age, the Vδ2+T-
25 cell compartment becomes increasingly oligoclonal, but different individuals

1 may still display diverse or 'private' Vγ9Vδ2+ clonal expansions with distinct
2 effector phenotypes (32), potentially including variable expression of gut
3 homing markers. It is therefore possible that the TGN1412 recipients with gut
4 symptoms (A, B, and E) featured Vδ2+ clonotypes that were absent from the
5 blood of those without (C, D and F), hence these cells may have exhibited
6 different thresholds for pAg activation and intestinal recruitment in the context
7 of cytokine storm. Indeed, rapid expansion of Vγ9JP+ γδ T-cells has also been
8 observed during immune reconstitution of patients receiving allogeneic
9 hematopoietic stem cell transplantation (alloHSCT), but the clonotypes
10 generated after treatment are substantially different from the hosts' pre-
11 transplantation repertoires (33). Patient γδ T-cell repertoire and microbiome
12 composition of pAg-producing bacteria may therefore prove to be important
13 determinants of clinical outcome in future studies of novel immunotherapies.
14 Indeed, while $V\delta2^{+}$T-cells constitute only a minor fraction of total circulating
15 lymphocytes in healthy individuals, their number and activation state in
16 peripheral blood and body tissues have previously been correlated with
17 therapeutic/clinical outcomes (34-36). Work from our own laboratory has also
18 demonstrated that activated blood $V\delta2^{+}$T-cells rapidly up-regulate β7 and can
19 populate human gut lamina propria where they induce substantial mucosal
20 production of IFNγ (3). In patients with Crohn's disease, gut-homing potential
21 and pro-inflammatory properties of $V\delta2^{+}$T-cells are enhanced (36), suggesting
22 that they play a key role in human gut immunity and inflammation.

23 The $V\delta2^{+}$ lineage is absent in rodents and does not recognize antigen in
24 the context of MHC (37). Instead, $V\delta2^{+}$T-cells respond to butyrophilin (BTN)
25 proteins, considered as part of the B7 family of costimulatory receptors (37),

with critical roles recently identified for both BTN3A1 and BTN2A1 (38). $V\delta2^{+}$T-cells lack alloreactivity while displaying potent anti-tumor and anti-microbial functions, such that reconstitution of this lineage after chemotherapy-induced lymphopenia may reduce infection rates in patients receiving hematopoietic stem cell transplantation, without increased incidence of graft versus host-disease (33, 39). Expansion of blood $V\delta2^{+}$T-cells has also been observed in CRS, most notably in healthcare workers exposed to SARS-CoV-1 in the 2003 outbreak; these individuals displayed strikingly similar features including relatively stable αβT-cell numbers and TCR-Vβ repertoire, whereas marked expansions of $V\delta2^{+}$T-cells with IFNγ-producing capacity were still evident three months after disease onset (40). These findings resemble data from nonhuman primate models in which phosphoantigen injection stimulates blood $V\delta2^{+}$T-cell expansion *in vivo* (41), leading to accumulation of an IFNγ-producing subset both in lungs and intestinal mucosa (20), accompanied by robust Th1 immune protection against a range of different pathogens.

$V\delta2^{+}$T-cells undergo expansion in response to a variety of microbial infections and can dominate the blood lymphocyte pool for extended periods (10). It is also now widely recognized that $V\delta2^{+}$T-cells display tissue-tropic phenotypes consistent with trafficking to barrier sites where phosphoantigen-producing microbes and tumors frequently originate (29, 30). In particular, $V\delta2^{+}$T-cells are associated with effective host immunity to phosphoantigen-producing mycobacteria and robust responses to bacillus Calmette-Guérin (BCG) vaccination (42, 43), which induces population expansion and upregulation of CD69 and IFNγ expression *in vitro*. Notably, these responses are enhanced in BCG-responders compared with non-sensitized controls (44,

1 45), and the pool of Vδ2$^+$T-cells generated lacks lymph node homing receptors
2 while displaying homogenous expression of CD28 (45). Therefore, TGN1412
3 may have directly stimulated Vδ2$^+$T-cells in the trial patients, and prior microbial
4 exposures such as BCG may have influenced subsequent responses to
5 mucosal pathogens and/or cytokine storm (as also postulated in the context of
6 COVID-19 (46)). Indeed, while previous studies have primarily linked Vδ2$^+$T-
7 cell expansion with host protection against bacterial pathogens, these
8 lymphocytes can also lyse stressed host cells infected with viruses including
9 influenza (47) and SARS-CoV-1 (40). Together, these data suggest that
10 monitoring of gut-homing αβ and γδT-cell populations is likely to shed important
11 new light on the initiation, propagation, monitoring, and resolution of mucosal
12 symptoms in human subjects with irAEs or suffering CRS as a result of
13 immunotherapy or severe infections such as COVID-19.
14

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31

1 **FIGURE LEGENDS**

2

3 **Fig. 1 Blood T-cell expression of β7 integrin and CD103 following**
4 **TGN1412-induced cytokine storm.** $CD3^+$ T-cells were identified in whole
5 blood and β7 expression on memory ($CD45RA^-$) and naïve ($CD45RA^+$) subsets
6 was assessed. **(a)** Representative data for a healthy control and two patients
7 are shown, one in whom $β7^+$ cells were prominent in the memory T-cell
8 population (patient B) and one in whom $β7^+$ 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 for
11 memory T-cells showing the ratio of $β7^+$:$β7^-$ 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 $β7^+$:$β7^-$ cells was significantly higher at 8.6 and 10.2 months
14 than was observed in healthy controls. **(c)** Proportion of $β7^+$ 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 $β7^+$:$β7^-$ memory
17 T-cells and lowest percentage of $CD103^+$ cells, suggesting a selective
18 expansion of $α4β7^+$ '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 γδT-cells (putative Vδ2+) in**
21 **patients with gut irritability after TGN1412-induced cytokine storm.**
22 Peripheral blood $CD3^+$ T-cells **(a – R1)** included a $CD3^{hi}$ subset (black
23 histogram; identified by γδTCR-specific mAb used in **2h**) which exhibited a
24 $CD4^-CD8^-$ 'double negative' phenotype characteristic of unconventional
25 lymphocytes (data not shown). This discrete population uniformly expressed

CD45RO and was clearly identifiable in the blood of patients A, B, and E **(b – R2**; representative example from patient A), but not in patient C, D, or F **(c – R2**; representative example from patient C), or in 24 healthy controls analysed in parallel (**d – R2**; representative example). Example analyses in **(b)**, **(c)** and **(d)** are taken from 10 days post-TGN1412 infusion. $CD3^{hi}$T-cells in the patients with gut symptoms uniformly expressed β7 but not CD103/αE **(e and f)** indicating that these cells displayed the α4β7 heterodimer which mediates homing to the intestine (representative example from patient B). Staining with a specific mAb confirmed that the $CD3^{hi}$ cells were $\alpha\beta$-$TCR^{-}$ **(g – R3)** but strongly expressed γδ-TCR (**h**; black histogram - unfilled trace indicates isotype control) and mapped to the $CD3^{hi}$ population observed previously **(a – R1**; black histogram). While total T-cell numbers in the patients were comparable at early time points **(i)** the $CD3^{hi}$ γδT-cells subset was present only in patients A, B, and E, and persisted for up to two years post-TGN1412 infusion, decreasing slowly over time **(j)**. In patient E, symptoms of gut irritability and diarrhea worsened at month 21, accompanied by an increase in CD3hi γδT-cells up to month 24, at which time symptoms had started to improve and he had more formed stool (although still not normal). Peak population size of $CD3^{hi}$ γδT-cells (16% of the total T-lymphocyte pool) was reached approximately 1.5 months post-TGN1412 exposure. Between 5-25% of this population displayed an activated ($CD69^{+}$) phenotype that decreased steadily over seven months **(k)**. The median values and inter-quartile ranges of data obtained from healthy subjects (n = 24) are provided for reference (horizontal dashed lines).

**Fig. 3 The expanded γδT-cell population spontaneously produced IL-10 and expressed IFNγ upon reactivation.** To assess the functional potential of

1 $CD3^{hi}\beta7^{+}\gamma\delta$T-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. $CD3^{hi}CD45RO^{+}\gamma\delta$T-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 $CD3^{hi}\gamma\delta$T-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).

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