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The role of CD8^{+} T cell clones in immune thrombocytopenia

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Abstract:
Immune thrombocytopenia (ITP) is traditionally considered an antibody-mediated disease. However, a number of features suggest alternative mechanisms of platelet destruction. In this study, we use a multi-dimensional approach to explore the role of cytotoxic CD8^{+} T cells in ITP. We characterised patients with ITP and compared them to age-matched controls using immunophenotyping, next-generation sequencing of T cell receptor (TCR) genes, single-cell RNA sequencing, and functional T cell and platelet assays. We found that adults with chronic ITP have increased polyfunctional, terminally differentiated effector memory CD8^{+} T cells (CD45RA^{+}CD62L^{-}) expressing intracellular interferon-g, tumour necrosis factor-a, and Granzyme B defining them as TEMRA cells. These TEMRA cells expand when the platelet count falls and show no evidence of physiological exhaustion. Deep sequencing of the T cell receptor showed expanded T cell clones in patients with ITP. T cell clones persisted over many years, were more prominent in patients with refractory disease, and expanded when the platelet count was low. Combined single-cell RNA and TCR sequencing of CD8^{+} T cells confirmed that the expanded clones are TEMRA cells. Using *in vitro* model systems, we show that CD8^{+} T cells from patients with ITP form aggregates with autologous platelets, release interferon-g and trigger platelet activation and apoptosis through TCR-mediated release of cytotoxic granules. These findings of clonally expanded CD8^{+} T cells causing platelet activation and apoptosis provide an antibody-independent mechanism of platelet destruction, indicating that targeting specific T-cell clones could be a novel therapeutic approach for patients with refractory ITP.

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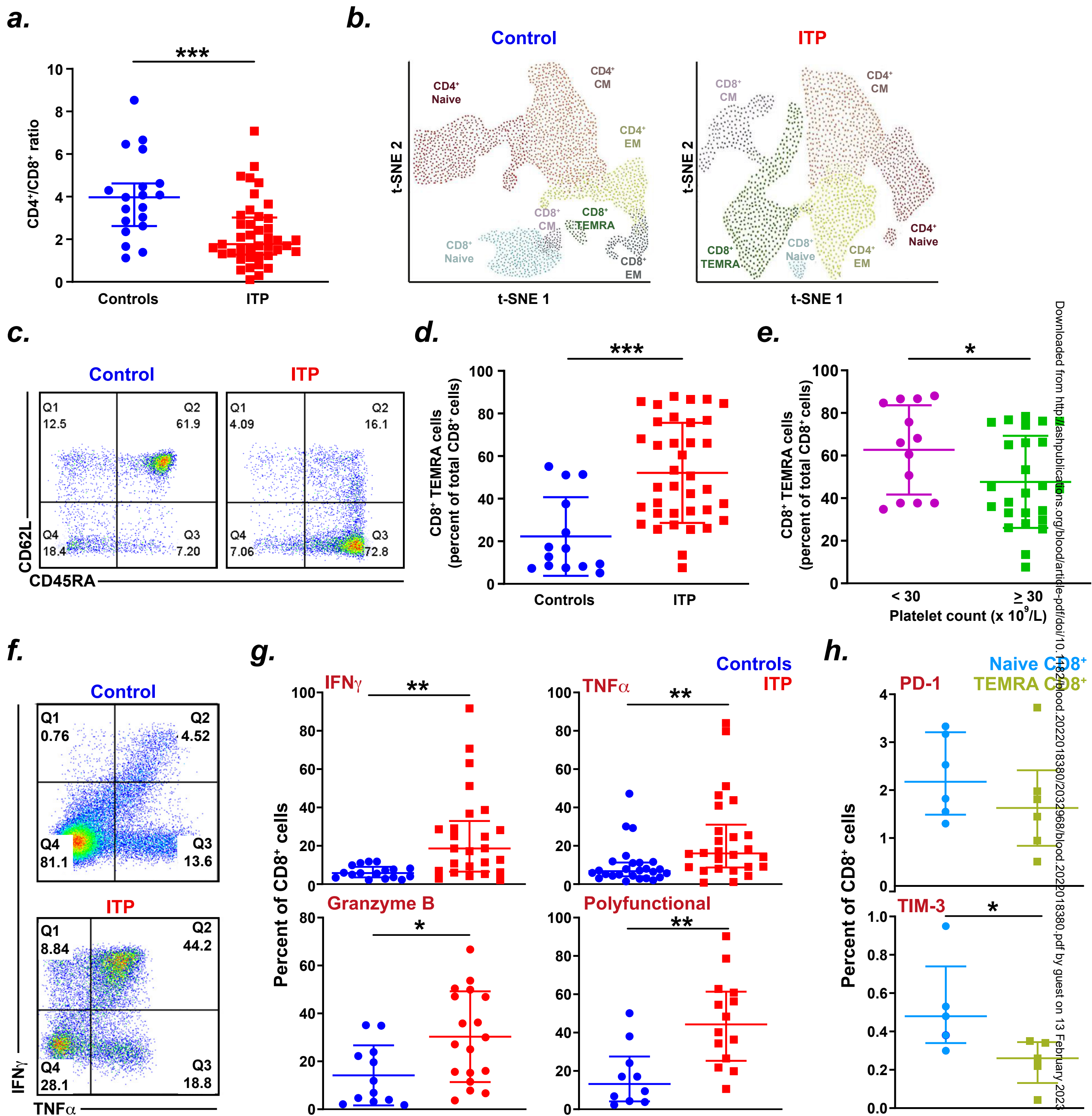

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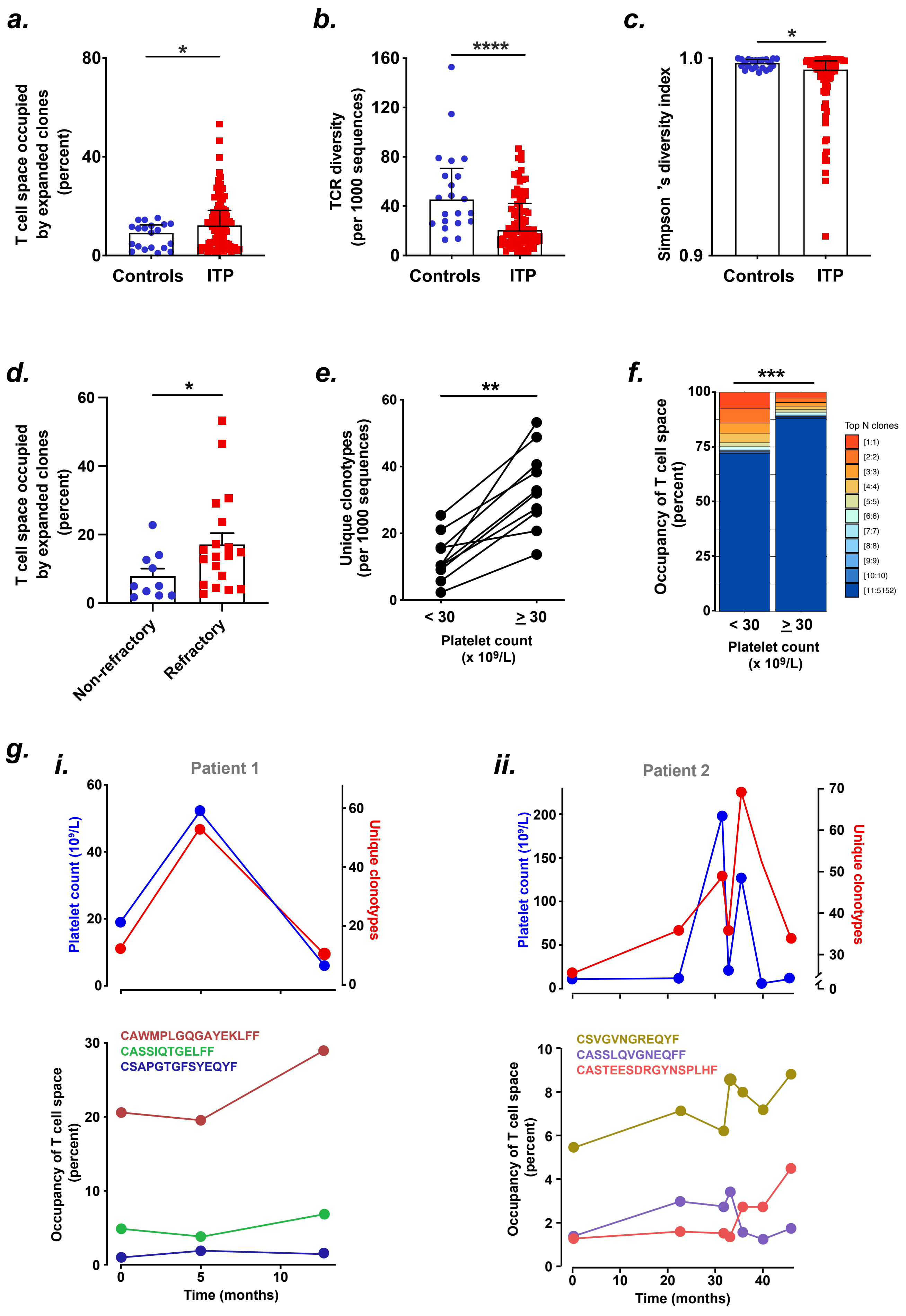

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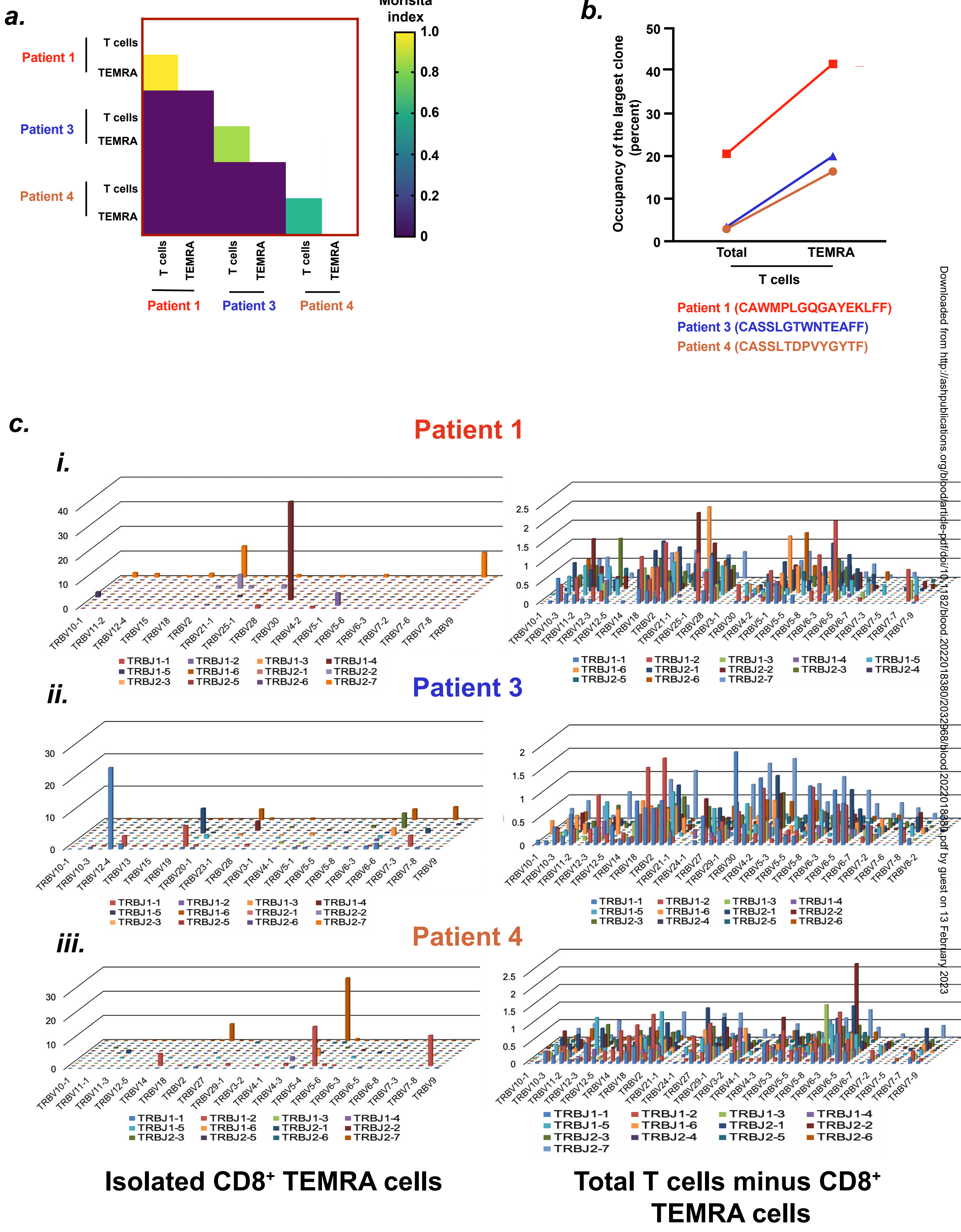
Figure 3



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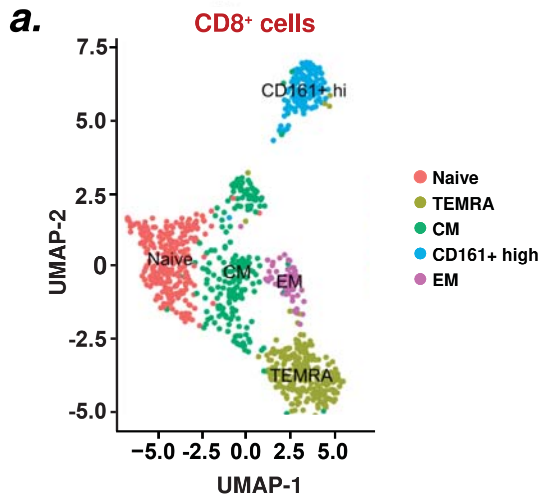

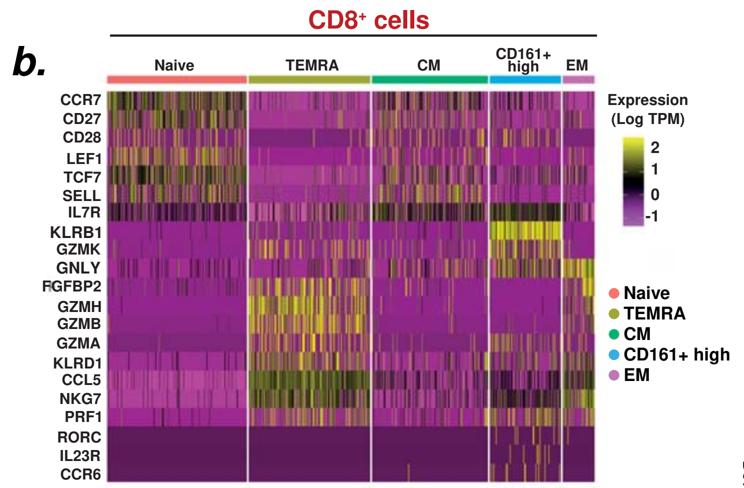

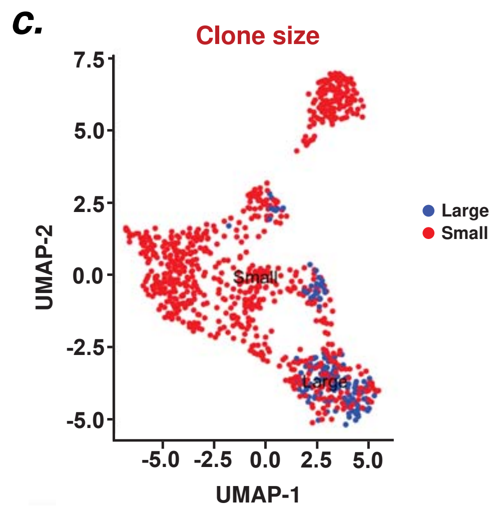

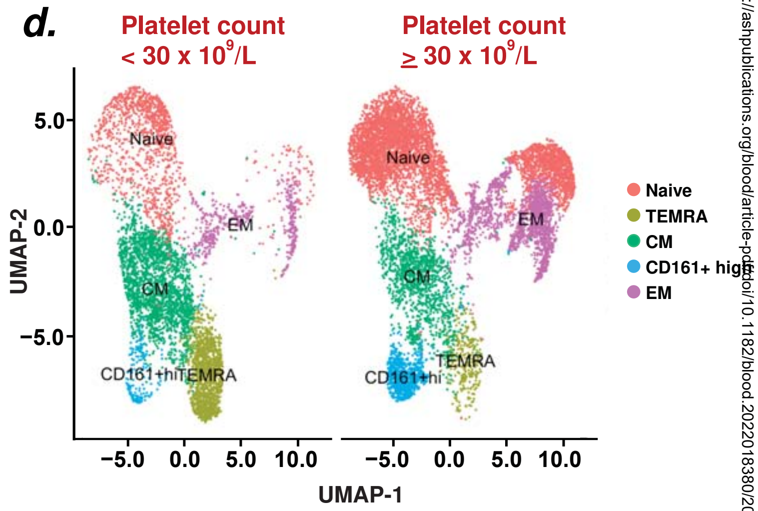

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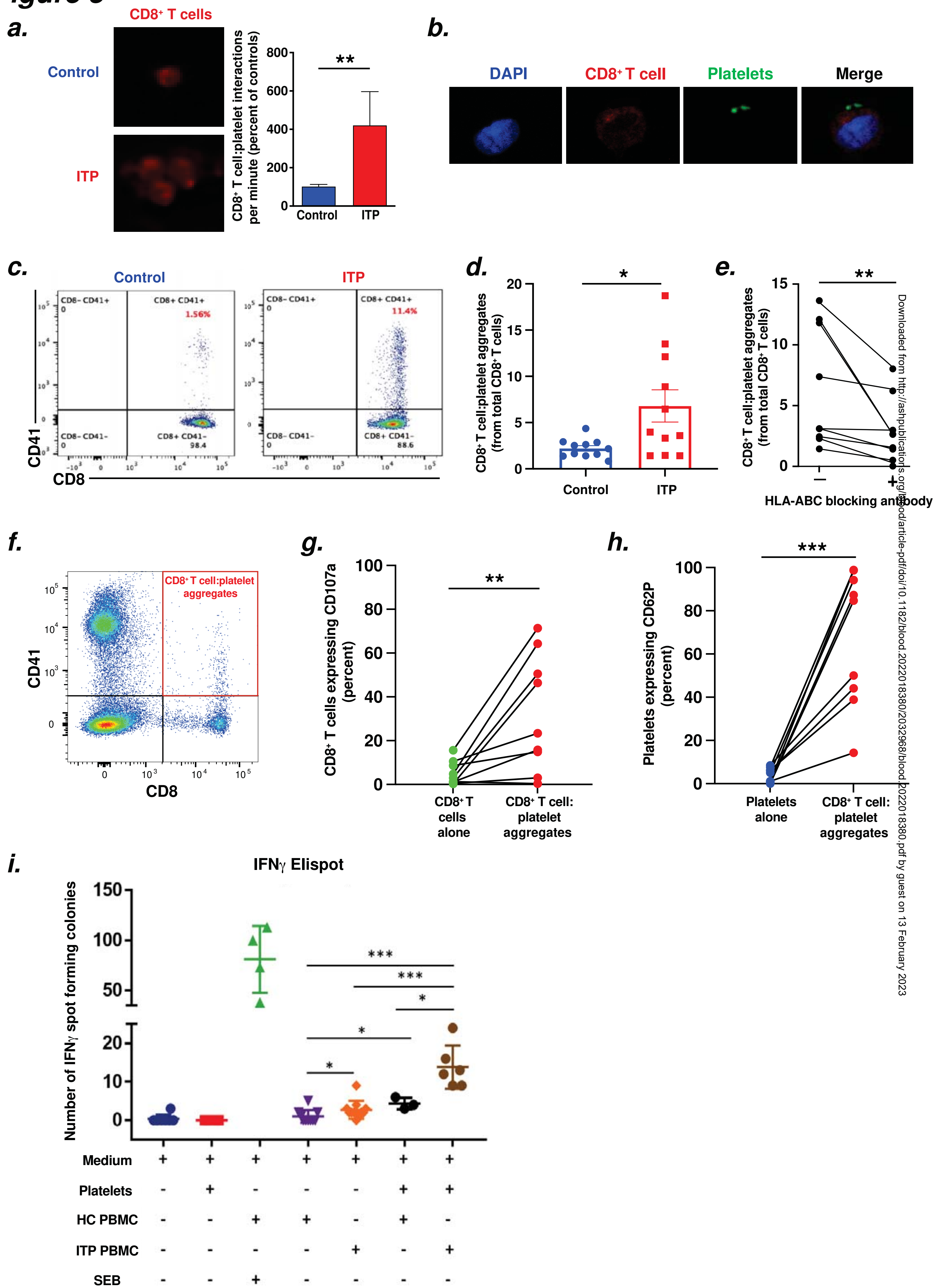
Figure 5



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1 **Article Title: The role of CD8$^+$ T cell clones in immune thrombocytopenia**

2 **Short Title: CD8$^+$ T cell clones in immune thrombocytopenia**

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39 **Key Points**

40 1. Patients with chronic ITP had clonal expansions of disease-associated
41 terminally differentiated effector memory (TEMRA) $CD8^+$ T cells

42 2. $CD8^+$ T cells bind to platelets and cause platelet activation and apoptosis
43 defining an antibody-independent mechanism of platelet destruction

44

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45 **Abstract**

46 Immune thrombocytopenia (ITP) is traditionally considered an antibody-mediated
47 disease. However, a number of features suggest alternative mechanisms of platelet
48 destruction. In this study, we use a multi-dimensional approach to explore the role of
49 cytotoxic $CD8^+$ T cells in ITP. We characterised patients with ITP and compared
50 them to age-matched controls using immunophenotyping, next-generation
51 sequencing of T cell receptor (TCR) genes, single-cell RNA sequencing, and
52 functional T cell and platelet assays. We found that adults with chronic ITP have
53 increased polyfunctional, terminally differentiated effector memory $CD8^+$ T cells
54 ($CD45RA^+CD62L^-$) expressing intracellular interferon-γ, tumour necrosis factor-α,
55 and Granzyme B defining them as TEMRA cells. These TEMRA cells expand when
56 the platelet count falls and show no evidence of physiological exhaustion. Deep
57 sequencing of the T cell receptor showed expanded T cell clones in patients with
58 ITP. T cell clones persisted over many years, were more prominent in patients with
59 refractory disease, and expanded when the platelet count was low. Combined single-
60 cell RNA and TCR sequencing of $CD8^+$ T cells confirmed that the expanded clones
61 are TEMRA cells. Using *in vitro* model systems, we show that $CD8^+$ T cells from
62 patients with ITP form aggregates with autologous platelets, release interferon-γ and
63 trigger platelet activation and apoptosis through TCR-mediated release of cytotoxic
64 granules. These findings of clonally expanded $CD8^+$ T cells causing platelet
65 activation and apoptosis provide an antibody-independent mechanism of platelet
66 destruction, indicating that targeting specific T-cell clones could be a novel
67 therapeutic approach for patients with refractory ITP.

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70 **Introduction**

71 Immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterised
72 by thrombocytopenia with increased morbidity and mortality due to bleeding, fatigue
73 and treatment-related complications[1–4]. International guidelines highlight a lack of
74 diagnostic and prognostic markers; limited data to guide treatment decisions; and
75 heterogeneity of responses to treatment[2,5,6].

76 The first biological studies in ITP focused on the role of autoantibodies, with passive
77 transfer experiments demonstrating a pathogenic role for autoantibodies against
78 platelet surface antigens[7–9]. Drug discovery efforts have therefore focused on
79 suppressing aberrant humoral immunity through B cell depletion (by targeting CD20
80 or B cell activating factor)[10,11], disrupting immunoreceptor signalling (by blocking
81 SYK[12] or BTK[13]), and inhibiting autoantibody activity (through the use of steroids,
82 intravenous immunoglobulin or neonatal Fc receptor inhibition)[14].

83 Nonetheless, antibody-independent mechanisms of thrombocytopenia such as T
84 cells are likely to play an important role in ITP since: anti-platelet antibodies are
85 difficult to detect in many patients[15]; they do not predict response to treatment[16]; B
86 cell-directed therapies are not effective in many patients[17]; and a proportion of
87 patients remain refractory to all existing therapies, suggesting other mechanisms of
88 disease. While abnormalities in $CD4^+$ T cells with a skew to Th1[18–20] and abnormal
89 number and function of T regulatory cells (Tregs)[20] are thought to drive the
90 autoimmune process, the role of $CD8^+$ T cells remains unclear.

91 Cytotoxic $CD8^+$ T cells were first implicated in in ITP in 2003[21], and murine models of
92 ITP subsequently suggested that $CD8^+$ T cells contribute to thrombocytopenia *in*
93 *vivo*[22,23]. However, the nature or importance of $CD8^+$ T cells in patients with ITP is
94 not known, and the role of platelet-specific $CD8^+$ T cells have not been characterised
95 in humans[24–28].

96 We therefore pursued several orthogonal approaches to identify $CD8^+$ T cell clones
97 and explore $CD8^+$ T cell mediated platelet destruction in patients with ITP.

98

99 **Methods**

100 **Patient recruitment**

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101 Patients were recruited from the Imperial College NHS Trust ITP centre or the Weil
102 Medical College of Cornell University, New York Presbyterian Hospital New York.
103 Patients were included if they had been diagnosed with primary ITP based on
104 established criteria[1]. Subjects with secondary ITP were excluded (patients were all
105 screened for HIV, hepatitis C, hepatitis B, ANA, blood film or flow analysis when
106 indicated). A total of 83 patients and 51 age-matched healthy controls were included
107 in the study.

108 We categorised patients on their clinical phenotype to correlate findings with severity
109 of disease. We used the following definitions: A platelet count of less than 30 x 10^9/L
110 was considered more active disease as this is the platelet count recommended for
111 treatment in most international guidelines. Patients with chronic ITP lasting more
112 than 1 year and who were refractory to at least two prior therapies were defined as
113 refractory.

114 The study was done in accordance with The Multi Centre Research Ethics
115 Committee in Wales guidelines MREC Wales reference 07/MRE09/54: R12039,
116 R12033 and the Institutional Review Board for the Weil Cornell Medicine, New York.
117 Written consent was obtained for all participants in the study.

118 Peripheral blood mononuclear cells (PBMCs) and clinical metadata were taken
119 during clinic visits (not all samples were used for each experiment).

120 **PBMC Preparation**

121 18mL of venous blood was collected into lithium heparin vacutainers (BD
122 Biosciences, USA) and diluted with Dulbecco's phosphate-buffered saline (DPBS;
123 Sigma-Aldrich, USA) at a ratio of 1:1, and layered on top of Histopaque®-1077
124 (Sigma-Aldrich, USA) in SepMate™-50 (IVD) tubes (STEMCELL Technologies,
125 Canada). After centrifugation for 15 minutes at 1200g, the upper layer containing
126 plasma and PBMCs were washed twice with DPBS. PBMCs were then counted
127 using Trypan Blue (TB; Gibco, UK).

128 **Platelet Preparation**

129 Venous blood collected into 2.7mL trisodium citrate vacutainer was centrifuged
130 immediately at 100g for 20 minutes to obtain the platelet-rich plasma (PRP). PRP
131 was supplemented with 75mU/mL of Apyrase (Sigma-Aldrich, USA), 100nM of

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132 Prostaglandin E1 (PGE1; Sigma-Aldrich, USA), and 10%/V Trisodium citrate (ACD),
133 and further centrifuged at 1500g for 10 minutes to remove platelet-poor plasma
134 (PPP). Isolated platelet pellets were then washed with 5mL HEPES-Tyrode buffer
135 supplemented with 3.5mg/mL bovine serum albumin (HT-BSA; Sigma-Aldrich, USA),
136 PGE1 and Apyrase. Total platelet count was determined using flow cytometry.

137 **PBMC surface and intracellular staining**

138 T cell subsets were described based on the surface expression of CD45RA and
139 CD62L dividing the cells into four subsets; naïve ($CD45RA^+CD62L^+$), terminally-
140 differentiated effector memory or TEMRA ($CD45RA^+CD62L^-$), central memory (CM;
141 $CD45RA^-CD62L^+$) and effector memory (EM; $CD45RA^-CD62L^-$) (*Supplementary*
142 *Data Figure 1*).

143 Cells were stimulated with eBioscience Cell Stimulation Cocktail
144 (ThermoFisher Scientific), fixed and permeabilised using staining buffer set
145 (ThermoFisher Scientific) for intracellular cytokine staining. Cells were stained with
146 conjugated antibodies against Granzyme B, interferon-γ (IFNγ), interleukin 2 (IL-
147 2) (BD Bioscience) and tumour necrosis factor-α (TNFα) (BioLegend).

148 Stained cells were analysed using BD LSR II cytometer (BD Bioscience). BD
149 FACSDIVA software (BD Bioscience) was used to acquire events on the cytometer
150 and FlowJo software (Tree Star) and FCS Express 6 Flow Cytometry Research
151 Edition (De Novo Software) were used to analyse acquired data. 32 patients were
152 included in the immunophenotype analysis.

153 **DNA-Based TCR sequencing**

154 Next generation sequencing of the T-cell receptor β gene (*TRB*) was carried out as
155 previously described[29]. A total of 70 patients from London (n=38) and New York
156 (n=32) were included. Briefly, genomic DNA was extracted from patients' PBMC or
157 FACS-sorted TEMRA cells samples using DNeasy Blood & Tissue kit (Qiagen, UK),
158 quantified using a Qubit Fluorometer (ThermoFisher Scientific) and amplified by
159 multiplex-PCR of rearranged variable, diverse, joining (VDJ) segments of the *TCR*
160 genes, which encode the hypervariable CDR3 domain. The products were size
161 selected using Pronex beads (Promega, UK) and sequenced on a MiSeq (Illumina,
162 UK).

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163 Analysis of raw *TCR* sequences was performed using MiXCR[30]. VJ gene usage was
164 evaluated using VDJ tools. Clonal expansion, TCR Diversity (D), Simpson's Diversity
165 index and similarity of the TCR repertoire were calculated using tcR package
166 supported in R and numpy package supported in python.

167 Morisita-Horn similarity-index (CMH) was used to explore TCR sharing in ITP
168 patients. Pairwise comparisons were performed between all patients to measure
169 compositional similarity or overlap. CMH values lie between 0 (no overlap) and 1
170 (perfect overlap). The CMH value for every combination of two patients represents a
171 total of 2,485 pairwise combinations.

172 Samples were taken at multiple time points for TCR sequencing in 9 patients,
173 including two followed over a number of years. Clinical data for these patients are
174 characterised in *Supplementary Data Table 1* (Patient 1-9).

175 TCR sequencing was also performed on TERMA cells isolated based on their
176 surface expression ($CD45RA^{+}CD62L^{-}$) and compared to whole PBMCs in the same
177 individuals using CMH.

178 **Single cell immune profiling (RNA-seq and TCR-seq) library construction and**
179 **sequencing**

180 $CD8^{+}$ T cells from patients with expanded T cell clones were FACS sorted using a
181 FACSAria flow cytometer (Becton Dickinson, Mountain View, California,
182 USA). Library constructions were performed using Chromium system (10x
183 Genomics, Chromium Single Cell V(D)J and 5' Library kits). Reverse transcription,
184 amplification, and library preparation of both 5′ transcriptome and V(D)J libraries
185 were performed per published protocols (10x Genomics). The constructed library
186 was sequenced on the HiSeq platform (Illumina) with 150 × 2 paired-end reads for
187 gene expression and TCR libraries, respectively. With sequencing, we obtained
188 gene expression from 15,431 of 17,000 cells and additional TCR clonotype from
189 63.9% of these cells. A minimum of 8,384 reads per cell were collected for gene
190 expression and 6,939 reads for TCR profiling, respectively.

191 **ScRNA Analysis**

192 Cell Ranger (v.3.0.2) performed sample de-multiplexing, barcode processing and
193 single-cell 5′ unique molecular identifier (UMI) counting. The Cell
194 Ranger mkfastq pipeline was used to demultiplex the Illumina Base Call files into

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195 FASTQ files and aligned to the GCRhg38 genome. Cell Ranger count was applied to
196 each FASTQ file to produce a feature barcoding and gene expression library. Seurat
197 v.2.3.4 was used for gene expression analysis. The following criteria were applied to
198 obtain gene-barcode matrix: gene number between 313 and 2,500 and mitochondrial
199 gene percentage ≤ 10%. After filtering 7,391 cells were left for analysis.

200 **Functional Assays**

201 Functional assays were performed on patients with chronic ITP lasting more than 1
202 year which was refractory to at least two prior therapies, and who had expanded T
203 cell clones.

204 VenaFluoro8+ microchips (Cellix, Ireland) were coated with human von Willebrand
205 factor (VWF) to enable capture of platelets onto the microfluidic channels. Fresh
206 citrated whole blood from healthy individuals and ITP patients was stained with
207 DiOC6 (2.5μM) to label platelets and perfused on the microchip channel at 1,000s^{-1}
208 for 3.5 minutes using a Mirus Evo Nanopump and Venaflux64 software (Cellix) to
209 capture a monolayer of unactivated platelets, as previously described[31]. Isolated
210 PBMC or blood lysed with red blood cell lysis buffer (Biolegend) from the same
211 individual were stained with a conjugated anti-human $CD8^+$ antibody and perfused
212 on the autologous platelet-covered channels at 50s^{-1} for 5-10 minutes. Interactions
213 between $CD8^+$ T cells and platelets were monitored in real-time using an inverted
214 fluorescent microscope (Zeiss, Germany) or a SP5 confocal microscope (Leica,
215 Germany). The number of $CD8^+$ T cell:platelet interactions per minute was
216 derived by counting cells in one field of view over 5-10 minutes.

217 **$CD8^+$ T cell-platelet *ex vivo* co-culture system**

218 $CD8^+$ T cells were negatively selected from PBMC using Mojosort Human CD8 T cell
219 Isolation Kit (BioLegend). Autologous platelets were obtained from citrated whole
220 blood. $CD8^+$ T cells were co-cultured with autologous platelets at a ratio of 1:5
221 overnight, as previously described[21,32,33] at 37˚C/5% CO_2 in complete medium before
222 being harvested and stained with antibodies against CD8, CD41, CD107a and
223 CD62P (BioLegend). For MHC class I blocking assays, freshly isolated platelets
224 were incubated with 20μg/mL of purified anti-human HLA-A,B,C (BioLegend) for 15
225 minutes in 37˚C/5% CO_2 prior to co-culture with $CD8^+$ T cells.

226 **Confocal Imaging of $CD8^+$ T cell-platelet aggregates**

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227 After overnight co-culture, $CD8^+$ T cell-platelets were prepared for confocal imaging.
228 Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at 37˚C/5% CO_2,
229 washed and blocked with 2% bovine serum albumin (BSA, ThermoFisher Scientific)
230 for 1 hour. Cells were stained with purified anti-human CD42b mAb (BioLegend),
231 followed by goat anti-mouse IgG (H+L)-Alexa Fluor Plus 555 (ThermoFisher
232 Scientific) before staining for Alexa Fluor 594-conjugated anti-human CD8a
233 (BioLegend). Nucleic acid staining of cell was performed using Hoechst-33342
234 (ThermoFisher Scientific) and mounted in ProLong Glass Antifade Mountant
235 (ThermoFisher Scientific) on poly-L-lysine coated glass slides (Sigma Aldrich, UK).
236 Cells were analysed using a Stellaris 8 Inverted Confocal Microscope (Leica, UK).

237 **IFNγ Enzyme-Linked ImmunoSpot (ELISpot) Assay**

238 IFNγ ELISpot assay was performed by co-culturing PBMCs both with and without
239 platelets in a sterile condition on Millipore 96-well PVDF plates (ThermoFisher
240 Scientific) as per manufacturer's instructions (Mabtech, Sweden). Plates were
241 analysed using Zeiss Compact ELISpot Reader (Zeiss, Germany).

242 **Statistical analysis**

243 The distribution of the data in different data sets were determined using Shapiro Wilk
244 test. Unpaired and paired data sets were compared using two-tailed Mann-
245 Whitney *U* and Wilcoxon matched-pairs tests, respectively. For more than 2 groups,
246 Kruskal-Wallis test was used, and Bonferroni multiplicity correction was applied.
247 Strength of association between two variables was analysed by Spearman
248 (parametric) and Pearson (non-parametric) correlation.
249

250 All statistical analyses were performed using GraphPad Prism 7 (GraphPad, USA)
251 and a P value (P) of less than 0.05 was considered significant.

252 **Data Sharing Statement**

253 The raw FastQ files are deposited at the Short Read Archive
254 (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA930724.
255

256 **Results**

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257 Patient details are summarised in Table 1 (further clinical metadata, where available
258 is included in *Supplementary Data Table 1*). Platelet counts were taken at the time of
259 sample collection.

260 Compared to age-matched controls (n=19), patients with chronic ITP (n=32) had
261 lower $CD4^+/CD8^+$ ratios (1.77 vs 3.97; P ≤ 0.001; Figure 1a). $CD4^+$ and $CD8^+$ cells
262 were further analysed by quantifying surface CD45RA and CD62L expression
263 (*Supplementary Data Figure 1*). Using t-distributed Stochastic Neighbour Embedding
264 (tSNE) the most significant differences between patients and controls were an
265 expanded population of terminally-differentiated $CD8^+$ T cells, known as TEMRA
266 cells[34] (Figure 1b). TEMRA cells are polyfunctional cells, expressing multiple
267 inflammatory cytokines including TNFα, IFNγ and Granzyme B. They re-express
268 CD45RA and lose the expression CD62L ($CD45RA^+CD62L^-$). These cells express
269 high levels of CD57 and low CD27 and CCR7 (*Supplementary Data Figure 1*).

270 TEMRA cells were higher in patients with ITP compared to controls (66.30% vs.
271 8.56%; P ≤ 0.001; Figure 1b-d & *Supplementary Data Figure 1*). Correspondingly,
272 patients with ITP had reduced naive $CD8^+$ T cells compared to controls (19.85% vs.
273 56.06%, P ≤ 0.001). TEMRA cells were further increased in patients with more active
274 disease with a platelet count of less than 30 x 10^9/L when compared to those with
275 platelet counts equal or greater than 30 x 10^9/L; (66% vs. 44%; P ≤ 0.05; Figure 1e).

276 $CD8^+$ T cells from patients with ITP had increased TNFα (15.8% vs. 7.9%; P ≤ 0.01),
277 IFNγ (28.9% vs. 6.6%; P ≤ 0.01), and Granzyme B levels (28.6% vs. 9.4%; P ≤ 0.05).
278 Polyfunctional $CD8^+$ T cells expressing the combination of IFNγ, TNFα and
279 Granzyme B were substantially higher in patients with ITP than controls (44.3% vs.
280 13.2%; P ≤ 0.01; Figure 1f-g). IL-2-expressing $CD8^+$ T cells were also increased
281 (26.4% vs. 5.5%; P ≤ 0.01) and the highest cytokine levels were found within the
282 TEMRA cells.

283 TEMRA cells had reduced Tim-3 when compared to naïve $CD8^+$ T cells (0.26% vs.
284 0.48%; P ≤ 0.05) and unchanged PD-1 expression. Such low/unchanged expression
285 of these molecules indicates their continued activation with no evidence of
286 progressing into T cell exhaustion[35,36] (Figure 1h).

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287 To establish whether these expanded cells were clonal in nature, we sequenced the
288 T cell receptor (TCR) using Illumina Miseq platform, allowing us to identify single T
289 cell clones and look for shared clones that target a common antigen.
290 We observed a higher number of T cell clones occupying more than 5% of the T cell
291 repertoire in patients with ITP (n=70) when compared to age-matched controls
292 (n=21) ($P \leq 0.05$; Figure 2a). This expansion of T cell clones was associated with
293 reduced T cell repertoire diversity ($P \leq 0.0001$) and lower Simpson's diversity index
294 ($P \leq 0.05$) in ITP compared to age-matched controls (Figures 2b-c). This is
295 consistent with established models of T cell homeostasis: expansion of a clone
296 occurs at the expense of overall T cell repertoire diversity, thus maintaining
297 approximately static T cell numbers[37]. Patients from London and New York were
298 similar in terms of their T cell repertoire diversity (median of 21 vs 20, respectively)
299 and Simpson's diversity index (median of 0.9938 vs 0.9959, respectively).

300 We found no evidence for viral driven clonal expansion: all patients were negative for
301 known viral causes of ITP (HIV, hepatitis B and C) and 791 out of 815 (97%) of the
302 top 10 clones from each patient were private clones (unique to individual patients)
303 with no known viral, bacterial, autoimmune or tumour antigen specificities when
304 mapped to available databases of TCR sequences[38].

305 Patients with ITP showed no over-representation of any Vβ family or any shared
306 clones across patients: the average Morisita-Horn similarity-index (CMH) value was
307 0.0005 indicating that ITP patients had about 0.05% similarity in their TCR repertoire
308 compared to 0.1% similarity found amongst controls. When we compared the top 10
309 clones across all ITP patients, there were neither any shared T cell clones across
310 patients nor any CDR3 motif enrichment in the expanded clones of patients
311 indicating that expanded T cell clones are not driven by the same antigenic
312 exposure.

313 Patients with more refractory disease (chronic ITP lasting more than 1 year which
314 was refractory to at least two prior treatments) had higher number of T cell clones
315 occupying more than 5% of the T cell repertoire (median of 13.8% vs 4.9%, $P \leq 0.05$;
316 Figure 2d). In one patient with refractory ITP (Patient 1), an individual T cell clone
317 occupied more than 30% of the T cell repertoire (Figure 2gi).

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318 Longitudinal samples were analysed in 9 patients. Paired analysis of T cell clones
319 when platelet counts were < 30 x 10^9/L compared to platelet counts ≥ 30 x 10^9/L
320 showed expanded clones and reduced T cell repertoire diversity when the platelet
321 count was lower (P ≤ 0.01, Figure 2e; P ≤ 0.001, Figure 2f).

322 In two patients with chronic ITP followed with longitudinal sampling (Patient 1 and 2),
323 expanded clones persisted over several years. In these patients, TCR clonality was
324 inversely correlated with the platelet count (P ≤ 0.05; r = 0.56; Figure 2g).

325 Using Morisita-Horn similarity-index (CMH) we found strong overlap between clones
326 in isolated $CD8^+$ TEMRA cells and whole T cell compartment (Figure 3a). For
327 example, the top 3 expanded clones in the T cell compartment were also the
328 dominant clones in the $CD8^+$ TEMRA cell compartment (Figure 3b). By comparing
329 the distribution of TRB V-J usage in the TEMRA compartment with the remaining T
330 cells, we show that TEMRA cells of patients are dominated by high abundance
331 TRBV/TRBJ gene rearrangements (highest TRB clonotype frequency at 41.7%,
332 25.2%, and 26.1%, in patients 1, 3 and 4, respectively). In contrast, the remaining T
333 cell subsets ($CD4^+$ and the rest of the $CD8^+$ T cell compartment) showed polyclonal
334 distribution of TRB V/J rearrangements with no predominant clones (Figure 3c).

335 To determine the nature of the expanded clones, we combined scRNA and TCR
336 sequencing in isolated whole $CD8^+$ T cells from patients with ITP and expanded T
337 cell clones. Based on their signature genes, we identified five distinct clusters in
338 $CD8^+$ T cells from patients with ITP: naïve, TEMRA, central memory (CM), effector
339 memory (EM) and $CD161^+$ high (hi) cells (Figures 4a-b). By mapping $CD8^+$ TCR
340 sequencing to gene expression, we found that the largest unique clones showed an
341 aggregative distribution and were mostly comprised of TEMRA subtype (Figure 4c).
342 Expanded TEMRA cell clones were more prominent in a patient with platelet count <
343 30 x 10^9/L compared to a platelet count ≥ 30 x 10^9/L (Figure 4d). The cytotoxic
344 features of the clonally expanded cells, both by flow and by single cell analysis,
345 indicate their potential for killing.

346 To explore whether these TEMRA cells from the patients with refractory ITP and
347 expanded T cell clones could interact with platelets and cause thrombocytopenia we
348 set up a series of co-culture experiments.

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349 We first simulated a blood vessel by perfusing PBMCs stained with anti-CD8
350 antibody through microfluidic channels onto which a monolayer of autologous
351 platelets had been captured via VWF at a shear rate of $50s^{-1}$ (equivalent to that seen
352 in venous blood flow)[31]. Sustained interactions between $CD8^+$ T cells and platelets
353 occurred 4x more frequently in blood from patients with refractory ITP than controls
354 ($P \leq 0.01$) and occasionally formed aggregates of $CD8^+$ T cells (Figure 5a &
355 *Supplementary Data Video*).

356 To determine whether $CD8^+$ T cell-platelet interaction could kill platelets, we cultured
357 isolated $CD8^+$ T cells with autologous platelets overnight. Confocal imaging
358 confirmed stable $CD8^+$ T cell:platelet aggregates in a patient with ITP (Figure 5b).
359 $CD8^+$ T cell:platelet aggregates ($CD8^+CD41^+$) were more frequent in the co-cultures
360 from patients than controls ($P \leq 0.05$; Figures 5c-d) and were partially inhibited by
361 blocking MHC Class I (HLA-ABC) on platelets prior to co-culture ($P \leq 0.01$; Figure
362 5e) indicating these interactions were TCR mediated. CD107a expression was
363 significantly higher in $CD8^+$ T cell:platelet aggregates in co-cultures when compared
364 to $CD8^+$ T cells cultured alone. CD107a is expressed on both activated platelets and
365 $CD8^+$ T cells. Within $CD8^+$ T cells, the expression of CD107a co-localises with the
366 expression of Granzyme B (*Supplementary Data Figure 2*) indicating cytolytic activity
367 and release of Granzyme and Perforin ($P \leq 0.01$; Figures 5f-g). Platelets in the
368 aggregates showed increased CD62P expression ($P \leq 0.001$; Figure 5h), together
369 with Annexin V expression (*Supplementary Data Figure 3),* consistent with platelet
370 activation and apoptosis[39,40]. In controls the $CD8^+$ T cell:platelet interactions resulted
371 in very minimal increase in CD62P or CD107a expression (*Supplementary Data*
372 *Figure 4*).

373 To confirm platelet specificity, we cultured PBMCs and autologous platelets in an
374 IFNγ ELISpot assay. PBMCs from patients with ITP showed higher spot forming
375 colonies compared to PBMCs from controls when cultured with autologous platelets
376 (12.50 vs 4; $P \leq 0.05$) (Figure 5i; *Supplementary Data Figure 5*).

377

378 **Discussion**

379 Using a number of orthogonal approaches, we have described expanded terminally-
380 differentiated effector $CD8^+$ (TEMRA) T cell clones in patients with ITP. These
381 TEMRA cells are polyfunctional, primed for killing, and demonstrate no features of

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382 exhaustion. While polyfunctional TEMRA cells are usually described in the context of
383 viral or vaccine responses[41,42], there was no evidence of infection in our cohort. By
384 deep sequencing the T cell receptor, we identified long-lived expanded T cell clones,
385 which were more expanded in patients with refractory ITP, and varied in frequency
386 with disease state, supporting a causal role in the pathobiology of ITP. Unlike other
387 autoimmune conditions such as diabetes and multiple sclerosis[43,44], there was no
388 TCR sharing between individuals with ITP which could be due to the heterogeneity of
389 MHC class I molecules and/or because of the variable platelet epitopes involved
390 across our patient cohort.

391 Using a model of venous blood flow and *ex vivo* co-culture experiments, we show
392 that $CD8^+$ T cells from patients with chronic ITP (and refractory to at least two prior
393 treatments) directly interact with autologous platelets forming aggregates. The
394 expression of CD107a and Granzyme B, as well as CD62P and Annexin V within
395 aggregates indicate degranulation of cytotoxic granules from $CD8^+$ T cells and
396 platelet activation and apoptosis[39,40]. These features, together with increased
397 secretion of IFNγ by T cells in ITP patients cultured with platelets, and the inhibition
398 of aggregates through MHC class I blockade, all support TCR-mediated autoimmune
399 platelet destruction by activated clonal $CD8^+$ T cells.

400 Platelets and megakaryocytes express T cell co-stimulatory molecules enabling
401 them to activate T cells in a platelet MHC class I dependent manner[45,46].
402 Megakaryocytes have been shown to be potent antigen presenting cells[47,48] and
403 platelet interactions with $CD8^+$ T cells have been described in the context of blood
404 transfusions and infections[49,50]. These interactions are thought to provide a
405 protective role regulating CD8 T cells during sepsis[49]. We also observed transient
406 interactions between platelets and $CD8^+$ T cells in blood from healthy controls,
407 consistent with these previous reports[50–52]. In contrast to the interactions we see in
408 ITP, we found very little activation of platelets during interactions with healthy control
409 $CD8^+$ T cells.

410 Platelet lysis by $CD8^+$ T cells in ITP was first suggested from studies measuring the
411 lysate from co-cultures of Indium labelled platelets added to $CD8^+$ T cells *in vitro*[21,25].
412 Later, mouse models of ITP, using lymphocyte transfer experiments and a mixture of
413 depletion studies, inferred $CD8^+$ T cells-mediated thrombocytopenia *in vivo*[22].

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414 However, the direct effect of these cells on platelets in patients with ITP remained
415 uncertain.

416 Our study is the first to identify individual disease associated T cell clones and to
417 culture isolated $CD8^+$ T cells from ITP patients with autologous platelets without
418 artificially stimulating the $CD8^+$ T cells demonstrating $CD8^+$ T cell mediated platelet
419 activation and apoptosis. Further work is now needed to understand the dynamics of
420 these interactions *in vivo* and to explore the role of autoreactive T cells in other
421 thrombocytopenic disorders.

422 Our findings provide novel and critical insights into the pathophysiology of ITP.
423 Current T cell therapies, such as azathioprine, mycophenolate and cyclosporin show
424 efficacy in ITP[6], but they are often poorly tolerated, and may be inadequate in
425 patients who have refractory ITP with very expanded T cell clones. Deciding on
426 treatment escalation in patients with refractory ITP remains challenging. Although
427 our study was not designed to evaluate TCR as a biomarker, it is tempting to
428 speculate that the persistence of expanded T cell clones highlights a possible target
429 in patients refractory to current therapies and could lead to more directed and more
430 effective treatments.

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443 **Authorship contribution**

444 Contribution: A.M., A.S., N.C. and B.M.Z. designed the study interpreted data, and
445 wrote the paper; A.M., A.S, P.H, M.T., R.S, E.W.,Y.D, A.CB., A.C., W.AP, A.K., J.C,
446 I.S-C performed experiments; E.T, A.T, A.H. collated clinical metadata and PBMC
447 collection; J.B, J.Z, N.I. and J.C interpreted data and wrote the paper. All authors
448 edited and approved the paper for submission.

449 **Conflicts of Interest Disclosures**

450 No authors have conflicts of interest which are relevant to this manuscript

451

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630 **Tables**

631 **Table 1 – Cohort Characteristics**

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	ITP	Controls
No of samples	83	51
Age		
Median	45	54
IQR	22 – 86	23 – 87
Platelet count		
Median	37	N/A
IQR	7 – 249	
Gender (M:F)	1.2:1	1:1
Longitudinal samples	24	N/A

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636 **Figure Legends**

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638 **Figure 1: TEMRA cells without features of exhaustion are expanded in patients**
639 **with ITP and correlate with disease activity**

640 **a,** Peripheral $CD4^+/CD8^+$ ratio is significantly lower in patients with ITP compared
641 to controls indicating CD8 mediated disease. **b,** A t-distributed Stochastic Neighbour
642 Embedding (tSNE) plot of the T cell $CD4^+$ and $CD8^+$ subsets based on their surface
643 expression of CD45RA and CD62L in a control and an patient with ITP. Expanded
644 TEMRA cells are shown in a patient with ITP. **c,** An example of a dot plot from flow
645 cytometry analysis of a control vs an ITP patient using CD45RA and CD62L.
646 **d,** Patients with ITP compared to controls have significantly higher numbers of
647 TEMRA cells. **e,** Patients with platelets < 30 x 10^9/L have higher numbers of TEMRA
648 cells compared to those with platelets ≥ 30 x 10^9/L. **f,** An example of a dot plot from
649 flow cytometry analysis of a control vs an ITP patient showing expression of
650 IFNγ and TNFα **g,** $CD8^+$ T cells in patients with ITP have increased
651 IFNγ, TNFα and Granzyme B; polyfunctional $CD8^+$ T cells (expressing
652 IFNγ, TNFα and Granzyme B) are also increased in ITP. **h,** PD-1 expression has not
653 changed, and Tim-3 expression is reduced in TEMRA cells.

654 * P value ≤ 0.05, ** P value ≤ 0.01, *** P value ≤ 0.001

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656 **Figure 2. High throughput T cell receptor sequencing reveals expansion of**
657 **private clones associated with decreased TCR diversity in ITP vs HC: as**
658 **clones expand, the platelet count falls**

659 **a,** The percentage of space occupied by the expanded clones (defined as clones
660 occupying more > 5% of the repertoire) is significantly higher in ITP. **b,** Number of
661 productive unique CDR3 sequences (unique clones) per 10^3 unique clones is
662 significantly lower in ITP. **c,** Simpson's diversity index is significantly lower in ITP. **d,**
663 In refractory ITP patients, the percentage of space occupied by the expanded clones
664 is significantly higher. **e,** The number of productive unique clones per 10^3 unique
665 CDR3 sequences (diversity) is reduced in patients with platelet count < 30 x 10^9/L
666 and recovers in individual patients as the count increases (platelet count $\geq$ 30 x
667 10^9/L). **f,** The amount of the T cell repertoire/space taken up by expanded clones is
668 higher in patients with platelet count < 30 x 10^9/L, and falls in individual patients as
669 the count recovers, reflecting the changes in T cell repertoire. **g**, In two patients with
670 chronic ITP, individual clones are followed over a number of years (point zero is the
671 first time TCR is measured) and compared to the overall T cell diversity and the
672 platelet count. T cell receptor diversity falls with the platelet count and increases as it
673 recovers. Correspondingly, the individual clones expand as the platelet count falls,
674 and contract as the platelet count increases.

675 * P value ≤ 0.05, ** P value ≤ 0.01, *** P value ≤ 0.001, **** P value ≤ 0.0001

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681 **Figure 3: Expanded unique T cell clones originate in the TEMRA compartment**

682 **a,** Morisita's Overlap Index (0 = the samples are entirely different to 1 = the samples
683 are identical) shows the overlap between clones detected in the overall T cells and
684 clones detected in the TEMRA cells from the same patients. The mean C_{MH} value of
685 the TCR repertoires was over 0.4 between T cell and TEMRA cell compartments. In
686 comparison, there is no overlap between patients. **b,** Percentage space occupied by
687 the largest clonotype among T cell and TEMRA compartment in three ITP patients.
688 **c,** 3D-plot visualisation of the composite TRBV and TRBJ repertoire of the TEMRA
689 compartment compared to non-TEMRA $CD8^+$ T cell and $CD4^+$ cells combined.

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694 **Figure 4. Combined single-cell RNA and TCR sequencing defines expanded**
695 **clones as terminally-differentiated effector memory (TEMRA) cells**
696 **a,** Sorted $CD8^+$ T cells are clustered using their gene expression profile into naïve,
697 TEMRA, central memory (CM), effector memory (EM) and CD161+ hi (high)
698 cells. **b,** Heatmap of genes in sorted $CD8^+$ T cells differentially expressed according
699 to clusters including naïve, TEMRA, CM, EM and CD161+ hi cells. **c,** Large clones
700 (top 10 clones) are predominantly within the TEMRA subset shown in red. **d,** Patient
701 with platelet count $< 30 \times 10^9$/L have more TEMRA T cells compared to patients with
702 platelet count $\geq 30 \times 10^9$/L.

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703 **Figure 5: $CD8^+$ T cells interact with platelets causing T cell activation, IFNγ**
704 **release, CD107a and platelet activation. $CD8^+$ T:platelet aggregates are**
705 **inhibited with MHC class I blocking on platelets.**
706 **a,** When $CD8^+$ T cells flow along a chamber coated with platelets, $CD8^+$ T cells from
707 ITP patients were 4x more likely to slow down and stop along the platelet coated
708 surface than controls. **b,** Confocal imaging of $CD8^+$ T cell interactions with platelets
709 in a patient with ITP**. c,** Example of dot plot from flow cytometry analysis of a control
710 vs patient with ITP when $CD8^+$ T cells are co-cultured with platelets, showing $CD8^+$ T
711 cell:platelet aggregates ($CD8^+CD41^+$) from total $CD8^+$ T cells**. d,** $CD8^+$ T cell:platelet
712 aggregates are higher in patients with ITP and **e,** are inhibited when HLA-ABC (MHC
713 class I) receptors on platelets are blocked. **f,** In $CD8^+$ T cell:platelet co-culture, **g,**
714 CD107a is increased in the $CD8^+$ T cell:platelet aggregates compared to $CD8^+$ T
715 cells cultured alone, consistent with release of Granzyme B, **h**, platelets in the $CD8^+$
716 T cell:platelet aggregates show increased CD62P, consistent with platelet activation.
717 **i,** IFNγ ELISpot assay of T cells cultured with autologous platelets shows that T cells
718 from patients with ITP have increased secretion of IFNγ when cultured with platelets
719 (detected by IFNγ forming spots)**.**
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721 * P value ≤ 0.05, ** P value ≤ 0.01, *** P value ≤ 0.001
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