Exaggerated in vivo IL-17 responses discriminate recall responses in active TB

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- Supplementary figures S1-7
- Supplementary tables S1-7

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The authors have declared that no conflict of interest exists.

Abstract

Background

Host immune responses at the site of *Mycobacterium tuberculosis* (Mtb) infection serve to contain the pathogen, but also mediate the pathogenesis of tuberculosis (TB) and onward transmission of infection. Based on the premise that active TB disease is predominantly a manifestation of immunopathology, we tested the hypothesis that immune responses at the site of host-pathogen interactions would reveal enrichment of immunopathologic responses in patients with active TB that were absent in individuals with equivalent immune memory for Mtb but without disease.

Methods

In cohorts of patients with active TB and cured or latent infection, we undertook molecular profiling at the site of a tuberculin skin test to model in vivo host-pathogen interactions in Mtb infection. Genome-wide transcriptional differences were identified by differential gene expression analyses. Enrichment of immune cells and cytokine activity was derived using specific transcriptional modules. Findings were validated in independent cohorts of patients with active TB, as well as Mtb infected tissues.

Results

Active TB in humans is associated with exaggerated IL-17A/F expression, accumulation of Th17 cells and IL-17A bioactivity, including increased neutrophil recruitment and matrix metalloproteinase-1 expression directly implicated in TB pathogenesis. These features discriminate recall responses in patients with active TB from those with cured or latent infection, and are also evident at the site of TB disease.

Conclusions

Our data are consistent with a model in which elevated Th17 responses within tissues drive immunopathology and transmission in active TB, and support targeting of the IL-17A/F pathway in host-directed therapy for active TB.

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Introduction

Mycobacterium tuberculosis (Mtb) infection results in a spectrum of clinical outcomes, from asymptomatic latent infection to symptomatic disease. The focus of host-pathogen interactions is characterised histologically by granuloma formation, a chronic inflammatory process that may contain the infection, but can also result in tissue damage that promotes transmission of infection to other individuals (1, 2). The distinctions that tip the balance between protective and pathogenic immune responses remain a fundamental question in tuberculosis research. This knowledge is expected to inform rational vaccine design and development of host-directed therapies (3).

Chronic inflammatory pathology at the site of human tuberculosis has been the subject of extensive descriptive studies, but discriminating between protective and pathological immune responses has been limited to comparing leucocyte phenotype and function in blood from Mtb-exposed patients with and without active disease (1). We have shown that genome-wide transcriptional profiling of biopsies of the tuberculin skin test (TST) can be used to make comprehensive molecular and systems level assessment of in vivo immune responses at the site of standardised host-pathogen interactions (4–7). Importantly, the transcripts enriched within the TST reflect the genome-wide variation in molecular pathology at the site of tuberculosis (TB) disease (5, 7), suggesting the TST represents a valuable surrogate for assessing TB immunopathogenesis in vivo.

On the premise that active TB disease is predominantly a manifestation of immunopathology, in this study we aimed to test the hypothesis that immune responses at the site of host-pathogen interactions, modelled by the TST, would reveal enrichment of immunopathologic responses in patients with active TB that were absent in individuals with equivalent immune memory for Mtb but without disease.

Results

Immune responses at the site of TST in active and cured TB

The TST has been most extensively used to identify patients with T cell memory for mycobacterial antigens, but the clinical response does not differentiate infected individuals with and without active disease (1). We sought to test the hypothesis that molecular profiling of the TST may identify elements of the recall response which are specifically associated with disease. We undertook 48-hour TSTs in patients with microbiologically-confirmed TB disease within the first month of treatment ('active TB') to identify disease associated responses, and compared these to TST responses in patients within one year of curative TB treatment ('cured TB') to identify non-disease

86 associated recall responses (table S1). Age, gender and site of TB disease were comparable between
87 the two groups (table S2). As expected, clinical induration in response to the TST was not
88 significantly different between the two groups (fig 1A), hitherto interpreted to reflect comparable
89 cell mediated immune memory.

90 In comparison to skin biopsies from the site of control saline injection, 1910 genes were significantly
91 enriched in response to the TST in at least one study group. Of these, 1251 were enriched in both
92 groups (fig 1B). Bioinformatic systems level assessment of the shared response revealed many
93 prototypic cell mediated immune responses which we had previously described in the TST (fig 1C)
94 (5). Pairwise assessment of the integrated list of transcripts that were enriched in either group
95 revealed statistically significant covariance, consistent with the hypothesis that the majority of
96 responses do not discriminate between the two groups (fig 1D).

97 Differential gene expression in the TST in active TB

98 A proportion of genes were differentially enriched between the two groups (fig 1D & tables S3-4). 44
99 genes were expressed significantly more in patients with active TB (table S3) compared to patients
100 with cured TB. Amongst these, pathway analysis identified statistically significant enrichment of
101 transcripts involved in extracellular matrix (ECM) remodelling, such as matrix metalloproteinase 1
102 (MMP-1), and beta defensins that both exert antimicrobial functions and also provide a chemotactic
103 gradient for CCR2-expressing cells, including neutrophils (8) (figs 2A & S1A). MMP-1, previously
104 implicated in pathogenic degradation of the ECM in TB (9), was the most over-expressed gene in
105 active TB compared to cured TB (fig 2B & table S3). This difference was validated at protein level by
106 immunohistochemistry, which also revealed that the differences in MMP-1 expression between
107 patients with active and cured TB was restricted to the inflammatory infiltrates within the TST (fig
108 2C-E).

109 Elevated IL-17 responses in active TB

110 We hypothesised that the genes over-expressed in active TB were regulated by common upstream
111 signals in the tissue environment. To test this hypothesis, we compared the predicted upstream
112 regulators of differentially expressed transcripts in the TST of active and cured TB patients using
113 Ingenuity Pathway Analysis (6). This analysis suggested that IL-17A induced the expression of genes
114 over-expressed in active TB and not those over-expressed in cured TB (fig 3A). In contrast, IFNγ was
115 predicted to be an upstream signal for gene expression enriched in both active and cured TB (fig 3A).
116 IFNγ responses, largely attributed to T helper (Th)-1 polarised CD4+ T cells are necessary for
117 immunological protection against Mtb (10), but they are insufficient and do not discriminate

118 between people who do and do not develop disease (1). The role of IL-17A in TB is less clear. This
119 cytokine belongs to a family of six structurally related cytokines and shares greatest sequence
120 homology with IL-17F. These bind the same receptor, and consequently exert the same functions,
121 particularly increased neutrophil recruitment via upregulation of chemokine expression (11).

122 There is unequivocal evidence that IL-17A/F contribute to host defence against bacterial and fungal
123 pathogens (12). Importantly however, they are also strongly indicated in the immunopathology of
124 chronic inflammatory diseases (12, 13). This includes evidence for IL-17A dependent neutrophil
125 mediated pathology in mouse models of Mtb infection (14–16). Our bioinformatics analysis
126 suggested increased enrichment of transcripts in the human in vivo recall response of patients with
127 active TB may be driven by IL-17A/F activity. Therefore, we sought to test the hypothesis that IL-
128 17A/F activity is exaggerated in active TB. Consistent with this hypothesis the expression of both IL-
129 17A and IL-17F were enriched in the TST of people with active TB compared to cured TB (fig 3B). In
130 contrast, IFNγ transcript levels representing the prototypic molecule in cell mediated immune recall
131 responses was not significantly different (fig 3B). Interestingly, the expression of IL-22, a cytokine
132 with closely related biological function to IL-17A and IL-17F (17), was also not elevated in active TB
133 (fig 3B). The differences revealed at the transcriptional level were also reflected by increased
134 immunofluorescence of IL-17F protein in the TST of people with active TB (fig 3C & 3D).

135 In order to test the functional significance of the differences in IL-17A/F expression between active
136 and cured TB, we evaluated differences in the bioactivity of IL-17A between these groups. We
137 addressed this by generating cellular response modules from the transcriptomes of
138 cytokine-stimulated keratinocytes (KC) (18, 19). We confirmed that these modules were both
139 sensitive and specific for their cognate stimuli by assessing their expression in other independent
140 datasets (fig S2). We then compared the geometric mean expression of these cytokine-specific
141 transcriptional modules in the TST transcriptomes. The IL-17A-induced gene module was significantly
142 increased in the TST of people with active TB compared to that of cured TB, but expression of IFNγ,
143 type I IFN or TNF-inducible gene modules was not significantly different between the two groups (fig
144 4A). We extended our approach to evaluating the functional bioactivity of other specific cytokines
145 using transcriptional modules for IL-10- and IL-4/IL-13-inducible gene expression, described in a
146 previous report (5). Neither of these were significantly different in the TST of people with active and
147 cured TB (fig S3A). The IL-4/IL-13 bioactivity module was used as a measure of Th2 responses
148 normally associated with allergy and immune responses to helminths. Another member of the IL-17
149 family, IL-17E contributes to Th2 responses (11). We found no enrichment of IL-17E expression in the
150 TST of people with active TB consistent with IL-4/IL-13 bioactivity and distinct from IL-17A/F
151 bioactivity (fig S3B).

152 Focusing on mechanisms that may contribute to pathogenesis, enrichment of MMP-1 expression in
153 the TST of patients with active TB (fig 2) can also be attributed to increased IL-17A/F bioactivity,
154 through induction of MMP expression by stromal cells (20). Neutrophils can also contribute to the
155 immunopathology of TB (14–16, 21, 22), and a key function of IL-17A/F is to promote neutrophil
156 recruitment via induction of neutrophil chemokines (12). Therefore, we tested the hypothesis that
157 the TST in active TB will also reveal increased neutrophil recruitment, compared to that of patients
158 with cured TB. We compared the expression of two independently derived transcriptional modules
159 that have been extensively validated to reflect neutrophil frequency in tissues including the TST (23,
160 24). We found significantly higher expression of the neutrophil modules in people with active TB
161 compared to cured TB (fig 4B). These differences were mirrored by gene expression levels of IL-17A-
162 inducible CXCL1, CXCL8 and S100A9 that drive neutrophil recruitment to sites of inflammation (11,
163 15) (fig 4C). In contrast, accumulation of monocytes and T cells assessed by their respective gene
164 expression modules (23), and the expression of the monocyte chemoattractant, CCL2, did not differ
165 in the TST of people with active and cured TB (fig 4B&C).

166 Increased frequency of Th17 cells in active TB TST responses

167 IL-17A/F are predominantly produced by Th17 cells and neutrophils (11). Immunohistochemistry
168 revealed that in the TST of people with active TB, IL-17F, originated predominantly from
169 mononuclear cells, rather than from polymorphonuclear cells (fig 5A). Therefore, we tested the
170 hypothesis that Th17 cells were enriched in the TST of people with active TB compared to that of
171 cured TB. We derived transcriptional modules specific for differentially polarised CD4+ Th subsets
172 from a published dataset (25) and demonstrated their specificity in an independent dataset (26) (fig
173 S4A). We further validated the Th17 module by showing that this correlated closely with the
174 expression of the IL-17A/F bioactivity module within skin biopsies of patients with psoriasis vulgaris,
175 representing an alternative Th17-mediated inflammatory condition (27) (fig S4B). The expression of
176 the Th1- and Th2-associated transcriptional modules in the TST was comparable between patients
177 with active and cured TB, but expression of the Th17 associated module was significantly increased
178 in patients with active TB (figs 5B & S3).

179 In order to investigate the mechanism for increased Th17 responses in the TST of patients with
180 active TB, we tested the hypothesis that active TB was also associated with increased circulating
181 Th17 cells. We assessed the expression of the Th subset modules in the transcriptome of PPD-
182 stimulated PBMC from an independent cohort of individuals with active TB disease and latent TB
183 infection (28). In contrast to that observed in the TST, patients with active TB revealed enrichment
184 for the Th1-associated gene expression module in PBMC but showed no difference in the

185 Th17-associated module (fig 5C). As a result, we explored the alternative hypothesis that the
186 inflammatory environment generated at the site of TST promotes the Th17 differentiation observed
187 in patients with active TB. We investigated the expression of cytokines implicated in Th17
188 differentiation, IL-1β, IL-6, IL-23 and TGFβ (11). The expression of each of these was significantly
189 correlated with enrichment for Th17 cells in the TST of active TB. In contrast there was no
190 correlation with IL-12 and IL-4 expression that drive Th1 and Th2 cell differentiation respectively (fig
191 5D). These data support a model in which the local tissue microenvironment may promote Th17
192 differentiation within tissues in active TB.

193 IL-17 activity is not a feature of latent TB and is not confounded by demographic 194 background, extrapulmonary disease or time on treatment

195 In order to validate our findings, we compared the TST transcriptome of a second independent
196 cohort of people with active TB with that of individuals with latent TB infection. Consistent with our
197 previous results, this comparison revealed significant enrichment for IL-17A/F bioactivity and
198 neutrophil infiltration in people with active TB (fig 6A). Furthermore, Th17 cells were enriched in
199 those with active TB despite there being no overall difference in T cell numbers compared to the TST
200 in individuals with latent TB (fig 6A). Of note, the comparison to latent TB in this analysis, also
201 excluded the possibility that lower levels of IL-17A/F bioactivity in the TST of people with cured TB
202 compared to active TB was an off-target effect of the antimicrobial treatment for active TB.

203 Taken together our data show that active TB is associated with exaggerated Th17 recall responses
204 and IL-17 bioactivity within the tissue microenvironment of host-pathogen interactions. Our findings
205 were replicated using two different methods for transcriptional profiling (microarray figs 4-5 and
206 RNA-Seq fig 6), and were consistent in geographically distinct cohorts (fig S5A). Significantly
207 exaggerated Th17 recall and IL-17A/F bioactivity in people with active TB compared to either cured
208 or latent TB were preserved in analyses including only UK cohorts (figs S5B & S5C), confirming that
209 the differences were not due to confounding by other variables in the different cohorts. In addition,
210 we found no difference in these responses between people with pulmonary and extrapulmonary TB
211 disease (fig S6). Of note, people with active TB were assessed at different time points within the first
212 month of treatment (median 11 days, IQR 5-28 days). Rapid changes in the peripheral blood
213 transcriptome associated with active TB have been reported (29, 30), but we found no diminution of
214 the exaggerated Th17 recall responses and IL-17A bioactivity invoked by the TST challenge in this
215 time frame (fig S7), suggesting that the mechanisms that drive these responses in active are not
216 swiftly reversed by treatment.

IL-17 activity is present at the site of TB disease

Having established that IL-17A/F production by T cells was a prominent feature of the in vivo tissue recall response to Mtb stimulation, we sought to determine whether this was also evident at the site of TB disease. The transcriptome of human TB granuloma (31) showed an enrichment of T cells, Th17 cells and IL-17A bioactivity compared to normal lung tissue (fig 6B). In addition, we examined the transcriptome of human Mtb-infected lymphadenitis compared to other 'reactive' causes of lymphadenopathy devoid of granulomatous inflammation or malignancy (32). Interestingly, despite the fact that reactive lymph nodes were enriched for the T cell transcriptional module, the Th17 and IL-17A/F bioactivity modules were enriched within Mtb infected lymph nodes (fig 6C), further confirming enrichment for IL-17A/F bioactivity to be a feature at the site of TB disease.

Discussion

Active TB disease is characterised by chronic inflammation that can result in significant tissue destruction, necessary for the onward transmission of Mtb (2). Identifying the processes that govern this immunopathology offers the opportunity to intervene therapeutically, limiting tissue damage and transmission of infection. Pathogenic immune pathways have been difficult to identify because most components of the immune response to Mtb do not discriminate between different clinical outcomes of infection (1). The present study provides compelling new evidence that IL-17A/F responses may mediate immunopathology in active TB. The inclusion of multiple cohorts and diverse demographic backgrounds increased the generalisability of our findings and circumvented the limitations of our cross-sectional study design. The findings are consistent with the well-established role of IL-17A/F in the immunopathology of chronic inflammatory human disease exemplified by psoriasis, for which blockade of the IL-17A/F axis provides an effective treatment (13).

Importantly, the primary physiological role of IL-17A/F is to promote host defence against bacterial and fungal infection, cogently demonstrated by mice deficient for IL-17A/F or IL-17 receptors, and by humans with inborn errors of IL-17 immunity (12). Mouse models also suggest a protective role for IL-17A/F following BCG vaccination (33) and in the early stages of Mtb infection, particularly in the context of more virulent Mtb strains (34, 35), through localising T cells near Mtb-infected macrophages (34) and by preventing formation of necrotic granuloma (36). However, in mice rendered susceptible to TB disease as a result of IFNγ deficiency or through receiving repeated BCG vaccinations, IL-17A/F responses drive neutrophil-mediated pathology (14–16, 21). We infer from these data that IL-17A/F responses may play a dichotomous role in TB by contributing to protection in early infection, but to the pathology of disease if early infection is not controlled and multibacillary bacterial replication and chronic immune activation ensues. Additional support for this

250 model is evident in IL-27R deficient mice, which exhibit exaggerated Th17 responses because they
251 lack IL-27 inhibition of RORγT (37). These mice show enhanced clearance of Mtb, but also increased
252 immunopathology dependent on IL-17A. Interestingly, a human candidate gene study identified host
253 genetic variation associated with increased secretion of IL-17A to be correlated with both protection
254 from incident TB but also more severe TB disease (38). Taken together, we hypothesise that
255 exaggerated IL-17A/F recall responses are the consequence of chronic multibacillary infection, but in
256 turn mediate increased pathology in established disease. Of the other IL-17 family members that
257 signal via alternative receptors, much less is known about the functional role of IL-17B, IL-17C and IL-
258 17D (11). These cytokine responses were not specifically tested in the present study. IL-17E, known
259 to promote Th2 responses (11), was not differentially enriched in active TB. Equally, we found no
260 evidence of elevated IL-10 responses in active TB, indicating an uncoupling of IL-17A/F activity from
261 regulatory responses, driving immunopathology rather than the control of Mtb replication (39).
262 Another cytokine, IL-22, does have functional overlap with IL-17A/F (17), but did not discriminate
263 between people with and without disease in our study.

264 Our data support the hypothesis that exaggerated IL-17A/F responses arise from Th17 cells, but they
265 do not unequivocally exclude other cellular sources. Our immunohistochemical analysis did not show
266 any clear evidence for IL-17F production by polymorphonuclear cells in the TST, but we were not
267 able to test whether other lymphocyte populations, such as γδ T cells, made a significant
268 contribution (35, 40, 41). Future studies will require single cell resolution to definitively confirm the
269 source of exaggerated IL-17A/F responses in this model, as well as to determine whether active TB
270 shows enrichment for 'pathogenic' Th17 cells that express both IFNg and IL-17A/F (42). Consistent
271 with previous studies (40, 43), we found no evidence of increased circulating Th17 cells in active TB,
272 although this was measured in a separate active TB cohort to the ones that underwent TST
273 assessment. Nevertheless, this observation underscores the unique value of molecular level
274 assessment of immune responses in the tissue microenvironment at the site of host-pathogen
275 interactions, modelled by the TST. Importantly, it also suggests the model that differential Th17
276 responses within the TST are governed by immune signalling networks within the tissue
277 microenvironment after T cell recruitment. In active TB patients, the tissue expression of cytokines
278 that promote Th17 differentiation correlated with the transcriptional module for Th17 cells. The
279 source of these cytokines is likely to be myeloid cells (11, 13, 44). Although we cannot prove a causal
280 relationship at this stage, our data are consistent with a model in which infiltrating monocytes from
281 patients with active TB secrete elevated levels of cytokines that promote the differentiation of
282 recruited T cells to a Th17 phenotype. Of note, active TB patients have higher frequency of

circulating CD14+ CD16+ non-classical monocytes (45), which can potentiate the differentiation of CD4 T cells to a Th17 phenotype in patients with chronic inflammatory conditions (46).

Our findings challenge the long-established view that curative treatment of TB does not lead to contemporaneous changes to immunological recall responses (47). Our data are consistent with the hypothesis that IL-17A-inducible neutrophil chemotaxis and expression of MMP-1 represent key mediators of immunopathology in TB by promoting granuloma formation, bacterial replication and matrix degradation that can lead to cavitation and onward transmission (9, 48). Our results support future studies to evaluate the impact of modulating IL-17A/F activity to ameliorate the pathology associated with chronic TB disease. The availability of therapies that block the IL-17A/F cytokine axis, developed and licensed for chronic inflammatory diseases (13), offers invaluable opportunities to transition from proof of concept pre-clinical studies, for example in non-human primate models, to first in man experiments.

Methods

Study populations

The study comprised recruitment of several different populations. The discovery 'Active TB' group that formed the basis of most of the analyses in figs 1-5 was the HIV seronegative patients from the same cohort described in our previous publication (5), who were recruited from TB clinics in London, UK and Cape Town, South Africa, and were all within one month of commencing antibiotic therapy. The comparator 'Cured TB' group was an independent cohort recruited from TB clinics in London, UK and Lima, Peru who fulfilled the inclusion/exclusion criteria (table S1). All 'Cured TB' patients were less than 2 years after completion of curative anti-TB antibiotic therapy for drug sensitive disease. In addition, a separate validation cohort of patients with active TB was recruited from TB clinics in London (fig 6). These patients were also within one month of commencing antibiotic therapy. This population was compared to individuals with latent TB recruited from TB clinics in London and Lima, who fulfilled inclusion/exclusion criteria (table S1). All study participants were HIV seronegative and for those with active or cured TB, the presence of Mtb infection was confirmed by routine culture or molecular based methods according to local clinic protocols. The demographic, clinical and laboratory data for each study group is summarised in table S2.

Study approval

314 Recruitment of patients with cured and latent TB was approved by UK National Research Ethics
315 Committee (reference number: 14/LO/0505) and Universidad Peruana Cayetano Heredia
316 Institutional Ethics Committee (reference number: 62349). Recruitment of patients for the validation
317 active TB cohort was approved UK National Research Ethics Committee (reference number:
318 16/LO/0776).
319

320 ## Study schedule and sampling

321 On recruitment to the study, all participants received 0.1 mL intradermal injection of two units
322 tuberculin (Serum Statens Institute) or saline in the volar aspect of one forearm, and this site was
323 marked with indelible ink. At 48 hours, the clinical response at the injection site was evaluated by
324 measurement of the maximum diameter of inflammatory induration and two 3 mm adjacent punch
325 biopsies were obtained from marked TST or saline injection site as previously described (4). One
326 biopsy was placed in RNAlater (Thermo Fisher) and stored at -70°C, and the other biopsy was placed
327 in 10% formalin neutral buffered solution (Sigma-Aldrich) and stored at room temperature for at
328 least 1 week prior to paraffin embedding.
329

330 ## Sample processing

331 The TST transcriptome from active TB patients in the discovery cohort was derived directly from the
332 data repository E-MTAB-3254 (ArrayExpress - https://www.ebi.ac.uk/arrayexpress/). Skin samples
333 from all other participants was stored in RNAlater at -70oC after collection. For processing, TST
334 samples were equilibrated to room temperature for 30 minutes before being transferred to CK14
335 lysing kit tubes (Bertin Instruments) containing 350µl of Buffer RLT (Qiagen) supplemented with 1%
336 2-Mercaptoethanol (Sigma). Tubes were pulsed for 6 cycles on a Precellys Evolution homogeniser
337 (Bertin Instruments), each cycle consisting of 23 seconds of homogenisation at speeds of 6300 rpm.
338 Samples were rested on ice for 2 minutes between cycles. After homogenisation, cellular debris and
339 lysing beads were precipitated by centrifugation and RNA isolated from the supernatant using
340 RNeasy Mini Kit (Qiagen). Total RNA from TST samples of patients with cured TB was purified,
341 labelled and hybridised on Agilent 8x60k microarrays as previously described (5). For the validation
342 cohort of active TB and individuals with latent TB, the KAPA mRNA HyperPrep Kit (Roche Diagnostics)
343 was used to construct stranded mRNA-Seq libraries from up to 500 ng intact total RNA after which
344 paired-end sequencing was carried out using the 75 cycle high-output kit on the NextSeq 500
345 desktop sequencer (Illumina). Each run contained 24 samples and was demultiplexed using bcl2fastq

346 by Illumina (https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-
347 software.html). Paired end reads were mapped to the Ensembl human transcriptome reference
348 sequence (homo sapiens GRCh38, latest version available). Mapping and generation of read counts
349 per transcript were done using Kallisto (49). R/Bioconductor package tximport was used to import
350 the mapped counts data and summarise the transcripts-level data into gene level (50).
351

352 ## Whole genome transcriptional profiling and analysis software

353 Raw microarray data was processed and normalised as previously described (51). Raw RNASeq
354 counts were normalised within-sample into TPM (transcripts per million) to remove feature-length
355 and library-size effects (52). Log2 transformed TPM were used for further analysis. Data matrices
356 from non-TST datasets were obtained from processed data series downloaded at the ArrayExpress
357 repository. Probe identifiers were converted to gene symbols using platform annotations provided
358 with each dataset. In circumstances where downloaded datasets were not log2 transformed, this
359 was performed on the entire processed data matrix. Significant gene expression differences
360 between datasets were calculated from normalised expression matrices using MultiExperiment
361 Viewer v4.9 (http://www.tm4.org/mev.html). Pathway analysis was performed in InnateDB (53) and
362 visualized as network diagrams in Gephi v0.8.2 beta. Upstream regulator analysis was performed
363 using Ingenuity Pathway Analysis (Qiagen), focusing on cytokines with predicted activation z-score
364 >2. Genes predicted to be regulated in either active or cured TB formed the basis of the network
365 diagram in fig 3. The expression of transcriptional modules was determined by calculating the
366 geometric mean expression of all the module constituent genes found in the dataset being analysed,
367 using R scripts generated in our previous publication (23), and which are available to download and
368 use from the Github repository (https://github.com/MJMurray1/MDIScoring). Venn diagrams were
369 constructed using the BioVenn tool (http://www.biovenn.nl/).

370

371 ## Transcriptomic data repositories

372 All transcriptional datasets used in this study are described in table S5. Accession numbers refer to
373 datasets in the ArrayExpress repository (https://www.ebi.ac.uk/arrayexpress/). The TST
374 transcriptome of the discovery cohort of patients with active TB was derived from dataset E-MTAB-
375 3254, the cured TB TST transcriptome was derived from dataset E-MTAB-6815, and the validation
376 active TB and latent TB TST transcriptomes were derived from dataset E-MTAB-6816.

377 ## Module derivation and expression

378 Immune cell modules used were ones rated with the highest Module Discriminatory Index (MDI)
379 score for module sensitivity and specificity as determined in our previous publication (23). These
380 were "M19" (T cells), "M37.1" (neutrophils) and "Monocyte 2-fold" (monocytes). We also made use
381 of another neutrophil module "ImSig" that was derived and validated elsewhere (24). We have
382 previously generated and validated the specificity of macrophage response modules to IL-10 or
383 IL4/IL-13 stimulation (5), and these are also utilised in this manuscript.

384 To derive keratinocyte (KC) cytokine-response modules, we made use of previously published
385 transcriptomic data (GSE12109 & GSE36287) from primary human keratinocytes (KCs) stimulated
386 with a selection of cytokines (18, 19). Significant transcriptional responses (paired t-test with α of
387 $p<0.05$ and no multiple testing correction) of genes over-expressed >4-fold in the cognate cytokine
388 condition relative to unstimulated KC were initially identified. Modules were then derived from
389 genes that were not also upregulated > 2-fold by non-cognate cytokine conditions compared to
390 unstimulated KC. The KC IL-17 response module utilised a cut-off of 2-fold between IL-17 stimulated
391 KC and unstimulated KC as too few genes were upregulated >4-fold compared to unstimulated KC.
392 The constituent genes of the KC modules are shown in table S6. The KC TNFa response module
393 generated in this way has already been published (6), but the other cytokine modules are newly
394 described. The gene components of the KC cytokine-response modules are available in table S6, and
395 their specificity was evaluated in both datasets in which they were derived and in independent
396 datasets of in vitro cytokine-stimulated KC (fig S1A).

397 The transcriptome of CD4+ T cells polarised towards different T helper phenotypes was derived from
398 dataset GSE54627 (25). To derive specific modules for each differentiation state, we used gene
399 expression from cells stimulated with anti-CD3 and anti-CD28, identifying genes over-expressed in
400 the phenotype of interest compared to all other conditions by paired t-test with α of $p<0.05$ and no
401 multiple testing correction. Each module was derived from the unique genes over expressed by
402 more than 1.5-fold in the cognate condition compared to all other stimulation conditions (except for
403 Th1 module where a 2 -fold cut-off was used). The gene components of T helper modules are
404 available in table S7, and their specificity was evaluated in the dataset from which they were
405 derived, in an independent dataset of polarised CD4 T cells, and in skin biopsies of patients with
406 psoriasis vulgaris (fig S1B) (25–27).

407

408 ## Immunostaining

409 Immunostaining of IL-17F was performed on 10 µm sections as previously described (54). Briefly,
410 monoclonal mouse-anti-IL-17F, 1:50, code MA5-16229 [Thermo Fisher Scientific, Waltham, MA, USA]
411 and DAPI (sc-24941, Santa Cruz Biotechnology, Texas, Dallas, USA) for detecting nuclei were used.
412 Irrelevant primary antibodies from Sigma (Milan, Italy; irrelevant mouse, 1:50, code I8765) were
413 applied at the same concentration of the related specific primary antibodies for immunostaining of
414 negative control slides, and as a further negative control, secondary antibody (AF568, goat anti-
415 mouse; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) alone was used (data not shown).
416 Light-microscopic analysis was performed at a magnification of 40x with a Leica DM4000B
417 microscope equipped with DFC-320 Leica digital camera (Leica Microsystems, Wetzlar, Germany).
418 Zeiss confocal microscope (LSM800, Carl Zeiss, Germany) was used to acquire confocal images.
419 Quantification of IL-17F was performed on 10x confocal images of samples through the
420 measurement of mean grey value (MGV) using software ImageJ (https://imagej.net/Fiji).

421 Immunostaining of MMP-1 was performed as previously described (55, 56). Scanned slide images
422 were obtained with use of NanoZoomer Digital Pathology System (Hamamatsu, Japan.
423 Quantification of MMP-1 staining was performed blindly by extracting MMP-1 associated 3, 3 -
424 diaminobenzidine (DAB) stain using standard deconvolution protocols in ImageJ. Cellular infiltrates
425 were manually selected as depicted in Fig 2 and DAB stain quantified by staining intensity as
426 proportion of the area selected using ImageJ. The selected region was moved without resizing to
427 adjacent tissue that did not contain cellular infiltration to calculate background MMP-1 intensity.
428 Three cellular infiltrates and background tissue regions were analysed for each tissue samples. Six
429 TST samples were quantified in each group.

Author contributions

Substantial contributions to the conception or design of the work;	GP, CT, GST, LCB LFP, TM, CU, DAM, BMC, MN
Substantial contributions to the acquisition, analysis, or interpretation of data for the work;	GP, CT, GST, LCB, AK, LFP, AF, AA, CV, TB, FLR, TM, BMC, MN
Drafting the work or revising it critically for important intellectual content;	GP, CT, GST, LCB, BMC, MN
Final approval of the version submitted for publication	All authors
Accountability for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved	GP, MN

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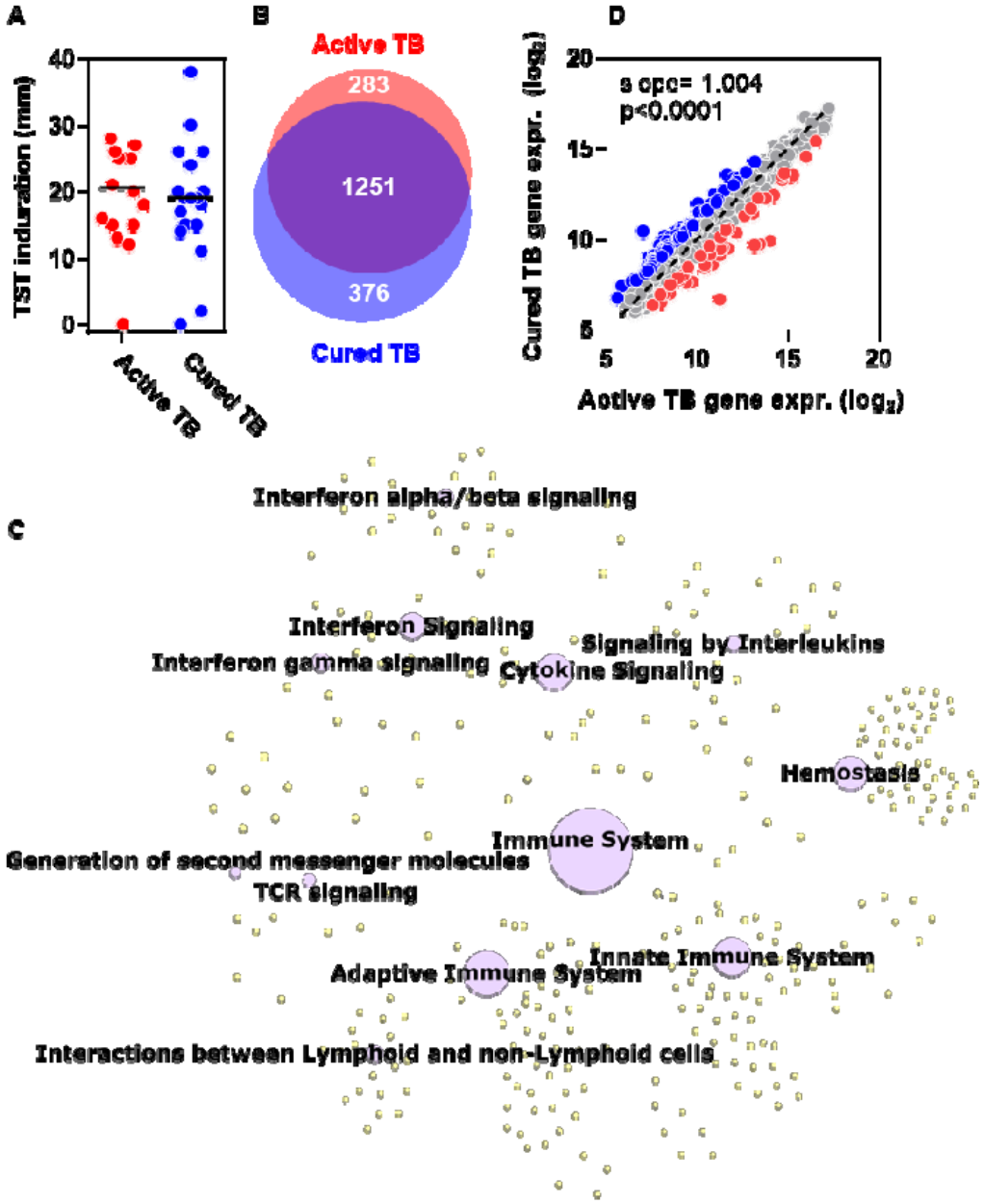


Figure 1. TST transcriptome in patients with active and cured TB disease. A) Induration at the site of TST was recorded by routine clinical assessments in both populations (mm) and was not different between the two groups (Mann-Whitney test). B) Venn diagram depicting genes significantly upregulated 2-fold in the TST of patients with active or cured TB relative to control saline injection. ($p<0.01$ by t-test with no multiple testing correction). C) TST transcriptome common to both active and cured TB summarised as a network diagram. Purple nodes represent Reactome database functional pathways, yellow nodes represent genes and edges reflect relationship between pathways and genes. Pathway node diameters are proportional to the respective pathway –log10 p value enrichment statistic. D) Pairwise dot-plot of 1910 gene integrated TST signature in patients with either active or cured TB. Dotted line reflects line of perfect covariance. p value derived from linear regression model between the two variables. Red and blue dots represent genes >2 fold

581 differentially expressed ($p<0.01$ by t-test with no multiple testing correction) between active and
582 cured TB.

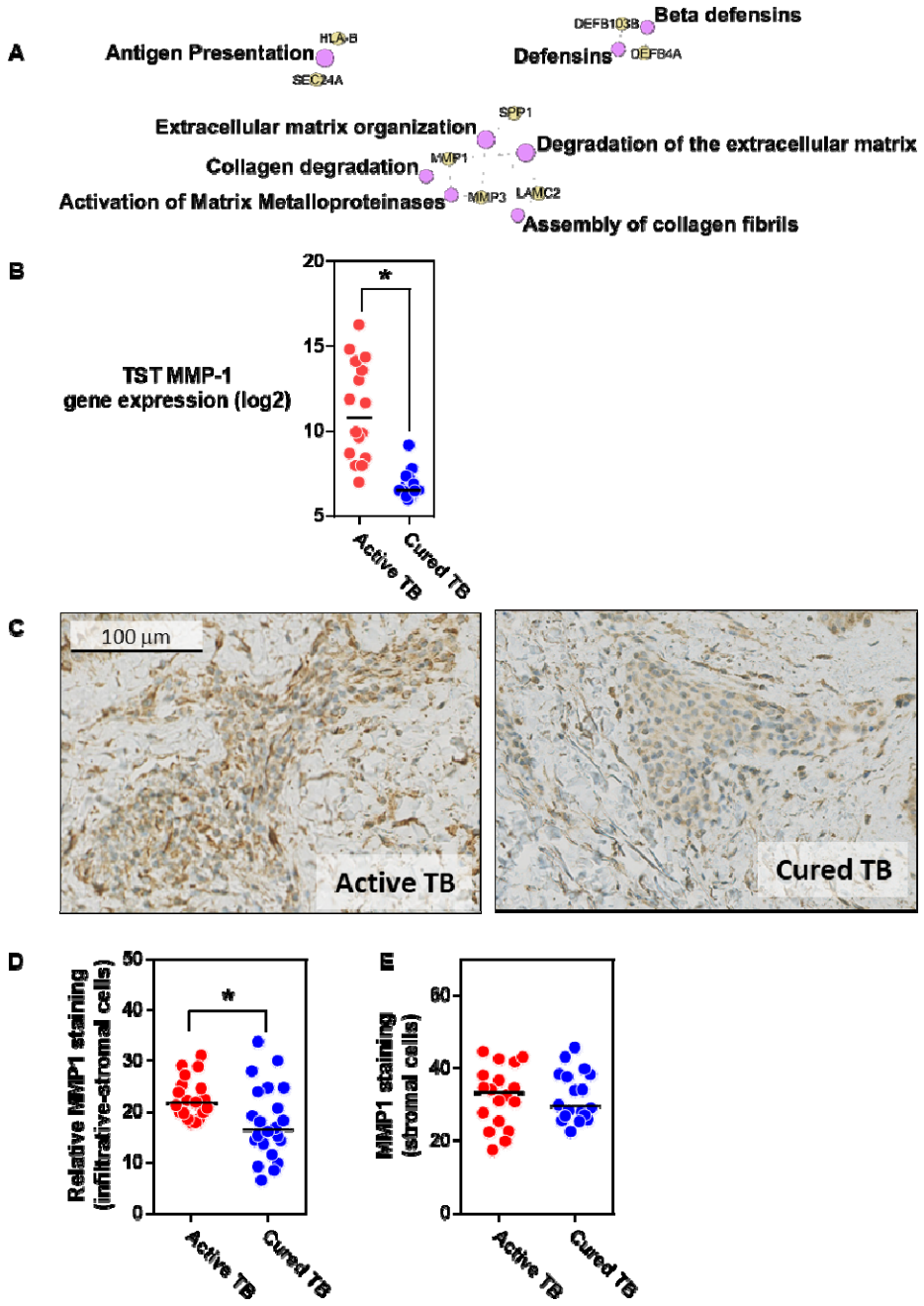


Figure 2. MMP-1 over-expression in active TB. A) Network diagram of genes and Reactome pathways over-expressed in the TST of patients with active TB compared to cured TB. Purple nodes represent Reactome database functional pathways, yellow nodes represent genes and edges reflect relationship between pathways and genes. Pathway node diameters are proportional to the respective pathway –log10 p value enrichment statistic. B) mRNA expression in TST of patients with active and cured TB of MMP1 gene. C) Representative MMP-1 immunohistochemistry staining in inflammatory infiltrates with TST samples from patients with active and cured TB. D) Differential MMP-1 staining intensity for 18 cellular infiltrates in each group of patients relative to adjacent zones of skin with no cellular infiltration. E) MMP-1 staining intensity in TST zones outside inflammatory infiltrates. * = $p<0.01$ by Mann-Whitney test.

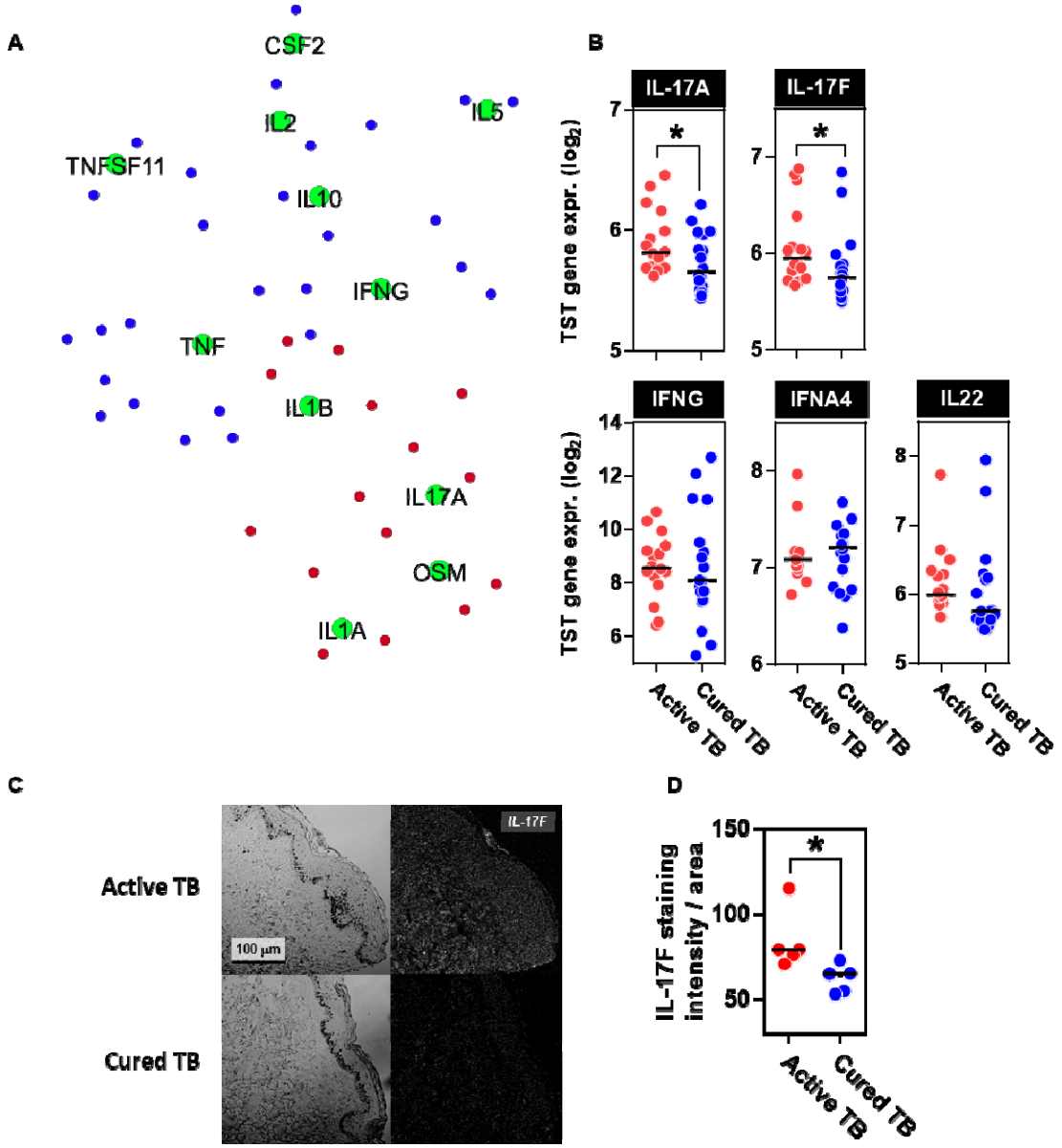
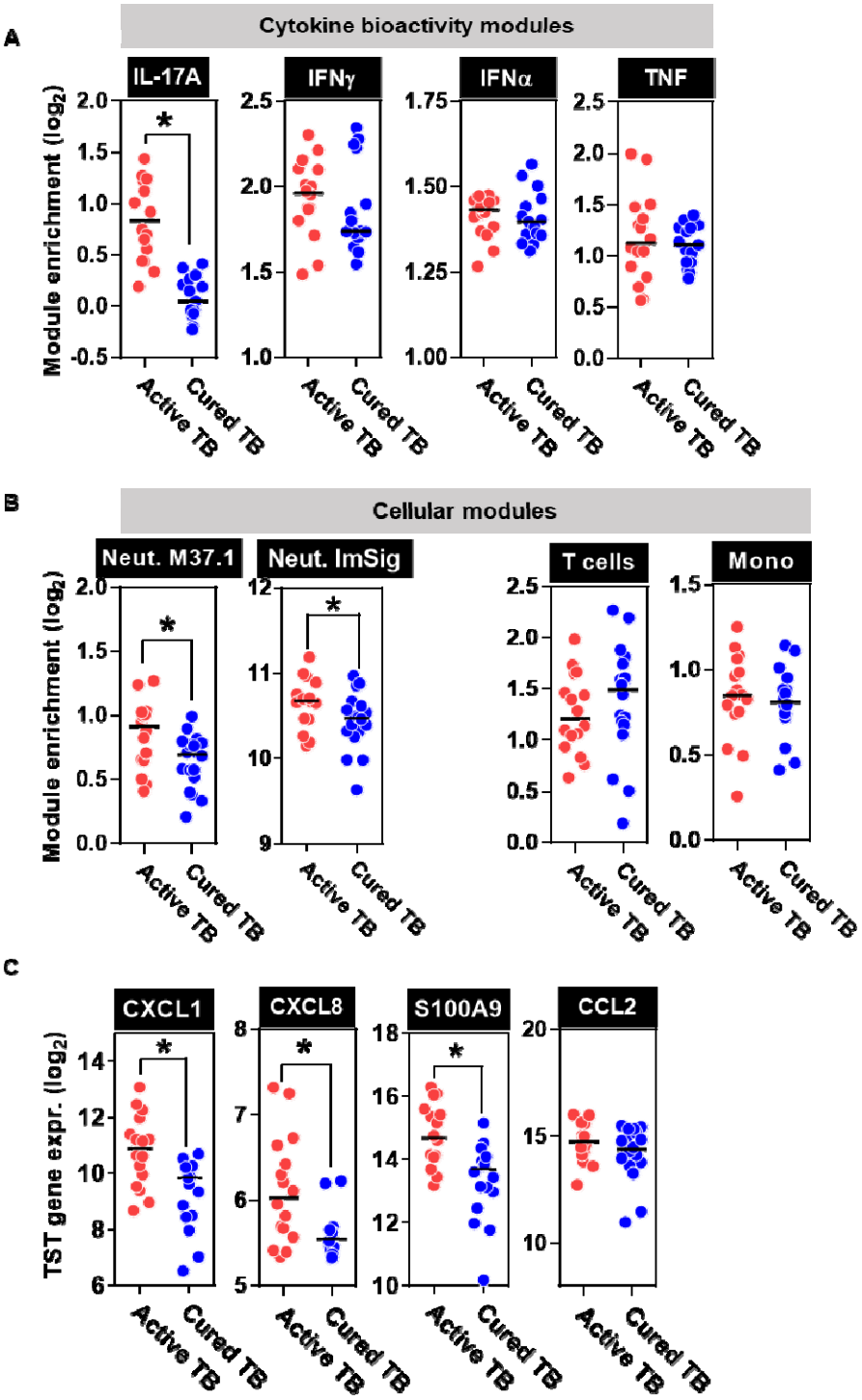


Figure 3. IL-17 is over expressed in active TB. A) Network diagram depicting upstream cytokine analysis of genes differentially expressed in TST of patients with active and cured TB. Red and blue nodes represent significant genes overexpressed in active and cured TB respectively as identified in fig 1D. Green nodes represent predicted cytokines regulating the gene expression of red and blue nodes. Edges depict relationship between upstream regulators and differentially expressed genes. B) Expression of selected cytokine transcript within the TSTs of active and cured TB patients. C) Expression of IL-17F by immunofluorescence in TST of patients with active TB. Left panel = phase contrast image, right panel = IL-17F positivity (white). D) Quantification of IL-17F staining throughout TST sections from patients with active and cured TB, determined by pixel intensity as a proportion of area sampled. Each dot represents IL-17F expression in the cross-section of an entire TST biopsy from one patient. * $p<0.01$ by Mann-Whitney test.



607

608 **Figure 4. TST challenge in active TB is characterised by enrichment of IL-17 responses.** Enrichment
609 in TST relative to saline injection of A) keratinocyte response modules of cytokine bioactivity, B)
610 immune cell modules and C) CXCL1, CXCL8, S100A9 and CCL2 genes. Neutrophil (Neut.) modules
611 M37.1 and ImSig validated for sensitivity and specificity in references (23, 24) respectively. * =
612 $p<0.01$ by Mann-Whitney test.

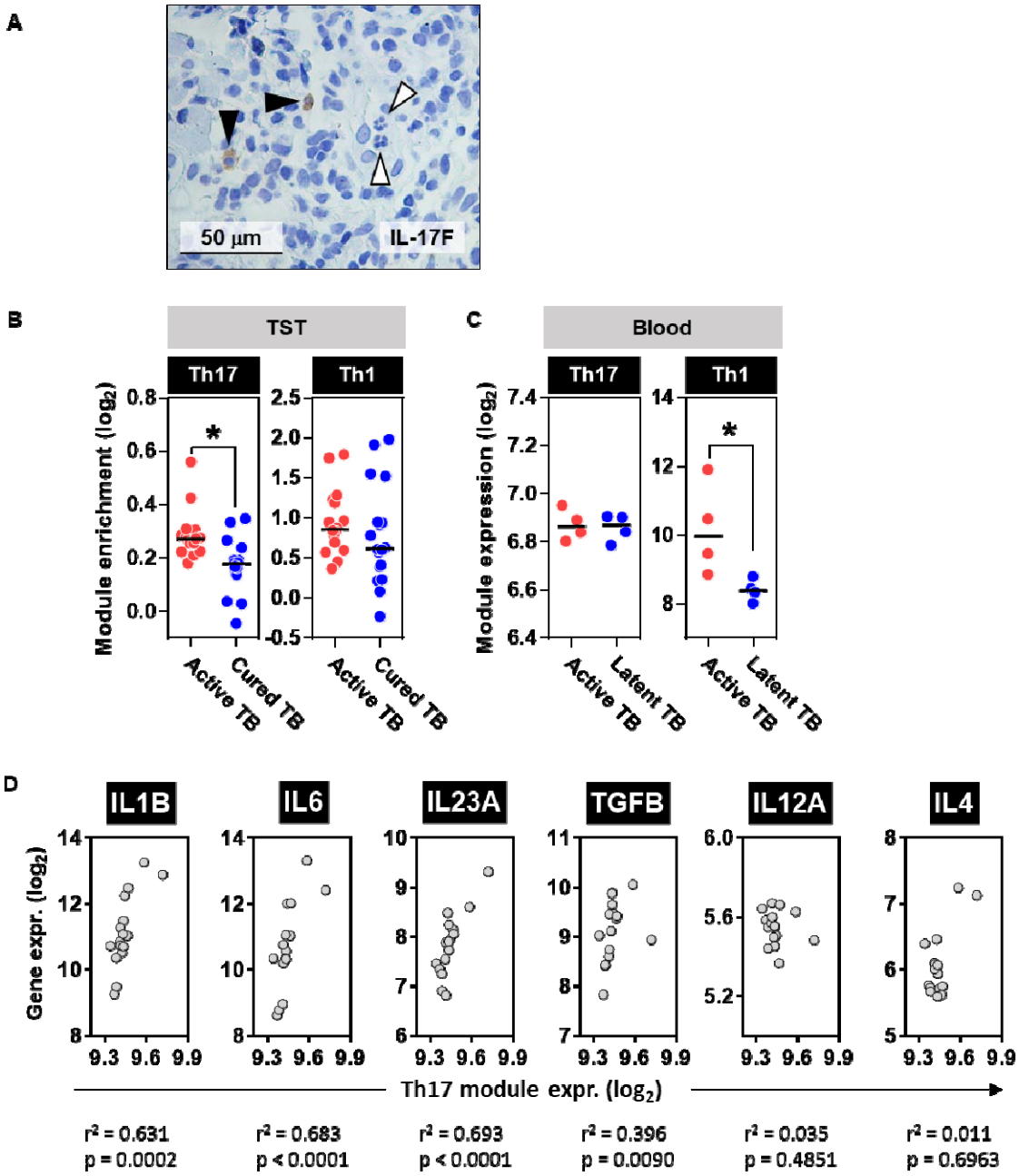
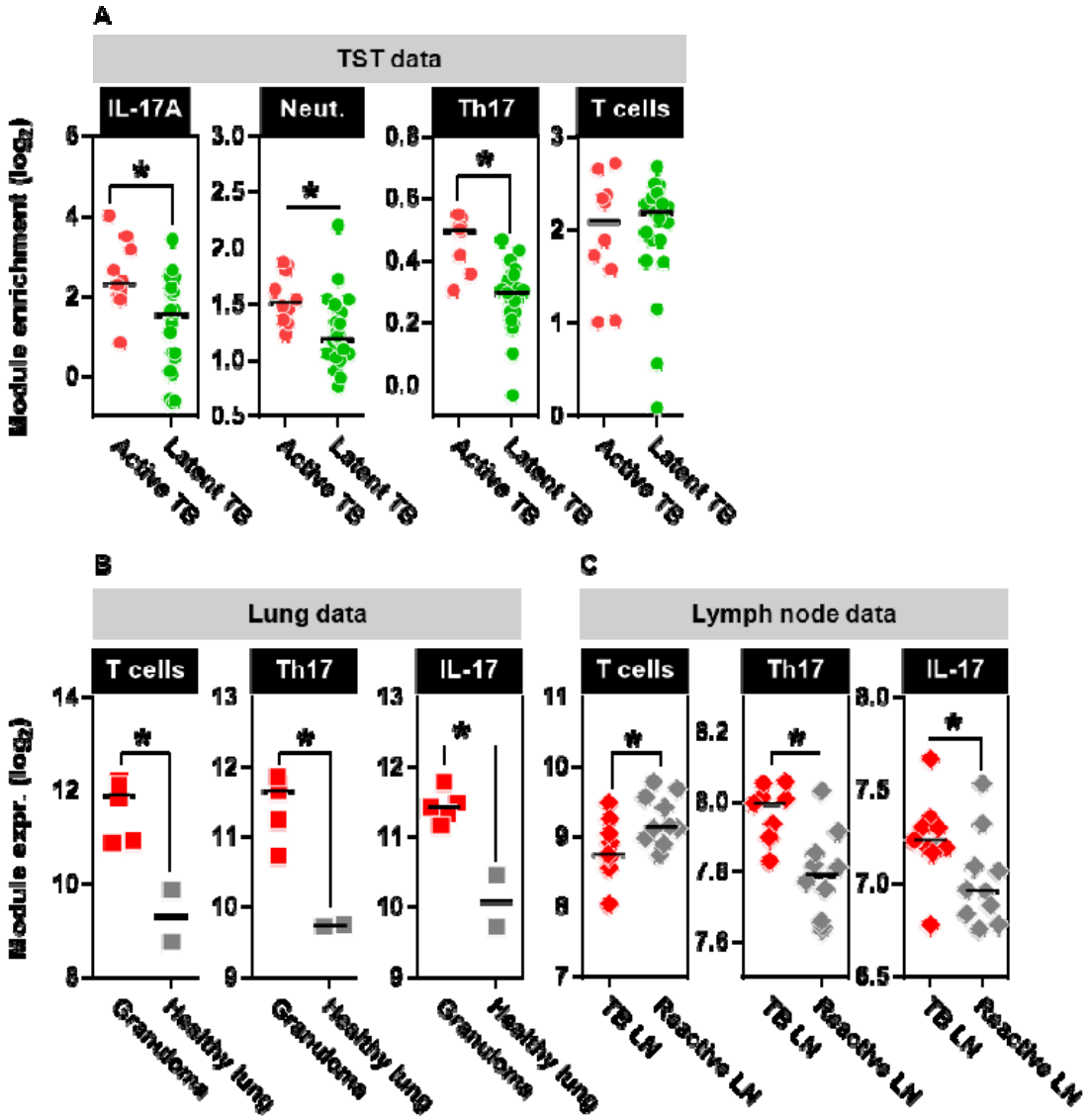


Figure 5. Active TB is characterised by elevated Th17 cells. A) IL-17F immunohistochemistry in TST of patients with active TB. Black arrows point to mononuclear cells that express IL-17F and white arrows point to polymorphonuclear cells that do not express IL-17F. B) Th17 and Th1 cell module enrichment in TST relative to saline injection. C) Expression of Th17 and Th1 modules in PPD-stimulated PBMC from patients with active or latent TB (data originated from dataset GSE27984). * = $p<0.01$ by Mann-Whitney test. D) Relationship between expression of Th17 transcriptional module and individual cytokine gene expression in TST of cytokines implicated in polarising CD4+ T cells to a Th17 phenotype. Data points represent patients with active TB, Spearman rank correlation coefficients (r^2) and p values.



624

625 **Figure 6. Th17 cells and IL-17 activity are a feature of both TST reactions and sites of human TB**
626 **disease.** A) Enrichment in TST relative to saline injection of transcriptional modules for IL-17
627 bioactivity, neutrophils, Th17 cells and T cells in TST of independent cohort of patients with active TB
628 and a separate cohort of individuals with latent TB infection. B) Expression of T cells, Th17 cells and
629 IL-17 bioactivity modules from the site of human TB granuloma relative to healthy lung tissue
630 (dataset GSE20050) and C) in human TB infected lymph nodes (LN) relative to reactive lymph nodes
631 that do not display evidence of granulomatous inflammation or cancer (dataset E-MTAB-2547). * =
632 $p<0.01$ by Mann-Whitney test.