

1 Classification: Biological Science/Immunology and Inflammation

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3 **Evasion of MAIT cell recognition by the African *Salmonella***
4 **Typhimurium ST313 pathovar that causes invasive disease.**

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KEY WORDS

33 *Salmonella* Typhimurium, sequence type 313, ST313, invasive nontyphoidal
34 *Salmonella*, MR1, MAIT cells, riboflavin, RibB.

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36 **SUMMARY**

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38 Mucosal-associated invariant T (MAIT) cells are innate T lymphocytes activated by
39 bacteria that produce vitamin B2 metabolites. Mouse models of infection have
40 demonstrated a role for MAIT cells in antimicrobial defence. However, proposed
41 protective roles of MAIT cells in human infections remain unproven and clinical
42 conditions associated with selective absence of MAIT cells have not been identified.
43 We report that typhoidal and non-typhoidal *Salmonella enterica* strains activate MAIT
44 cells. However, *S. Typhimurium* sequence type 313 (ST313) lineage 2 strains, which
45 are responsible for the burden of multidrug-resistant non typhoidal invasive disease in
46 Africa, escape MAIT cell recognition through overexpression of *ribB*. This bacterial
47 gene encodes the 4-dihydroxy-2-butanone-4-phosphate synthase enzyme of the
48 riboflavin biosynthetic pathway. This MAIT cell-specific phenotype did not extend to
49 other innate lymphocytes. We propose that *ribB* overexpression is an evolved trait that
50 facilitates evasion from immune recognition by MAIT cells and contributes to the
51 invasive pathogenesis of *S. Typhimurium* ST313 lineage 2.

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55 **STATEMENT OF SIGNIFICANCE**

56 Non-typhoidal *Salmonella* serotypes are a common cause of self-limiting
57 diarrhoeal illnesses in healthy adults. However, recently, a highly invasive multi-drug
58 resistant *Salmonella* Typhimurium sequence type 313 has emerged as a major cause
59 of morbidity and mortality in sub-Saharan Africa, particularly in children and
60 immunosuppressed individuals. In this paper we describe escape from MAIT cell
61 recognition as an additional mechanism of immune evasion of *S. Typhimurium* ST313.
62 As MAIT cells represent an early defence mechanism against pathogens at mucosal
63 surfaces, and their frequency and function are altered in immunosuppressed
64 individuals in sub-Saharan Africa, harnessing their function may offer an important
65 therapeutic strategy to improve mucosal immunity.

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67

68 **body**

69 **INTRODUCTION**

70

71 The Gram-negative bacterium *Salmonella enterica* spp. comprises many serovars
72 which are closely related phylogenetically but cause very different disease
73 presentations and distinct immune responses in immunocompetent hosts [1], [2].
74 Infection by the human restricted *Salmonella* typhoidal serovars (*S. Typhi* and *S.*
75 *Paratyphi*) results in a severe systemic disease called enteric fever. In contrast,
76 nontyphoidal serovars originating from zoonotic reservoirs such as *S. Typhimurium*
77 and *S. Enteritidis*, cause self-limiting diarrhoeal disease in healthy individuals [1]–[3].
78 Multi-drug resistant *S. Typhimurium* strains of a distinct multilocus sequence type 313
79 (ST313) recently emerged in sub-Saharan Africa. *S. Typhimurium* ST313 is associated
80 with invasive blood stream infections in immunocompromised individuals and is distinct
81 from the *S. Typhimurium* strains that cause gastroenteritis globally.

82

83 Since it was first reported in 2009 [4], the *S. Typhimurium* ST313 clade has become
84 the major cause of invasive nontyphoidal *Salmonella* (iNTS) disease in Africa [5], [6],
85 and comprises two sub-clade lineages [6], termed lineages 1 and 2. Bacteraemia by
86 iNTS causes an estimated 77,500 deaths annually worldwide [7], primarily in Africa,
87 among young children with recent malaria, malarial anaemia or malnutrition and in
88 adults afflicted with HIV, among whom recurrent disease is also common [1], [5], [8]–
89 [11]. *S. Typhimurium* ST313 isolates have rarely been reported outside of Africa [4]
90 and African ST313 blood isolates are genetically distinct from rare diarrhoeal ST313
91 isolates found in the United Kingdom [12] or Brazil [13]. Genotypic and phenotypic
92 analyses of several clinical isolates of the two well-described ST313 lineages identified
93 signatures of metabolic adaptation and unique enteropathogenesis in animal models,
94 consistent with adaptation to invasive disease in an immunocompromised human
95 population [4], [14], [15].

96

97 B and T cell responses can mediate a protective role in mouse models of *Salmonella*
98 infection. B cells provide the first line of defence at mucosal sites to restrain systemic
99 dissemination, while T cells are needed for *Salmonella* clearance [16]–[18]. Cross-
100 reactive and serovar-specific MHC-restricted T cell responses have been well
101 characterised in humans [19]–[24]. *Salmonella* can also induce activation of non-MHC-
102 restricted T cells, specifically $\gamma\delta$ T cells, invariant Natural Killer T cells (iNKT) and
103 Mucosal-associated invariant T (MAIT) cells [25]–[27], although their protective role
104 remains undetermined.

105

106 MR1-restricted MAIT cells comprise a highly conserved class of semi invariant T cells,
107 bridging innate and adaptive immunity [28]. The MHC class I-like molecule MR1,
108 bound to derivatives of vitamin B2 intermediates, activates MAIT cells [29]. This
109 process can drive antibacterial activity, *in vitro* and *in vivo*, and correlates with the
110 presence of the vitamin B2 biosynthetic pathway in several commensal and pathogenic
111 bacteria and fungal species (reviewed in [30]). Similar to iNKT and $\gamma\delta$ T cells, MAIT
112 can be activated by cytokines (IL-12, IL-18, type I IFN) independently of their TCR
113 engagement [31]. The ability of MAIT cells to recognise *S. Typhimurium*-infected
114 targets [32] prompted the identification, within bacterial supernatants, of the potent
115 MAIT cell agonists (lumazine and pyrimidines), derivatives of the vitamin B2
116 intermediate 5-A-RU [29], [33]. Following intranasal infection with *S. Typhimurium*,
117 murine MAIT cells become activated and accumulate in the lungs [26]. Human
118 challenge studies with typhoidal serovars (*S. Typhi* and *S. Paratyphi A*) also
119 demonstrated sustained MAIT cell activation and proliferation at peak of infection [34],
120 [35].

121

122 While many commensal and pathogenic bacteria possess the riboflavin biosynthetic
123 pathway, the levels of resulting MAIT stimulation varies [36], [37], perhaps reflecting
124 the influence of microenvironment on bacterial metabolism and antigen availability.
125 The ability of MAIT cells to recognise and respond to several isolates of the same
126 pathogen may also vary depending on metabolic differences between isolates [38].

127

128 We hypothesized that MAIT cells contributed to the cellular response to *Salmonella*
129 *enterica* serovars responsible for invasive disease, and examined the ability of MAIT
130 cells to recognise and respond to different *S. enterica* serovars associated to invasive
131 disease in Africa. Here, we demonstrate that *S. Typhimurium* ST313 lineage 2 isolates
132 escape MAIT cell recognition through overexpression of RibB, a bacterial enzyme of
133 the riboflavin biosynthetic pathway. Our results suggest that MAIT cell immune
134 protection represents an important 'evolutionary bottleneck' for the pathogen.

135

136

137 **RESULTS**

138

139 **Identification of cellular responses to multiple *Salmonella enterica* subsp**
140 ***enterica* serovars.**

141

142 To identify potential differences in the response of innate and adaptive T cells to
143 distinct *Salmonella* pathovars, we focused on two pathovariants of *S. Typhimurium*
144 that are responsible for different types of human disease. *S. Typhimurium* ST313 is
145 associated with invasive disease among immunocompromised individuals in Africa
146 and a representative isolate is D23580 (STM-D23580). *S. Typhimurium* sequence type
147 19 (ST19) causes non-invasive diarrhoeal infections in immunocompetent individuals
148 globally (a representative isolate is LT2, designated STM-LT2). Peripheral blood
149 mononuclear cells (PBMC) isolated from healthy donors were infected with both
150 *S. Typhimurium* pathovariants, and *S. Typhi* strain Ty2 (ST-Ty2) was used to represent
151 a more distantly related serovar that causes invasive disease in immunocompetent
152 individuals. *Escherichia coli* (*E. coli*) was included as unrelated control. Upon infection,
153 PBMC were incubated overnight in the presence of brefeldin A to permit intracellular
154 cytokine accumulation. T lymphocytes were stained with a panel of fluorescently
155 labelled antibodies to simultaneously identify different T cell populations (MAIT, $\gamma\delta$,
156 CD4 and CD8), and determine their activation status (CD69) and cytokine production
157 (IFN- γ and TNF- α).

158

159 We first defined the heterogeneity of T cell responses to *Salmonella*, by performing an
160 unsupervised clustering analysis on all CD3⁺ T cells expressing the activation marker
161 CD69 following overnight incubation with the *Salmonella* pathovariants. Dimensionality
162 reduction analysis by *t*-SNE revealed 22 populations of CD3⁺ CD69⁺ T cells, some of
163 which differed in frequency according to the infecting *Salmonella* pathogen. Clusters
164 were then annotated and assigned to MAIT (identified as CD3⁺ V α 7.2⁺ CD161^{high}), $\gamma\delta$,
165 CD4⁺ or CD8⁺ T cell subsets based on the expression of distinct phenotypic markers.

166

167 We discovered that a group of clusters of MAIT cells (clusters 6, 15, 18, 21) was under-
168 represented among all CD69⁺ cells upon infection with STM-D23580, compared with
169 STM-LT2, ST-Ty2 and *E. coli* (Figure 1A). Next, we analysed expression of IFN- γ and
170 TNF- α in CD69⁺ activated T cells. We determined that the under-represented clusters
171 of CD69⁺ MAIT cells represented IFN- γ and TNF- α producing MAIT cells (Figure 1B).

172 MAIT cells were next analysed using Uniform Manifold Approximation and Projection
173 (UMAP), a neighbouring dimensionality reduction technique that preserves embedding
174 and global distances better than t-SNE [39], and clearly defined the trajectory of the
175 distinct subpopulations of *Salmonella*-activated MAIT cells (Figure 1C). STM-LT2
176 stimulated MAIT cells clustered close to MAIT cells stimulated with ST-Ty2 and *E. coli*,
177 which were characterised by elevated expression of CD69 and the presence of single
178 and double producers of TNF- α and IFN- γ . In contrast, STM-D23580 stimulated MAIT
179 cells clustered closer to unstimulated cells, away from MAIT cells stimulated with ST-
180 Ty2 and *E. coli* (Figure 1D, top left panel). STM-D23580 stimulated MAIT cells
181 expressed low levels of CD69, with only a small TNF- α producing subpopulation and
182 almost no IFN- γ producing cells (Figure 1D).

183

184 **S. Typhimurium ST313 lineage 2 fails to elicit MAIT cell activation in healthy and** 185 **susceptible individuals.**

186

187 To validate our unsupervised analysis, we infected PBMC with the different *Salmonella*
188 strains at increasing multiplicity of infection (MOI), and then assessed MAIT cell
189 activation by flow cytometry. Infection by STM-D23580 consistently induced limited
190 MAIT cell responses across a range of MOIs and in every healthy donor tested. In
191 comparison with STM-LT2, ST-Ty2 or *E. coli*, STM-D23580 stimulated MAIT cells
192 significantly expressed less CD69 and produced less IFN- γ and TNF- α (Figure 2A-D).
193 This effect was not dependent on a selective loss of MAIT cells, as STM-D23580 did
194 not have a detrimental effect on MAIT cell viability (SI Appendix, Figure S1A). $\gamma\delta$ T
195 cells, a subset of innate T lymphocytes also present in PBMC, were strongly activated
196 by STM-D23580, indicating that the lack of activation is a phenomenon limited to MAIT
197 cells (Figure 2A, 2C).

198

199 In culture, *Salmonella spp.* secrete vitamin B2 intermediates that can bind to MR1 on
200 antigen presenting cells (APCs), to trigger MAIT cell activation [29]. To examine
201 whether STM-D23580 secretes MAIT cell agonists, we collected supernatants from
202 single-colony cultures to stimulate PBMC. Supernatants from STM-LT2 and *E. coli*
203 induced a dose-dependent production of IFN- γ and TNF- α by MAIT cells, whereas
204 STM-D23580 supernatants did not (SI Appendix, Figure S1B).

205

206 To validate such findings, we assessed MAIT cell responses to a broader selection of
207 bacterial isolates, including two *S. Typhi* strains (ST-Ty2 and ST-Quailes) and one *S.*

208 Paratyphi A strain; in addition two differently sourced stocks of STM-D23580 were
209 tested, to ensure that genuine sequence type 313 isolates were being used. At two
210 different MOIs, STM-D23580 elicited the lowest levels of MAIT cell activation of the
211 group (SI Appendix, Figure S1C-S1D). In contrast, $\gamma\delta$ T cell responses were
212 comparable across all *Salmonella* pathovars (SI Appendix, Figure S1E-S1F).

213

214 We next determined whether the lack of MAIT cell activation was caused by the entire
215 *S. Typhimurium* ST313 clade or was a unique characteristic of ST313 lineage 2 which
216 is currently causing most clinical disease in Africa [14]. STM-D23580 and additional
217 isolates of ST313 lineage 2, were compared with closely-related isolates that were
218 members of ST313 lineage 1 or the UK ST313 group that is associated with
219 gastroenteritis (Figure 2E). To examine MR1-independent MAIT cell activation, we
220 used *Enterococcus faecalis* as a negative control as it lacks the vitamin B2 biosynthetic
221 pathway [29].

222

223 Remarkably, only the ST313 strains belonging to lineage 2, such as D23580, D37712
224 and U60, failed to elicit MAIT cell activation (Figure 2F-2G). All other *Salmonella* ST313
225 lineages tested (strains U2, U5 and D25248) triggered the same level of MAIT cell
226 responses as *S. Typhimurium* 4/74 (STM-4/74) [40], a sequence type 19 strain that is
227 closely related to STM-LT2 and is associated with non-invasive diarrhoeal infections.

228

229 To validate our observations in a relevant population, we performed a series of assays
230 on blood samples obtained from healthy adult residents of Malawi, an endemic area
231 for iNTS infections caused by ST313 strains. We further expanded our investigation
232 into the distinct ST313 clusters by infecting PBMC with four strains from lineage 1, four
233 strains from lineage 2 and four strains from lineage 2.2. Lineage 2.2 comprises
234 multidrug-resistant strains that differ from lineage 2 by 27 single nucleotide
235 polymorphisms [41] and by 4 plasmids [42]. In comparison to the sequence type 19
236 representative strain STM-4/74, PBMC infected with ST313 lineage 1 strains elicited
237 similar MAIT cell responses, whereas infection with ST313 lineages 2 and 2.2 induced
238 significantly lower levels of MAIT cell responses (Figure 3A and SI Appendix, Fig S2).

239

240 Lastly, we extended our findings to a clinically susceptible cohort of HIV-infected adults
241 living in Malawi. In comparison with a cohort of healthy samples from the UK and
242 Malawi, and consistent with previous reports (reviewed in [43]), the overall percentage
243 of MAIT cells was reduced among HIV-infected individuals, particularly in those not

244 receiving antiretroviral therapy (ART) (Figure 3B). In line with the data obtained with
245 healthy volunteers, MAIT cells from HIV⁺ adults also failed to produce IFN- γ and TNF-
246 α following *ex vivo* stimulation with STM-D23580, regardless of their ART status
247 (Figure 3C). In contrast, MAIT cells from HIV⁺ individuals responded strongly to *S.*
248 Typhi and STM-4/74.

249

250 These findings suggest that evasion of MAIT cell recognition by sequence type 313
251 *Salmonella* strains may be a critical factor during the course of natural iNTS disease
252 in endemic populations and in clinically-susceptible groups.

253

254 **STM-D23580 does not affect MR1-dependent antigen presentation.**

255

256 To define the molecular mechanisms underlying the lack of MAIT stimulation by STM-
257 D23580, we first investigated whether MAIT cell activation was MR1-dependent.
258 Adding the anti-MR1 blocking antibody 26.5 [44] completely abrogated the MAIT cell
259 activation induced by STM-LT2 and *E. coli*, as well as the minimal activation induced
260 by STM-D23580 (Figure 4A-4B).

261

262 We next examined whether STM-D23580 either failed to produce stimulatory MR1
263 ligands or actively inhibited MAIT cell activation. MAIT cell activation was restored
264 following the addition of the canonical MAIT cell ligand 5-amino-6-D-ribitylaminouracil
265 (5-A-RU) and methylglyoxal (MG) [33] to infected PBMC (Figure 4C-4D),
266 demonstrating that a dominant antagonistic MR1 ligand was not released by STM-
267 D23580. Consistent with these results, a combination of supernatants from overnight
268 cultures of both STM-D23580 and STM-LT2 (added simultaneously or one hour apart)
269 fully restored MAIT cell activation (SI Appendix, Figure S3A-S3B). Likewise, co-
270 infection of PBMC with STM-D23580 plus either STM-LT2, ST-Ty2 or *E. coli* also
271 restored MAIT cell activation to the levels observed with single infections (Figure 4E-
272 4F). Taken together, our data show that STM-D23580 neither interferes nor blocks
273 MAIT cell activation in the presence of stimulatory MR1 ligands.

274

275 To exclude the possibility that the lack of MAIT cell activation arose from an insufficient
276 infection of APCs, we exposed Monocyte-derived Dendritic Cells (MoDCs) to
277 fluorescently labelled STM-D23580 or STM-LT2. Live *Salmonella*-containing MoDCs
278 were FACS-sorted and co-cultured with enriched autologous CD3⁺ T lymphocytes, as
279 described previously [45]. In contrast to STM-LT2 infected MoDCs, STM-D23580

280 infected MoDCs did not stimulate effector MAIT cells (SI Appendix, Figure S3C). Using
281 an MR1-overexpressing antigen presenting cell line, we found that STM-D23580
282 supernatants do not cause downregulation of surface MR1 expression, thus excluding
283 this as a possible cause of the lack of MAIT cell activation (SI Appendix, Figure S3D).

284

285 Cytokines, such as IL-12, IL-18 and type I IFN, released by APCs upon bacterial or
286 viral infection can also activate MAIT cells in a MR1-independent manner [31]. We
287 confirmed that when MoDCs were co-cultured with purified MAIT cells, equal amounts
288 of bioactive IL-12p70 were secreted upon infection with STM-D23580, STM-LT2 and
289 *E. coli* (SI Appendix, Figure S3E).

290

291 Taken together, these observations refuted the hypothesis that lack of MAIT cell
292 activation is caused by an impaired MR1-dependent antigen-presentation following
293 STM-D23580 infection.

294

295 **STM-D23580 evades MAIT cell recognition by overexpression of RibB.**

296

297 The observation that STM-D23580 and related ST313 isolates do not interfere with
298 MR1 presentation or cytokine production suggests that these bacteria might not
299 produce the MR1 binding ligands that are generated by other *S. Typhimurium* or *S.*
300 *Typhi* pathovariants. The major source of natural antigens driving MAIT cell activation
301 derives from by-products of microbial riboflavin synthesis [33]. We depict the
302 *Salmonella* riboflavin biosynthetic pathway in Figure 5A. A comparison of the coding
303 sequences (CDS) of the enzymes involved in the riboflavin biosynthesis pathway found
304 only one nucleotide change (SNP) between the sequence type 19 STM-4/74 and the
305 sequence type 313 STM-D23580 strains. This synonymous coding variant was located
306 in the *ribD* gene (Glu316Glu) [46], suggesting that there were no biochemical
307 differences between the riboflavin biosynthesis pathways of the sequence type 313
308 and sequence type 19 isolates.

309

310 To determine whether the enzymes of the riboflavin pathway of the sequence type 19
311 STM-4/74 and sequence type 313 STM-D23580 strains were expressed at different
312 levels, we investigated the transcriptomic and proteomic data from our recent
313 comparative analysis [46]. Strains STM-4/74 and STM-D23580 are closely-related,
314 sharing 92% of coding genes [46]. Differential gene expression analysis of the *rib*
315 genes at the transcriptomic level identified significant up-regulation of *ribB* (≥ 2 fold-
316 change, FDR ≤ 0.001) in STM-D23580 in four out of the five experimental conditions

317 (Figure 5B). We then examined data from a quantitative proteomic approach which
318 showed that RibB protein levels were up-regulated in STM-D23580 compared to STM-
319 4/74, during growth in rich medium at early stationary phase (ESP) (Figure 5C).

320

321 We searched for a molecular explanation for the high levels of *ribB* expression in STM-
322 D23580 compared to STM-4/74. In STM-D23580, *ribB* and its 5' untranslated region
323 (5'UTR) are transcribed as a single transcript that is initiated from a single gene
324 promoter which we identified previously [46]. By analogy with the genetic mechanism
325 identified for over-expression of the PgtE virulence factor in STM-D23580 [47] we
326 searched for nucleotide polymorphisms that distinguished the *ribB* regions of the two
327 strains. There were no differences between the promoter sequences of the *ribB* genes
328 or the 5' UTR of the strains STM-D23580 and STM-4/74.

329

330 To determine whether the MAIT cell activation phenotype was linked to the
331 overexpression of the RibB enzyme (4-dihydroxy-2-butanone 4-phosphate synthase),
332 we created a derivative of STM-4/74 that expressed high levels of RibB. Since
333 deletions in the riboflavin biosynthetic pathway genes are lethal without high dose
334 riboflavin supplementation [33][48] and *ribB* is essential for *Salmonella in vivo*
335 virulence [49], we used a gene cloning approach to overexpress the *ribB* gene of STM-
336 D23580 in STM-4/74 from a recombinant plasmid (STM-4/74 RibB⁺⁺). The expression
337 of high levels of the RibB enzyme by STM-4/74 ablated MAIT cell activation induced
338 by wild-type STM-4/74. Infection with STM-4/74 RibB⁺⁺ induced low levels of cytokine
339 production and CD69 expression by MAIT cells, recapitulating the phenotype of STM-
340 D23580 (Figure 5D-5E). Importantly, $\gamma\delta$ T cells responded equally to both STM-4/74
341 wild type and STM-4/74 RibB⁺⁺ (SI Appendix, Figure S4).

342

343 We next evaluated the bacterial growth rate, as well as the infection efficiency of the
344 STM-4/74 RibB⁺⁺ bacterial strain. Mid-log phase curves demonstrated no significant
345 differences in the growth rate between STM-4/74 wild type, STM-4/74 RibB⁺⁺ and other
346 related strains (SI Appendix, Figure S5A). Human monocyte-derived dendritic cells
347 and human monocyte-derived macrophages were infected *in vitro*, and the number of
348 intracellular bacterial colony-forming units (cfu) was measured as a readout of
349 internalisation. In both cell types tested, the number of recovered intracellular STM-
350 4/74 RibB⁺⁺ was comparable to the number obtained from other *Salmonella* strains (SI
351 Appendix, Figure S5B). These data exclude the possibility that the lack of MAIT
352 activation by STM-4/74 RibB⁺⁺ reflects slow growth or poor bacterial internalisation into
353 antigen-presenting cells.

354

355 MAIT activator ligands such as 5-OP-RU (5- (2-oxopropylideneamino)-6-D-
356 ribitylaminouracil) or 5-OE-RU (5- (2-oxoethylideneamino)-6-D-ribitylaminouracil),
357 products of the riboflavin pathway, cannot be measured due to their unstable nature
358 [33]. Therefore, to investigate whether overexpression of RibB altered the balance of
359 downstream products from the riboflavin pathway, we measured the amount of
360 riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) by
361 HPLC. Following growth to early stationary phase, intracellular samples and culture
362 supernatants from STM-4/74 and STM-4/74 RibB⁺⁺ contained larger amounts of
363 riboflavin than the sequence type 313 strains STM-D23580 and STM-D37712 (Figure
364 6A-6B). The STM-4/74 RibB⁺⁺ supernatant with the highest level of riboflavin also
365 contained the largest amount of FMN, compared with STM-4/74, STM-D23580 and
366 STM-D37712 (Figure 6C).

367

368 Overall, the data show that the RibB over-producing strain (4/74 RibB⁺⁺) produced the
369 highest level of extracellular riboflavin and FMN, whereas had the lowest level of
370 intracellular FMN. In contrast, the STM-4/74 wild-type strain had the largest
371 intracellular amount of FMN, compared with 4/74 RibB⁺⁺ and both of the sequence type
372 313 strains (Figure 6D). While intracellular FAD levels were similar between STM-4/74,
373 STM-D23580 and STM-D37712, STM-4/74 RibB⁺⁺ contained the lowest level of
374 intracellular FAD (Figure 6E). We conclude that the lowest levels of intracellular FMN
375 was found in the STM-D23580, STM-D37712 and STM-4/74 RibB⁺⁺ strains that
376 overexpress the RibB enzyme.

377

378 **Reduced MAIT cell antibacterial activity against *S. Typhimurium* ST313 lineage**
379 **2 infected macrophages.**

380

381 To test the role of MAIT cells in clearing *Salmonella* infections, we developed an *in*
382 *vitro* assay where human monocyte-derived macrophages were infected with the
383 different *Salmonella* strains, either in presence or absence of purified MAIT cells. As
384 observed with PBMC, we measured less IFN- γ in supernatants from co-cultures of
385 purified MAIT cells and macrophages infected with STM-4/74 RibB⁺⁺ and sequence
386 type 313 lineage 2 strains, as compared to the control sequence type 19 strains (SI
387 Appendix, Figure S6A).

388

389 In these experiments, the number of intracellular cfu recovered from infected
390 macrophages was used as a surrogate of bactericidal activity induced by MAIT cells.

391 In the majority of the biological replicates and in comparison with macrophages alone,
392 we observed a modest reduction of cfu numbers (25% or less) when MAIT cells and
393 sequence type 313 lineage 2 infected macrophages were put in co-culture (SI
394 Appendix, Figure S6B). In contrast, almost all biological replicates infected with
395 sequence type 19 *Salmonella* had a reduction of more than 25% in the number of cfu
396 when MAIT cells were added to the infected macrophages (SI Appendix, Figure S6B).
397 No differences in bactericidal activity were observed between STM-4/74 RibB⁺⁺ and
398 STM-4/74. While macrophages pre-treated with IFN- γ acquired bactericidal activity
399 against all *Salmonella* isolates (SI Appendix, Figure S6C), MAIT cell anti-microbial
400 activity in these *in vitro* co-cultures was not directly correlated to the amount of IFN- γ
401 found in supernatants (SI Appendix, Figure S6D).

402 **DISCUSSION**

403

404 Here we investigated the ability of MAIT cells to recognise and respond to diverse
405 invasive *Salmonella enterica* serovars. We found that MAIT cells isolated from the
406 blood of healthy individuals were not activated by exposure to invasive disease-
407 associated *S. Typhimurium* sequence type 313 (ST313) lineage 2 strains. Our data
408 demonstrated how *S. Typhimurium* ST313 lineage 2 evades MAIT cell recognition by
409 overexpressing *ribB*, a bacterial gene encoding the RibB enzyme involved in the
410 riboflavin pathway. Our results lead us to propose that this RibB-mediated mechanism
411 provides an evolutionary advantage that allows invasive *S. Typhimurium* ST313
412 lineage 2 bacteria to escape cell immune responses by overexpressing a single
413 riboflavin bacterial gene.

414

415 MR1-restricted MAIT cells are highly abundant in the gut mucosa [50], where they
416 reach their final maturation upon recognition of vitamin B2 metabolites derived from
417 gut commensals presented by MR1 expressing mucosal B cells [50]. MAIT cells show
418 antimicrobial activity *in vivo* and *in vitro* [51], [52] through MR1 dependent and
419 independent interactions. Microorganisms must express the riboflavin biosynthesis
420 pathway to be able to activate MAIT cells [29], [53]. Mutations in key enzymes of the
421 riboflavin biosynthetic pathway in both Gram positive and negative bacteria can
422 abrogate MAIT cell activation [33], [54]. Different bacteria that possess the riboflavin
423 biosynthetic pathway induce varying levels of MAIT stimulation [36], [37], possibly
424 through the influences of the microenvironment on bacterial metabolism and antigen
425 availability, or the known short half-life of the potent MAIT cell antigens, 5-OP-RU and
426 5-OE-RU [55]. The ability of MAIT cells to recognise and respond to several isolates
427 of the same pathogen may also vary to reflect bacterial metabolic differences. For
428 example, riboflavin metabolism variation among clinical isolates of *Streptococcus*
429 *pneumoniae* produces different measurable levels of riboflavin and FMN that correlate
430 with differential activation of MAIT cells [38].

431

432 *Salmonella* spp. possess an active riboflavin biosynthetic pathway, which generates
433 MAIT cell agonists [29], [33]. MAIT cells recognise and kill *S. Typhimurium*-infected
434 targets *in vitro* [32], and activated MAIT cells accumulate in murine lungs following
435 intranasal infection with *S. Typhimurium* [26]. However, bacterial lung clearance was
436 independent of MAIT cells in this infection model, possibly due to the non-physiological
437 route of infection. While some mouse models of infection with *Salmonella* sequence
438 type 313 strains have been published [14], [56], these models present limited utility as

439 mice have very low frequencies and absolute numbers of MAIT cells compared with
440 humans [28]. Human studies demonstrated sustained MAIT cell activation and
441 proliferation at the peak of infection with *S. Typhi* and *S. Paratyphi* [34], [35]. Among
442 immunocompetent humans, the clinical outcomes of infection by *Salmonella enterica*
443 spp. depend on the infecting serovar. Human-restricted typhoidal serovars, such as *S.*
444 *Typhi* induce the most severe form of systemic disease, typhoid fever; while the broad-
445 host *S. Typhimurium* sequence type 19 causes self-limiting gastroenteritis. The
446 recently documented multidrug resistant *S. Typhimurium* ST313 clade causes the
447 majority of iNTS cases among immunocompromised adults and malnourished young
448 children living in sub-Saharan Africa.

449

450 Several bacterial factors have been reported to enhance invasiveness of *S.*
451 *Typhimurium* ST313, suggesting a multifactorial adaptation of this African lineage to a
452 systemic lifestyle. These include interference with the complement cascade [47],
453 interference with DC function [45], reduced inflammasome activation [57],
454 dissemination through CD11b⁺ migratory DC [58] among others.

455

456 Here we found that *S. Typhi*, *S. Paratyphi* A and most *S. Typhimurium* pathovars
457 potently elicited *ex vivo* MR1-dependent MAIT cell activation, but all tested isolates
458 from the invasive *S. Typhimurium* ST313 lineage 2 barely induced cytokine secretion
459 or CD69 upregulation. This suboptimal activation was restricted to MAIT cells, as $\gamma\delta$
460 cell activation was comparable across all isolates tested. We excluded differences in
461 infection efficiency, MR1 expression, co-stimulatory cytokines (IL-12), the genetic
462 sequence of the riboflavin encoding enzymes of *S. Typhimurium* and the presence of
463 dominant inhibitory ligands as potential mechanisms for these results. Following
464 comparative proteomic and transcriptomic analyses, we discovered that invasive *S.*
465 *Typhimurium* ST313 lineage 2 pathovars escape MAIT cell recognition by
466 overexpressing *ribB*, a bacterial gene encoding the riboflavin biosynthetic enzyme
467 RibB. By overexpressing this single riboflavin gene in a sequence type 19 *S.*
468 *Typhimurium* strain, we revealed that up-regulation of this single riboflavin gene was
469 sufficient to abrogate MAIT cell responses.

470

471 Transcriptional control of the bacterial *ribB* gene is controlled by a conserved FMN
472 riboswitch, which is located in the 5' untranslated region (5' UTR) of *ribB* [59], and is
473 negatively regulated by FMN and other flavins at the transcriptional and translational
474 levels in *E. coli* [60]. The SroG small RNA (sRNA) is derived from the *ribB* 5' leader
475 sequence, although the function of this sRNA remains unknown [61]. In addition to the

476 riboswitch-mediated regulation, RibB expression is induced by growth in a low pH
477 environment [62]. In STM-D23580, both *sroG* and *ribB* are transcribed as a single
478 transcript that is initiated from a single gene promoter which we identified previously
479 [46].

480

481 While the precise molecular mechanism responsible for *ribB* overexpression remains
482 to be established, a single noncoding nucleotide polymorphism in the promoter of the
483 *pgtE* gene of the ST313 lineage 2 strain STM-D23580 is known to be responsible for
484 high expression of the outer membrane PgtE virulence factor, which promotes
485 bacterial survival and dissemination during mammalian infection [47]. The lack of
486 nucleotide differences between the promoter sequences of the *ribB* genes or the
487 riboswitch of the two strains suggests that the high level of expression of *ribB* in STM-
488 D23580 is caused by a novel and uncharacterised regulatory mechanism.

489

490 It has been proposed that genomic changes in ST313 isolates that confer altered
491 metabolism and increased anaerobic metabolic capacity are linked to adaptation of the
492 extra-intestinal niche [14]. Riboflavin and its derivatives are important cofactors for
493 flavoproteins involved in cellular redox metabolism and several biochemical pathways,
494 proposed to be essential for the metabolic adaptation of the ST313 clade. Riboflavin
495 promotes intracellular microbial survival and virulence during *in vivo* infection with
496 *Histoplasma capsulatum* and *Brucella abortus* [63], [64]. In addition, accumulation of
497 riboflavin is a candidate virulence factor in *Pseudogymnoascus destructans* skin
498 infection [65].

499

500 The metabolomic measurements of the end-products of the riboflavin pathway showed
501 a correlation between lower levels of intracellular FMN and increased expression of
502 RibB. Because FMN is a negative regulator of *ribB* gene expression [60], we speculate
503 that the lower levels of intracellular FMN observed in the ST313 strains D23580 and
504 D37712 are linked to the high levels of expression of RibB in these African *S.*
505 *Typhimurium* strains.

506

507 Overall, our findings suggest that MAIT cells play a crucial role in defence against
508 invasive *Salmonella* disease in humans and that evasion from MAIT cell recognition is
509 a critical mechanism for the invasiveness of *S. Typhimurium* ST313 lineage 2 isolates,
510 including those belonging to the lineage 2.2 cluster [41]. We propose that differences
511 in MAIT cells activation may associate to distinct diseases caused by closely related
512 microorganisms. The increased susceptibility of immunocompromised patients to the

513 *S. Typhimurium* ST313 lineage 2 strains suggests that MAIT cells might play a
514 particularly relevant role in the context of waning CD4⁺ T cell mediated protective
515 adaptive immunity, where protection relies mostly on the innate immune response. For
516 example, following HIV co-infection and/or malnutrition, among individuals suffering
517 from recurrent gut infections secondary to intestinal barrier dysfunction [66], [67],
518 microbiota dysbiosis [68], [69] and multiple innate and adaptive immune defects [70].
519 Our findings may be of major relevance during the initial phase of infection, in the gut,
520 where it is expected that resident immune cells such as MAIT cells should prevent
521 systemic infection by encountering and responding rapidly to bacterial signals. We
522 propose that the ability of MAIT cells to target gastrointestinal pathogens represents a
523 key immunological evolutionary bottleneck that has been effectively countered by
524 *Salmonella*, resulting in the current epidemic of invasive disease in Africa.

525

526

527

528 **METHODS**

529

530 **Bacterial strains and preparation of stocks.**

531 This study included representative strains of *Salmonella enterica* serovar
532 Typhimurium, from both the sequence type 19 and the sequence type 313. *S. Typhi*
533 and *S. Paratyphi A* serovars were utilised as comparative Typhoidal invasive strains,
534 while *Escherichia coli* (DH5 α) was used as unrelated bacterial control. Supplementary
535 Table 1 lists bacterial strains used in this study.

536 Overnight bacterial cultures from a single colony origin were used to inoculate LB
537 Lennox broth (Sigma) supplemented with sucrose (Sigma) at a final concentration of
538 10%. Inoculated cultures were incubated at 37°C under constant shaking for
539 approximately 3 hours, until reaching mid logarithmic phase. Bacterial aliquots were
540 prepared and immediately frozen at -80°C for long-term storage. Bacterial viability of
541 frozen aliquots was monitored periodically in order to maintain experimental
542 reproducibility. The number of viable colony forming units (cfu) was determined with
543 the Miles and Misra method, by plating 10-fold dilutions of the bacterial suspension
544 onto LB Lennox agar (Sigma). On the day of the experiment, a single aliquot was
545 thawed, washed twice with PBS and re-suspended in RPMI 1640 media to obtain the
546 desired multiplicity of infection (MOI).

547 In the case of bacterial supernatants, these were taken from late exponential phase
548 cultures, grown from a single colony following 18 hours incubation under constant
549 shaking. Supernatants were filter sterilised before using.

550

551 **Construction of *S. Typhimurium* 4/74 pP_L-*ribB***

552 To construct pP_L-*ribB*, the *ribB* gene was amplified from genomic DNA of *S.*
553 *Typhimurium* 4/74 using primers *ribB*_FW and *ribB*_RV. The PCR product was used
554 for a linear amplification reaction with plasmid pJV300 (pP_L) using Phusion DNA
555 polymerase (New England Biolabs), and the resulting product was digested with DpnI.
556 The plasmid was transformed into *E. coli* TOP10 and selected on LB plates
557 supplemented with 100 μ g/mL ampicillin. Plasmid presence was confirmed by PCR
558 and DNA sequencing using oligonucleotides pP_L_Seq_FW and pP_L_Seq_RV. The
559 pP_L-*ribB* plasmid was subsequently purified and transformed into *S. Typhimurium*
560 4/74. Supplementary Table 2 lists plasmids and oligonucleotides used in this study.

561

562 **Isolation of human cells from peripheral blood from healthy volunteers in the**
563 **UK.**

564 Leukocyte Reduction System cones were obtained from the UK National Blood Centre
565 with informed consent following local ethical guidelines (NHSTB account T293). Blood
566 was diluted in PBS and separated by gradient centrifugation using Lymphoprep™
567 (AxisShield). Peripheral Blood Mononuclear Cells (PBMC) were collected from the
568 interface, washed with PBS, resuspended in complete medium and counted. Complete
569 medium used throughout was RPMI 1640 (Sigma), supplemented with 10% heat-
570 inactivated FCS (Sigma), 2 mM L-glutamine, 1% nonessential amino acids and 1%
571 sodium pyruvate (all from Gibco).

572

573 **Isolation of PBMC from healthy and HIV-infected volunteers in Malawi.**

574 Blood samples were obtained at Queen Elizabeth Central Hospital (Blantyre, Malawi)
575 following local ethical guidelines (P09/17/2284). Adults presenting for HIV testing at
576 the voluntary testing clinic, the HIV outpatient clinic, and the medical inpatient wards
577 at the Queen Elizabeth Central Hospital were recruited. Based on the use of
578 antiretroviral therapy, these patients were classified as ART naïve (without) or ART
579 treated (with). Upon appropriate consent and medical authorisation, a blood sample
580 was collected and PBMC were isolated and used in *ex vivo* infection assays, as
581 described below.

582

583 **Ex vivo infection assays with PBMC**

584 PBMC were seeded in 96 well plates round bottom (5 to 8 x10⁵ cells per well) and
585 infected with the different *Salmonella* strains from frozen mid-log phase stocks, at the
586 indicated MOI. Upon 80 minutes incubation at 37°C, 100 µg/mL gentamicin (Lonza)
587 was added to kill extracellular bacteria. 200 µg/mL of gentamicin was required for the
588 experiments carried out in Malawi using ST313 strains from lineages 1, 2 and 2.2.

589 At 180 minutes post-infection, 5ng/mL brefeldin A (BioLegend) solution was added to
590 every well in order to achieve accumulation of intracellular cytokines. Samples were
591 incubated overnight at 37°C for no more than 15 hours.

592 For MR1 blocking experiments, infection was performed in the presence of 30 µg/mL
593 MR1 blocking antibody 26.5 [44] or mouse IgG2a isotype control (ATCC). The MAIT
594 agonists 5-Amino-6-D-ribitylaminouracil (5-A-RU) was synthesised as described [71]
595 and 1 µg/mL was combined to 50 µM methylglyoxal (MG, Sigma).

596

597 **Assessment of cytokine production and MAIT cell activation by flow cytometry.**

598 Following incubation in the presence of brefeldin A, cells were harvested, washed and
599 stained with a viability dye (live/dead Zombie Aqua, BioLegend) for 20 minutes.

600 Fixation was performed for 30 minutes at 4°C using the Foxp3
601 Fixation/Permeabilization buffers (eBioscience). Fixed cells were permeabilised and
602 stained with an antibody cocktail for 40 minutes at room temperature, washed and
603 stored protected from light at 4°C in PBS with 0.5% bovine serum albumin (FACS
604 buffer) until acquisition. The following antibodies were used for extracellular and
605 intracellular staining as 2 different panels: anti-CD3 Alexa700 (clone UCHT1;
606 BioLegend), anti-CD3 PerCp Cy5.5 (clone UCHT1; BioLegend), anti-CD4 APCef780
607 (clone RPA-T4; eBioscience), anti-CD4 Alexa700 (clone RPA-T4; BioLegend), anti-
608 CD8 BV785 (clone RPA-T8; BioLegend), anti-TCR γ/δ APC (clone B1; BioLegend),
609 anti-CD161 BV605 (clone HP-3G10; BioLegend), anti-CD161 BV421 (clone HP-3G10;
610 BioLegend), anti-V α 7.2 PE (clone 3C10; BioLegend), anti-V α 7.2 PE-Cy7 (clone 3C10;
611 BioLegend), anti-CD69 FITC (clone FN50; BioLegend), anti TNF- α PECy7 (clone
612 Mab11; BioLegend), anti TNF- α APC (clone Mab11; BioLegend), anti-IFN- γ FITC
613 (clone 4S.B3; BioLegend) and anti-IFN- γ PE Dazzle (clone 4S.B3; BioLegend).
614 Samples from the UK were acquired on a FortessaX20 (BD), whilst samples from the
615 case-control study in Malawi were acquired on a LSR Fortessa cytometer (BD). All
616 data was analysed with the same gating strategy on FlowJo (v.10.6.1).

617

618 **Unsupervised analysis of flow cytometry data.**

619 Two dimensionality reduction methods based on a neighbouring graph approach were
620 implemented, t-Distributed Stochastic Neighbour Embedding (t-SNE) [72] and Uniform
621 Manifold Approximation and Projection (UMAP) [73]. t-SNE algorithm was performed
622 on the Cytokit platform [74] using up to 5,000 cells from each sample. UMAP was run
623 as a plugin on FlowJo (v.10.4.1) using 15 nearest neighbours, a minimum distance of
624 0.5 and Euclidean distance for selected parameters. Files with .fcs extension from
625 related experimental conditions were concatenated before UMAP analysis.

626

627 **Co-culture of *Salmonella* infected monocyte-derived dendritic cells and purified** 628 **T cells.**

629 Monocyte-derived dendritic cells (MoDCs) were obtained from PBMC by enrichment
630 of CD14⁺ monocytes using magnetic beads (Miltenyi). Differentiation was achieved
631 with recombinant human GM-CSF (40 ng/mL) and human IL-4 (40ng/mL), both from
632 PeproTech. After 5 days, MoDCs were infected with violet-labelled (CellTracker™, Life
633 Technologies) *Salmonella* strains, either STM-D23580 or STM-LT2 at an MOI of 10,
634 as reported elsewhere [45].

635 At six hours post-infection, *Salmonella*-containing MoDCs were FACS sorted as single
636 cells. Sorted MoDCs were co-cultured with magnetically enriched (Miltenyi) CD3⁺ T
637 cells obtained from the same donor, at a ratio of 1 MoDC per 6 T cells. Following 12
638 hours incubation in the presence of brefeldin A, T cells were harvested and stained for
639 intracellular cytokines as described above.

640

641 **Co-culture of *Salmonella* infected MoDCs and expanded MAIT cells.**

642 Human MoDCs were obtained as described above. Human MAIT cells were isolated
643 by sorting CD2 MACS-enriched (Miltenyi) leukocytes with CD161 and V α 7.2
644 antibodies (BioLegend). MAIT cells were grown for around 6 weeks in complete RPMI
645 media supplemented with IL-2, as described elsewhere [71].

646 40,000 MoDCs and 20,000 MAIT cells (2:1 ratio) were seeded in 96 well plates flat
647 bottom and were infected at MOI of 3.5. After 80 minutes, 100 μ g/mL gentamicin was
648 added and supernatants were harvested following 26 hours incubation. IL-12 p70 was
649 measured by ELISA (R&D systems) in triplicates and following manufacturer's
650 instructions.

651

652 **Co-culture of *Salmonella* infected monocyte-derived macrophages and
653 expanded MAIT cells.**

654 Monocytes were obtained from leukocyte reduction system cones by enrichment of
655 CD14⁺ cells using magnetic beads (Miltenyi), according to manufacturer's protocol.
656 Monocytes were seeded in 24-well plates (450,000 - 500,000 cells/well) and
657 differentiated into macrophages using recombinant human M-CSF at 100 ng/mL
658 (PeproTech). After 6 days of incubation at 37°C and 5% CO₂, the adherent
659 macrophages were carefully washed to remove M-CSF containing-media and fresh
660 antibiotic-free medium was added. Next, macrophages were infected with the different
661 *Salmonella* strains at a MOI of 15. After 30 minutes post-infection, cells were washed
662 and incubated for further 30 minutes with 100 μ g/mL of gentamicin-containing medium
663 to kill any remaining extracellular bacteria. At 1 hour post-infection, macrophages were
664 washed again and MAIT cells were added (ratio 1 MAIT cell per 5 macrophages) into
665 the respective wells. From this point and onwards, media contained gentamicin at 30
666 μ g/mL as maintenance dose. Cells were incubated until completing 6 hours post-
667 infection before being washed twice with PBS and lysed with 2% saponin. The number
668 of intracellular viable bacterial cfu was determined with the Miles and Misra method as
669 described above. IFN- γ in the supernatants was measured with a commercial ELISA
670 (BD-Pharmingen), as per manufacturer instructions.

671

672 **MR1 over-expressing cell line**

673 THP-1 cells were transduced with an MR1 encoding lentivirus [34]. MR1
674 overexpressing cells were seeded in 96 well plates flat bottom and incubated overnight
675 in the presence of 50 μ L of supernatants from bacterial cultures at late exponential
676 phase, or in the presence of 5-A-RU as positive control. Cells were harvested, washed
677 and stained for surface expression of MR1 (clone 26.5; BioLegend) by flow cytometry.
678 Expression of MHC-I (clone G46-2.6, BD Biosciences) was also monitored as
679 unrelated control.

680

681 **Transcriptomic and proteomic analyses of riboflavin enzymes.**

682 RNA-seq and proteomic data for genes involved in the riboflavin biosynthetic pathway
683 were extracted from a recent work [46]. Briefly, a differential expression comparative
684 analysis between strains STM-D23580 and STM-4/74 was performed at the
685 transcriptomic level in five *in vitro* infection-relevant conditions: ESP (early stationary
686 phase), anaerobic growth, NonSPI2 (SPI2-non-inducing PCN), InSPI2 (SPI2-inducing
687 PCN) and inside murine RAW264.7 macrophages (ATCC TIB-71). Specific details
688 about growing bacteria in these conditions had been previously described [40], [75].
689 For a comparative proteomic analysis, bacteria were grown to ESP in the LB rich
690 medium.

691 The RNA-seq-based comparative approach between STM-D23580 and STM-4/74 was
692 based on Voom/Limma analysis from three different biological replicates for each
693 strain. A detailed pipeline for the analysis can be found in Canals *et al.* [46].

694 Proteomic data for strains STM-D23580 and STM-4/74 were obtained using an LC-
695 MS/MS (Q Exactive Orbitrap, 4h reversed phase C18 gradient) platform. Samples
696 included six biological replicates for each strain. Label-free quantification and
697 differential expression analyses between the two strains were performed using the
698 Progenesis Q1 software (Nonlinear Dynamics) [46].

699

700 **Measurement of riboflavin, FMN and FAD in supernatants and pellets of bacterial**
701 **cultures.**

702 Bacterial pellets and supernatants from early stationary phase cultures ($OD_{600} \sim 2$),
703 were prepared in triplicate and frozen at -80 °C. For extraction of cellular flavins, pellets
704 were resuspended in 100 μ L of 100 mM ammonium formate, 100 mM formic acid, 25%
705 (v/v) methanol and heated at 80 °C for 10 min. Insoluble material was removed by
706 centrifugation. For analysis 5 μ L of this material or the culture supernatants was

707 separated by HPLC on a Dionex UPLC system with a Kinetex C18 column
708 (Phenomenex; 1.7 μ m, 150 x 2.1 mm). Separation was achieved at 45 °C and 0.2
709 mL/min isocratically using 20 mM potassium phosphate buffer (pH 2.5) with 25%
710 methanol (v/v) over 8 min followed by a 1 min wash step in 100% methanol. Flavins
711 were detected with fluorescence (450 nm excitation, 520 nm emission) and peaks were
712 quantified by comparison to known standards. For normalisation the total pmol of flavin
713 for each culture was divided by the measured OD₆₀₀ of the cultures to give a final
714 pmol/OD₆₀₀ value.

715

716 **Statistical analysis**

717 Statistical analyses were performed using GraphPad-Prism8 (GraphPad Software;
718 San Diego, United States). Differences among groups were determined by paired one-
719 way, two-way ANOVA or Kruskal-Wallis as appropriate. Post-hoc corrections were
720 applied, Dunnett's or Dunn's for comparisons to a control data set and Bonferroni's,
721 Tukey's or Sidak's for comparisons of selected pairs tests, as appropriate. A p -value
722 <0.05 was considered statistically significant ($*p <0.05$, $**p <0.01$, $***p <0.001$ and
723 $****p <0.0001$).

724

725 **Data Availability**

726 All data has been made available in the manuscript.

727

728

729 **FIGURE LEGENDS**

730

731 **Figure 1. Identification of cellular responses to multiple *Salmonella enterica***
732 **subsp *enterica* serovars.**

733 PBMC were left unstimulated or were infected at MOI of 5 with STM-D23580, STM-
734 LT2, ST-Ty2 or *E. coli*. Intracellular staining was performed to detect CD69 expression
735 and cytokine production (TNF- α and IFN- γ), as correlates of T cell activation. **(A)** *t*-
736 SNE plots on gated CD69⁺ CD3⁺ T cells infected with STM-D23580, STM-LT2, ST-Ty2
737 or *E. coli*. Four CD3⁺ T cell populations (CD4⁺, CD8⁺, $\gamma\delta$ ⁺ and MAIT) were annotated
738 based on the expression of distinct phenotypic markers. CD4, CD8, TCR $\gamma\delta$, V α 7.2⁺,
739 CD161, IFN- γ and TNF- α were the parameters included for *t*-SNE analysis. Plots
740 correspond to one representative donor. **(B)** *t*-SNE plots as in (A) showing relative
741 expression of TNF- α and IFN- γ on CD69⁺ CD3⁺ T cells. **(C)** UMAP analysis on
742 concatenated CD3⁺ V α 7.2⁺ CD161⁺ MAIT cells from the same donor as in (A) and (B).
743 Calculated UMAPs are shown for each experimental condition. CD69, IFN- γ and TNF-
744 α were the parameters included for analysis. **(D)** Top left panel showing UMAP as an
745 overlay of concatenated MAIT cell populations from (C): unstimulated in light grey,
746 STM-D23580 in blue, STM-LT2 in green, ST-Ty2 in red and *E. coli* in dark grey. Top
747 right and bottom panels: UMAPs showing expression of CD69, TNF- α and IFN- γ in
748 pink. **(A-D)** Data from one donor representative of four biological replicates.

749

750 **Figure 2. *S. Typhimurium* ST313 lineage 2 fails to elicit MAIT cell activation.**

751 PBMC were left unstimulated (U) or were infected with a variety of *Salmonella* strains
752 at the indicated MOI. *E. coli* was included as positive control and *E. faecalis* as negative
753 control. **(A)** Production of TNF- α and IFN- γ by MAIT and $\gamma\delta$ ⁺ T cells was detected by
754 intracellular staining. Representative flow cytometry plots from one volunteer are
755 shown. **(B)** Percentage of TNF- α and/or IFN- γ producing MAIT cells when stimulated
756 at increasing MOI, from 0.5 to 20 bacteria per cell. Data represented as mean \pm SEM,
757 two-way ANOVA + Dunnet's, *n*=4. **(C)** CD69 staining profile of stimulated MAIT and
758 $\gamma\delta$ ⁺ T cells. Representative histograms from one volunteer are shown. **(D)** CD69
759 expression on MAIT cells when stimulated as in (B). Data represented as geometric
760 mean \pm SEM, two-way ANOVA + Dunnet's, *n*=4. **(E)** Phylogenetic relationships
761 between strains used in these experiments (red) within the context of *Salmonella*
762 *enterica* phylogeny. **(F)** Percentage of TNF- α and/or IFN- γ producing MAIT cells,
763 treated with bacterial strains at MOI of 2.5 and 5. Data represented as mean \pm SEM,
764 two-way ANOVA + Dunnet's, *n*=4. **(G)** Levels of CD69 expression on MAIT cells

765 treated as in (E). Data represented as geometric mean \pm SEM, two-way ANOVA +
766 Dunnet's, $n=4$.

767

768 **Figure 3. Characterisation of MAIT cell responses to *Salmonella* spp. in relevant**
769 **and susceptible cohorts of individuals.**

770 **(A)** PBMC were isolated from ten healthy individuals living in Malawi. Cells were
771 infected at MOI of 7 with various strains from sequence type 313 (ST313) lineages 1,
772 2 and 2.2 or with the sequence type 19 (ST19) reference strain STM-4/74. Each dot
773 represents the average of TNF- α and/or IFN- γ producing MAIT cells per individual
774 upon stimulation with 4 strains from each lineage (see Figure S2). Median \pm IQR,
775 Friedman + Dunn's, $n=10$ for each group. **(B)** Percentage of MAIT cells (CD3⁺ V α 7.2⁺
776 CD161⁺) in PBMC isolated from healthy ($n=12$) and HIV-infected individuals living in
777 Malawi, with ($n=6$) or without ART ($n=6$). Data represented as percentage of live CD3⁺
778 T cells, median with IQR, Kruskal-Wallis + Dunn's. **(C)** PBMC isolated from HIV⁺
779 patients with or without ART were infected at MOI of 7 with either STM-D23580, STM-
780 4/74, STy-H58 or *E. coli*. PMA/ionomycin was used as positive control. Data
781 represented as percentage of TNF- α and/or IFN- γ producing MAIT cells, mean \pm SEM,
782 one-way ANOVA + Dunnet's, $n=6$ for each group.

783

784 **Figure 4. STM-D23580 does not affect MR1-dependent antigen presentation.**

785 **(A)** PBMC were infected (at MOI 2.5 and 7.5) with STM-D23580 (blue), STM-LT2
786 (green) or *E. coli* (grey), and incubated in the presence of anti-MR1 blocking antibody
787 or the equivalent isotype control. Data are represented as percentage of TNF- α and/or
788 IFN- γ producing MAIT cells, box-and-whisker plot, two-way ANOVA + Dunnet's, $n=4$.

789 **(B)** Representative example of cytokine production by stimulated MAIT cells treated
790 as in (A). **(C- D)** PBMC were left unstimulated or were infected (at MOI 2.5 and 5)
791 with either STM-D23580 or STM-LT2, in the presence (pink bars) or absence (grey bars)
792 of the MR1 ligands 5-A-RU and MG. Percentage of cytokine producing MAIT cells and
793 their CD69 expression are shown. Data represented as mean and geometric mean \pm
794 SEM, two-way ANOVA + Bonferroni's, $n=4$. **(E)** PBMC were infected with D23580 at
795 MOI of 2.5, alone or in combination with STM-LT2 (green), ST-Ty2 (orange) or *E. coli*
796 (grey), at two different MOI (2.5 and 5). Data represented as percentage of TNF- α
797 and/or IFN- γ producing MAIT cells, mean \pm SEM, one-way ANOVA + Sidak's, $n=5$. **(F)**
798 Representative example of cytokine production by stimulated MAIT cells treated as in
799 (E).

800

801 **Figure 5. STM-D23580 evades MAIT cell recognition by overexpression of *ribB*.**

802 (A) Schematic representation of *Salmonella*'s riboflavin pathway, adapted from
803 Soudais et al. [54]. (B) The relative transcriptional expression levels of the *ribABDEFH*
804 genes were derived from our published RNA-seq data set [46]. The gene expression
805 values from STM-D23580 and STM-4/74 were determined in five infection-relevant
806 conditions: ESP (early stationary phase), anaerobic growth, NonSPI2 (SPI2-non-
807 inducing PCN), InSPI2 (SPI2-inducing PCN), and macrophage (intra-RAW264.7
808 murine macrophage environment). Values indicate fold-change (FC) and false
809 discovery rate (FDR), calculated from a Voom/Limma analysis (using Degust) for the
810 RNA-seq data comparison of STM-D23580 versus STM-4/74. Data represent three
811 biological replicates. PCN = phosphate carbon nitrogen minimal medium. (C) The
812 relative expression levels of the RibABDEFH proteins were derived from our published
813 proteomic data set for the ESP condition [46]. The heat map shows differential
814 expression analysis following comparison between STM-D23580 and STM-4/74.
815 Results from LC-MS/MS were analysed using the Progenesis Q1 software (Nonlinear
816 Dynamics) for label-free quantification analysis. Each sample represents six biological
817 replicates. Data represented in panels B and C were extracted from Canals et al. [46].
818 (D) PBMC were infected at two different MOI (2 and 5) with STM-D23580 (blue), STM-
819 4/74 (green) or STM-4/74 RibB⁺⁺ (pink). Data represented as percentage of TNF- α
820 and/or IFN- γ producing MAIT cells, mean \pm SEM, two-way ANOVA + Tukey's, $n=6$. (E)
821 CD69 staining profile of stimulated MAIT cells treated as in (D). Representative
822 histograms from one volunteer are shown, MFI=Median Fluorescence Intensity.

823

824 **Figure 6. STM-4/74 RibB⁺⁺ has the lowest intracellular levels of FMN, a negative**
825 **regulator of *ribB* gene expression.**

826 Supernatants from early stationary phase and bacterial pellets were harvested and
827 analysed by HPLC using riboflavin, FMN and FAD standards. Data is reported in pmol
828 and has been normalised to the absorbance (OD₆₀₀) from each culture. (A) Riboflavin
829 levels in supernatants. (B) Intracellular riboflavin levels. (C) FMN levels in
830 supernatants. (D) Intracellular FMN levels. (E) Intracellular FAD levels. Measurements
831 were obtained from 3 biological replicates, mean \pm SEM, one-way ANOVA + Dunnet's.

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833

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847

848 **AUTHOR CONTRIBUTIONS**

849

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851 J.C.D.H., G.N., M.A.G. and A.S.; Methodology, L.P-L., M.S., R.C., A.A., P.M. and G.N.;
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855

856 **CONFLICT OF INTEREST**

857 Rocío Canals was employed by the University of Liverpool at the time of the study and
858 is now an employee of the GSK group of companies. The other authors declare no
859 conflict of interest.

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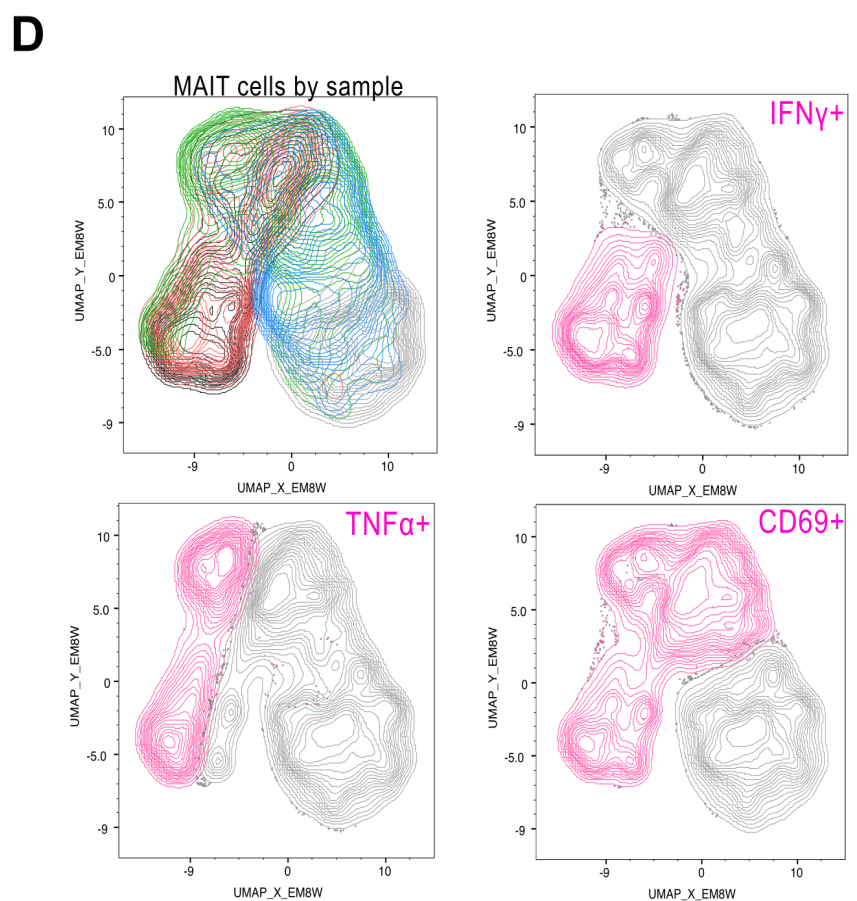
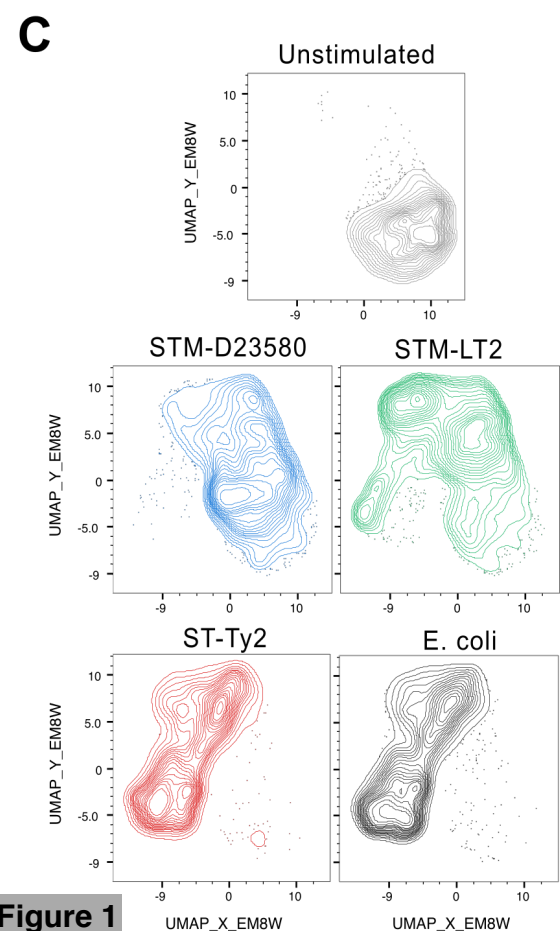
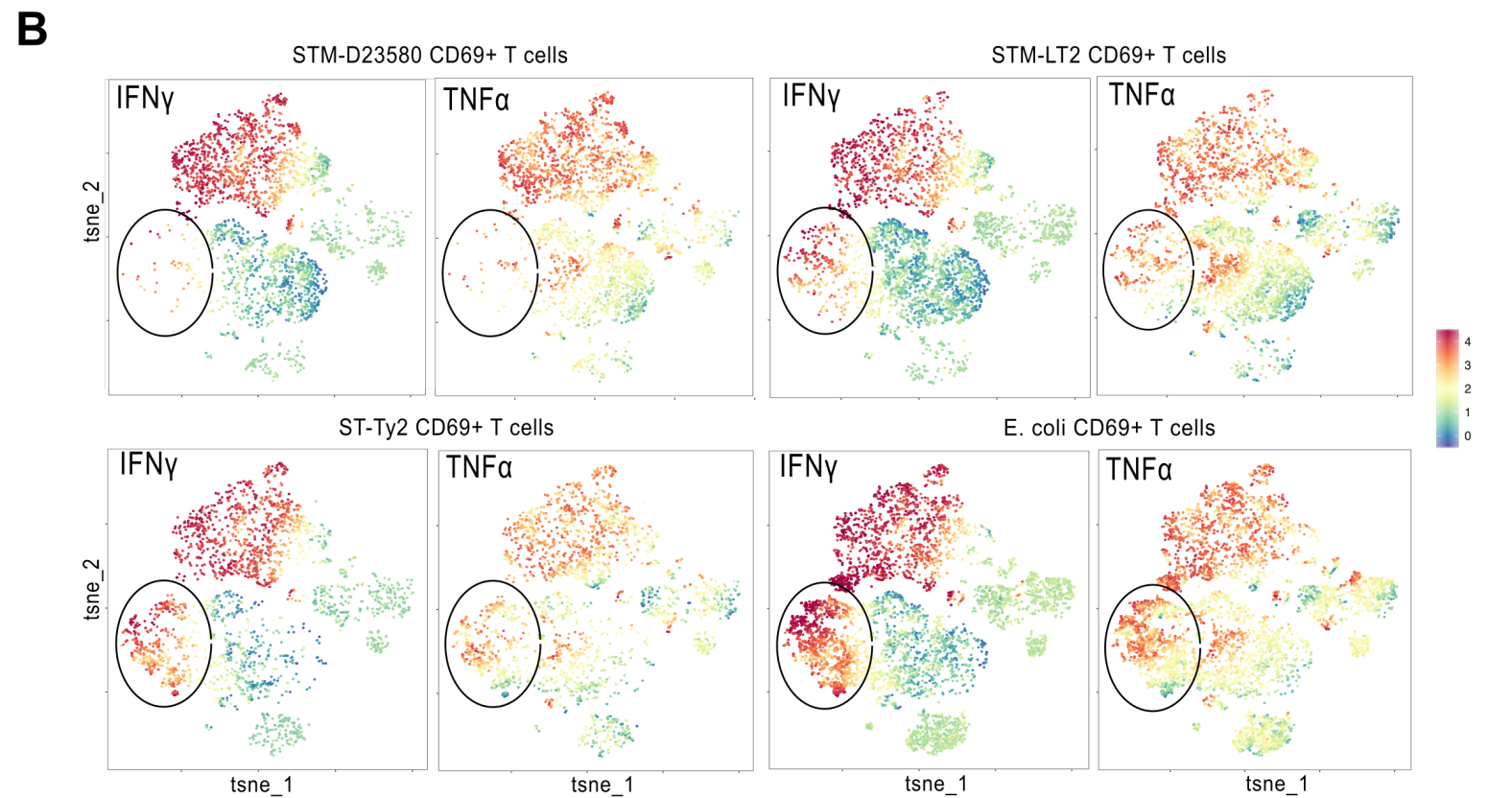
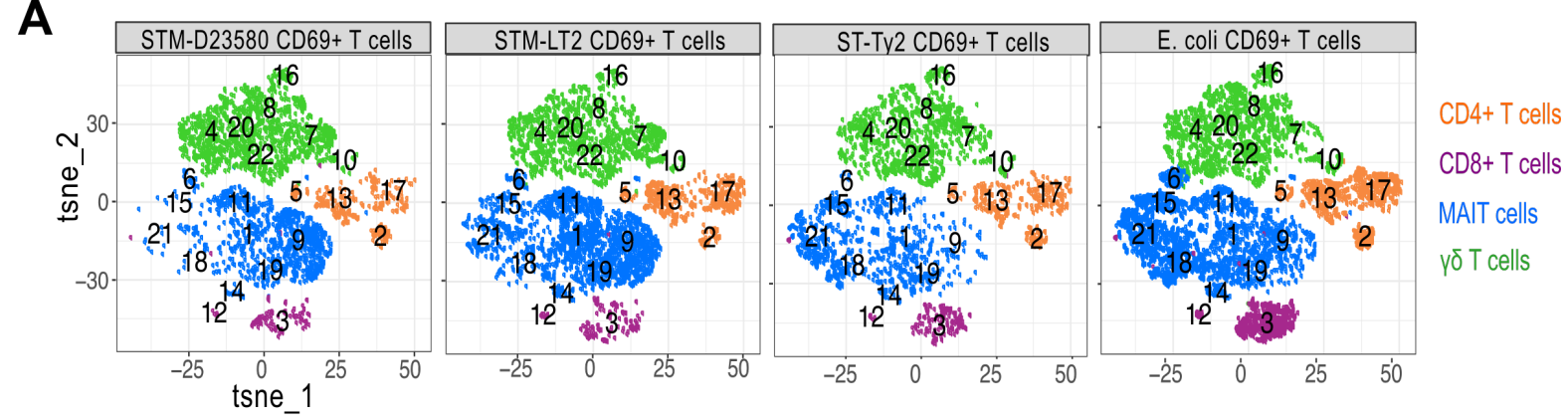


Figure 1

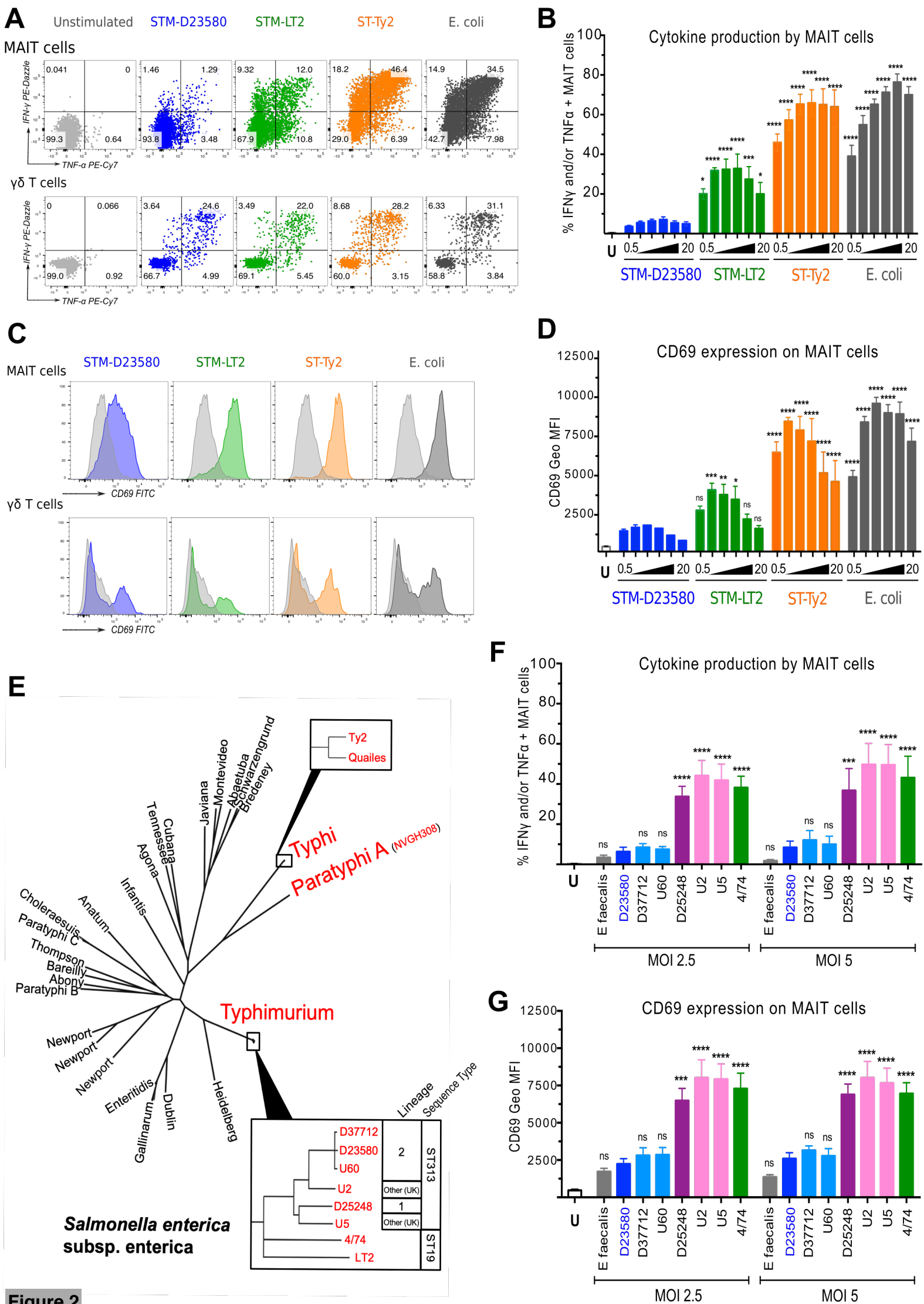
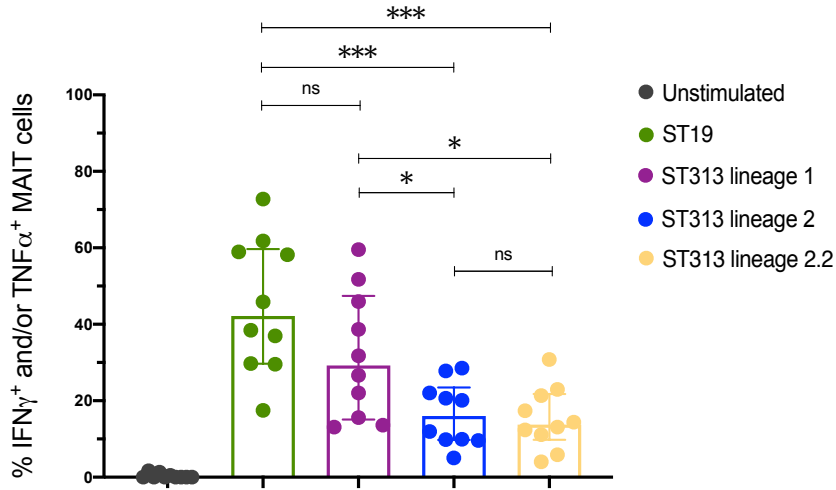


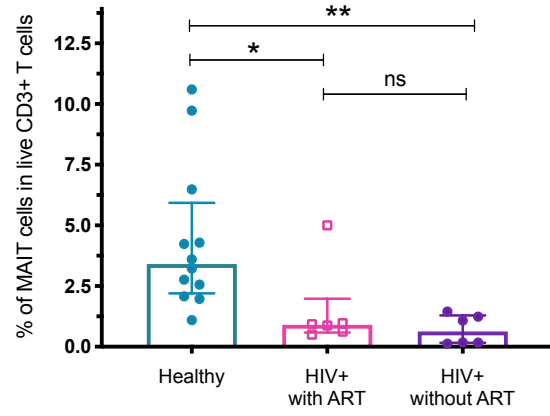
Figure 2

A

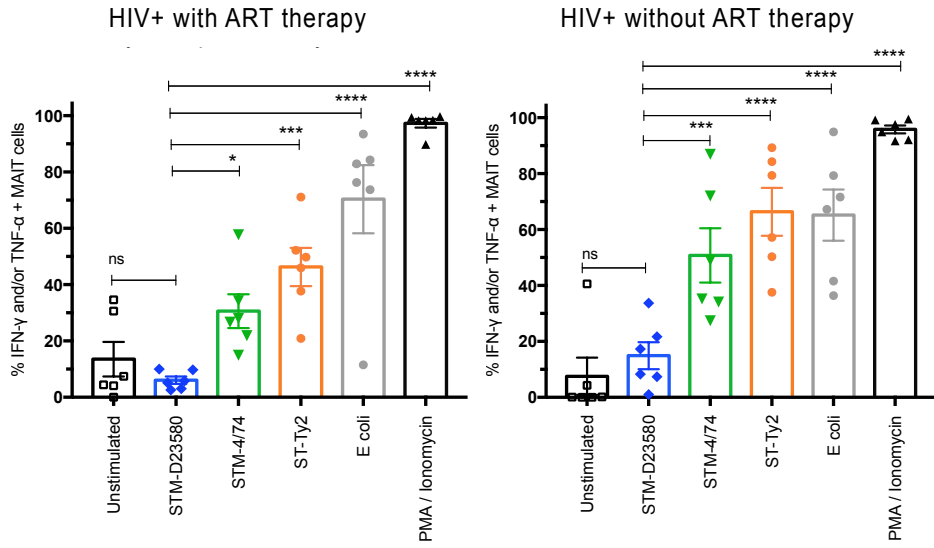
Cytokine production by MAIT cells

**B**

Frequency of MAIT cells

**C**

Cytokine production by MAIT cells



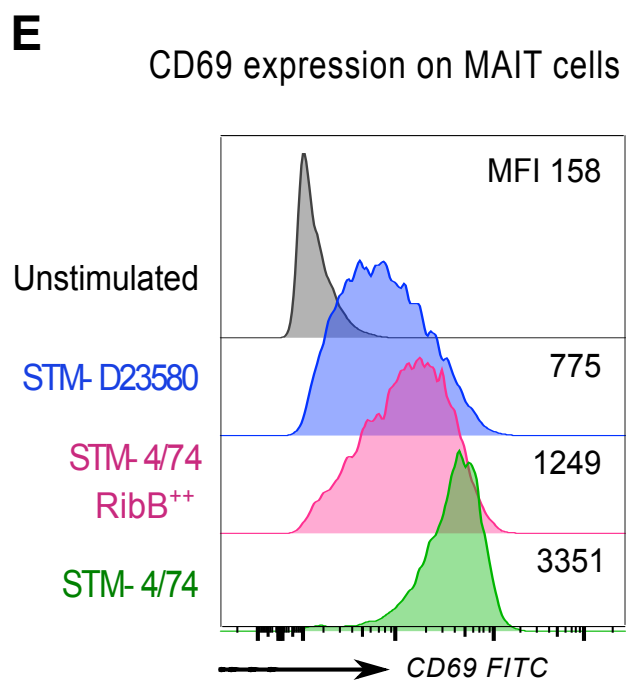
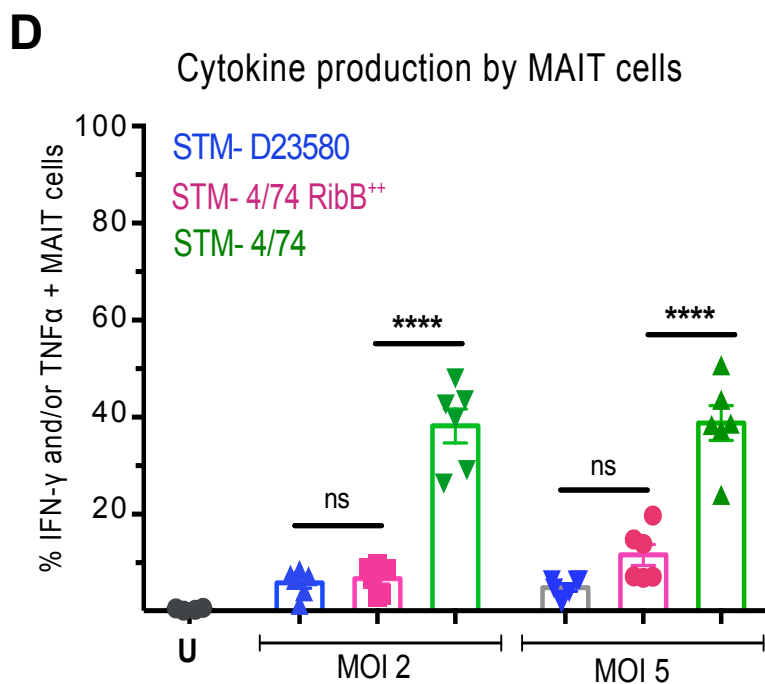
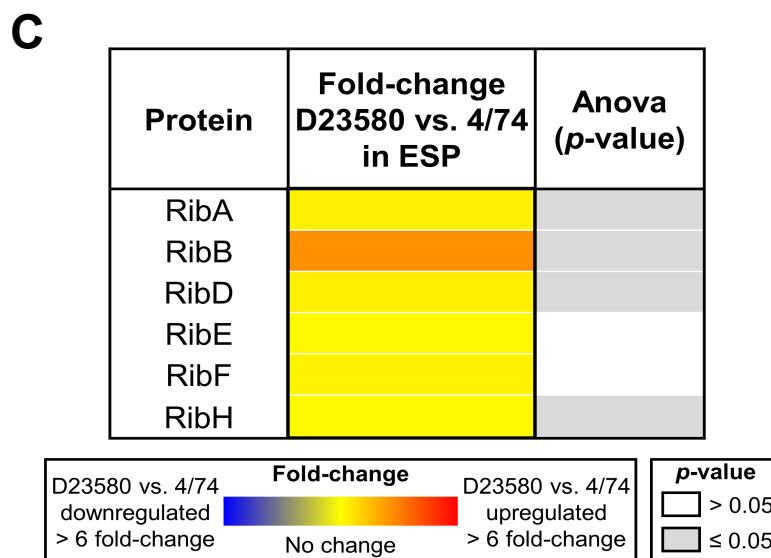
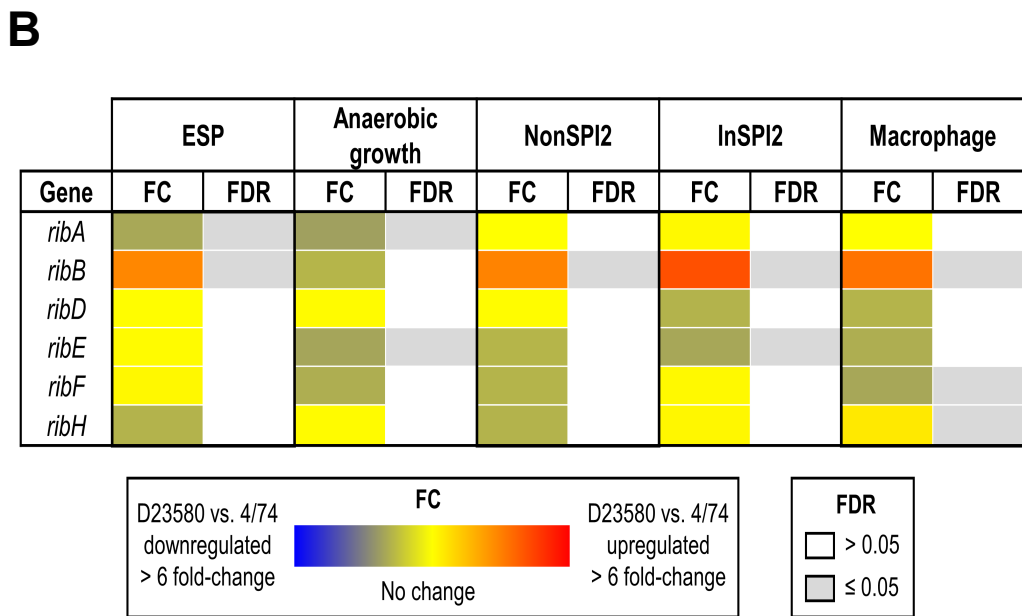
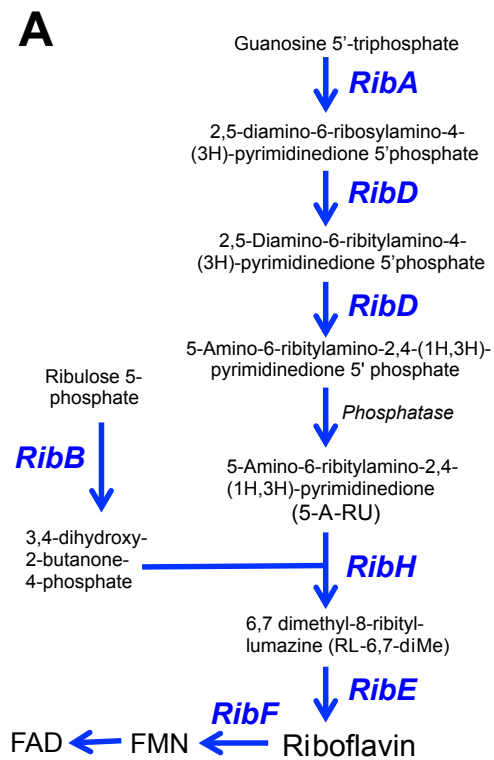
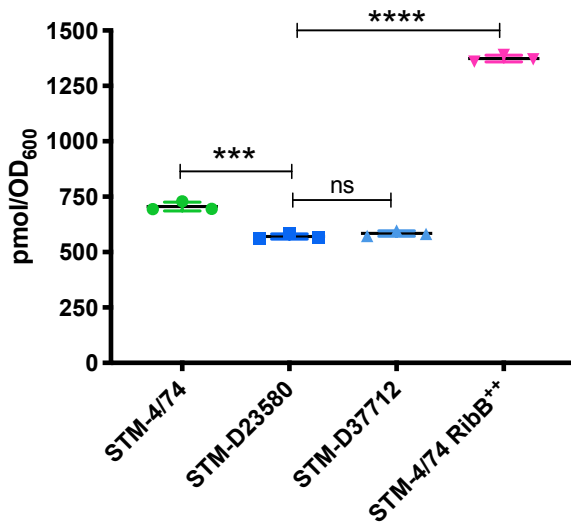
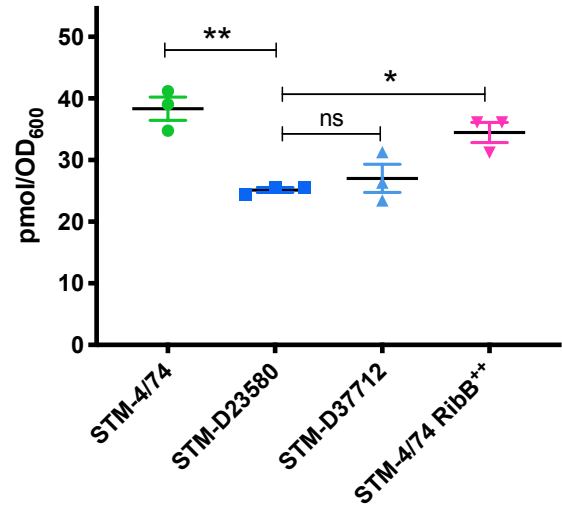
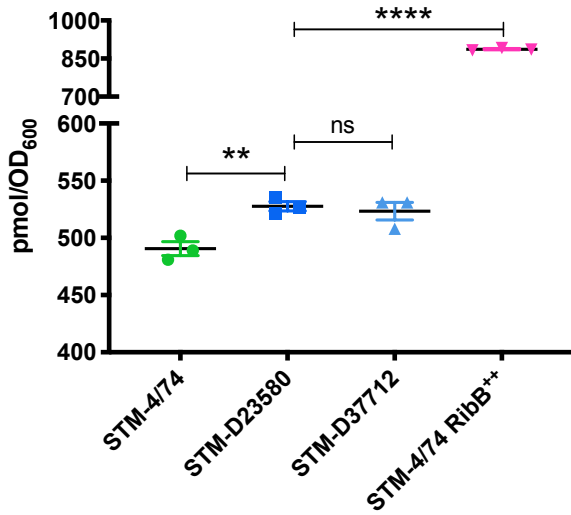
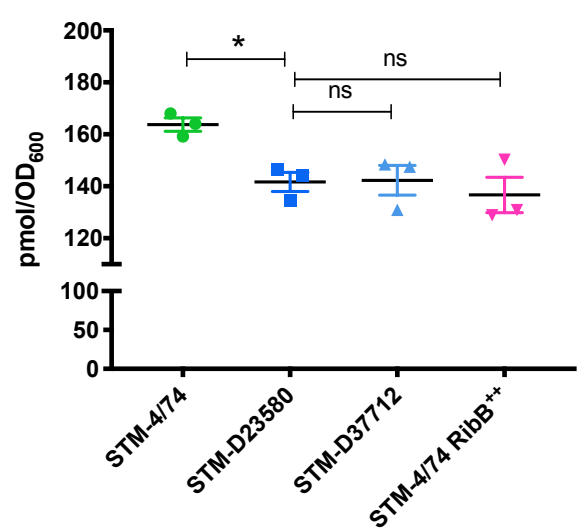


Figure 5

A**Riboflavin (supernatants)****B****Riboflavin (intracellular)****C****FMN (supernatants)****D****FMN (intracellular)****E****FAD (intracellular)**