γδ T cell IFNγ production is directly subverted by *Yersinia pseudotuberculosis* outer protein YopJ in mice and humans

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**Abstract**

*Yersinia pseudotuberculosis* is a foodborne pathogen that subverts immune function by translocation of *Yersinia* outer protein (Yop) effectors into host cells. As adaptive γδ T cells protect the intestinal mucosa from pathogen invasion, we assessed whether *Y. pseudotuberculosis* subverts these cells in mice and humans. Tracking Yop translocation revealed that the preferential delivery of Yop effectors directly into murine Vy4 and human Vδ2+ T cells inhibited anti-microbial IFNγ production. Subversion was mediated by the adhesin YadA, injectosome component YopB, and translocated YopJ effector. A broad anti-pathogen gene signature and STAT4 phosphorylation levels were inhibited by translocated YopJ. Thus, *Y. pseudotuberculosis* attachment and translocation of YopJ directly into adaptive γδ T cells is a major mechanism of immune subversion in mice and humans. This study uncovered a conserved *Y. pseudotuberculosis* pathway that subverts adaptive γδ T cell function to promote pathogenicity.

**Author summary**

Unconventional γδ T cells are a dynamic immune population important for mucosal protection of the intestine against invading pathogens. We determined that the foodborne pathogen *Y. pseudotuberculosis* preferentially targets an adaptive subset of these cells to subvert immune function. We found that direct injection of *Yersinia* outer proteins (Yop) into adaptive γδ T cells inhibited their anti-pathogen functions. We screened all Yop effectors and identified YopJ as the sole effector to inhibit adaptive γδ T cell production of IFNγ. We determined that adaptive γδ T cell subversion occurred by limiting activation of the transcription factor STAT4. When we infected mice with *Y. pseudotuberculosis*...
expressing an inactive YopJ, this enhanced the adaptive γδ T cell response and led to greater cytokine production from this subset of cells to aid mouse recovery. This mechanism of immune evasion appears conserved in humans as direct injection of Y. pseudotuberculosis Yop into human γδ T cells inhibited cytokine production. This suggested to us that Y. pseudotuberculosis actively inhibits the adaptive γδ T cell response through YopJ as a mechanism to evade immune surveillance at the site of pathogen invasion.

Introduction

Pathogens in the genus Yersinia include three species (Y. pestis, Y. pseudotuberculosis, and Y. enterocolitica) that can cause human disease. Y. pseudotuberculosis and Y. enterocolitica cause enteric infections [1,2] while Y. pestis is the causative agent of bubonic, septicemic, and pneumonic plague that has claimed over 200 million human lives [3,4]. Bubonic and septicemic plague is transmitted by blood sucking fleas while aerosols spread the pneumonic plague. Despite vaccine availability [5], and sensitivity to antibiotic treatment, pneumonic plague commonly results in fatality in part due to the rapid course of the infection [6].

Pathogenic Yersinia spp. harbor a virulence plasmid that encodes numerous virulence factors to subvert host immune responses, including IFNγ production [7–9]. Immune cell subversion requires Yersinia adherence to host cells through bacterial adhesins and translocation of Yersinia outer proteins (Yop) effectors into the host cell cytoplasm by a type III secretion system (T3SS). Yersinia spp. predominantly target host phagocytes like macrophages, dendritic cells (DC), neutrophils, and B cells to subvert immune function during infection, but injection into other immune populations like conventional T cells has been reported, albeit to a lesser degree than their phagocytic counterparts [10–12]. Yersinia virulence factors include components of the T3SS (e.g., YopB) and translocated effectors (e.g., YopJ and YopH). YopB forms a pore in the host cell membrane necessary for translocation of Yop effectors [13,14]. Numerous Yop effectors translocate into host cells to inhibit immune responses and promote Yersinia spp. pathogenesis. One notable example is YopJ, an acetyl transferase and a possible cysteine protease that inhibits the mitogen-activated protein kinase (MAPK) pathway and tumor necrosis factor receptor-associated factor (TRAF) ubiquitination [15–19]. YopJ is the major Yop effector responsible for the induction of pyroptosis in macrophages during infection [20] and limits toll-like receptor 4 (TLR4) dependent signaling pathways [21]. While YopJ has no known direct effects on conventional T cell activation, YopP (a Yop homolog in Y. enterocolitica) indirectly inhibits T cell priming via DC subversion [22]. YopH has been reported to have direct effects on conventional T cells in vitro. Transfection of a YopH expression plasmid into Jurkat or human T cells inhibited T cell receptor (TCR) signaling and promoted T cell apoptosis [23,24]. Additionally, stimulation of Jurkat cells with a YopH deficient Y. pseudotuberculosis restored T cell signaling and IL-2 production [25,26]. Even in this context, it is notable that many of the downstream targets in the αβ TCR signaling pathway were inhibited at an excessively high (>50) multiplicity of infection (MOI) and in vivo relevance is unclear [23,26]. Thus, the role of direct subversion of T cell function, especially among unconventional T cells, by Yersinia spp. remains largely unexplored.

γδ T cells make up a large proportion of lymphocytes at barrier surfaces and mucosal tissues including the intestines of mice and humans [27,28]. This is particularly pertinent to infections caused by Y. pseudotuberculosis, which has evolved to invade the intestinal barrier. The activity of γδ T cells can be modulated by numerous cell-intrinsic and environmental factors like the γδ TCR, cytokines, and co-stimulatory or inhibitory receptors [29]. For example, IL-12 and
Results

Viable Y. pseudotuberculosis inhibits IFNγ production by adaptive γδ T cells in a YopB- and YadA-dependent manner

Initial experiments were carried out to determine if Y. pseudotuberculosis inhibits adaptive γδ T cell function ex vivo. To overcome the extremely low number of Vγ4 T cells in gut-associated lymphoid tissues of naïve specific pathogen free (SPF) mice, a previously established in vivo methodology was utilized to generate a sizable population of adaptive Vγ4 T cells for in vitro manipulation. As such, naïve Balb/c mice were exposed to foodborne L. monocytogenes and MLN enriched in adaptive γδ T cells were isolated 9 days after infection [37], several days after mice typically clear Vγ4 T cells for infection [42]. Interestingly, IFNγ but not IL-17A production from type-3 innate lymphoid cells is critical for the control of foodborne L. monocytogenes infection [43]. As such, unconventional T cells like Vγ4 T cells that are ideally placed to provide protection against pathogen invasion at mucosal sites may be particularly relevant to Yersinia infections that invade mucosal barriers of the lungs (pneumonic Y. pestis) and gut (Y. pseudotuberculosis and Y. enterocolitica).

Despite a foundational understanding of Yersinia pathogenesis, physiologically robust evidence linking Yersinia pathogenesis to direct subversion of T cell function is lacking. Here, we uncovered a novel YopJ-dependent immunomodulatory pathway used by Y. pseudotuberculosis to directly subvert a murine Vγ4Vδ1 anti-microbial response to aid Y. pseudotuberculosis pathogenesis. Y. pseudotuberculosis also directly subverted a human Vδ2+ T cell IFNγ response, suggesting that this pathway may function similarly in human infection to aid Y. pseudotuberculosis pathogenesis.

IL-18 may promote IFNγ production from some γδ T cell subsets whereas IL-1β and IL-23 predominantly drive IL-17A production from other γδ T cell subsets [30–35]. Vγ4Vδ1 (German nomenclature [36]) T cells have traditionally been considered an innate-like cell. However, our group recently characterized a long-lived CD27+ CD44hi Vγ4Vδ1 T cell memory population in the context of foodborne Listeria monocytogenes infection [37,38]. While Vγ4 T cells are typically programmed for IL-17A production, this subset has the multifunctional capacity to produce both IL-17A and IFNγ [37]. Similar observations of IFNγ production were made in clonally expanded Vγ4 T cells in response to Staphylococcus aureus in the skin [39]. IFNγ activates macrophages to kill intracellular pathogens or phagocytosed bacteria and induces chemokines that attract immune cells to the site of infection. IFNγ is a critical cytokine in protection from Y. enterocolitica infection [2,40], Y. pestis intranasal challenge [41], and associated with protection from Y. pseudotuberculosis [42]. Interestingly, IFNγ but not IL-17A production from type-3 innate lymphoid cells is critical for the control of foodborne Y. enterocolitica infection [43]. As such, unconventional T cells like Vγ4 T cells that are ideally placed to provide protection against pathogen invasion at mucosal sites may be particularly relevant to Yersinia infections that invade mucosal barriers of the lungs (pneumonic Y. pestis) and gut (Y. pseudotuberculosis and Y. enterocolitica).

**Viable Y. pseudotuberculosis subversion of γδ T cell function**
Table 1. *Y. pseudotuberculosis* strains and mutants used in this study.

| *Y. pseudotuberculosis* | Notation | Relevant Characteristics | References |
|-------------------------|----------|--------------------------|------------|
| 32777                   | WT       | Yptb wild-type serogroup O:1 strain | [47] |
| 32777c                  | WT2777c  | Virulence pYV-cured derivative of 32777 that lacks the T3SS | [47] |
| 32777 YopF[172A]        | YopF[172A] | Catalytically inactive YopF | [45] |
| 32777 YopHR409A         | YopHR409A | Catalytically inactive YopH | [45,48,49] |
| 32777 ΔYopB            | ΔYopB    | Deletion of YopB | [45,46] |
| 32777 YopE[444A]        | YopE[444A] | Catalytically inactive YopE | [45] |
| 32777 YopT[139A]        | YopT[139A] | Catalytically inactive YopT | [45] |
| 32777 ΔYopM            | ΔYopM    | Deletion of YopM | [50] |
| 32777 ΔYopK            | ΔYopK    | Deletion of YopK | [51] |
| 32777 ΔYopK            | ΔYopK    | Frameshift mutation in YopK | [52] |
| 32777 YopE/β-lac        | WT Yptb-βla | YopE TME-1 β-lactamase fusion protein | [53–55] |
| 32777 ΔYopB YopE/β-lac | ΔYopB Yptb-βla | Deletion of YopB in the YopE/β-lac | [53–55] |
| IP2666                 | WT       | Yptb wild-type serogroup O:3 strain | [47] |
| IP40                   | ΔYopB    | IP2666 yopB40 (a stop codon at codon 8 of YopB followed by a frameshift) | [56] |
| IP2666 ΔInv            | ΔInv     | Deletion of adhesin and invasin | [12,57] |
| IP2666 ΔYadA           | ΔYadA    | Deletion of adhesin and YadA | [12,57] |
| IP2666 ΔInv ΔYadA      | ΔInv ΔYadA | Deletion of adhesin, invasion, and YadA | [12,57] |
| IP40 ΔInv ΔYadA        | ΔInv ΔYadA | Deletion of invasion and YadA in IP40 and pMMB207 mCherry | [12,57] |

[45–47]. γδ T cell function was assessed 24 hours later as described above. Stimulation with live *Y. pseudotuberculosis* strains ΔYopB or 32777c restored the IFNγ response of *Vγ1.1/2 CD44hi CD27− γδ T cells* (Fig 1B), similar to levels seen after stimulation with heat-killed WT *Y. pseudotuberculosis* (Fig 1A). These data indicate that *Y. pseudotuberculosis* inhibits IFNγ production by *Vγ4 T cells* in a manner that requires the T3SS and translocation of Yop effectors.

*Y. pseudotuberculosis* adheres to host cells with the bacterial adhesins invasin (Inv) and YadA to translocate effectors through the T3SS [58–60]. For *Y. enterocolitica*, both Inv and YadA bind β₁-integrin either directly or indirectly through the extracellular matrix, respectively [61]. Additionally, β₁-integrin expressed on host cells is a known adhesion target for *Y. pseudotuberculosis* [60,62]. To evaluate the role of these adhesins in the inhibition of γδ T cell function, live *Y. pseudotuberculosis* with a deletion of Inv (ΔInv), YadA (ΔYadA), or both (ΔInv ΔYadA) (Table 1) were utilized to infect MLN cell suspensions. *Vγ1.1/2 CD44hi CD27− γδ T cells* stimulated with ΔInv bacteria produced only minimal IFNγ, comparable to unstimulated cells or cells stimulated with WT (Fig 1C). In contrast, ΔYadA or ΔInv ΔYadA stimulation led to partial restoration of IFNγ production, and stimulation with ΔYopB or ΔYopB ΔInv ΔYadA bacteria led to full restoration of IFNγ production (Fig 1C). Thus, YadA but not Inv contributes to translocation dependent inhibition of IFNγ production by *Vγ4 T cells*.

**Translocation of Yop effectors into adaptive γδ T cells by *Y. pseudotuberculosis* is associated with IFNγ inhibition**

To determine if *Y. pseudotuberculosis* can translocate Yop effectors into adaptive *Vγ4 T cells*, a WT strain expressing a YopE-β-lactamase fusion protein (*Yptb-βla*) in conjunction with a FRET-based β-lactamase reporter assay was used [63]. YopE translocation into target cells can be readily assessed by a change in fluorescence using flow cytometry. Thus, translocation of the YopE-β-lactamase fusion protein reports Yop effector translocation by emission in the blue range (Yop+) or lack thereof by emission in the green range (Yop−) | 0.1371/journal.ppat.1010103.t001 |

https://doi.org/10.1371/journal.ppat.1010103.t001
deficient β-lactamase. *Y. pseudotuberculosis* reporter (ΔYopB *Yptb*-βla) was used as a translocation deficient control. Stimulation of MLN cell suspensions with WT or ΔYopB *Yptb*-βla confirmed reporter activity at various MOI (S1A and S1B Fig). Two hours post stimulation with WT *Yptb*-βla, the majority of Vγ1.1/2 CD44hi CD27- γδ T cells were positive for Yop effector.
translocation (Fig 2A). Yop translocation into Vγ1.1/2 CD44hi CD27+ γδ T cells was comparable to known DC and macrophage targets (Fig 2A). Additionally, Yop translocation was more efficient into Vγ1.1/2 CD44hi CD27+ γδ T cells than CD4 or CD8 T cells (Fig 2A). Y. pseudotuberculosis also preferentially targeted Vγ1.1/2 CD44hi CD27+ γδ T cells over CD44+ γδ T cells and activated phenotype CD4 or CD8 T cells for Yop translocation (S1C Fig). YadA and Inv promote Yersinia adherence by direct or indirect interactions with the β1-integrin [66–69]. Analysis of β1-integrin expression on Vγ1.1/2 CD44hi CD27+ γδ T cells, CD4 T cells, and CD8 T cell revealed that most Vγ1.1/2 CD44hi CD27+ γδ T cells expressed the β1-integrin (S2A Fig). In contrast, most conventional CD4 and CD8 T cells did not express the β1-integrin. In addition, use of the WT Yptb-βla reporter for Yop translocation demonstrated that Yop translocation was associated with higher β1-integrin expression among γδ T cells (S2B Fig). Thus, Y. pseudotuberculosis selectively targets adaptive γδ T cells for Yop translocation among a diverse group of immune populations assessed in an ex vivo culture system.

As adaptive Vγ4 T cells were directly targeted with Yop effector translocation, WT Yptb-βla was utilized to determine whether Vγ1.1/2 CD44hi CD27+ γδ T cells that contained Yop effectors were functionally impaired. An MOI of 1 was used as it provided similarly sized populations of Vγ1.1/2 CD44hi CD27+ γδ T cells that did or did not contain translocated effectors from the same culture conditions (S1B Fig). Among WT Yptb-βla stimulated cells, Yop+ Vγ1.1/2 CD44hi CD27+ γδ T cells had reduced IFNγ production as compared to their Yop− counterparts (Fig 2B). To extend these results, the ability of Y. pseudotuberculosis to translocate Yop effectors into human γδ T cells and inhibit IFNγ production was assessed in peripheral blood mononuclear cells (PBMC) cultures stimulated with the WT Yptb-βla reporter. Approximately 8% of human Vδ2+ T cells were Yop+ and these cells had significantly reduced IFNγ production as compared to the Yop− counterparts (Fig 2C and 2D). These data indicate that Y. pseudotuberculosis is capable of translocating Yop effectors into γδ T cell subsets and inhibiting IFNγ production in mice and humans.

YopJ is necessary for Y. pseudotuberculosis to inhibit IFNγ production in adaptive γδ T cells

As multiple effectors are translocated into target cells, a panel of yop mutant Y. pseudotuberculosis (Table 1) [45] was screened to determine if individual Yop effectors inhibit IFNγ production. Similar to the ΔYopB mutant, stimulation with a catalytically inactive YopJ (YopC172A) mutant that lacks acetyl transferase activity, but not other mutant Y. pseudotuberculosis, restored IFNγ production in Vγ1.1/2 CD44hi CD27+ γδ T cells (Fig 3A). The C172A mutation in YopJ prevents YopJ mediated inhibition of MAPK and NF-κB signaling pathways by abolishing its serine and threonine acetylation activity [70]. A similar restoration of IFNγ production was observed in human Vδ2+ T cells from PBMC of healthy donors upon YopC172A Y. pseudotuberculosis stimulation (Fig 3B). Thus, the YopJ effector is responsible for inhibition of IFNγ production from murine Vγ4 and human Vδ2+ T cells.

YopJ inhibits expression of multiple genes, including ifng, in adaptive γδ T cells

To uncover mechanisms by which YopJ inhibits IFNγ production, the transcriptome of cell sorter-purified Vγ1.1/2 CD44hi CD27+ γδ T cells after WT or YopC172A Y. pseudotuberculosis stimulation of MLN cells was assessed by RNA-Seq. Principal component analysis revealed unique gene expression clustering, and approximately 900 genes were expressed at higher levels in the YopC172A stimulation as compared to WT Y. pseudotuberculosis (Fig 4A and 4B). These differentially expressed genes may include genes that are directly inhibited by YopJ.
activity in Vγ1.1/2 CD44hi CD27− γδ T cells or indirectly inhibited by YopJ activity in other cells such as DC or macrophages. To resolve this, the WT Yptb-βla reporter provided an opportunity to evaluate the molecular changes elicited by the activity of translocated Yop in adaptive γδ T cells. The transcriptome of sort purified Yop+ and Yop+ Vγ1.1/2 CD44hi CD27− γδ T cells after WT Yptb-βla stimulation was assessed by RNA-Seq. Principal component analysis revealed unique gene expression clustering, and approximately 900 genes were more highly expressed in Yop+ vs Yop+ Vγ1.1/2 CD44hi CD27− γδ T cells after WT Yptb-βla stimulation (Fig 4C and 4D). Overlapping gene expression profiles from the two datasets were assessed to narrow the analysis to direct YopJ effects on Vγ1.1/2 CD44hi CD27− γδ T cells. This comparison revealed 130 genes that were differentially expressed in both datasets, suggesting they are regulated directly by translocated YopJ in adaptive Vγ4 T cells (Fig 4E). These genes were categorized into different groups depending on their known functions. Some differentially expressed genes play a particular role in anti-infection functions (3.9%), stress sensing (1.6%), and lymphocyte activation/regulation (7.9%), genes that may be important for protective T cell responses (Fig 4E and 4F). Among these genes, IFNγ was the single most significant differentially expressed gene suggesting it is a major target of direct YopJ-mediated inhibition of adaptive γδ T cell function (Fig 4F). Differentially expressed genes among those that promote antimicrobial function included several that are important in augmenting type-1
and -3 inflammation in T cells. For example, Ptgs2 (encodes cyclooxygenase-2, COX2), Nkg7 (natural killer cell granule protein 7), Prf1 (perforin-1), and Il17a (IL-17A) appear to be regulated directly by translocated YopJ in adaptive Vγ4 T cells (Fig 4F) [71–73]. However, analysis of IL-17A protein after stimulation of MLN cell suspensions with YopJ C172A, WT, and ΔYopB Y. pseudotuberculosis demonstrated that YopJ did not regulate IL-17A production from Vγ1.1/2-CD44hiCD27-γδ T cells (S4 Fig). Some of the observed differentially expressed genes are important in the activation status of T cells (e.g., Il2ra, Ctla4, and Cd69) and suggest that translocated YopJ may limit the activation of adaptive Vγ4 T cells. There was also a notable impact (48.0%) on genes associated with cell proliferation, metabolism and energy, mitosis and cell cycle, RNA/DNA processing, and ER/Golgi processing (Figs S3A and S3B and 4E), suggesting that YopJ influences the adaptive γδ T cell transcriptional profile more broadly than just targeting the IFNγ pathway. Genes that were differentially expressed upon WT Y. pseudotuberculosis stimulation or among Yop+ cells also suggest that many of the processes associated with immune responses and cellular activity were regulated by YopJ (S3C–S3E Fig). Collectively, YopJ appears to regulate the expression of many genes associated with T cell function in Vγ1.1/2-CD44hiCD27-γδ T cells, suggesting that adaptive Vγ4 T cells are broadly constrained in their immune functions by Y. pseudotuberculosis.

YopJ inhibits the IL-12p40-mediated STAT4 pathway in adaptive γδ T cells

To gain potential mechanistic insights into YopJ inhibition of IFNγ production and other Vγ1.1/2-CD44hiCD27-γδ T cell functions, a motif discovery algorithm designed for regulatory element analysis was utilized to assess our RNA sequencing results [74]. Several transcription factor binding motifs related to IFNγ signaling were differentially expressed after YopJ C172A Y. pseudotuberculosis but not WT Y. pseudotuberculosis stimulation including members of the E twenty-six (ETS)-domain family, Krüppel-like factor and specificity protein (KLF/SP) transcription factor gene family, and the interferon regulatory factors (IRF) family
of transcription factors (S5A Fig). IRF8 protein was validated after WT, ΔYopB, and YopJ<sup>C172A</sup> stimulation. Indeed, a higher percentage of Vγ4 T cells expressed IRF8 protein after stimulation with YopJ<sup>C172A</sup> compared to WT and ΔYopB.

Fig 4. YopJ translocation leads to the inhibition of a broad anti-microbial gene response from Vγ4 T cells. (A and B) MLN suspensions from L. monocytogenes infected mice were stimulated with 10 MOI of WT or YopJ<sup>C172A</sup> Y. pseudotuberculosis for 24 hours. Antibiotics were given 2 hours post-stimulation. Five hundred Vγ1.1/2-CD44<sup>hi</sup>-CD27<sup>-</sup> γδ T cells from each stimulation were flow sorted and processed for RNA sequencing. (A) PCA plots are depicted for similarity of groups YopJ<sup>C172A</sup> and WT Y. pseudotuberculosis stimulated Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells. (B) Heat maps are depicted for differentially expressed genes of YopJ<sup>C172A</sup> or WT Y. pseudotuberculosis stimulated Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells. (C and D) MLN suspension from L. monocytogenes infected mice were stimulated with 1 MOI of WT Yptb-βla. Five hundred Yop<sup>-</sup> or Yop<sup>+</sup> Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells were flow sorted and processed for RNA sequencing. (C) PCA plots are depicted for similarity of Yop<sup>-</sup> or Yop<sup>+</sup> Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells. (D) Heat maps are depicted for differentially expressed genes of Yop<sup>-</sup> or Yop<sup>+</sup> stimulated Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells. (E-G) A Venn diagram of differentially expressed genes (higher) that overlapped between RNA sequencing analyses favoring YopJ<sup>C172A</sup> Y. pseudotuberculosis stimulation or Yop<sup>+</sup> cells is displayed. Shared genes were categorized by gene function. The heat map highlights differentially expressed genes among Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells from the indicated stimulations and categories. Homer motif analysis was performed on the RNA sequencing dataset. Motifs and associated genes to YopJ<sup>C172A</sup> stimulated Vγ1.1/2 CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells are highlighted. Each experiment was performed with 3 biologic samples per group. Cutoffs for significant genes are p < 0.05 and FDR < 0.10.

https://doi.org/10.1371/journal.ppat.1010103.g004

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protein after stimulation with ΔYopB compared to WT Y. pseudotuberculosis stimulation (S5B Fig). Stimulation with YopC172A Y. pseudotuberculosis was also able to partially restore IRF8 levels to those seen after ΔYopB Y. pseudotuberculosis stimulation (S5B Fig). IRF8 was also impacted by IL-12p40 blockade, which signals through signal transducer and activator of transcription 4 (STAT4) (S5B Fig). Interestingly, a number of transcription factor binding motifs downstream of STAT4 signaling were enriched including Etv5, Runx3, and Tead1 (Fig 4G) [75–78]. The RNAseq and homer motif analyses were also compared to an existing STAT4 ChIP-on-chip [79]. 7 genes identified from our main analyses (Figs 4F and 4G and S5B) were STAT4 target genes (S5C Fig). In summary, transcriptional profiling revealed a global subversion of anti-pathogen immune functions that may be associated with YopJ subversion of STAT4 activity.

IL-12 signaling leads to STAT4 phosphorylation and formation of STAT4-STAT4 homodimers that re-localize to the nucleus where they directly bind to the Ifig promoter to induce IFNγ expression [79–81]. To determine whether YopJ inhibits IFNγ production by interfering with the STAT4 pathway, STAT4 protein and phosphorylation were assessed by flow cytometry of MLN cells stimulated with Y. pseudotuberculosis. STAT4 phosphorylation was analyzed 6 hours after stimulation with WT, ΔYopB, or YopC172A Y. pseudotuberculosis. Consistent with suppression of IFNγ production and the RNA-Seq analysis, WT Y. pseudotuberculosis significantly reduced the percentage of pSTAT4+ CD44hi CD27−γδ T cells as compared to ΔYopB and YopC172A Y. pseudotuberculosis (Fig 5A). However, STAT4 protein levels were the same in all three infection conditions (WT, ΔYopB, and YopC172A Y. pseudotuberculosis) (Fig 5B). Flow cytometry antibodies for STAT4 protein were validated by comparing STAT4 from WT and STAT4 KO splenocytes (S5D Fig). These data suggest that STAT4 phosphorylation but not protein is decreased upon YopJ translocation. STAT4 phosphorylation was also evaluated using the Yptb-βla reporter system described above. Among CD44hi CD27−γδ T cells, YopJ cells had a higher percentage of pSTAT4+ cells compared to Yop+ cells suggesting intrinsic YopJ-mediated inhibition of STAT4 phosphorylation levels (Fig 5C). Additionally, as STAT4 phosphorylation is downstream of IL-12 signaling, an anti-IL-12/23p40 subunit antibody (anti-p40) was used to determine whether IL-12 signals in the environment regulated STAT4 phosphorylation after Y. pseudotuberculosis stimulation. Indeed, IL-12/23p40 neutralization abrogated STAT4 phosphorylation levels regardless of Yop translocation (Fig 5C). As IL-12/23p40 was required to elicit IFNγ production from adaptive Vγ4 T cells in the culture conditions, we assessed whether YopJ C172A Y. pseudotuberculosis stimulation modulated IL-12p70. The concentration of IL-12p70 was comparable between WT and YopJ C172A Y. pseudotuberculosis stimulated cultures (Fig 5D). Thus, changes in IL-12 were unlikely to contribute to adaptive Vγ4 T cell subversion in vitro. To understand the role of YopJ and IL-12 on Vγ1.1/2 CD44hi CD27−γδ T cells in a more simplified system, purified γδ T cells were stimulated with YopJ C172A Y. pseudotuberculosis in the presence of excessive IL-12p70. Adaptive Vγ4 T cells were unable to produce IFNγ in response to YopJ C172A Y. pseudotuberculosis and IL-12p70 (S6A Fig). Finally, we assessed whether the addition of IL-12p70 could overcome the YopJ-mediated inhibition of IFNγ production after WT Y. pseudotuberculosis stimulation. While a supraphysiologic level of IL-12p70 (50 ng/ml) was able to partially overcome YopJ-mediated inhibition, lower levels of IL-12p70 addition (2 and 10 ng/ml) were unable to overcome YopJ-mediated inhibition (S6B Fig). Importantly, these latter concentrations were orders of magnitude higher than those detected in our culture conditions. Thus, IL-12 is not sufficient to induce IFNγ production from adaptive Vγ4 T cells. Collectively, these results suggest that Y. pseudotuberculosis stimulation elicits IL-12 production to promote adaptive Vγ4 T cell IFNγ responses, and that YopJ translocation into adaptive Vγ4 T cells inhibits IL-12 mediated STAT4 phosphorylation.
Foodborne infection with YopJ<sup>C172A</sup> Y. pseudotuberculosis induces IFNγ production in adaptive Vγ4 T cells

To determine whether YopJ subverts adaptive γδ T cell function in vivo, foodborne infection with WT and YopJ<sup>C172A</sup> Y. pseudotuberculosis was performed on naïve Balb/c mice. As YopJ<sup>C172A</sup> Y. pseudotuberculosis is attenuated in vivo [82], a one log higher (2-4x10<sup>8</sup> CFU) infection dose of YopJ<sup>C172A</sup> Y. pseudotuberculosis was administered to normalize the internal bacteria burdens in the MLN between infection groups (S7A Fig). While mice infected with WT and YopJ<sup>C172A</sup> Y. pseudotuberculosis lost a similar amount of weight, mice infected with YopJ<sup>C172A</sup> Y. pseudotuberculosis recovered slightly faster (Fig 6A). MLN were isolated 9 days after infection to evaluate adaptive γδ T cell function. Consistent with the ex vivo stimulation of L. monocytogenes-elicited Vγ4 T cells with Y. pseudotuberculosis, Vγ1.1/2 CD44<sup>hi</sup>CD27<sup>-</sup>γδ T cells from the MLN of YopJ<sup>C172A</sup> Y. pseudotuberculosis infected mice displayed enhanced IFNγ production when stimulated ex vivo compared to their WT Y. pseudotuberculosis infected counterparts (Fig 6B and 6C). When the same infectious doses were used for both WT and YopJ<sup>C172A</sup> Y. pseudotuberculosis (5x10<sup>7</sup> CFU), Vγ1.1/2 CD44<sup>hi</sup>CD27<sup>-</sup>γδ T cells from the MLN of YopJ<sup>C172A</sup> Y. pseudotuberculosis infected mice also displayed enhanced IFNγ production.
production compared to their WT Y. pseudotuberculosis infected counterparts (S7B Fig).

These experiments demonstrate that the increased IFNγ observed was not a result of a higher infectious dose nor of a higher bacteria burden at the time of analysis. As IL-12/23p40 was required for adaptive Vγ4 T cell IFNγ production in vitro (Fig 5), the impact of IL-12/23p40 was assessed in vivo. Naïve Balb/c mice were infected with WT or YopJ<sup>C172A</sup> Y. pseudotuberculosis and treated with an IL-12/23p40 neutralizing antibody or isotype control. All YopJ<sup>C172A</sup> Y. pseudotuberculosis infected mice treated with anti-IL-12/23p40 succumbed by day 8 post
infection (Fig 6D). This data suggests that IL-12 promotes the protective capacity of catalytically inactive YopJ. Similarly, IL-12 contributed to the protection of mice infected with WT Y. pseudotuberculosis. Serum was also collected on days 2, 4 and 6 after infection to assess circulating IL-12p70 levels. IL-12p70 was detectable 6 days after YopJ infection but was mostly below the limit of detection after WT Y. pseudotuberculosis infection (Fig 6F). Collectively, these data suggest that IL-12 is important in the protection of mice infected with WT or YopJ Y. pseudotuberculosis.

Foodborne infection with WT Yptb-βla was performed to determine whether Y. pseudotuberculosis could target adaptive Vγ4 T cells with Yop translocation in vivo. Balb/c mice were foodborne infected with L. monocytogenes to elicit a population of Vγ1.1/2 CD44hi CD27 γδ T cells as described previously [37,83]. After a return to homeostasis at 30 days post L. monocytogenes infection, mice were foodborne infected with WT Yptb-βla. Y. pseudotuberculosis burden was assessed in the MLN 3 days post foodborne infection to determine whether WT Yptb-βla could establish a productive infection where Vγ4 T cells reside. Indeed, infected mice contained detectable Y. pseudotuberculosis in the MLN 3 days after foodborne infection (Fig 6F).

Analysis of the translocation of Yop into Vγ1.1/2 CD44hi CD27 γδ T cells, myeloid cells (CD11b+), and CD4 and CD8 T cells was performed. Consistent with our in vitro observations, Vγ1.1/2 CD44hi CD27 γδ T cells and myeloid cells contained translocated Yop in vivo (Fig 6G). Y. pseudotuberculosis was relatively inefficient at Yop translocation into CD4 and CD8 T cells (Figs 6G and S7C). Collectively, these data show that foodborne Yop Y. pseudotuberculosis infection of naïve mice elicits IFNγ production in adaptive γδ T cells and that Yop can be translocated into adaptive Vγ4 T cells in vivo.

Discussion

In this study, we assessed the subversion of an adaptive subset of γδ T cells specialized in the promotion of pathogen resistance at the intestinal mucosa through the production of anti-inflammatory cytokines like IFNγ and IL-17A [37]. While limited evidence suggests that Yop effectors directly target T cells to subvert their function, we identified that the Y. pseudotuberculosis effector molecule YopJ directly inhibits IFNγ production from adaptive CD44hi CD27 γδ T cells to subvert host immunity in mice. Additionally, we demonstrate that circulating human Vδ2+ T cells are similarly inhibited by direct translocation of YopJ, demonstrating that the direct targeting of γδ T cells by Y. pseudotuberculosis to inhibit IFNγ production is a conserved pathway of immune evasion in humans. Thus, Y. pseudotuberculosis Yop effectors translocated into murine Vγ4 T cells and human Vδ2+ T cells directly subvert their anti-microbial functions and host immunity by limiting IFNγ production.

While Yersinia mediated inhibition of conventional T cells has been previously reported, studies have largely focused on the indirect subversion of T cells that is mediated by translocation of Yop effectors into myeloid cells [55,82]. For example, YopJ/P appears to primarily subvert conventional T cell function through indirect mechanisms associated with inhibiting DC [22,26]. On the contrary, the study of γδ T cells in the context of Y. pseudotuberculosis infection has been primarily limited to the potential antigens that drive γδ T cell recognition of infection [84–89].

After phagocytosis of pathogens, activated DC migrate to lymph nodes and present antigen to T cells. APC-derived IL-12 further shapes T cell responses by providing a critical signal during T cell activation [90]. Interestingly, Y. pestis can limit both the migratory capacity of DC and the production of IL-12 [91], and Y. enterocolitica can induce programmed death of DC and inhibit antigen presentation [22]. Y. pseudotuberculosis YopJ can also indirectly limit NK cell function by interfering with DC TLR4 signaling pathways and YopP in Y. enterocolitica.
can limit NK cell function through STAT4 inhibition [21,92]. Given that IL-12 signaling promotes STAT4 phosphorylation and IFNγ production in γδ T cells [29,93] and *Yersinia spp.* can inhibit DC, a potential extrinsic mechanism emerges for *Y. pseudotuberculosis* to inhibit γδ T cell responses by suppressing DC functions. In line with these observations, IL-12p40 was critical for the induction of phospho-STAT4 in γδ T cells after stimulation with *Y. pseudotuberculosis* in *in vitro* cultures. In contrast, *Y. pseudotuberculosis* and IL-12 were unable to directly elicit IFNγ production from a highly enriched population of *in vitro* expanded γδ T cells suggesting that IL-12 is required but not sufficient for γδ T cell IFNγ production. However, stimulation of MLN cell suspensions with ΔYopB or YopJΔC127A *Y. pseudotuberculosis* elicited STAT4 phosphorylation among γδ T cells suggesting that translocation of Yop effectors and YopJ in particular subverts γδ T cell function. Tracking Yop translocation *in vitro* revealed that γδ T cells that contain Yop had reduced pSTAT4 levels and inhibited IFNγ production. Importantly, γδ T cells that did not contain Yop effectors from the same cultures expressed higher pSTAT4 levels and comparable IFNγ production as γδ T cells stimulated with ΔYopB-βla Yptb. Additionally, IL-12 levels were comparable between cultures stimulated with WT and YopJΔC127A *Y. pseudotuberculosis*. Collectively, these data suggest that functional impairment of γδ T cells was mediated by direct translocation of YopJ into γδ T cells and cell intrinsic mechanisms *in vitro*.

The YopJ effector family has been increasingly described by their acetyltransferase function on serine, threonine, and lysine amino acid residues [19,94]. Serine and threonine are common targets of phosphorylation to propagate signaling cascades or elicit functional consequences. For example, phosphorylation of STAT4 leads to dimerization and transport to the nucleus to promote transcription of STAT4 target genes. Acetylation of these target residues may inhibit phosphorylation and downstream signaling events [70]. Thus, a potential mechanism of YopJ subversion of γδ T cells is through acetylation of STAT4 to inhibit the phosphorylation or dimerization of STAT4. Other potential targets of YopJ acetyltransferase activity are the IL-12R and Janus kinases upstream of STAT4 activation. YopJ has also been reported to have cysteine protease, lysine acetyltransferase, ubiquitin-like protein protease, and deubiquitinase activity that may provide other potential avenues for YopJ to modulate the function of γδ T cells through STAT4 [94–96].

An important aspect of *Y. pseudotuberculosis* pathogenesis unveiled by this work is the preferential targeting of a specialized subset of γδ T cells for delivery of inhibitory Yop effector molecules. *Y. pseudotuberculosis* injected Yop effectors into adaptive γδ T cells in a similar proportion as macrophages and DC and to a much greater extent than conventional CD4 or CD8 T cells. Of note, *Y. pseudotuberculosis* has been reported to translocate Yop effectors more efficiently into T_reg cells than conventional CD4 T cells at high MOI to modulate their function [65]. The preferential targeting of γδ T cells in this system is associated with expression of the β1-integrin by adaptive γδ T cells. Additionally, the majority of adaptive γδ T cells are anatomically segregated from conventional T cells in the paracortex by their localization in the interfollicular and medullary areas of the gut draining lymph nodes [38]. The distinct localization of adaptive γδ T cells may facilitate interactions with *Y. pseudotuberculosis* in *vivo*. Loss of the adhesin YadA but not Inv abrogated YopJ mediated γδ T cell inhibition, suggesting that *Y. pseudotuberculosis* utilizes YadA to target adaptive γδ T cells for Yop translocation, consistent with previous studies suggesting that the adhesin Inv is largely dispensable for *Y. enterocolitica* virulence [61,97]. Interestingly, this appears distinctly from the targeting of conventional CD4 T cells that relies on Inv and is enhanced in the absence of YadA [11,65].

While our data demonstrates that STAT4 phosphorylation is inhibited by YopJ, our RNA-Seq analysis suggests that other targets may also be affected. One of YopJ’s known targets is the MAPK family of proteins that can have broad effects on cell proliferation, differentiation,
survival, and apoptosis [18]. The MAPK pathway in CD4 T cells and NK cells may also promote STAT4 activity and downstream IFNγ mRNA stabilization, respectively [98,99]. Our data demonstrate a broad impact of YopJ on adaptive γδ T cell proliferation, metabolism, cell cycle, RNA/DNA processing, and ER/Golgi processing gene expression networks. These pathways may be regulated by MAPK family member activity [100–105]. Homer motif analysis identified other potential means by which YopJ may regulate IFNγ production. For example, Ets-1 is a T-bet cofactor and necessary for Th1 IFNγ responses [106]. Increases in ETS-domain family of transcription factor motifs were associated with type 3 innate lymphoid cells (ILC3) but not Th17 cells [107], which may suggest that one of the potential mechanisms of YopJ-mediated inhibition may target conserved pathways in unconventional lymphocyte populations. Many NK cell receptors are also expressed on γδ T cells and may facilitate TCR independent effector functions [108–110]. In line with this, our profiling demonstrates that YopJ limits gene expression of Nkg7, which has recently been reported to promote cytotoxic granule release and inflammation during infection and cancer [72], and Prf1, which encodes the pore forming molecule perforin necessary to deliver lytic machinery into target cells [111]. Finally, interactions with *Y. pseudotuberculosis* YopJ led to the upregulation of Ulbp1, which encodes a stress-induced NKG2D ligand, and Idil, which encodes an enzyme in the mevalonate pathway. As human γδ T cells respond to phospho-antigens derived from the non-mevalonate pathway in bacteria and mammalian mevalonate pathway in humans [112], this may limit the removal of translocated cells through NK or γδ T cell sensing mechanisms and suggests a broad mechanism to subvert human immunity. Thus, our findings suggest that adaptive Vγ4 T cells provide dynamic anti-infectious immunity that is subverted by direct translocation of YopJ.

A number of studies have highlighted the importance of IFNγ production from conventional CD4 and CD8 T cells, NK cells, and ILC3 for *Yersinia* resistance [40,43,113,114], although the *in vivo* relevance of Yop inhibition of conventional T cells has not been addressed. Foodborne infection with Yop<sup>C172A</sup> *Y. pseudotuberculosis* led to an enhanced response from Vγ4 T cells, including increased IFNγ production, that was associated with a more rapid recovery of weight. As IL-12 was critical for Vγ4 T cell derived IFNγ production *in vitro*, the role of IL-12 after foodborne infection of Balb/c mice was assessed. Consistent with previous studies [22,40,115,116], IL-12 appeared critical for protection against foodborne WT and Yop<sup>C172A</sup> *Y. pseudotuberculosis* infection. As serum IL-12 was only readily detectable after Yop<sup>C172A</sup> *Y. pseudotuberculosis* infection, increased IL-12 may also contribute to the enhanced IFNγ response from Vγ4 T cells *in vivo*. Finally, assessment of cell populations targeted for Yop translocation *in vivo* was comparable to the results from the *ex vivo* MLN cultures. The highest percentage of Yop<sup>+</sup> cells were among CD11b<sup>+</sup> cells and Vγ4 T cells. Given the low MOI used in our *ex vivo* studies with the WT Yptb-<b>β</b>la reporter and the lack of inhibition observed in Vγ4 T cells that lack Yop effectors from the same culture conditions, it is likely that intrinsic Yop effects on Vγ4 T cells is a mechanism of inhibiting Vγ4 T cell function *in vivo*. Thus, while *Y. pseudotuberculosis* can use γδ T cell intrinsic mechanisms to subvert the γδ T cell IFNγ response, multiple mechanisms may be available for *Yersinia spp.* to subvert γδ T cell functions to aid pathogenesis *in vivo*. As we previously demonstrated that foodborne but not i.v. infection led to adaptive Vγ4 T cell responses [37], our physiologic foodborne *Y. pseudotuberculosis* infection model revealed novel aspects of *Yersinia* pathogenesis and adaptive Vγ4 T cell biology.

In summary, the *Y. pseudotuberculosis* effector YopJ directly inhibits essential anti-effective functions of murine Vγ4 T cells and human Vδ2<sup>+</sup> T cells. *Y. pseudotuberculosis* targeted Vγ4 T cells in a T3SS- and YadA-dependent process to deliver Yop effectors directly into Vγ4 T cells. *Ex vivo* whole tissue cultures revealed that direct inhibition of Vγ4 T cell function was the major mechanism of Vγ4 T cell subversion. YopJ translocation led to a dramatic reduction in
STAT4 phosphorylation levels and IFNγ production, which is important for protection from *Yersinia*. YopJ also inhibited a broad anti-infective gene signature. Thus, these findings add substantial insight into YopJ effector functions on murine and human γδ T cells and the pathogenesis of foodborne *Y. pseudotuberculosis* infection.

**Materials and methods**

**Ethics statement**

All animal experiments were conducted in accordance with the Stony Brook University Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Blood collection from healthy human donors was approved by the Institutional Review Board at Stony Brook University.

**Mice**

Female 8–12 week old BALB/cJ mice were purchased from the Jackson Laboratory. Mice were euthanized by CO2 inhalation.

**Human studies**

Blood was sampled from a total of 6 adult healthy human donors of either gender between the ages of 20 and 40. Studies were designed so no randomization to experimental groups was necessary. Donors provided written informed consent.

**Bacteria**

Bacteria strains used in this study include: *Y. pseudotuberculosis* on the 32777 background WT strain, WT32777c, YopJ<sup>C172A</sup>, YopH<sup>R409A</sup>, ΔYopB, YopE<sup>R144A</sup>, YopT<sup>C139A</sup>, ΔYopM, ΔYpkA, ΔYopK, WT Yptb-βla, and ΔYopB Yptb-βla. *Y. pseudotuberculosis* on the IP2666 background WT strain, ΔYopB, ΔInv, ΔYadA, ΔInv ΔYadA, and ΔYopB ΔInv ΔYadA. See Table 1 for details. All strains were stored in 25% glycerol stocks at -80˚C. For stimulations, *Y. pseudotuberculosis* strains were cultured overnight at 28˚C and 220 RPM in LB media. The following morning, *Y. pseudotuberculosis* was sub-cultured 1:10 in LB and 50 mM CaCl<sub>2</sub> at 37˚C and 220 RPM for approximately 2 hours. Stimulation doses were based on the OD600.

**Foodborne *L. monocytogenes* immunization**

*L. monocytogenes* (EGDe strain) expressing a mutation in the internalin A gene (InlAm<sup>M</sup>) was used for foodborne infection to facilitate epithelial cell invasion [117]. InlAm<sup>M</sup> *L. monocytogenes* was cultured overnight at 37˚C and 220 RPM in BHI media. The following morning, *L. monocytogenes* was sub-cultured 1:10 in BHI at 37˚C and 220 RPM for approximately 2 hours. Infection doses were based on the OD600. Mice were food and water deprived for 4 hours. Approximately 0.5 cm<sup>3</sup> bread pieces were inoculated with 2x10<sup>9</sup> CFU *L. monocytogenes* in 50 μL. Mice were monitored to ensure the inoculated bread was consumed within 1 hour. Mice that did not fully consume bread were removed from the study. Bacterial infection doses were confirmed by plating inoculum on BHI.

**Foodborne *Y. pseudotuberculosis* infection**

*Y. pseudotuberculosis* strains (Table 1) were cultured overnight at 28˚C and 220 RPM in LB media. Infection doses were based on the OD600. Mice were food and water deprived for 4 hours. Approximately 0.5 cm<sup>3</sup> bread pieces were inoculated with 2-4x10<sup>7</sup> CFU for WT32777.
Y. pseudotuberculosis, 2-4x10^7–2-4x10^8 CFU for YopC\textsuperscript{172A} Y. pseudotuberculosis, or 2x10^9 CFU for WT Yptb-βla infection in 50 μL. Mice were monitored to ensure the inoculated bread was consumed within 1 hour. Mice that did not fully consume bread were removed from the study. Bacterial infection doses were confirmed by plating inoculum on LB.

**Single cell preparations, Y. pseudotuberculosis stimulations, and flow cytometry**

MLN from L. monocytogenes immunized mice were harvested 9 days after immunization and mechanically dissociated using a syringe plunger through a 70 μm cell strainer into a single cell suspension. Cells were resuspended in IMDM (Gibco) supplemented with 10% fetal bovine serum, 0.01 M HEPES, 100 μM non-essential amino acids (Gibco), 2 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl or 1x Glutamax (Gibco), and 1 mM sodium pyruvate. Cells were counted using a Vi-CELL Viability Analyzer (Beckman Coulter). Cells were stimulated in 96 well round-bottom tissue culture treated plates with various strains of Y. pseudotuberculosis at 1 or 10 MOI (1 MOI for WT or ΔYopB Yptb-βla and 10 MOI for all other Y. pseudotuberculosis stimulations, unless otherwise indicated) at 37˚C/5% CO\textsubscript{2}. 100 U/mL of penicillin and 100 μg/mL of streptomycin were added to cells 2 hours post-stimulation. Cells were stimulated for a total of 24 hours or as indicated. BD GolgiPlug (BD Biosciences) was added 5 hours prior to the end of stimulation. If translocation was assessed, β-lactamase Loading Solutions kit (Invitrogen) was used to load CCF4-AM by incubating CCF4-AM at RT with cells for 1 hour in the dark. Cells were then processed for surface staining via incubation with live/dead stain, antibody, and Fc block (BioXcell) for 20 min in the dark at 4˚C. Antibodies used included antibodies specific to CD45, CD3, TCRδ, CD8, CD4, Vγ1.1, Vγ2, CD44, CD27, F4/80, CD11b, MHCII, CD11c, and CD29 (BioLegend). Cells were fixed, permeabilized, and stained with anti-IFNγ, anti-IRF8, or anti-STAT4 using BD Cytofix/Cytoperm kit (BD Biosciences) for intracellular cytokine staining. Functional γδ T cell analysis was done by stimulation with BD leukocyte activation cocktail (containing PMA, ionomycin, and brefeldin A; BD Pharmingen) for 4 hours prior to staining. Flow cytometry data were acquired using a BD LSRFortessa and analyzed by FlowJo software (BD Biosciences). Cell culture supernatant was analyzed for IL-12p70 using the BioLegend ELISA MAX Deluxe Set Mouse IL-12 (p70) kit per manufacturer instructions.

**Human γδ T cell response**

Blood was drawn and collected from healthy human donors in BD Vacutainer sodium heparin tubes (BD Biosciences). Blood was diluted 1:1 with 1x PBS at room temperature. Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat of Ficoll-paque PLUS gradient centrifugation (GE Healthcare) for 20 min at 1,400 x g without a brake. PBMC were washed with 1x PBS at room temperature and resuspended in IMDM supplemented with 10% fetal bovine serum, 0.01 M HEPES, 100 μM Non-essential amino acids (Gibco), 2 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl or 1x Glutamax (Gibco), and 1 mM sodium pyruvate. Y. pseudotuberculosis strains were cultured overnight at 28˚C and 220 RPM in LB media the night prior. The following morning, Y. pseudotuberculosis was sub-cultured 1:10 in LB and 50 mM CaCl\textsubscript{2} at 37˚C and 220 RPM for approximately 2 hours. Stimulation doses were based on the OD600. Cells were counted using a Vi-CELL Viability Analyzer (Beckman Coulter). Cells were stimulated in 96 round-bottom tissue culture treated plates with various strains of Y. pseudotuberculosis at 1 MOI at 37˚C/5% CO\textsubscript{2}. 1x penicillin and streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin) were added to cells 2 hours post-stimulation. Cells were stimulated for a total of 24 hours or as indicated. BD GolgiPlug (BD Biosciences) was
added 5 hours prior to the end of stimulation. If translocation was assessed, β-lactamase Loading Solutions kit (Invitrogen) was used to load CCF4-AM by incubating CCF4-AM at RT with cells for 1 hour in the dark. Cells were then processed for surface staining via incubation with live/dead stain, antibody, and Fc block (BioXcell) for 20 min in the dark at 4°C. Antibodies used included antibodies specific to Vδ2, CD3, TCRδ, (BioLegend). Cells were fixed, permeabilized, and stained with anti-IFNγ using BD Cytofix/Cytoperm kit (BD Biosciences) for intracellular cytokine staining. Flow cytometry data were acquired using a BD LSRFortessa and analyzed by FlowJo software (BD Biosciences).

**Phospho-flow cytometry**

After surface staining for flow cytometry, cells were washed and stained for pSTAT4 using a methanol-based approach. Cells were fixed in 4% PFA/1.5% methanol for 30 minutes in the dark at 4°C. Cells were then washed and incubated in methanol in the dark at 20°C for 45 minutes. After washing, cells were stained with anti-pSTAT4 (Y693)-PE (BD Biosciences) and washed once more. Flow cytometry data were acquired using a BD LSRFortessa and analyzed by FlowJo software (BD Biosciences).

**Enumeration of Y. pseudotuberculosis burden**

MLN were crushed and diluted in media prior to plating on LB agar. Total Y. pseudotuberculosis burden per organ was calculated.

**Sequencing and analysis**

Samples were prepared after Y. pseudotuberculosis stimulation as described above. Cell preparations were stimulated with 10 MOI of WT or YopJ<sup>C172A</sup> Y. pseudotuberculosis or 1 MOI of WT Yptb-βla for 24 hours. 500 Vγ1.1/2<sup>+</sup> CD44<sup>hi</sup> CD27<sup>-</sup> γδ T cells were flow sorted directly into a tube with NEBNext Cell Lysis Buffer and Murine RNase Inhibitor and processed for RNA sequencing using NEBNext Single Cell/Low Input RNA Library Prep Kit (Illumina). Sequencing was performed at the Cold Spring Harbor Laboratory sequencing core on a NextSeq500. Fastq files were produced as an output of the sequencing files. Fastq were run through FastQC to perform quality control of transcripts prior to alignment. Fastq files were pair-ended aligned to GRCm38/mm10 by way of HISAT2 and output as .BAM files [118]. Raw counts of aligned transcripts were quantified with FeatureCounts [119]. Dimensionality reduction was performed with PCA analysis with the axes PC1 and PC2 in R-studio [120]. To determine differential expression between samples, FeatureCounts raw count matrix was analyzed through DESeq2 with a parametric fitting normalized to the geometric mean of each individual gene across samples [121]. Cutoff values for significance and quality control were a p-value of <0.05 and FDR-value of <0.10, respectively. Significantly differentially expressed genes were visualized on a heatmap with a dendrogram that was clustered through average linkage. The distance measurement on the dendrogram used was through the Euclidean method. Overlapping expressions between gene differential expression sets were filtered with R-studio. Upregulated and downregulated genes from the differential expression analysis were separated with R-studio and these Gene IDs were used for HOMER motif analysis [74]. Parameters of analysis of each gene used were 400bp preceding the initiation site and 100bp after the initiation site. The length of the motifs analyzed was set between 8 and 10.
\(\gamma\delta\) T cell purification

\(\gamma\delta\) T cells were expanded \textit{in vitro} according to published protocols [122]. The MLN and spleen were isolated and processed into a single cell suspension 9 days post foodborne \textit{L. monocytogenes} infection of Balb/c mice. Red blood cells were lysed with red blood cell lysis buffer or ammonium chloride for 1 minute and cells from the MLN and spleen were combined. \(\gamma\delta\) T cells were enriched by negative selection using the following rat IgG primary antibodies from BioLegend: anti-CD4 (clone GK1.5), anti-CD8\(\alpha\) (clone 53–6.7), anti-B220 (clone RA3–6B2), anti-MHC-II (clone M5/114.15.2), and anti-CD11b (clone M1/70). The MACS goat anti-rat IgG kit (Miltenyi Biotec) was used per manufacturer instructions with MACS LD columns and a QuadroMACS magnet. Enriched cells were cultured in 48-well plates coated overnight with 5 µg/ml anti-TCR\(\delta\) (clone GL3). Cells were cultured in RPMI 1640 supplemented with 25 mM HEPES (Gibco), 1x glucose (Gibco), 10 g/ml folate (Sigma Aldrich), 1x sodium pyruvate (Gibco), 5x10^5 M 2 beta-mercaptoethanol (Sigma Aldrich), 1x Glutamax (Gibco), 1x penicillin-streptomycin (Gibco), and 10% FBS with 100 U/ml recombinant human IL-2. After 2 days of culture, cells were transferred into new wells with the same culture media to rest for 5 days. Cells were then stimulated with 10 MOI YopJ\textsuperscript{C172A} \textit{Y. pseudotuberculosis} with 0.1, 1, or 10 ng/ml of recombinant murine IL-12p70 (Peprotech).

\textit{In vivo} anti-IL-12p40 antibody treatment and serum collection

On the day of foodborne WT or YopJ\textsuperscript{C172A} \textit{Y. pseudotuberculosis} infection and on day 2, 4, and 6 after infection, mice were treated with 0.2 mg of anti-IL-12p40 (clone C17.8; BioLegend) by i.p. injection. Blood was collected via tail vein on day 2, 4, and 6 after infection. Blood was incubated at ambient temperature for 30 minutes before being spun down at 1500G for 10 minutes at 4°C. Serum was isolated and analyzed for IL-12p70 with the BioLegend ELISA MAX Deluxe Set Mouse IL-12 (p70) kit.

\textit{Ex vivo} anti-IL-12p40 and recombinant IL-12p70 treatments

At the start of \textit{Y. pseudotuberculosis} stimulation of MLN cell suspensions, cultures were treated with 10 µg/ml anti-IL-12p40 (clone C17.8; BioLegend) for neutralization. In other conditions, recombinant murine IL-12p70 (Peprotech) was added at the start of \textit{Y. pseudotuberculosis} stimulation of MLN cell suspensions at 2, 10, or 50 ng/ml.



Statistical analysis

GraphPad Prism 6 software (GraphPad Software Inc.) was used for statistical analysis. The differences between the means were compared using the statistical analysis described in the associated figure legends. All the data are presented as mean ± SEM and \(p < 0.05\) was considered significant. * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\), **** \(p < 0.0001\).

Supporting information

S1 Data. Excel spreadsheet containing the underlying numerical data for Figs 1A–1C, 2A and 2B, 3A, 5A–5D and 6A–6F.

(XLSX)

S1 Fig. Use of the \textit{Yptb-\beta la} reporter to track Yop translocation. (A) MLN suspensions from \textit{L. monocytogenes} infected mice were stimulated with WT or \(\Delta\text{YopB} \textit{Y. pseudotuberculosis}\) containing a \(\beta\)-lactamase translocation reporter (\textit{Yptb-\beta la}) for 2 hours and given antibiotics. Cells were loaded with CCF4-AM dye to measure \(\beta\)-lactamase activity. FITC indicates CCF4-AM
loaded cells without translocation (Yop) and BV421 indicates CCF4-AM loaded cells with Yop translocation (Yop⁺). Vγ1.1/2 CD44hi CD27γδ T cells were analyzed for Yop translocation 2 hours post stimulation at an MOI of 10. Representative contour plots are displayed. (B) MLN from L. monocytogenes infected mice were stimulated with Yptb-βla for 2 hours and given antibiotics. Yop translocation was detected as described above. The indicated cell populations were analyzed for Yop translocation 2 hours after stimulation. Representative contour plots are displayed. (C) MLN from L. monocytogenes infected mice were stimulated with Yptb-βla for 2 hours and given antibiotics. Vγ1.1/2 CD44hi CD27γδ T cells were analyzed for Yop translocation 2 hours post stimulation at the indicated MOI and quantified for Yop translocation. Data consists of one experiment with 2–10 mice/group and the graphs depict the mean ± SEM in (A–C). ***p < 0.0001, **p < 0.001, and *p < 0.01. A t-test was used for (A), and a repeated measures one-way ANOVA was used for (C). Comparisons were performed to ΔYopB Y. pseudotuberculosis in (A) and as depicted in (C).

(TIF)

S2 Fig. The majority of Vγ1.1/2 CD44hi CD27γδ T cells and γδ T cells containing Yop express β1-integrin. (A) MLN from L. monocytogenes infected mice were isolated and processed into single cell suspensions. Vγ1.1/2 CD44hi CD27γδ T cells, CD4 T cells, and CD8 T cells were analyzed for β1-integrin expression. (B) MLN suspensions from L. monocytogenes infected mice were loaded with CCF4-AM dye and stimulated with 10 MOI WT Y. pseudotuberculosis containing a β-lactamase translocation reporter. CCF4-AM dye reports the occurrence of β-lactamase activity and Yop translocation. γδ T cells that contain Yop (Yop⁺) or do not contain Yop (Yop⁻) were analyzed for β1-integrin expression 2 hours after stimulation. Representative histogram plots are displayed. Data is pooled from two experiments with a total of 7 mice/group and the graphs depict the mean ± SEM in (A–C). ****p < 0.0001 and ***p < 0.001. A repeated measures one-way ANOVA was used for (A) and a t-test was used for (B), and. Comparisons were done to adaptive γδ T cells in (A) and as depicted in (B).

(TIF)

S3 Fig. YopJ regulates the transcriptional profile of Vγ1.1/2 CD44hi CD27γδ T cells. (A and B) MLN from L. monocytogenes infected mice were stimulated with 10 MOI of WT Y. pseudotuberculosis (WT) or mutant YopJ Y. pseudotuberculosis (YopJ(C172A)) for 24 hours. Antibiotics were given 2 hours post-stimulation. Five hundred Vγ1.1/2 CD44hi CD27γδ T cells from each stimulation were flow sorted and processed for RNA sequencing. The heat map depicts upregulated genes in Vγ1.1/2 CD44hi CD27γδ T cells after YopJ(C172A) Y. pseudotuberculosis stimulation and individual genes are listed. (C–E) Genes differentially expressed (downregulated) that overlapped between RNA sequencing analyses as displayed in the Venn diagram in (C) to select for direct effects of YopJ on Vγ1.1/2 CD44hi CD27γδ T cells. The heat map depicts downregulated genes in Vγ1.1/2 CD44hi CD27γδ T cells from the analysis in (C). Individual genes are listed in (E). Each experiment was performed once with biologic replicates. The cutoff for gene significance was p < 0.05 and FDR < 0.10.

(TIF)

S4 Fig. YopJ does not inhibit IL-17A production in Vγ1.1/2 CD44hi CD27γδ T cells. MLN cell suspensions from L. monocytogenes infected mice were stimulated with 10 MOI of WT, YopJ(C172A), or ΔYopB Y. pseudotuberculosis for 24 hours. Antibiotics were given 2 hours after stimulation and brefeldin A was added for the last 5–6 hours. Vγ1.1/2 CD44hi CD27γδ T cells were analyzed for IL-17A production after stimulation. Representative histograms are displayed. The graph depicts mean ± SEM and represents two independent experiments with
4 mice per group. A repeated measures one-way ANOVA was used. * p < 0.05.

(TIF)

**S5 Fig. YopJ impacts IFNγ related transcription factor motifs but not STAT4 protein.** (A) Homer motif analysis was performed on the RNA sequencing results for the YopJ\(^{C172A}\) and WT Y. pseudotuberculosis comparison from Fig 5. The panel highlights the top transcription factor motifs of the ETS, SP/KLF, and IRF family of proteins identified in YopJ\(^{C172A}\) stimulated Vγ1.1/2 CD44\(^{hi}\) CD27\(^{ γ δ}\) T cells. (B) MLN from L. monocytogenes infected mice were stimulated with 10 MOI of WT, YopJ\(^{C172A}\), or ΔYopB Y. pseudotuberculosis for 6 hours. Antibiotics were given 2 hours post-stimulation. Vγ1.1/2 CD44\(^{hi}\) CD27\(^{ γ δ}\) T cells were analyzed for IFNγ levels with or without anti-IL12p40 neutralizing antibody. The graph depicts the percentage of IFNγ protein expression among Vγ1.1/2 CD44\(^{hi}\) CD27\(^{ γ δ}\) T cells after WT, YopJ\(^{C172A}\), or ΔYopB Y. pseudotuberculosis stimulation. Data depict two pooled experiments with a total of 8 mice/group and represents the mean ± SEM. (C) The genes from the RNAseq and Homer motif analysis in Figs 4F and 4G and S3B and S5A were compared to an existing STAT4 ChIP-on-chip dataset to identify common genes. Genes from our dataset that were represented in the top 1000 genes of the Chip-on-chip dataset are displayed. (D) STAT4 KO spleens are shown in maroon and WT spleens are shown in black in representative histograms. The graph depicts the MFI of STAT4 protein expression in bulk γδ T cells. Data depicts one experiment with 4 mice/group and represents the mean ± SEM. **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05. A repeated measures one-way ANOVA was used for (B), and a t-test was used for (D). Comparisons were performed as depicted in (B) and to Naïve WT in (D).

(TIF)

**S6 Fig. IL-12 is insufficient to induce IFNγ and does not readily overcome the inhibition of YopJ.** (A) γδ T cells enriched from the MLN and spleen of L. monocytogenes infected mice were expanded with plate bound γδTCR antibody for 2 days and rested for 5 days. After expansion, ~ 50% of cells were γδ T cells, and the majority of those were Vγ4 T cells. The enrichment summary reflects the mean enrichment from 4 samples. Afterwards, γδ T cells were isolated from cultures and stimulated with YopJ\(^{C172A}\) Y. pseudotuberculosis with 0.1, 1, or 10 ng/ml IL-12p70 for 24 hours. Antibiotics were added 2 hours after stimulation and brefeldin A was added for the last 5–6 hours. Histograms display IFNγ production from Vγ1.1/2 CD44\(^{hi}\) CD27\(^{ γ δ}\) T cells under different culture conditions. Data depicts one experiment with 4 mice pooled and split into the indicated stimulation conditions. (B) MLN cell suspensions from L. monocytogenes infected mice were stimulated with 10 MOI of WT Y. pseudotuberculosis in the presence of 2, 10, or 50 ng/ml IL-12p70 or 10 MOI of YopJ\(^{C172A}\) Y. pseudotuberculosis for 24 hours. Antibiotics were given 2 hours after stimulation and brefeldin A was added for the last 5–6 hours. Vγ1.1/2 CD44\(^{hi}\) CD27\(^{ γ δ}\) T cells were analyzed for IFNγ production. Representative histograms of IFNγ production from Vγ1.1/2 CD44\(^{hi}\) CD27\(^{ γ δ}\) T cells are displayed. The graph depicts mean ± SEM from one experiment with 4 mice per group * p < 0.05. A repeated measures one-way ANOVA was used for comparisons to YopJ\(^{C172A}\) Y. pseudotuberculosis in (B).

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**S7 Fig. The impact of foodborne infection of mice with Y. pseudotuberculosis.** (A) Balb/c mice were foodborne infected with the indicated doses of WT or mutant YopJ\(^{C172A}\) Y. pseudotuberculosis and tissues were analyzed 9 days post-infection. Bacteria burden was quantified from the MLN. Data reflect 3–5 mice per group pooled from 3 independent experiments and the graphs depict the mean ± SEM. (B) Balb/c mice were foodborne infected with the indicated doses of WT or mutant YopJ\(^{C172A}\) Y. pseudotuberculosis. Nine days post infection, MLN...
suspensions from WT or Yop\textsuperscript{C172A} Y. pseudotuberculosis infected mice were stimulated with PMA/ionomycin and brefeldin A for 4 hours. V\textgamma1.1/2 CD44\textsuperscript{hi} CD27\textsuperscript{−} γδ T cells were analyzed for IFN\gamma production. Representative histograms are displayed and quantified. Data depicts one experiment with 3 mice per group. (C) Balb/c mice were foodborne infected with WT (2-4x10\textsuperscript{7} CFU) or Yop\textsuperscript{C172A} Y. pseudotuberculosis (2-4x10\textsuperscript{8} CFU) and treated with 0.2 mg/mouse of anti-IL12p40 on days 0, 2, 4, and 6 post infection. IL-12p70 concentrations were determined from serum at days 2, 4, and 6 post infection. Data represent 2 independent experiments with a total of 9 mice per group. Serum samples were pooled into groups of 3 per experimental condition. (D) Balb/c mice were foodborne infected with 2x10\textsuperscript{9} CFU L. monocytogenes to elicit a V\textgamma1.1/2 CD44\textsuperscript{hi} CD27\textsuperscript{−} γδ T cell population in vivo. 30 days post infection, adaptive V\textgamma1.1/2 CD44\textsuperscript{hi} CD27\textsuperscript{−} γδ T cells from the MLN of immune mice were analyzed for Yop translocation using the CCF4-AM assay. Representative contour plots are shown. Yop translocation (Yop\textsuperscript{+}) among the indicated populations represents background staining as a negative control for Fig 6F. The graph depicts the mean ± SEM and is pooled from 2 experiments with a total of 4 mice per group. * * * * p < 0.0001, * p < 0.05. A one-way ANOVA was used for (A), and an unpaired t-test was used for (B). Comparisons were performed to uninfected in (A) and to 5x10\textsuperscript{7} WT Y. pseudotuberculosis in (B).

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