Streptococcus agalactiae and *Escherichia coli* Induce Distinct Effector γδ T Cell Responses During Neonatal Sepsis and Neuroinflammation

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Abstract

The neonatal phase of life is a time during which susceptibility to infection is particularly high, with prematurely born neonates being especially vulnerable to life-threatening conditions such as bacterial sepsis. While *Streptococcus agalactiae*, also known as group B *Streptococcus* (GBS) and *Escherichia coli* are frequent causative pathogens of neonatal sepsis, it is still unclear how the neonatal adaptive immune system responds to these pathogens. In the present study, we find that γδ T cells in neonatal mice rapidly respond to single-organism sepsis infections of GBS and *E. coli*, and that these infections induce distinct activation and effector functions from IFN-γ and IL-17 producing γδ T cells, respectively. We also report differential reliance on γδTCR signaling to elicit effector cytokine responses during neonatal sepsis, with IL-17 production during *E. coli* sepsis being associated with TCR signaling, whereas IFN-γ production during GBS sepsis is TCR-independent. Furthermore, we report that the divergent effector responses of γδ during GBS and *E. coli* sepsis impart distinctive neuroinflammatory phenotypes on the neonatal brain. The present study sheds light on how the neonatal adaptive immune response responds differentially to bacterial stimuli and how these responses impact neonatal sepsis-associated neuroinflammation.

Introduction

Late-onset neonatal sepsis remains a leading cause of neonatal morbidity and mortality worldwide, particularly amongst preterm infants (Bergin et al., 2015; Stoll et al., 2011). The neonatal period, defined as the first 28 days of life in humans, is a time during which risk of infection is especially high, due in part to the relative immaturity of the neonatal immune system (Bergin et al., 2015; Knoop et al., 2020; Segura-Cervantes et al., 2016). Premature neonates are highly vulnerable to life-threatening conditions such as sepsis (Bizzarro et al., 2005; Dong and Speer, 2015; Wynn et al., 2015). Gram-negative bacilli such as *Klebsiella*, *Pseudomonas* and *E.*

coli are prevalent in the gastrointestinal tract of premature neonates and are capable of translocating from the gut and causing sepsis (Basu, 2015; Carl et al., 2014; Knoop et al., 2020). Increased bacterial translocation from the neonatal gut is facilitated in part by selective deficiencies in gut barrier defense mechanisms, including decreased production of protective factors such as mucous, anti-microbial peptides, and IgA (Basu, 2015).

Conversely, Gram-positive neonatal sepsis is frequently caused by *Staphylococcus aureus* and *Streptococcous agalactia*e, or group B *Streptococcus* (GBS). GBS colonizes the neonate via vertical transmission during birth, often as the result of the neonate aspirating GBS-infected amniotic fluid (Heath and Jardine, 2014; Stoll et al., 2011). Although rates of GBS sepsis are declining due to improved early detection methods and prophylactic maternal antibiotic administration, the mortality rate of GBS sepsis can be as high as 10%, with 30-50% of survivors going on to experience neurological comorbidities in early childhood (Mynarek et al., 2021).

Although both GBS and *E. coli* can be found as components of a healthy microbiota, they have the potential to cause severe disease in vulnerable populations, such as neonates (Remington et al., 2010; Tavares et al., 2022). The increased risk of sepsis development amongst preterm neonates is further compounded by deficiencies in several innate immunological defense mechanisms, including decreased levels of circulating complement proteins, impaired neutrophil function, and reduced secretion of pro-inflammatory cytokines by dendritic cells compared to adults (Tsafaras et al., 2020). Similar to the innate immune system, the adaptive immune system in neonates bears several striking differences to that of adults (Tsafaras et al., 2020). Compared to adults, neonates have impaired memory T cell formation and are largely skewed toward Th2 over Th1 differentiation, thereby impairing their ability to mount a proper immune response to microbial infections (Barrios et al., 1996; Basha et al., 2014; Li et al., 2004). Neonates also have deficiencies in humoral immunity, such as delayed germinal center formation and impaired antibody responses to both T cell dependent and independent antigens (Semmes et al., 2021)

58 Despite their relative impairments in the conventional T and B cell compartments, neonates have
59 a significant population of γδ T cells (Basha et al., 2014). γδ T cells are innate-like lymphocytes
60 that are abundant in barrier sites and act as early immune sentinels during infection (Chien et al.,
61 2014; Vantourout and Hayday, 2013). In contrast to conventional CD4+ and CD8+ T cells, γδ T
62 cells are exported from the thymus as functionally mature cells and are poised to rapidly deploy
63 their effector functions upon the detection of microbial ligands or pro-inflammatory cytokines
64 (Ribot et al., 2021). As the first T cells to develop in the embryonic thymus, γδ T cells are critical
65 players in the neonatal immune response during a time when CD4+ and CD8+ T cells, and B cells
66 are still developing and maturing (Dimova et al., 2015; Gibbons et al., 2009; Vantourout and
67 Hayday, 2013). Indeed, γδ T cells have been found to play a critical role in host protection during
68 neonatal influenza (Guo et al., 2018) and *Clostridium difficile* infection (Chen et al., 2020),
69 underscoring their importance during early life.

70 In the present study, we characterize the immune responses to two major neonatal sepsis
71 pathogens, *Streptococcus agalactiae* (Group B *Streptococcus*) and *Escherichia coli.* We report
72 that these two pathogens induce distinct effector cytokine responses from gamma delta (γδ) T
73 cells in postnatal day 7 (P7) pups and that these responses differentially impact mortality. We
74 also report that these two pathogens drive distinct neuroinflammatory phenotypes in neonatal
75 mice. This study sheds light on how distinct sepsis pathogens drive differential adaptive immune
76 responses in neonatal mice, impacting sepsis mortality and neuroinflammation.

77 **Results**

78 *γδ T cells Respond to* E. coli *and GBS Neonatal Sepsis and Differentially Drive Mortality*

79 While group B *Streptococcus* (GBS) and *Escherichia coli* are frequent causative pathogens of
80 neonatal sepsis, it is still unclear how features of these bacteria differentially drive the neonatal
81 adaptive immune response (Tsai et al., 2014). Therefore, we infected C57BL/6 P7 mice with a

82 single-organism infection of either 10^6 CFU *Streptococcus agalactiae* (GBS) or $2x10^4$ CFU *E. coli.*
83 Both *E. coli* and GBS septicemia induced robust activation from γδ T cells in the spleen 18 hours
84 post-infection, as measured by an increase the proportion of CD69+ CD62L- γδ T cells compared
85 to uninfected controls (Fig. 1a). A modest increase in the activation status of conventional CD4+
86 and CD8+ T cells, and B cells was also observed (Fig. S1a-c). Paralleling clinical findings, P7
87 mice infected with Gram-negative *E. coli* experienced decreased survival compared to Gram-
88 positive GBS (Tsai et al., 2014), despite the higher bacterial burden in GBS-infected pups at 18
89 hours post-infection (Fig.1b-d). Similarly, greater γδ T cell activation was also observed in *E. coli*
90 compared to GBS-infected pups (Fig. 1a). Interestingly, antibody-mediated depletion of γδ T
91 cells in *E. coli* infected mice was sufficient to completely rescue mortality (Fig. 1c), however no
92 differences in survival were observed in GBS- infected pups upon depletion of γδ T cells (Fig. 1b).
93 Of note, depleting γδ T cells did not impact either GBS or *E. coli* bacterial burden in neonatal pups
94 (Fig. 1d), suggesting that γδ T cells do not impact anti-microbial immune defenses during
95 neonatal sepsis. These data indicate that although γδ T cells undergo activation during both GBS
96 and *E. coli* neonatal sepsis infection, those responses differentially impact mortality independent
97 of systemic bacterial burden.

98 *E. coli and GBS Neonatal Sepsis Drive Distinct Effector Cytokine Responses from γδ T cells*

99 Due to the differential impact of γδ T cells on mortality during GBS vs. *E. coli* neonatal sepsis, we
100 next sought to further characterize the responses of γδ T cells during these two single-organism
101 sepsis infections. γδ T cells undergo developmental programming in the thymus and exist in the
102 periphery as either those that produce either IL-17 or IFN-γ (Haas et al., 2009; Muñoz-Ruiz et al.,
103 2017; Ribot et al., 2009). These distinct effector programs can also be discerned based on the
104 expression of certain surface markers, such as CCR6, restricted to IL-17 producing γδ T cells,
105 and CD27, restricted to IFN-γ producing γδ T cells (Ribot et al., 2009). We therefore asked if
106 different subsets of γδ T cells were being activated during GBS and *E. coli* infection in neonates.

107 Cytokine staining of γδ T cells revealed a robust increase in IL-17 expression during *E. coli*, but
108 not GBS infection whereas GBS induced increased IFN-γ, but not IL-17, expression from γδ T
109 cells (Fig. 2a-c). These findings were validated with serum ELISA, showing systemic increases in
110 these cytokines during GBS and *E. coli* sepsis infections (Fig. 2d,e). Accordingly, γδ T cell
111 activation during GBS infection was restricted to those expressing CD27, whereas during *E. coli*
112 infection, γδ T cell activation was restricted to the CCR6+ expressing fraction (Fig. 2f,g),
113 suggesting GBS and E. coli infections induce discrete activation of γδ T cell subsets. Therefore,
114 single-organism GBS and *E. coli* infections in neonates induces specific activation of different γδ
115 T cell populations, resulting in distinct effector cytokine profiles.

116 *Neuroinflammation is a Feature of E. coli and GBS Neonatal Sepsis*

117 Adverse neurologic outcomes are associated with inflammatory events in early life (Mynarek et
118 al., 2021; Stoll et al., 2004) however, how the adaptive immune response contributes to these
119 outcomes remains poorly understood. Both *E. coli* and GBS were present in the brains of P7 pups
120 18 hours post-infection, with increased CFUs of GBS present compared to *E. coli* (Fig. 3a). Flow
121 cytometry analysis of $CD45^{hi}$ immune cells in the perfused brains of P7 pups revealed a significant
122 increase in monocytes and neutrophils in the brains of *E. coli* infected pups compared to
123 uninfected and GBS-infected pups (Fig. 3b,c). Interestingly, no significant increase in brain-
124 infiltrating monocytes or neutrophils was noted in GBS-infected pups compared to control mice,
125 despite the presence of GBS in the brain (Fig. 3b,c). To further investigate the neuroinflammatory
126 phenotype associated with GBS and *E. coli* sepsis infection in neonates, we measured mRNA of
127 immunological genes from bulk brain tissue on the Nanostring nCounter Gene Expression
128 platform. In the brains of both GBS and *E. coli* infected P7 pups, there was a significant increase
129 in the expression of monocyte and neutrophil chemotactic factors, such as *Ccl2* and *Cxcl1*,
130 respectively (Fig. 3d,e). Compared to uninfected pups, the brains of GBS-infected pups had
131 increased expression of genes involved in the innate immune response to Gram-positive bacteria,

132 such as *Tlr1* and *Tlr2* (Fig. 3d). *E. coli* brains had increased expression of genes associated with
133 TLR4 activation, such as *Cd14*, and the complement pathway, such as *C3* and *Marco* (Fig. 3e).
134 There were twenty-four differentially expressed genes between the brains of GBS and *E. coli*
135 infected mice including increased expression of *casp-3* during GBS infection, and increased *Il23*
136 expression during *E. coli* infection (Fig. 3e). These findings indicate that although both GBS and
137 *E. coli* can cause neuroinflammation during single-organism neonatal sepsis infection, they
138 induce distinct inflammatory phenotypes.

139 *TCR-Specific Activation of γδ T cells Occurs During E. coli, but not GBS, Septicemia and Sepsis-*
140 *Associated Neuroinflammation*

141 γδ T cells are capable of undergoing activation via multiple pathways, including MHC-independent
142 TCR activation by pathogen-derived non-peptide antigens (Constant et al., 1994). Nur77 is
143 transcription factor that is rapidly and specifically expressed during antigen-receptor mediated
144 signaling and activation in T and B cells (Ashouri and Weiss, 2017; Moran et al., 2011). Therefore,
145 to determine if γδTCR signaling was occurring in GBS and *E. coli* sepsis infection in mice, we
146 utilized P7 Nur77-GFP reporter pups. In the spleens of *E. coli* infected P7 pups, there was an
147 increase in the proportion of Nur77+ CD69+ γδ T cells (Fig. 4a), indicating TCR-mediated
148 activation of γδ T cells was occurring during *E. coli* neonatal sepsis infection. Importantly, nearly
149 all of these Nur77+ CD69+ cells in the spleen are CCR6+ (Fig. 4c), suggesting that the IL-17
150 signature during *E. coli* neonatal sepsis is associated with TCR signaling. Interestingly, there was
151 no change in the proportion of Nur77+ CD69+ γδ T cells in the spleens of GBS infected pups (Fig.
152 4a). These data suggest that during GBS infection, CD27+ γδ T cells undergo TCR-independent
153 activation. These data suggest that the CCR6+ γδ T cells responding to *E. coli* and CD27+ γδ T
154 cells responding to GBS neonatal sepsis undergo distinct pathways of activation to elicit their
155 effector cytokine responses.

156 γδ T cells play an important role in CNS homeostasis (Park et al., 2022; Ribeiro et al., 2019a) and
157 have been shown to have both a protective (Gentles et al., 2015) and detrimental (Gelderblom et
158 al., 2014; Welte et al., 2008) effect on the CNS under inflammatory conditions. We therefore
159 hypothesized that distinct γδ T cell responses in the periphery during GBS and *E. coli* sepsis
160 would impart a differential role for γδ T cells during sepsis-associated neuroinflammation. γδ T
161 cells within the CNS are highly skewed toward IL-17 production and CCR6 expression (Ribeiro et
162 al., 2019b; Wo et al., 2020). Similar to the spleen, we observed an increase in the proportion of
163 Nur77+ CD69+ γδ T cells in the brains of *E. coli*, but not GBS infected mice (Fig. 4b), with the
164 majority of Nur77+ CD69+ γδ T cells expressing CCR6 (Fig. 4c), suggesting that in the brain,
165 there is γδTCR specific signaling of IL-17 producing γδ T cells.

166 *γδ T cell Responses During E. coli and GBS Septicemia Differentially Impact Sepsis-Associated*
167 *Neuroinflammation*

168 In order to determine the contribution of γδ T cells to GBS and *E. coli* sepsis-associated
169 neuroinflammation, we compared immunological gene expression between BL/6 and TCRδ-/-
170 infected pups. TCRδ-/- were used in this instance due to the lack of depletion of brain-resident γδ
171 T cells during anti-TCRγδ antibody administration (Fig. S3a, b). Analysis of the brain by
172 Nanostring revealed sixty differentially expressed genes between BL/6 and TCRδ-/- *E. coli*
173 infected pups, and twenty-four differentially expressed genes between BL/6 and TCRδ-/- GBS
174 infected pups. Compared to BL/6 *E. coli* infected pups, TCRδ-/- pups had increased expression
175 of genes associated with apoptosis, such as *Casp-3,* and *Mapk1* (Fig. 4d). These findings
176 suggesting that during *E. coli* neonatal sepsis, γδ T cells may protect the neonatal brain from
177 inflammation-induced cell death. Compared to BL/6 GBS-infected mice, TCRδ-/- GBS infected
178 pups had decreased expression of genes involved in antigen presentation, such as *H2-Dmb2* and
179 genes involved in TGF-β signaling, such as *Tgfbr1* and *Smad5* (Fig. 4e). Genes involved in
180 apoptosis, such as *Casp-3*, were not significantly differentially expressed between TCRδ-/- and

181 BL/6 GBS infected pups, though it was increased in BL/6 GBS infected pups compared to BL/6
182 *E. coli* infected pups (Fig. 4e). Taken together, these data suggest that γδ T cells play a context-
183 specific role during sepsis-associated neuroinflammation.

184 **Discussion**

185 The present study reveals that γδ T cells undergo rapid activation and cytokine production during
186 a murine model of single-organism *Streptococcus agalactiae* (GBS) and *Escherichia coli* neonatal
187 sepsis infection. We also report that GBS and *E. coli* septicemia induce specific activation of IFN-
188 γ and IL-17 producing γδ T cells, respectively (Fig. 2). Although the γδ T cell compartment had
189 the highest proportion of cells undergoing activation in response to both *E. coli* and GBS sepsis
190 infections, a modest increase in activation status was observed for CD4+ and CD8+ T cells (Fig.
191 S1). This small proportion of activated CD4 and CD8 T cells may represent "virtual memory" (T_{VM})
192 T cells, which are abundant in neonates (Akue et al., 2012; Schüler et al., 2004). Increased
193 activation status of CD4+ and CD8+ T cells at the early timepoint of 18 hours post-infection may
194 further implicate T_{VM} cells, as they are capable of responding to infection faster naïve T cells due
195 to their memory-like capacity (Haluszczak et al., 2009; Lee et al., 2013).

196 In the context of *E. coli* infection, the presence of γδ T cells was found to be detrimental to the
197 survival of neonatal mice, whereas the presence of γδ T cells had no effect on survival of neonatal
198 pups during GBS infection (Fig. 1b, c). Furthermore, improved survival of pups in the absence of
199 γδ T cells during *E. coli* sepsis infection may be directly due to IL-17, the neutralization of which
200 has been shown to improve sepsis survival outcomes in other models of neonatal sepsis (Wynn
201 et al., 2016). This contrasts with other infections, such as neonatal murine influenza, in which
202 neonatal mice lacking γδ T cells rapidly succumbed to infection (Guo et al., 2018). The apparent
203 contradictory role of γδ T cells as pathogenic during sepsis vs. protective during other neonatal

204 infections could be due to the state of immune dysregulation and hyperinflammation that occurs
205 during sepsis.

206 Despite greater bacterial burden in the GBS sepsis infection, γδ T cells were more activated in
207 the context of *E. coli* sepsis infection (Fig. 1a,d), suggesting that specific bacterial factors, not just
208 bacterial burden, are more important in dictating the magnitude of a the neonatal γδ T cell
209 response. An additional outstanding question raised from this study is the extent to which γδ
210 cytokine responses depend directly on bacterial factors vs. the upstream immune response to
211 those bacterial factors, and whether γδ T cells are more likely to produce a certain cytokine in
212 response to specific virulence factors expressed by the bacteria. The clinical isolate of *E. coli*
213 used herein is an extraintestinal pathogenic *E. coli* (ExPEC) that expresses a number of virulence
214 genes, including the capsular polysaccharide K1 (Carl et al., 2014; Knoop et al., 2020). The K1
215 antigen has been implicated in various neonatal infections, including meningitis and sepsis
216 (Kaczmarek et al., 2014) and plays a critical role in the ability of *E. coli* to resist phagocytosis and
217 invade the central nervous system (Hoffman et al., 1999). Similarly, GBS COH-1 (ATCC) is a
218 highly virulent, encapsulated serotype III clinical isolate that expresses several virulence genes
219 involved in immune resistance and neuroinvasion. Included amongst these virulence factors is
220 the serine protease *CspA*, which has been implicated in the ability of GBS COH-1 to evade
221 opsonophagocytosis (Harris et al., 2003). GBS is also highly neuroinvasive and represents a
222 major causative pathogen of neonatal meningitis (Tavares et al., 2022). GBS COH-1 expresses
223 invasion associated gene (*IagA*), which has been implicated in the ability of GBS to infect human
224 brain microvascular endothelial cells (Doran et al., 2005). Furthermore, the extent to which
225 individual virulence factors expressed by *E. coli* and GBS induce specific γδ T cell activation
226 requires further investigation.

227 We also report that during *E. coli* and GBS sepsis infection, γδ T cells undergo distinct pathways
228 of activation in order to elicit their effector cytokine responses. While TCR-mediated activation

229 was associated with the production of IL-17 by γδ T cells during *E. coli* infection, we observed a
230 lack of Nur77 induction that during GBS infection, suggesting that γδ T cells likely use a TCR-
231 independent mechanism to undergo activation and produce IFN-γ (Fig. 4a). Similar to NK cells,
232 γδ T cells can produce IFN-γ in response to cytokines, such as IL-12, and IL-18 (Silva-Santos et
233 al., 2019), and in response to NKG2D ligands (Sedlak et al., 2014). Furthermore, the exact
234 mechanism by which CD27+ γδ T cells become activated and make IFN-γ during GBS infection
235 will be the subject of future studies.

236 In addition to the danger that sepsis poses to infants in the short-term, there are also several long-
237 term consequences that are common in survivors of neonatal sepsis. Poor neurological
238 outcomes, such as impaired neurologic growth, and the development of cerebral palsy, have been
239 associated with early life inflammatory events, including sepsis (Stoll et al., 2004). Although
240 mechanisms by which early life inflammation contributes to this phenomenon remain under
241 investigation, it is thought that inflammatory factors disrupt neuronal connectivity in the developing
242 brain (Cardoso et al., 2015). While both GBS and *E. coli* can cause severe neuroinflammation in
243 neonates (Kim, 2006; Tavares et al., 2022) how the neonatal adaptive immune response impacts
244 sepsis-associated neuroinflammation remains poorly understood. The present study therefore
245 sought to characterize the unique neuroinflammatory signatures associated with GBS and *E. coli*
246 systemic infection, and the extent to which these signatures are dependent upon γδ T cells. Both
247 GBS and *E. coli* were found in the brains of infected P7 pups, with slightly higher CFUs of GBS
248 compared to *E. coli*, similar to the periphery (Fig. 4a compared to 1d). *E. coli* infection also induced
249 greater infiltration of monocytes and neutrophils in the brain (Fig. 3b,c) despite both GBS and *E.*
250 *coli* showing increased gene expression of *Cxcl1* and *Cxcl10* (Fig. 3d,e). Therefore, this
251 recruitment may instead be due to the expression of bacterial factors, specific to *E. coli*, that act
252 as a direct chemotactic signal for these cells. Increased *casp-3* expression was observed in the
253 brains of *E. coli* infected TCRδ-/- pups compared to WT infected pups, suggesting that the γδ T

254 cell response in the brain during *E. coli* infection helps to prevent inflammation-induced cell death
255 in the developing brain (Fig. 4d). Interestingly, no significant increase in *casp-3* expression was
256 observed in GBS infected TCRδ-/- pups compared to WT infected pups (Fig. 3f). These data may
257 suggest that the specific γδ T cell responses during *E. coli* infection, possibly by IL-17 production,
258 promote cell survival in the developing brain. IL-17 has been shown to have an antiapoptotic effect
259 on tumor cells (Nam et al., 2008), and to help promote the survival of B cells and their
260 differentiation into plasma cells (Xu and Cao, 2010), but its specific role in this context remains
261 unknown. Furthermore, whether the induction of *Casp-3* and cell death in the brain is protective
262 or pathogenic requires further study.

263 During neonatal GBS infection, there was decreased expression of the *H2-Dmb2* gene in the
264 absence of γδ T cells. H2-Dmb2 is involved in the removal of CLIP from MHC class II molecules
265 and is critical for proper antigen presentation (Doebele et al., 2000; Santambrogio et al., 2019).
266 Although it is well-known that IFN-γ induces MHC II presentation (Kambayashi and Laufer, 2014;
267 Wijdeven et al., 2018), whether the decreased expression of molecules associated with MHC II
268 expression is dependent upon γδ T cell-derived IFN-γ during GBS infection also requires further
269 study. Similarly, *Il6ra*, *Tgfbr1*, and *Smad3* were also increased in the BL/6 compared to TCRδ-/-
270 GBS infected pups, suggesting that during GBS infection, γδ T cells may impact TGF-β signaling.

271 Overall, these findings present evidence of context-specific γδ T cell responses during neonatal
272 sepsis, with these responses differentially impacting survival and neuroinflammation. Taken
273 together, we report that γδ T cell responses during neonatal sepsis rely heavily on the initiating
274 pathogen. This work will help elucidate the contributions of neonatal γδ T cells to sepsis induced
275 mortality and neuroinflammation.

276

277 **Methods**

278 *Bacteria*

279 Clinical *E. coli* isolates were prepared as described previously (Knoop et al., 2020). GBS COH-1
280 was obtained from ATCC. Single bacterial colonies of GBS and *E. coli* were taken from a streak
281 plate and placed in a 15 mL conical (Fisher Healthcare) containing 5 mL of LB broth (Fisher
282 Healthcare) and placed into a 37°C incubator overnight. The following day, 10 mL of LB broth was
283 measured into a 50 mL conical tube (Fisher Healthcare) and a sterile dropper was used to place
284 2-3 drops of overnight bacterial stock into fresh LB broth. Bacteria was shaken at 150 rpm at 37°C
285 until an OD of 0.3 was reached. The bacterial culture was spun down at 12000 rpm for 10 minutes
286 (*E. coli*) or 30 minutes (GBS) and the LB supernatant was discarded. The bacterial pellet was
287 resuspended in 10mL of sterile PBS (Life Technologies) and a dose of $2x10^4$ CFU (*E. coli*) or 10^6
288 CFU (GBS) was intraperitoneally injected into the neonatal pups with an insulin syringe (Cardinal
289 Healthcare).

290 *Mice*

291 C57BL/6, TCRδ-/- and Nur77-GFP mice were purchased from Jackson Laboratory. All animals
292 were bred in accordance with the Institutional Animal Care and Use Committee at Mayo Clinic.
293 Anti-TCRγδ antibody (UC7-13D5, Biolegend) was given intraperitoneally for *in vivo* depletion of
294 γδ T cells (15 µg/g body weight).

295 *Infection Model*

296 Neonatal mice were infected on postnatal day 7 (P7). Spleens were harvested 18 hours post-
297 infection and blood was collected and allowed to clot for 45 minutes at room temperature and
298 spun down at 10,000xg for 2 minutes. Serum was collected and was stored at -20°C until use.
299 The liver was digested in 1mL of sterile PBS (Life Technologies) and 0.5 g of zirconium beads
300 (Fisher Healthcare) in a safe-lock snap cap tube (Fisher Healthcare) and placed into a tissue
301 homogenizer for 5 minutes. The liver homogenate was then serially diluted in PBS to achieve a

302 1:10^5 dilution (*E. coli*) and 1:10^6 dilution (GBS). Bacterial homogenate from *E. coli* and GBS
303 infected pups was plated on either MacConkey agar plates (Fisher Healthcare) or Tryptic Soy
304 Agar (DIFCO) respectively and CFUs were counted the following day.

305 *ELISA*

306 The following mouse ELISA kits were used: IL-17 DuoSet (Fisher Healthcare), IFN-γ ELISAmax
307 (Biolegend), ELISAs were performed according to manufacturer's instructions. Serum samples
308 were diluted 1:5 in ELISA diluent buffer (Biolegend).

309 *Flow Cytometry*

310 Spleens were harvested from mice and were placed in 1 mL of RPMI 1640 (VWR International
311 LLC), spleens were then mechanically homogenized using the frosted end of two glass slides
312 (Fisher Healthcare). Spleens were counted using a hemocytometer. Cells were then spun down
313 and resuspended in 500 µL FACS buffer (PBS containing 5% human serum, 0.5% BSA, 0.1%
314 sodium azide) and allowed to block for 20 minutes at 4°C. Surface master mix was made in FACS
315 buffer and staining was performed for 30 minutes in the dark at 4°C. Following this staining,
316 samples were washed twice with FACS buffer and samples were acquired on the CyTek Northern
317 Lights Spectral Flow Cytometer (Cytek Biosciences).

318 *Intracellular cytokine staining*

319 Cells were placed in 250 µL RPMI supplemented with 10% FBS, 2mM Glutamine (Gibco), 2mM
320 Pyruvate (BioWhittaker), 50 µg/mL Pen/Strep (BioWhittaker), and 0.55 mM 2-ME (Gibco). 1X
321 protein transport inhibitor (Fisher Healthcare) and 1:1000 PMA/Ionomycin (Biolegend) were
322 added, and samples were placed in an incubator at 37°C for four hours. Samples were spun down
323 and resuspended in FACS buffer to block for 15 minutes. Samples were then stained for surface
324 markers for 30 minutes at 4°C before 100 µL of fixative (Life Technologies) was added to each

325 sample for 30 minutes at room temperature. Samples were then washed once with FACS buffer
326 and once with 1X perm buffer (Biolegend) and spun down at 1500 rpm for 5 minutes. Samples
327 were then resuspended in 100 µL 1X perm buffer and intracellular antibodies and were placed at
328 4°C overnight. The following day, samples were washed twice with FACS buffer and acquired on
329 the CyTek Northern Lights Spectral Flow Cytometer.

330

331

332 The following antibodies were used for flow cytometry analysis:

Target	Fluorophore	Clone	Vendor	Titration
CD45	PE/Cy7, BV605	30-F11	Biolegend	1:500
CD4	BV421	GK1.5	Biolegend	1:500
CD8	BV510	53-6.7	Biolegend	1:250
CD11b	BV510	M1/70	Biolegend	1:250
TCRgd	BV421, PE	GL3	Biolegend	1:250
Viability	Zombie NIR, Violet	N/A	Biolegend	1:1000
Ly6G	APC	1A8	Biolegend	1:250
CD69	FITC, BV650	H1.2F3	Biolegend	1:250
CD62L	PE/Cy5	MEL-14	Biolegend	1:250
CCR6	BV785, APC	29-2L17	Biolegend	1:250
CD27	BV421	LG.3A10	Biolegend	1:250
Ly6C	PerCP	HK1.4	Biolegend	1:250
IL-17A	APC, PE	TC-11-18H10.1	Biolegend	1:100
IFN-g	PE, FITC	XMG1.2	BD Biosciences	1:100
TCRβ	BV711	H57-597	Biolegend	1:250
CD19	APC/Cy7	6D5	Biolegend	1:250
CD3	APC/Cy7	17A2	Biolegend	1:250

333 *Brain Isolation for Analysis by Flow Cytometry*

334 Mice were anesthetized with 10 ug/g of Ketamine/Xyalizine mixture and transcardially perfused
335 with 10 mL of cold, sterile PBS (Life Technologies). Brains were digested as previously described
336 (Cumba Garcia et al., 2016). In brief, brains were isolated and placed into a 50 mL conical tube
337 (Fisher Healthcare) containing 5 mL of RPMI (VWR International LLC). The RPMI containing the
338 brains of the mice were then transferred to a 7 mL glass Tenbroeck dounce homogenizer (Pyrex)
339 and homogenized until the brain was visibly digested (about 10 plunges). The homogenate was
340 then poured through a 70 µm filter into a new 50 mL conical tube, and 10 more mL of RPMI 1640
341 was added, along with 1 mL of 10X PBS and 9 mL of Percoll (Sigma-Aldrich INC). The 50 mL
342 conical tubes were then placed into a centrifuge and pelleted at 7840xg for 30 minutes at 4°C.
343 Following the spin, the supernatant was fully aspirated off and samples were washed with 50 mL
344 of fresh RPMI 1640 and spun again at 1500 rpm for 10 minutes. Samples were then blocked in
345 FACS buffer for 15 minutes before surface staining was performed at 4°C for 30 minutes.

346 *mRNA Isolation from Brains* Brains were homogenized in 1 mL of sterile PBS (Life Technologies)
347 using a dounce homogenizer (Pyrex). 100 µL of brain homogenate was used for mRNA isolation
348 with the Quiagen RNeasy Mini Kit according to manufacturer's instructions. RNA samples were
349 stored at -80°C until ready for use.

350 *Nanostring*

351 Nanostring nCounter Mouse Immunology Max Kit was used following mRNA isolation from the
352 brain. RNA hybridization was performed according to Nanostring manufacturer's instructions.
353 Samples were incubated for 24 hours at 65°C and then were run on the nCounter Prep Station
354 5s before being placed on the nCounter Digital Analyzer. The ROSALIND platform was used for
355 data analysis.

356 *Statistical Analysis*

357 Student's unpaired t-test, One-way ANOVA, and Kaplan-Meier tests were conducted using
358 GraphPad Prism (GraphPad Software, Inc.,)

359 *Author Contributions:* LTW and KAK conceived the studies and wrote the manuscript. LTW
360 performed animal experiments, ELISAs, flow cytometry, RNA analysis, and data analysis. KGG
361 assisted with animal husbandry and experiments. All authors reviewed the data and manuscript.
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Figure 1: γδ T cells Respond to *E. coli* and GBS Neonatal Sepsis and Differentially Drive Mortality

A) Gating scheme (L) and quantification (R) of CD69+, CD62L- splenic γδ+ T cells in postnatal day 7 (P7) 18 hours post-infection with either $2x10^4$ CFU *E. coli* or 10^6 CFU GBS COH-1 B) Survival curves of BL/6 P7 pups infected with *E. coli* or C) GBS treated with either isotype IgG or 15 µg/g anti-TCRγδ antibody D) Bacterial CFUs from the livers of GBS and *E. coli* infected pups treated with anti-TCRγδ antibody. Data shown is from four independent experiments, n>3 mice per group. Statistical tests used include one-way ANOVA (A, D), and Kaplan-Meier (B, C). with ns p>0.5

Figure 2: *E. coli* and GBS Neonatal Sepsis Drive Distinct Effector Cytokine Responses from γδ T cells

A) Flow cytometry gating scheme of IFN-y+ and IL-17+ splenic γδ+ T cells from uninfected, GBS, and *E. coli* infected BL/6 P7 pups. B) Mean fluorescent intensity (MFI) of IFN-y and C) IL-17 from splenic γδ+ T cells 18 hours post-infection. D) IFN-y and E) IL-17 serum ELISA. F) Proportion of activated CD27+ and G) CCR6+ γδ+ T cells from uninfected, GBS, and *E. coli* infected BL/6 P7 pups. Data shown is from three independent experiments, n>3 mice per group, statistical tests used include one-way ANOVA with ns p>0.5.

Figure 3: Neuroinflammation is a Feature of *E. coli* and GBS Neonatal Sepsis

A) GBS and *E. coli* CFUs from the brains of BL/6 P7 pups B) Absolute number of monocytes (CD45hi, CD11b+, Ly6C+, Ly6G-) and C) Neutrophils (CD45hi, CD11b+, Ly6C-, Ly6G+) in the perfused brains of *E. coli* and GBS infected BL/6 P7 pups 18 hours post-infection. D) Volcano plot of differentially expressed genes between BL/6 uninfected control vs. *E. coli* infected P7 pups and E) GBS infected P7 pups. Data shown is from four independent experiments, n>4 mice per group. Statistical tests used include Student's unpaired t-test (A), one-way ANOVA (B,C) with ns p>0.5.

Figure 4: TCR-Specific Activation of γδ+ T cells Occurs During E. coli, but not GBS, Septicemia and Differentially Drives Neuroinflammation

Proportion of Nur77+ CD69+ γδ+ T cells in the A) Spleen and B) Brain of GBS and *E. coli* infected Nur77-GFP P7 pups. C) CCR6 expression of Nur77+ CD69+ γδ+ T cells in the spleen and brain of *E. coli*-infected pups. D) Volcano plots of differentially expressed genes in the brains of *E. coli* infected, or E) GBS-infected BL/6 vs. TCRδ-/- P7 pups. Data shown is from three independent experiments, n>3 mice per group. Statistics used include one-way ANOVA (A,B) with ns p>0.5.

Fig. 1: γδ T cells Respond to *E. coli* and GBS Neonatal Sepsis and Differentially Contribute to Mortality

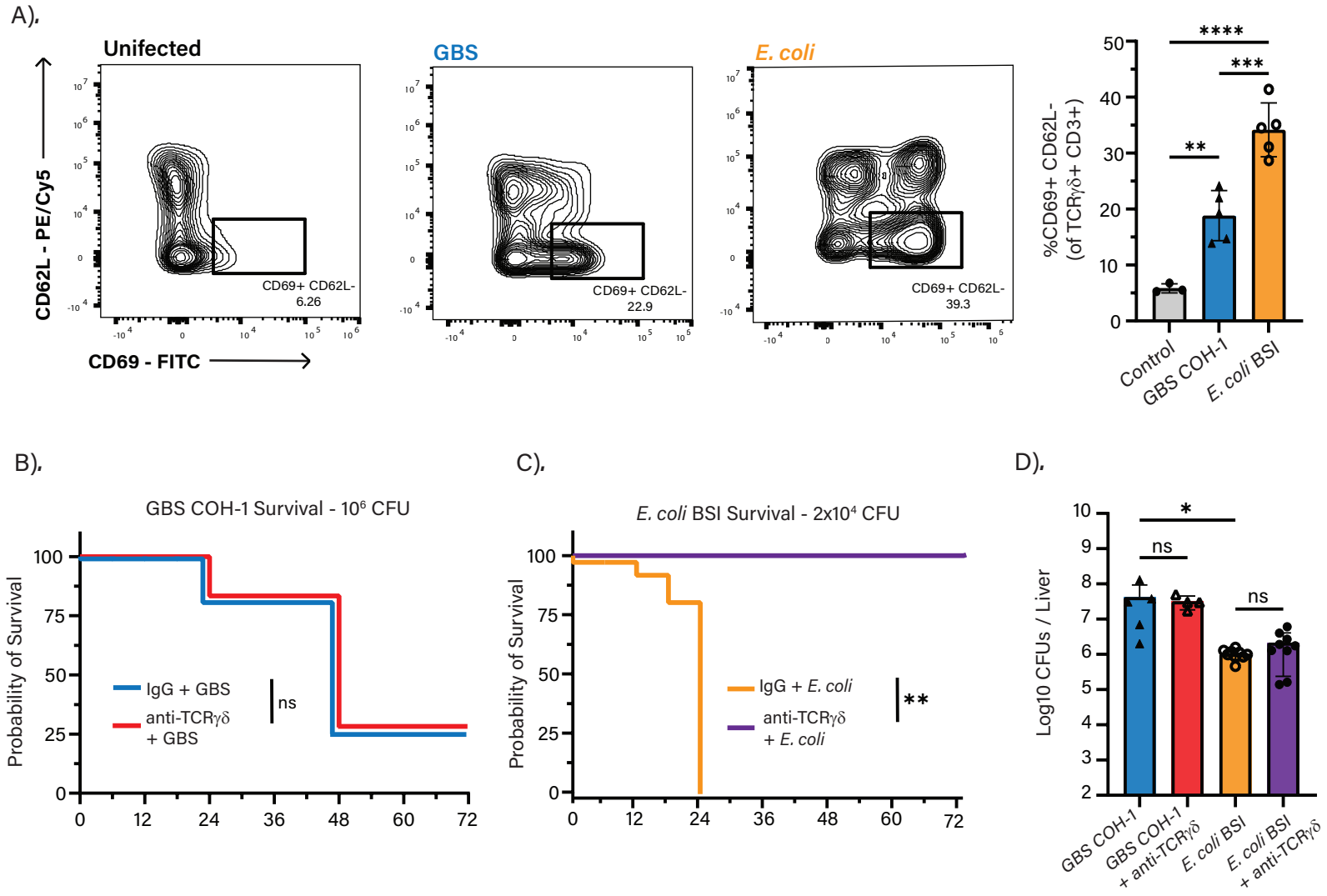


Fig. 2: *E. coli* and GBS Neonatal Sepsis Drive Distinct Effector Cytokine Responses from γδ T cells

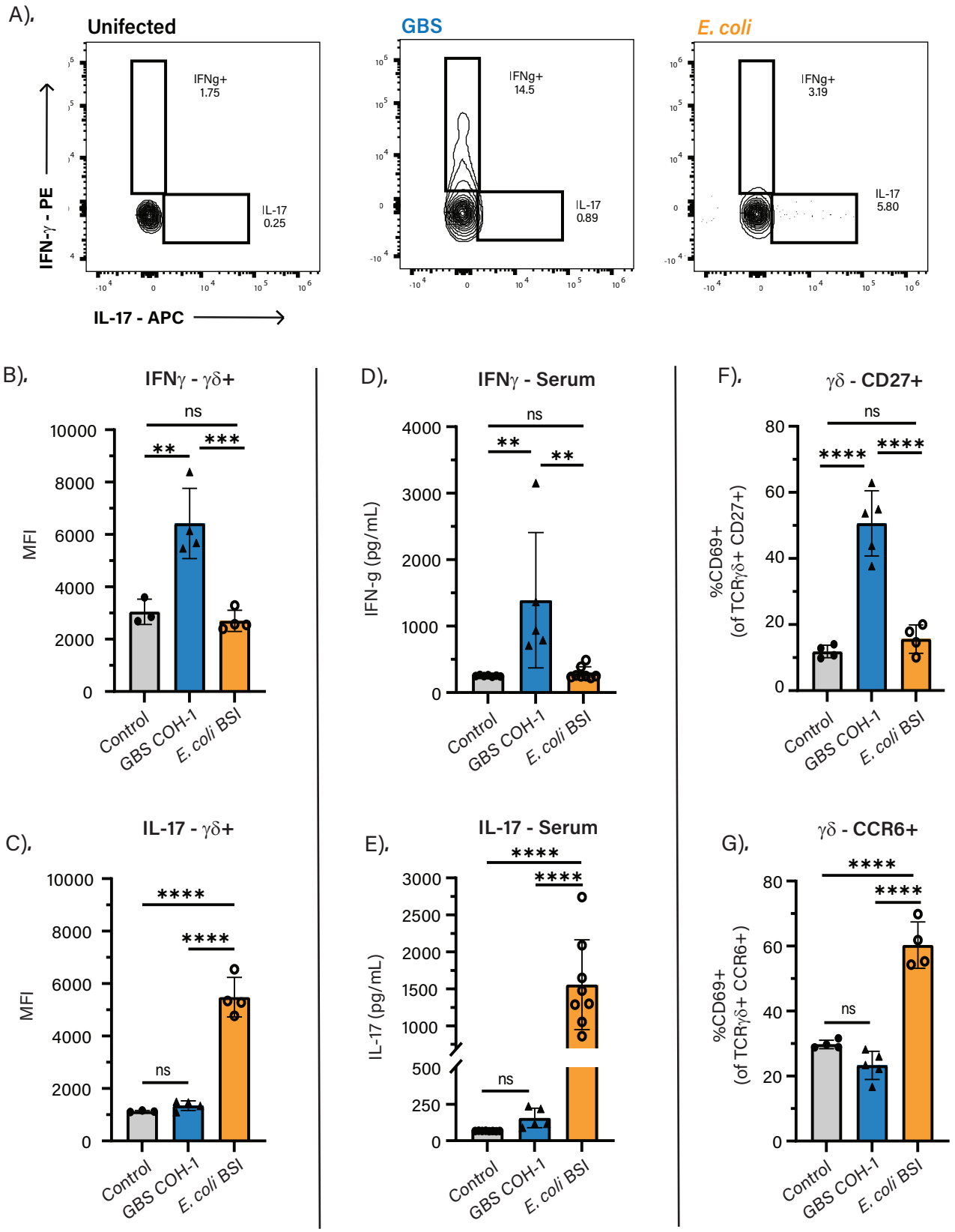
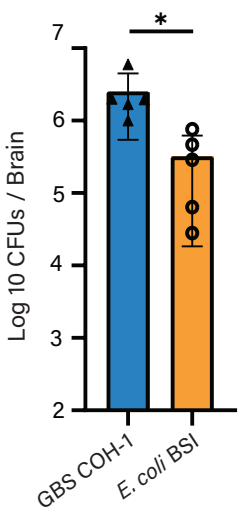


Fig. 3: Neuroinflammation is a Feature of *E. coli* and GBS Neonatal Sepsis

A).



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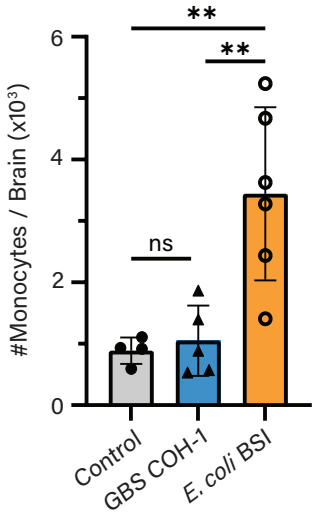

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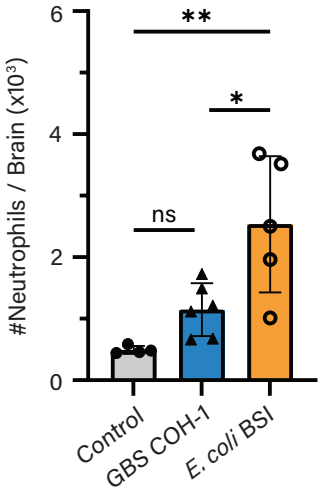
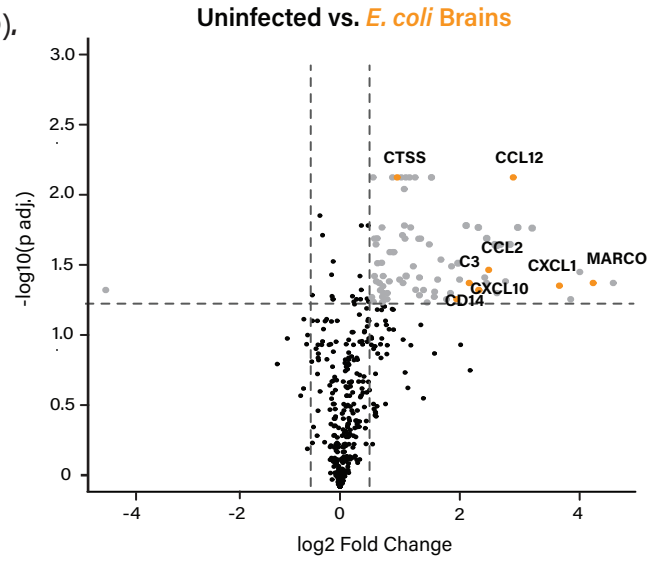
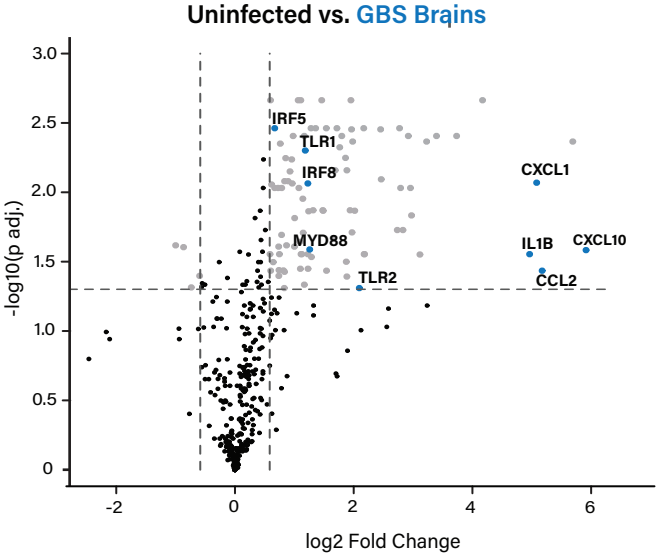


D).



E).



F).

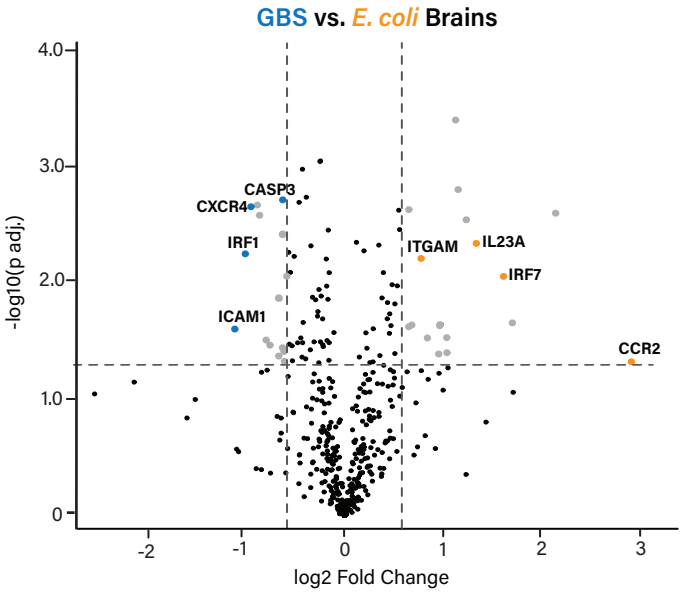


Fig. 4: TCR-Specific Activation of γδ T cells Occurs During *E. coli*, but not GBS, Septicemia and Differentially Drives Neuroinflammation

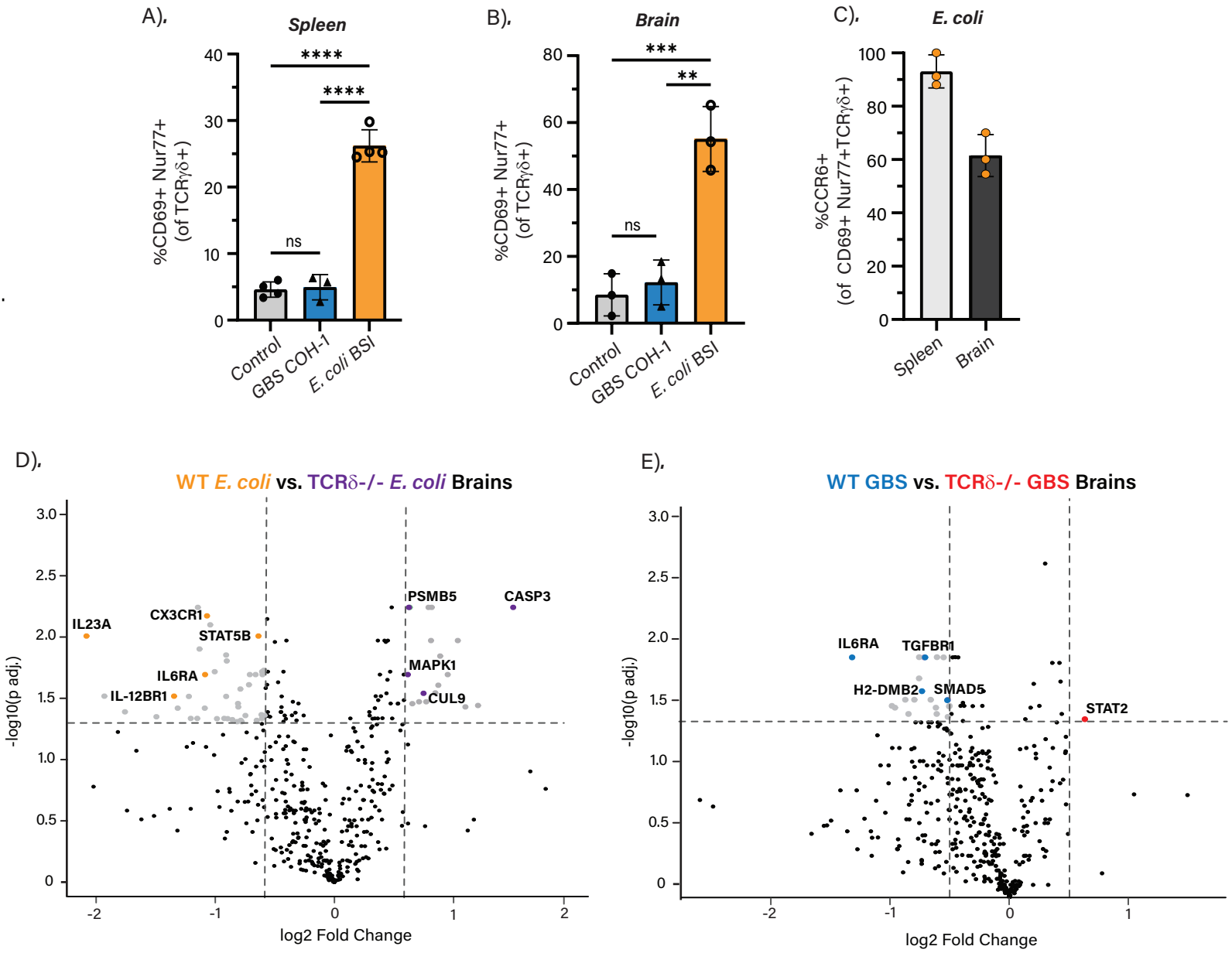


Figure S1: Responses of CD4+ and CD8+ T Cells, and B cells to GBS and *E. coli* Neonatal Sepsis

A) Flow cytometric staining of CD69+, CD62L- splenic CD4, B) CD8 T cells and C) B cells 18 hours-post *E. coli* or GBS infection in P7 BL/6 pups. Data shown is from two independent experiments, n>3 mice per group, statistical tests used include one-way ANOVA, ns p>0.5.

Figure S2: Flow Cytometry Gating Scheme of Neonatal Brain Immune Cell Populations

A) Live, single cells in the brain were gated on CD45, with CD45mid population representing microglia (CD11b+, CX3CR1+). The CD45hi compartment was gated into TCRγδ+ or CD11b+. CD11b+ cells were then gated into Ly6C+, Ly6G- (Monocytes), or Ly6C-, Ly6G+ (Neutrophils).

Figure S3: Lack of Depletion of Brain-Resident T Cells Upon Systemic anti-TCRγδ Antibody Administration

A) Absolute number of γδ+ T cells in the spleen and B) brain of BL/6 P7 pups intraperitoneally injected with 15 µg/g anti-TCRγδ antibody (clone: UC7-13D5). Data shown is from two independent experiments, n>4 mice per group. Statistical tests used include Student's unpaired t-test (A,B), with ns p>0.5.