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TIR-Domain-Containing Adapter-Inducing Interferon- β (TRIF) Regulates CXCR5+ T helper Cells in the Intestine

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Abstract

Objective: Establishing an effective humoral immunity is an important host defense mechanism in intestinal mucosa. T follicular helper (Tfh) cells are a spectrum of CXCR5 expressing T helper cells that induce antigen-specific B cell differentiation. Because the differentiation of T helper cells is largely regulated by innate immunity, we addressed whether TRIF signaling regulates Tfh cell differentiation and its ability to trigger humoral immune responses in the intestine.

Method: $CD4^+CXCR5^+$ T cells, B cells, and plasma cells in the Peyer's patches (PPs) of WT and TRIF-deficient ($Trif^{LPS2}$) mice were analyzed by flow cytometry at the baseline, 9 days post primary infection, and 7 days post-secondary infection with *Y. enterocolitica*. *Y. enterocolitica*-specific $CD4^+CXCR5^+$ T cells were generated *in vitro* by co-culturing peritoneal macrophages with splenic naïve T cells in the presence of *Y. enterocolitica* lysate. WT and $Trif^{LPS2}$ mice received $CD4^+CXCR5^+$ T cells isolated either from *Y. enterocolitica*-primed WT mice or generated *in vitro*. These mice were infected with *Y. enterocolitica* and followed up to 4 weeks. *Y. enterocolitica*-specific IgA and IgG were measured in stool and serum samples, respectively.

Results: At baseline, $CD4^+CXCR5^+$ T cell proportion was higher but the proportion of B cells and plasma cells was lower in the PPs of $Trif^{LPS2}$ mice compared to WT mice. After infection, the proportion of plasma cells also became higher in the PPs of $Trif^{LPS2}$ mice compared to WT mice. Corresponding increase of *Y. enterocolitica*-specific stool IgA but not serum IgG was found in $Trif^{LPS2}$ mice compared to WT mice. Both *in vivo* isolated and *in vitro* generated $CD4^+CXCR5^+$ T cells induced protective immunity against *Y. enterocolitica* infection.

Conclusion: Our results reveal a novel role of TRIF in the regulation of humoral immunity in the intestine that can be utilized as a basis for a unique vaccine strategy.

Keywords: Innate immunity; T follicular helper cells; Intestine; Bacterial infection; Enterocolitis; Plasma cells; B cells; Mucosal vaccine

Introduction

Most pathogens use the mucosa as the primary site of entry, and the gastrointestinal tract has the largest surface area inside our body. Because most of the mucosal surface of the gut is continuously exposed to foreign bodies like microorganisms and chemicals, it utilizes a unique immune defense mechanism: it induces a rapid and strong immune response to defend against pathogenic invasions, yet an immune response to commensal flora is actively suppressed [1-3]. Another unique aspect of gut mucosal immunity is the production of secretory IgA. Secretory IgA is the predominant immunoglobulin in the gut mucosa and it is considered to prevent pathogenic contact with the mucosal surface [4]. Such a precise selection of pathogens, rapid induction, immunoglobulin production, and easy accessibility, are all attractive features to explore in order to generate effective vaccines. Since the first attempt of live polio vaccination through oral-gastrointestinal rout in early 1950's, several mucosal vaccines have been established including Dukoral, Shanchol, and mORCVax for Vibrio Cholerae, Vivotif for Salmonella typhi, FluMist Quadrivalent for Influenza virus, Many for Poliovirus, RotaTeq and Rotarix for Rotavirus [5,6]. However, we have faced difficulties in developing strong immunization through the gastrointestinal mucosa, especially against bacterial pathogens [7,8]. The major obstacle of the gut mucosal vaccination is overcoming oral tolerance or the active suppression of immune response to the luminal contents [9]. In order to safely break the oral tolerance to a particular target, the action of the innate immunity has been a major focus as it works as an antigen-recognition system and triggers an adaptive immune response in both cellular and humoral arms [5]. However, our current ability to adequately utilize the gut mucosa as a platform to develop effective immunizations against a variety of pathogens requires a deeper understanding of the innate regulation of the gut immune defense mechanism, which induces protective rather than harmful immunity.

In the intestine, IgA secreting plasma cells are generated through T cell-dependent and T cell-independent pathways, which may directly or indirectly involve innate Toll-like receptor (TLR) signaling [10-13]. TLRs are pathogen recognition receptors that transduce intracellular

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signaling through either MyD88 (myeloid differentiation factor 88) or TRIF (Toll/interleukin-1 receptor domain-containing adapter inducing IFN-β) to initiate innate immune responses to pathogens and activate antigen-presenting cells. Mucosal B cells can be directly activated by antigens and TLR signaling which results in immunoglobulin class switching and rapid secretion of IgA in the gut mucosa without interacting with T cells [14]. In the T cell-dependent pathway, follicular B cells within PPs are activated through engagement with a specific type of helper T cells, called Tfh cells. Tfh cells are identified as a spectrum of CXCR5 expressing $CD4^+$ T cells that may co-express multiple surface markers including PD-1, ICOS, CD40L, OX40, and FR4, depending on their differentiation stages [15-17]. The expression of these surface markers allows Tfh cells to migrate into germinal centers (GCs), where they interact with B cells [18-20]. These receptor-ligand interactions between Tfh cells and B cells, together with specific cytokine expressions from both cell sides, lead to the terminal differentiation of B cells into plasma cells that predominantly secrete IgA after traveling into the gut mucosa [21,22]. We have shown that this traveling process from PPs to the intestinal mucosa is induced by TLR4 signaling [23]. Because IgAs generated by T cell-dependent pathways have stronger affinity to a specific antigen, promoting this pathway may contribute to the establishment of an improved mucosal vaccine strategy. Although a characteristic definition of gut-specific Tfh cells has not been established, a significant role of commensal bacteria has recently been demonstrated in the regulation of Tfh cells in the gut, which actually affect systemic humoral immunity [24]. More importantly, MyD88 signaling in T cells appear to be indispensable for the generation of gut-specific Tfh cells as it has been shown that the impaired Tfh generation in MyD88-deficient T cells resulted in an intestinal dysbiosis and an increased susceptibility to destructive inflammation [25]. These results indicate that multiple steps of the T cell dependent IgA secretion involve TLR signaling. Since the establishment of an oral vaccine is more complicated than the natural generation of humoral immunity [26], further research is needed to harness such multiple roles of TLR signaling for developing an improved mucosal vaccine strategy.

In this report, we examined the role of TRIF signaling in the regulation of Tfh cell differentiation and their ability to establish humoral immunity in the intestine. Our results indicate that TRIF signaling may involve multiple steps in the induction of humoral immunity in the intestine. TRIF-deficient ($Trif^{LPS2}$) mice have an increased number of $CD4^+CXCR5^+$ T cells in intestinal lymphoid organs compared to WT mice, even though this was not associated with increased IgA secretion at the baseline. However, $Trif^{LPS2}$ mice demonstrated a stronger antigen-specific IgA response than WT mice against primary as well as secondary infection with Gram-negative pathogen, *Y. enterocolitica*. We also show that pathogen specific $CD4^+CXCR5^+$ T cells can be generated *in vitro*, which confer protective immunity against enteric bacterial infection. These results provide a rationale for utilizing $CD4^+CXCR5^+$ T cells as an alternative vaccine strategy against enteric bacterial pathogens.

Materials and Methods

Mice and interventions

WT C57BL/6J and $Trif^{LPS2}$ mice were purchased from Jackson Lab and housed under specific pathogen-free conditions. All protocols were approved by the Cedars-Sinai Medical Center (CSMC) Institutional Animal Care and Use Committee. Eight to twelve weeks old male mice of each genotype were orogastrically inoculated with *Y. enterocolitica* (WA-314 serotype O:8) using a 22G, round-tipped feeding needle (Fine Science tools) [27]. For the primary infection, mice were given either 1 $\times 10^7$ CFU or 5×10^7 CFU of *Y. enterocolitica* and followed up to nine or fifteen days, respectively. In order to study the immunological memory, mice received 1×10^5 CFU of *Y. enterocolitica*, followed by reinfection (5×10^7 CFU) in 4 weeks.

In vitro $CD4^+CXCR5^+$ T cell differentiation

Splenic naïve $CD4^+$ T cells from WT were co-cultured with peritoneal macrophages from WT and $Trif^{LPS2}$ mice (5:1 ratio) in the presence of Yersinia lysate (100 μg/ml) for 3 days. Blocking anti-IFNAR1 antibody (20 μg/ml) or its isotype control was added to some wells to block type I IFNs. The differentiated $CD4^+$ T cells were analyzed by FACS or isolated by sorting in the ARIA III Cell Sorter.

In vitro generated $CD4^+CXCR5^+$ T cells, both PD-1^+ and PD-1^- components, were individually co-cultured with IgD^+ splenic naïve B cells isolated from WT mice by sorting (3.5×10^4 cells each) in the presence of Yersinia lysate (100 μg/ml) for 4 days. Supernatants were collected for immunoglobulin analysis.

Y. enterocolitica-specific $CD4^+CXCR5^+$ T cell transfer

WT and $Trif^{LPS2}$ mice were orogastrically inoculated with *Y. enterocolitica* (1×10^5 CFU). $CD4^+$ T cells were purified from the spleen, MLN, and the PPs 4 weeks post infection and injected into WT or $Trif^{LPS2}$ mice (i.p. 5×10^6 cells/mouse). Mice were orogastrically infected the next day with 5×10^7 CFU of *Y. enterocolitica*.

Cell preparation and purification

Single cell suspension from the spleen, MLN and the PPs were prepared by mechanical disruption with 70 μm nylon mesh. Peritoneal Mϕs were isolated as described previously [28]. Exclusion of floating cells after 48 hours incubation of peritoneal lavage allowed us to collect macrophages (Over 97% of adherent cells expressed F4/80). WT naïve $CD4^+$ T cells from the spleen were purified by magnetic sorting using the naïve $CD4^+$ T cells Isolation Kit (Miltenyi Biotec).

Cell staining and FACS analysis

Surface staining of CD4, CXCR5, PD-1, CD11c, B220, CD138 was performed according to the manufacturer's instructions (Biolegend, San Diego, CA). FACS analyses were performed on an LSRII flow cytometer with FACS Diva (BD) and FlowJo (Tree Star).

Histological analysis and Immunofluorescent staining

Freshly isolated PP and MLN samples were fixed in 10% neutral buffered formalin and embedded in paraffin. These samples were subjected to Hematoxylin and Eosin staining along with immunofluorescent staining. Four μm sections were incubated in 5% skim milk and 1% BSA containing PBS for 1 hour and stained with rabbit anti- mouse CXCR5 polyclonal antibody (1:100) (Bioss) overnight at 4°C followed by Alexa fluor 568 anti-rabbit IgG (1:200) (Invitrogen) for 90 min at room temperature. Sections were then re-incubated with 5% skim milk for one hour followed by staining with anti-mouse CD4 (1:200) (Coulter). Goat Anti-Mouse IgGAM - FITC (1:200) (Zymed) was used as the secondary antibody. Sections were counterstained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) and staining specificity was confirmed by isotype-matched antibodies. Stained slides were examined using ZEISS Observer. Z1 fluorescent microscope and analyzed using Zen software. $CD4^+CXCR5^+$ cells were separately counted inside and in the surroundings of a GC under 20x magnification. Total of 5 PPs from 3 WT and $Trif^{LPS2}$ mice each from baseline, and 7 PPs from 5 WT and $Trif^{LPS2}$ mice each from day 9 post infection group were analyzed.

Real-time PCR

Total RNA was isolated from the PP of WT and Trif^{LPS} mice one week post-secondary infection by using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A total of 1 μg RNA was used as the template for single strand cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed for IL-4, IL-5, IL-6, IL-10, IL-17A, IL-21, BAFF, APRIL, and β-actin (Supplemental Table S1). The cDNA was amplified using Maxima SYBR Green/ROX (Thermo scientific) on a Realplex Mastercycler (Eppendorf). Relative expression levels were calculated with the comparative 2^{-ddCt} method using β-actin as the endogenous control.

Measurement of immunoglobulin levels by enzyme-linked immunosorbent assay

Fecal pellets were disrupted in HBSS/0.02% sodium azide by vortexing for 5 minutes, and insoluble material was removed by centrifugation at 10,000 relative centrifugal force for 10 minutes at room temperature. These samples were diluted (1:200) for IgA analysis. Serum samples were also diluted (1:40) for Yersinia-specific IgG analysis. Culture supernatants from B cell - CD4$^+$CXCR5$^+$ T cell co-culture were diluted (1:2) for the analysis of total IgA and IgG. Total IgA and IgG concentrations were determined by using ninety-six-well ELISA plates that were coated with anti IgA antibody (Southern Biotech) and IgG antibody (Zymed), respectively, according to the manufacturer's instructions. Yersinia-specific IgA and IgG were measured by using SERION ELISA classic plates with anti-mouse IgA and anti-mouse IgG, respectively (Virion/Serion). The signal was developed using the peroxidase substrate (R&D Systems). Plates were analyzed using a 96-well plate absorbance reader. The values were expressed as OD per gram of stool or milliliter of serum.

The avidity of antibodies against Yersinia was determined by ELISA using the chaotropic agent sodium thiocyanate (NaSCN) as described previously [29]. The preparation of stool and serum samples was performed as described above. Plates were washed three times with 0.05% PBS–Tween 20, and the chaotropic agent NaSCN, diluted in PBS, was added to wells along with the samples. Subsequent ELISA steps were performed as detailed above. The avidity index for each mouse sample was recorded as the mean molarity of NaSCN that reduced the optical density at 650 nm (OD_{650}) by 50% from that of wells treated with PBS.

Statistical analysis

Kaplan Meier survival curve was generated for infected mice, and statistical differences were analyzed by Chi square test. Student's t test was used for 2 independent groups of samples. One-way ANOVA was used for more than 2 independent groups of samples, followed by Tukey's multiple comparison tests. All tests were performed with GraphPad Prism (Version 5.0b), and a *P* value of <0.05 was considered statistically significant.

Results

Trif^{LPS2} mice have an increased number of CD4$^+$CXCR5$^+$ T cells in intestinal lymphoid organs compared to WT mice

We have previously demonstrated that Trif^{LPS2} mice were more susceptible to enteric infection with *Y. enterocolitica* compared to WT mice, even though they generated more central memory T cells in the MLN than WT mice upon secondary infection [30]. We questioned whether Trif^{LPS2} mice have a defective humoral immunity. Because TRIF deficiency influences Th cell differentiation, we first analyzed the proportion of CD4$^+$CXCR5$^+$ T cells in the MLNs and the PPs by FCM. In the MLN, there was an increasing trend of CD4$^+$CXCR5$^+$ T cells in Trif^{LPS2} mice compared to WT mice but the difference was not statistically significant (Figure 1A). However, PD-1$^+$ component of CD4$^+$CXCR5$^+$ T cells was lower in Trif^{LPS2} mice than in WT mice (Figure 1A). A similar pattern of CD4$^+$CXCR5$^+$ T cell proportion and PD-1 positivity was found in the PP of Trif^{LPS2} mice in comparison to WT mice (Figure 1B). Those CD4$^+$CXCR5$^+$ T cells were located mainly in the surroundings of the germinal center (GC) of the PP and the MLN (Figure 1C). The distribution of CD4$^+$CXCR5$^+$ T cells was similar between Trif^{LPS2} and WT mice but the number of CD4$^+$CXCR5$^+$ T cells in the PPs was higher in TrifLPS2 mice than WT mice (Figure 1C).

Next, we compared the proportion of B cells and plasma cells in the PPs and the MLNs of WT and Trif^{LPS2} mice. Different from the finding with the CD4$^+$CXCR5$^+$ T cells, the proportion of both B cells and plasma cells in the PPs and the MLN of Trif^{LPS2} mice was lower than the MLNs and the PPs of WT mice (Figures 1D and 1E). This was not associated with the number of GCs in the PPs and the MLNs (Figure 1F). In addition, the differences in the populations of CD4$^+$CXCR5$^+$ T cells, B cells, and plasma cells did not result in an altered secretion of intestinal IgA, as we did not see any difference in stool IgA concentration between WT and Trif^{LPS2} mice (Figure 1G). These results indicate that TRIF deficiency populates the intestinal lymphoid tissues with more CD4$^+$CXCR5$^+$ T cells but that does not lead to B cell activation.

Trif^{LPS2} mice have stronger IgA response to enteric *Y. enterocolitica* infection than WT mice

In order to assess whether TRIF deficiency also leads to accelerated induction of antigen-specific CD4$^+$CXCR5$^+$ T cells in the intestinal lymphoid tissues in response to primary enteric bacterial infections, we analyzed a proportion of CD4$^+$CXCR5$^+$ T cells in the PPs and the MLNs nine days post *Y. enterocolitica* infection. Because Trif^{LPS2} mice are susceptible to enteric *Y. enterocolitica* infection [28], we adjusted the infection dose to 1×10^7 CFU per mouse which allowed us to keep over 40% of Trif^{LPS2} mice alive at day nine post infection (Figure 2A). Surviving Trif^{LPS2} mice showed almost double the number of CD4$^+$CXCR5$^+$ T cells in the PPs compared to WT mice (Figure 1B). PD-1 component of CD4$^+$CXCR5$^+$ T cell population was still lower in Trif^{LPS2} mice than WT mice (Figure 2B). Immunofluorescent analysis of CD4$^+$CXCR5$^+$ T cell distribution in the PPs showed more CD4$^+$CXCR5$^+$ T cells in GCs of Trif^{LPS2} mice compared to WT mice (Figure 2C).

While the B cell number in the PPs of Trif^{LPS2} mice was lower than in WT mice, Trif^{LPS2} mice had more plasma cells in the PPs compared to WT mice (Figure 2D). This increased number of plasma cells in Trif^{LPS2} mice was associated with an increased production of *Y. enterocolitica*-specific IgA measured in stool samples of the infected mice (Figure 2E). Therefore, Trif^{LPS2} mice may have a stronger ability to generate pathogen-specific CD4$^+$CXCR5$^+$ T cells that are sufficient to produce and secrete antigen-specific IgA in the intestine in response to primary *Y. enterocolitica* infection.

The accelerated antigen-specific humoral immunity in Trif^{LPS2} mice in response to primary infection with *Y. enterocolitica* leads to B cell memory

Given the stronger IgA response to primary *Y. enterocolitica* infection in Trif^{LPS2} mice, we tested whether this response leads to immunological memory. WT and Trif^{LPS2} mice were infected with *Y. enterocolitica* (1×10^5 CFU) to prime humoral immunity. 28 days later, these mice received a secondary infection challenge with *Y. enterocolitica* (5×10^7 CFU) [30]. At seven days post-secondary infection, the Trif^{LPS2} mice showed a

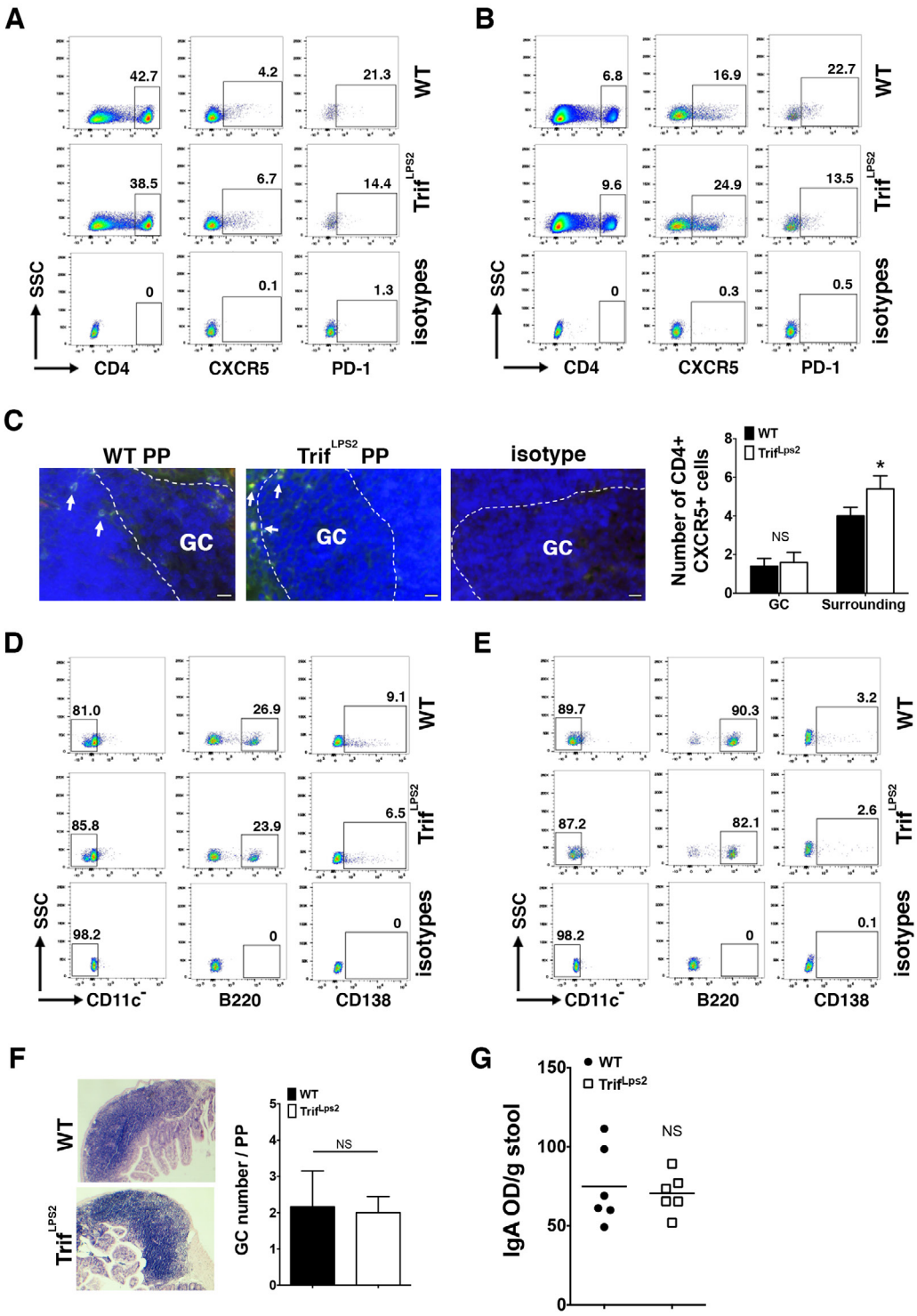


Figure 1: The larger population of CD4$^+$CXCR5$^+$ cells in the MLN and PP of Trif^{LPS2} mice compared to WT mice. **(A)** FCM analysis of CD4$^+$CXCR5$^+$PD-1$^+$ cells in the MLN. Each percentage is given in the gated population on the left side panel. Data from WT and Trif^{LPS2} mice at baseline and isotype control antibodies are shown. Representative picture of 6 independent experiments. **(B)** FCM analysis of CD4$^+$CXCR5$^+$PD-1$^+$ cells in the PP. Data from WT and Trif^{LPS2} mice at baseline and isotype control antibodies are shown. Representative picture of 6 independent experiments. **(C)** Immunofluorescent analysis of CD4$^+$CXCR5$^+$ cells in the PPs from WT and Trif^{LPS2} mice. CD4$^+$CXCR5$^+$ T cells are indicated by the arrows. GC was outlined with a dashed line. Representative picture of 6 mice each. Bars=10 μm. The graph shows the number of CD4$^+$CXCR5$^+$ T cells inside and in the surroundings of GCs (n=5 PPs each; *, P<0.05; NS: Not Significant, data are means ± SEM). **(D)** FCM analysis of CD11c-B220$^+$ and CD138$^+$, B cells and plasma cells respectively, in the MLN. Data from WT and Trif^{LPS2} mice at baseline and isotype control antibodies are shown. Representative picture of 6 independent experiments. **(E)** FCM analysis of CD11c-B220+ and CD138+, B cells and plasma cells respectively, in the PP. Data from WT and Trif^{LPS2} mice at baseline and isotype control antibodies are shown. Representative picture of 6 independent experiments. **(F)** Representative pictures of H&E staining of the PPs from WT and Trif^{LPS2} mice. Bar graph shows number of germinal centers found per PP (n=3 each, counted four PPs per mouse, data are means ± SEM). **(G)** Total IgA ELISA measurements of WT and Trif^{LPS2} stool samples. No differences found (the bar shows means; NS: Not Significant).

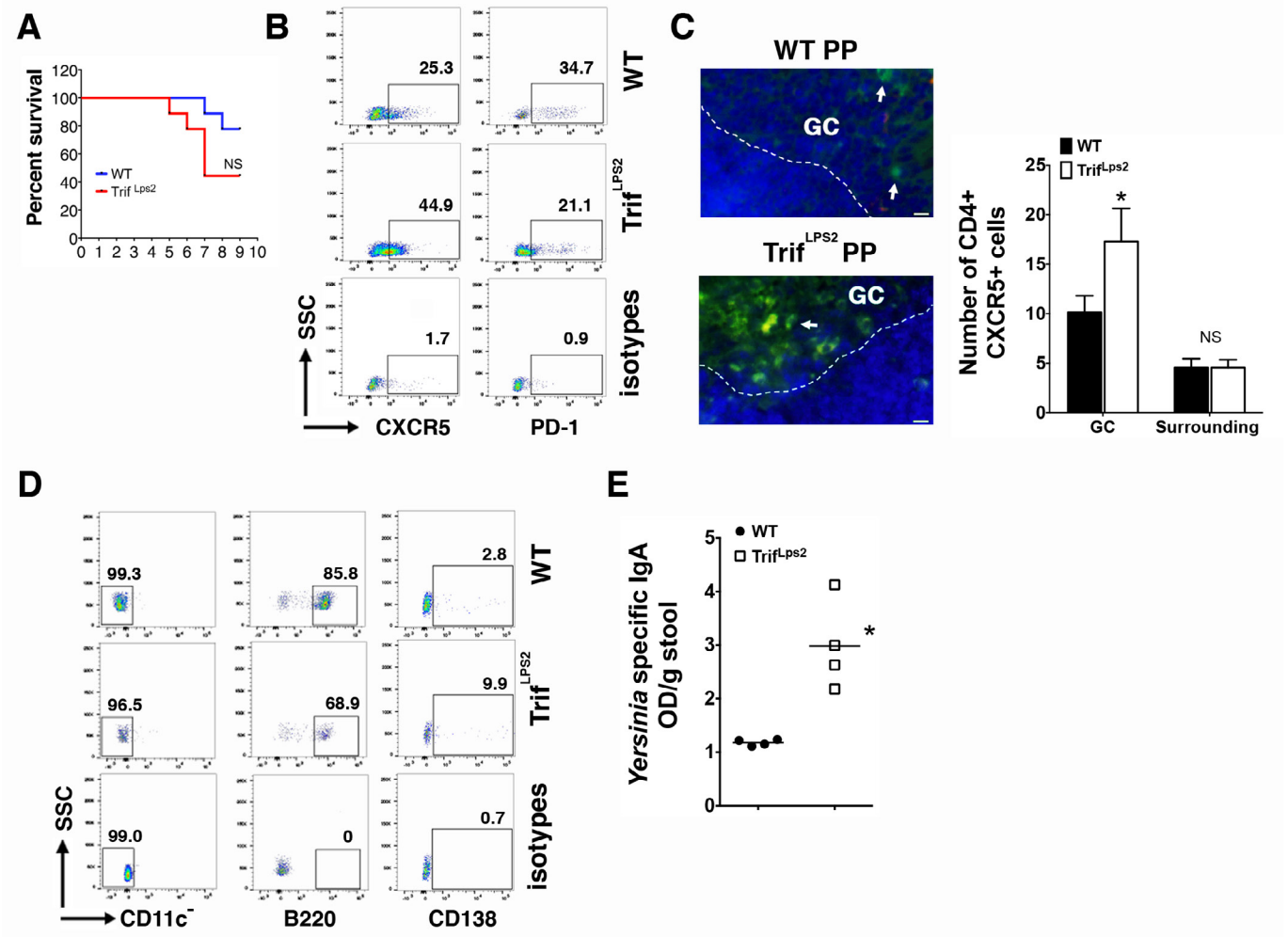


Figure 2: Trif^{LPS2} mice induce higher IgA response than WT mice against primary *Y. enterocolitica* infection. **(A)** Percent survival of WT and Trif^{LPS2} mice during 9 days of infection (1 × 10^7 CFU *Y. enterocolitica*; *n*=10 each). **(B)** FCM analysis of CD4$^+$CXCR5$^+$PD-1$^+$ cells in the PPs. Data from WT and Trif^{LPS2} mice nine days post *Y. enterocolitica* infection (1 × 10^7) and isotype control antibodies are shown. The percentages in CXCR5 gates are given in CD4$^+$ population, the percentages in PD-1 are given in CXCR5$^+$ population. Representative picture of 3 independent experiments. **(C).** Immunofluorescent analysis of CD4$^+$CXCR5$^+$ cells in the PPs from WT and Trif^{LPS2} mice nine days post *Y. enterocolitica* infection. CD4$^+$CXCR5$^+$ T cells are indicated by the arrows. GC was outlined with a dashed line. Representative picture of 6 mice each. Bars=10 μm. The graph shows the number of CD4$^+$CXCR5$^+$ T cells inside and in the surroundings of GCs (*n*=7 PPs each; *, *P*<0.05; NS: Not Significant, data are means ± SEM). **(D)** FCM analysis of CD11c-B220$^+$ and CD138$^+$ in the PP. Data from WT and Trif^{LPS2} mice nine days post *Y. enterocolitica* infection (1 × 10^7) and isotype control antibodies are shown. B220$^+$ percentages are given in the gated CD11c$^-$ population. Representative picture of 3 independent experiments. **(E)** *Y. enterocolitica*-specific IgA ELISA measurements from stool samples of the infected WT and Trif^{LPS2} mice (1 × 10^7) (the bar shows means; *, *P*<0.05).

greater proportion of CD4$^+$CXCR5$^+$ T cells in the PPs compared to WT mice (Figure 3A). Similar to their response to primary infection, Trif^{LPS2} mice showed a reduced B cell population but an increased proportion of plasma cells in the PPs compared to WT mice PPs seven days post-secondary infection (Figure 3A). This increase of plasma cell generation was associated with a higher concentration of *Y. enterocolitica* specific IgA in stools of Trif^{LPS2} mice relative to WT mice stools seven days post-secondary infection (Figure 3B). When we tested the systemic humoral immunity by measuring serum *Y. enterocolitica* specific IgG seven days post-secondary infection, Trif^{LPS2} mice showed a similar titer of IgG relative to WT mice (Figure 3C).

In spite of such a strong induction of antigen-specific humoral immunity in Trif^{LPS2} mice, they had a greater mortality rate than WT mice upon secondary infection with *Y. enterocolitica* (Supplementary Figure 1) [30]. To understand why Trif^{LPS2} mice were still susceptible to enteric *Y. enterocolitica* infection, we tested the avidity of *Y. enterocolitica* specific IgA and IgG taken from stool and serum of WT and Trif^{LPS2} mice seven days post-secondary infection. Although both IgA and IgG taken from Trif^{LPS2} mice tended to show higher avidity than Igs from WT mice, the difference was not statistically significant between them (Figure 3D). To further identify the underlying mechanism of increased humoral immunity in Trif^{LPS2} mice, we measured the mRNA expression of the genes that are involved in the T cell dependent and the independent class switch recombinations of B cells in the PPs seven days post-secondary infection (Figure 3E). Real-time PCR evidenced significantly higher expression of IL-6, IL-17A, and IL-21 in PPs of Trif^{LPS2} mice compared to PPs of WT mice (Figure 3E). These results suggest that Trif^{LPS2} mice have a strong induction of humoral immunity upon primary infection with *Y. enterocolitica,* which is limited to the intestine (IgA) but leads to immunological memory. A higher expression of IL-6, IL-17A, and IL-21 in the PPs of Trif^{LPS2} mice might be involved in this strong induction of humoral immunity in Trif^{LPS2} mice.

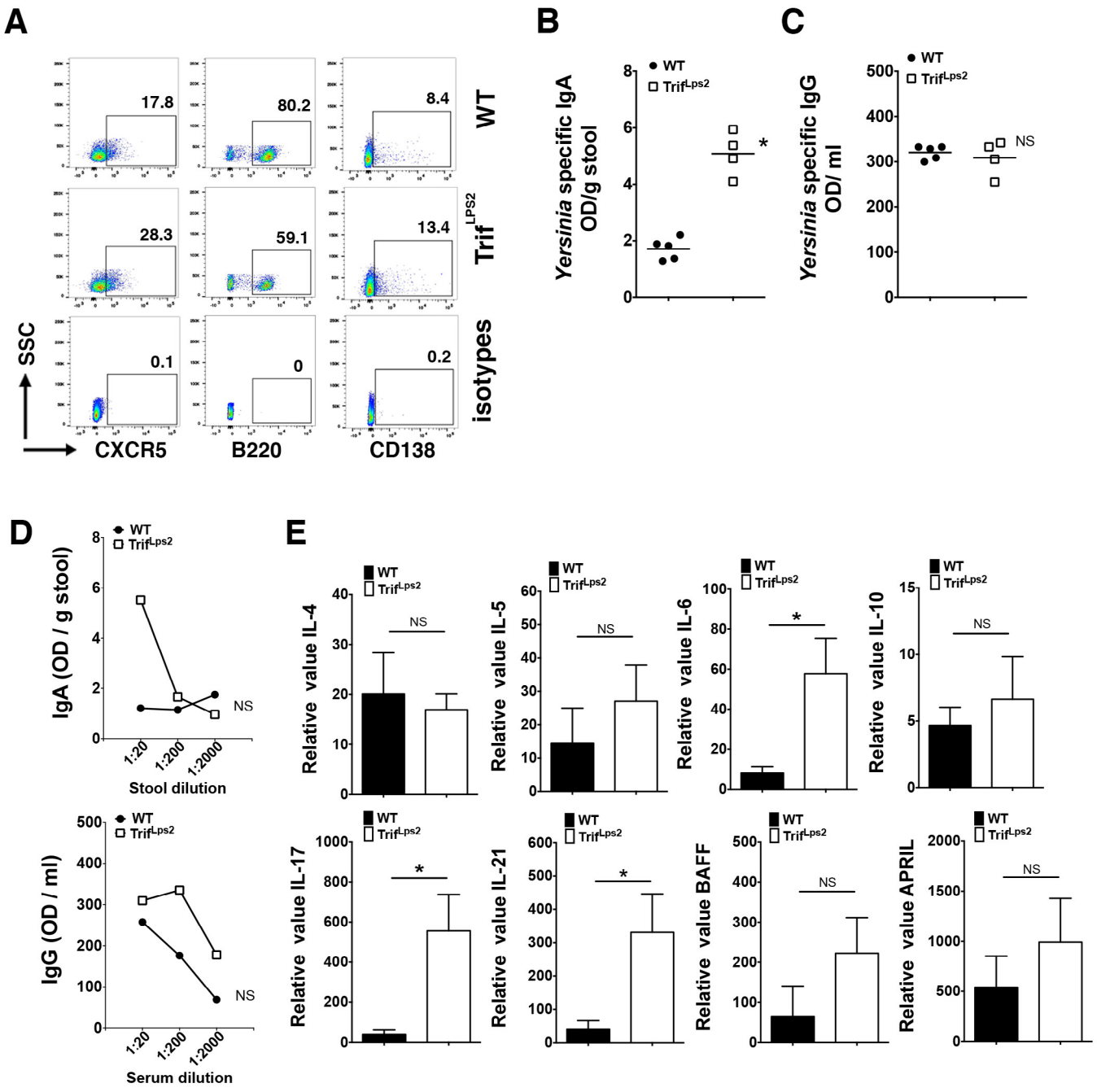


Figure 3: Strong induction of humoral immunological memory in Trif^{LPS2} mice in response to secondary *Y. enterocolitica* infection. **(A)** FCM analysis of CD4$^+$CXCR5$^+$PD-1$^+$ cells, B cells, and plasma cells in the PPs. Data from WT and Trif^{LPS2} mice seven days post-secondary infection (5 × 10^7 CFU) and isotype control antibodies are shown. The percentages in CXCR5 and B220 gates are given in CD4$^+$ and CD11c$^-$ populations, respectively. Representative picture of 3 independent experiments. **(B).** *Y. enterocolitica*-specific IgA ELISA measurements from WT and Trif^{LPS2} mice seven days post-secondary infection (the bar shows means; *, P<0.05). **(C)** *Y. enterocolitica*-specific IgG measurements from WT and Trif^{LPS2} mice seven days post-secondary infection (the bar shows means; NS: Not Significant). **(D)** Avidity of *Y. enterocolitica*-specific IgA and IgG from stool and serum samples of WT and Trif^{LPS2} mice seven days post-secondary infection. (n=4 each, NS: Not Significant) **(E)** Real- time PCR analysis of gene expression in the PP taken from WT and Trif^{LPS2} mice seven days post-secondary infection. (n=7 each; *, P<0.05; NS: Not Significant).

Transfer of Y. enterocolitica-primed CD4$^+$CXCR5$^+$ T cells protected naïve mice from primary infection with *Y. enterocolitica*

To test the ability of CD4$^+$CXCR5$^+$ T cells to induce humoral immunity, we isolated CD4$^+$CXCR5$^+$ T cells from the PPs and the MLNs of WT mice nine days post infection with *Y. enterocolitica* (1 × 10^7 CFU) and transferred them to naïve WT mice (5 × 10^6 cells / mouse). When we compared the mortality rate during primary *Y. enterocolitica* infection (high dose: 5 × 10^7 CFU), more mice that received CD4$^+$CXCR5$^+$ T cells survived compared to the control mice that received PBS (Figure 4A). Those mice that received CD4$^+$CXCR5$^+$

T cells showed higher proportions of $CD4^+CXCR5^+$ T cells and plasma cells in the PPs compared to those of the control mice 15 days post infection (Figure 4B). However, we could not see any difference in the stool concentration of *Y. enterocolitica* specific IgA between the control mice and the mice that received $CD4^+CXCR5^+$ T cells (Figure 4C). Instead of *Y. enterocolitica* specific IgA, the mice that received $CD4^+CXCR5^+$ T cells showed more serum *Y. enterocolitica* specific IgG, compared to the control mice (Figure 4D). Such protection against enteric *Y. enterocolitica* infection that was induced by $CD4^+CXCR5^+$ T cell transfer in WT mice could not be reproduced in $Trif^{LPS2}$ mice (Figure 4E). These results suggest that *Y. enterocolitica*-primed $CD4^+CXCR5^+$ T cells confer systemic protective immunity by IgG induction in WT mice, but this cannot be utilized in the absence of TRIF signaling.

In vitro generated *Y. enterocolitica* specific $CD4^+CXCR5^+$ T cells may induce protective immunity

To test whether antigen-specific $CD4^+CXCR5^+$ T cells generated *in vitro* can induce protective immunity *in vivo*, we incubated naïve T cells with peritoneal macrophages in the presence of *Y. enterocolitica* lysate [30]. With this co-culture, $CD4^+CXCR5^+$ T cells were generated within three days and the efficacy of $CD4^+CXCR5^+$ T cell generation was higher when we used $Trif^{LPS2}$ macrophages (Figure 5A). Similar to the data *in vivo*, PD-1 positivity in $CD4^+CXCR5^+$ T cells generated with $Trif^{LPS2}$ macrophages was lower than in CD4+CXCR5$^+$ T cells generated with WT macrophages (Figure 5A). These *in vitro* generated $CD4^+CXCR5^+$ T cells induced B cell production of IgA and IgG when they were co-cultured with naïve B cells, indicating B cell differentiation (Figure 5B).

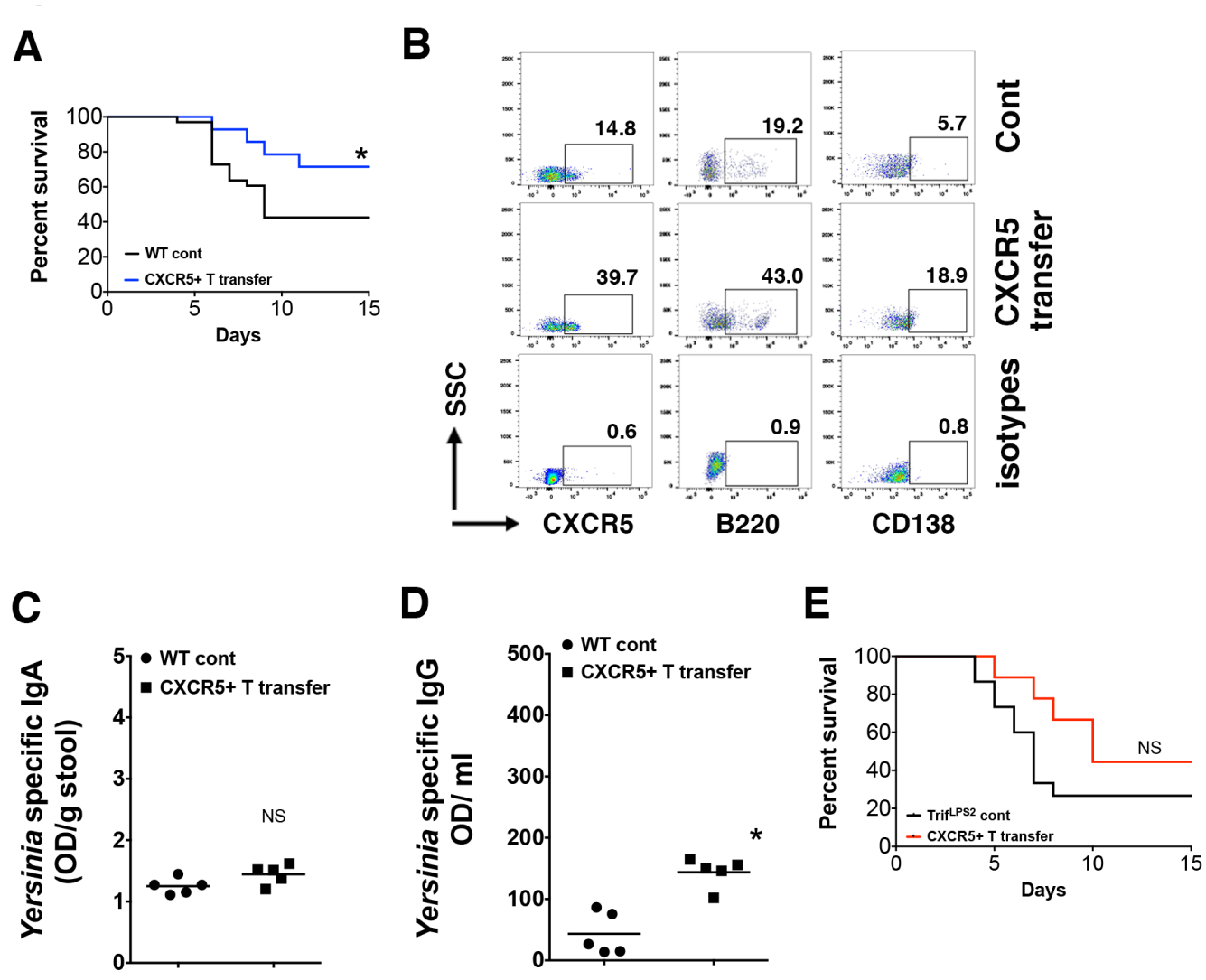
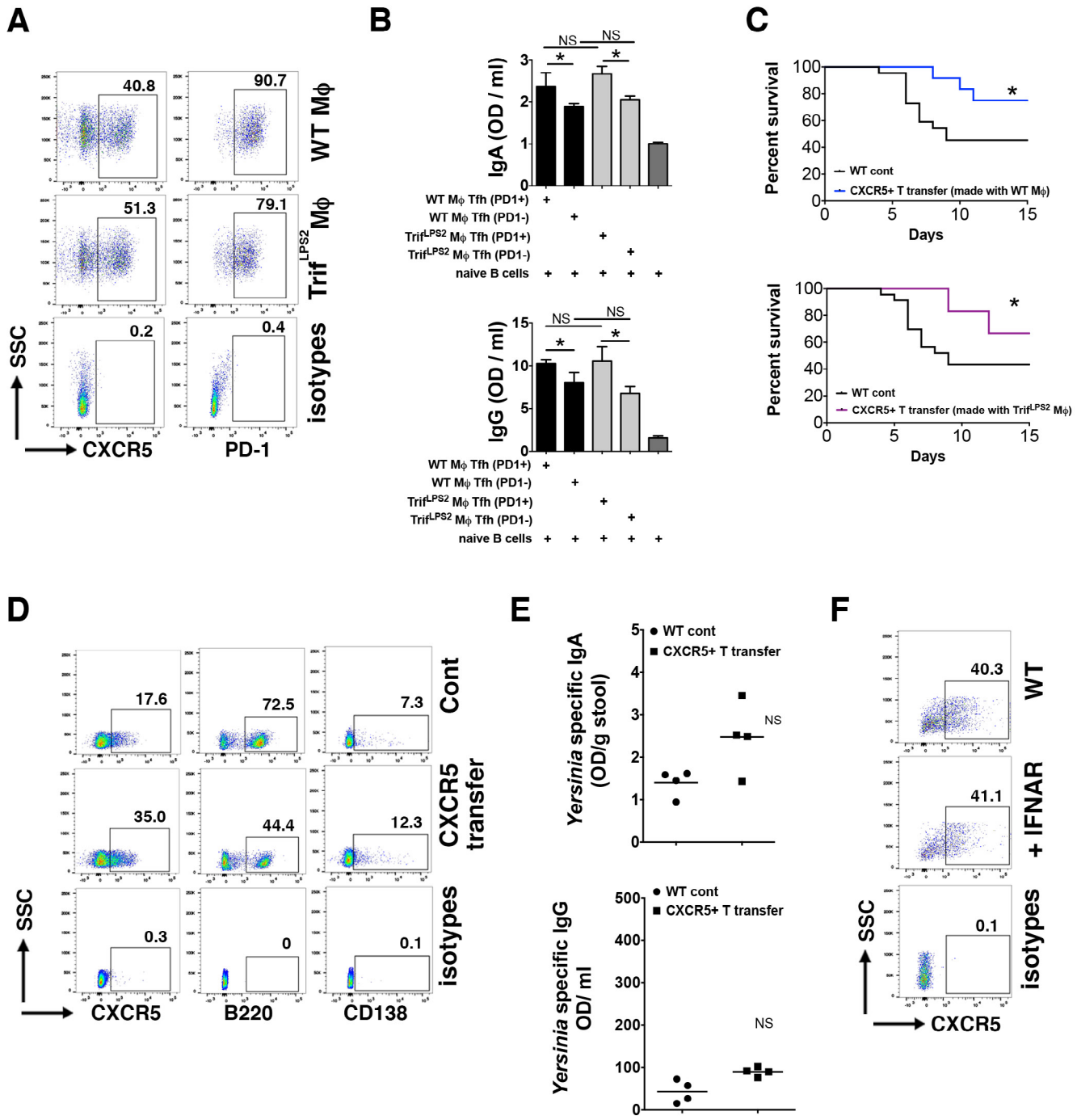


Figure 4: *Y. enterocolitica*-primed $CD4^+CXCR5^+$ T cells protected naïve mice from primary infection with *Y. enterocolitica*. **(A)** Percent survival of WT control and WT mice given $CD4^+CXCR5^+$ T cells (5×10^6 cells/mouse) from pre-infected mice (5×10^7 CFU *Y. enterocolitica*; n=9 each; *, P< 0.05). **(B)** FCM analysis of $CD4^+CXCR5^+$ cells, $CD11c^-B220^+$ B cells, and $CD138^+$ plasma cells. Data from WT control mice and WT mice that received $CD4^+CXCR5^+$ T cells from pre-infected mice are shown here along with the isotype controls. The percentages in CXCR5 and B220 gates are given in CD4+ and CD11c- populations, respectively. Representative picture of 3 independent experiments. **(C).** *Y. enterocolitica*-specific IgA ELISA measurements between WT control mice and WT mice that received $CD4^+CXCR5^+$ T cells from pre-infected mice stool samples (the bar shows means; NS: Not Significant). **(D)** *Y. enterocolitica*-specific IgG ELISA measurements between WT control mice and WT mice that received $CD4^+CXCR5^+$ T cells from pre-infected mice serum samples (the bar shows means; *, P<0.05). **(E)** Percent survival of $Trif^{LPS2}$ control and $Trif^{LPS2}$ mice given $CD4^+CXCR5^+$ T cells (5×10^6 cells/mouse) from pre-infected mice (5×10^7 CFU *Y. enterocolitica*; n=12 each; *, P<0.05).



Figures 5: Protective immunity conferred by *in vitro* generated *Y. enterocolitica*-specific CD4$^+$CXCR5$^+$ T cells. **(A)** CD4$^+$CXCR5$^+$ cells were generated by co-culturing WT naïve T cells with either WT macrophages (Mϕs) or Trif^{LPS2} Mϕs in the presence of *Y. enterocolitica* lysate. FCM analysis of CD4$^+$CXCR5$^+$PD-1$^+$ cells along with isotype controls are shown here. The percentages in CXCR5 gates are given in CD4$^+$ population, the percentages in PD-1 are given in CXCR5$^+$ population. Representative picture of 3 independent experiments. **(B)** B cell differentiation by *in vitro* generated Tfh cells. The concentration of IgA and IgG in supernatants of naïve B and Tfh co-culture in the presence of Yersinia lysate (n=4 each; *, P<0.05; NS: Not Significant, data are means ± SEM). **(C)** Percent survival between WT control vs. WT mice that received CD4$^+$CXCR5$^+$ T cells made with WT Mϕs (top), and WT control vs. WT mice that received CD4$^+$CXCR5$^+$ T cells made with Trif^{LPS2} Mϕs (bottom) (n=9 for control, n=5 for CD4$^+$CXCR5$^+$ cell given mice; *, P<0.05). **(D)** FCM analysis of CD4$^+$CXCR5$^+$ cells, CD11c$^-$B220$^+$ cells, and CD138$^+$ cells. Data from WT control mice and WT mice that received CXCR5$^+$ T cells made with WT Mϕs are shown here along with the isotype controls. The percentages in CXCR5 and B220 gates are given in CD4$^+$ and CD11c$^-$ populations, respectively. Representative picture of 3 independent experiments. **(E)** *Y. enterocolitica*-specific IgA and IgG ELISA measurements between WT control mice and WT mice that received CXCR5$^+$ T cells made with WT Mϕs (the bar shows means; NS: Not Significant). **(F).** CD4$^+$CXCR5$^+$ cells were generated by co-culturing WT naïve T cells with WT Mϕs in the presence of *Y. enterocolitica* lysate with and without anti-IFNAR antibody. FCM analysis of CD4$^+$CXCR5$^+$ cells along with isotype controls is shown. The percentages in CXCR5 gates are given in CD4$^+$ population. Representative picture of 3 independent experiments.

The ability to differentiate B cells was higher in PD-1$^+$ cells than PD-1$^-$ cells but similar between CD4$^+$CXCR5$^+$ T cells generated with WT macrophages and Trif^{LPS2} macrophages (Figure 5B). We then transferred those CD4$^+$CXCR5$^+$ T cells to naïve WT mice and subsequently infected them with *Y. enterocolitica*. The mice that received *Y. enterocolitica* specific CD4$^+$CXCR5$^+$ T cells showed a greater survival rate than the control mice that received PBS (Figure 5C). The protective effect of CD4$^+$CXCR5$^+$ T cells was similar in CD4$^+$CXCR5$^+$ T cells generated with WT macrophages and CD4$^+$CXCR5$^+$ T cells generated with Trif^{LPS2} macrophages (Figure 5C). The proportion of CD4$^+$CXCR5$^+$ T cells and plasma cells in the PPs of mice that received CD4$^+$CXCR5$^+$ T cells was twice as high as that of the control mice, but the proportion of B cells was smaller in the mice that received CD4$^+$CXCR5$^+$ T cells (Figure 5D). We then examined antigen-specific immunoglobulin production. While the mice that received CD4$^+$CXCR5$^+$ T cells tended to have higher concentrations of fecal IgA and serum IgG specific for *Y. enterocolitica*, the differences with IgA and IgG concentration in the control mice did not reach statistical significance (Figure 5E). Because type I IFNs are a group of the major effector molecules induced by TRIF signaling, we examined whether the increased generation of CD4$^+$CXCR5$^+$ T cells in absence of TRIF was associated with type I IFNs. Blocking type I IFN signaling by neutralizing antibody against IFNAR did not change the efficacy of *in vitro* generation of *Y. enterocolitica* specific CD4$^+$CXCR5$^+$ T cells (Figure 5F). These results indicate that pathogen specific CD4$^+$CXCR5$^+$ T cells can be generated *in vitro* and confer protective immunity by activating B cells.

Discussion

The gastrointestinal mucosa together with the MLNs and PPs constitute the largest immune system in our body, which has been co-evolved with and maintained by commensal microorganisms [31]. The process of developing mucosal immunity as well as maintenance of commensal microbial composition largely depends on TLR signaling [3,32-34]. Therefore, manipulation of TLR signaling in mucosal organs may have a variety of effects on both our immune function and the commensal microorganisms. Although this may be particularly beneficial for the improvement of mucosal vaccines, our current understanding in the regulation of mucosal immunity by TLR signaling has yet to open the ways to utilize it in a clinical setting. We have shown that the absence of TRIF in mice results in abnormal induction of memory T helper cell responses to enteric *Y. enterocolitica* infection [30]. Despite greater generation of central memory T cells that express IL-17A in the MLNs, these mice were more susceptible to enteric infection with *Y. enterocolitica* [30]. In this study, we addressed whether TRIF signaling is also involved in gut humoral immunity, and if so, if the mechanism of the regulation can be utilized to promote protective immunity. Trif^{LPS2} mice generated more CD4$^+$CXCR5$^+$ T cells in the PPs leading to a higher production of *Y. enterocolitica*-specific IgA compared to WT mice. These differences in gut humoral immunity became more pronounced when they received a secondary infection challenge, suggesting the acquisition of immunological memory. We also established the generation of *Y. enterocolitica*-specific CD4$^+$CXCR5$^+$ T cells *in vitro* that conferred protective immunity against enteric *Y. enterocolitica* infection. Since current oral vaccines especially against bacterial pathogens need to use whole bacterial organisms, either killed or attenuated, there is a difficulty to achieve a balance in the induction of strong systemic immunity in addition to a local IgA response without inducing adverse effects [5]. Our strategy of transferring pathogen-specific CD4$^+$CXCR5$^+$ T cells may help establish systemic immunization and enhance the efficacy of oral vaccines. Manipulation of TRIF signaling may also enhance effective generation of antigen-specific CD4$^+$CXCR5$^+$ T cells *in vitro*.

CD4$^+$CXCR5$^+$ Tfh cells are necessary to orchestrate humoral immunity in the intestine. It has been shown that CXCR5-deficient mice lack intestinal lymphoid tissues at birth and that they are unable to mount a humoral immune response against oral *Salmonella* infection as well as oral immunization with cholera toxin [35]. Despite such important roles played by CD4$^+$CXCR5$^+$ T cells in humoral immunity in the gut, their capacity to protect the host against enteric infection with bacterial pathogens remains obscure. It remains unanswered why Trif^{LPS2} mice succumbed to *Y. enterocolitica* infection in spite of the fact that they induced a large number of CD4$^+$CXCR5$^+$ T cells in the PPs and a strong IgA response to *Y. enterocolitica*. The role of IgA in the intestinal defense mechanism is mainly involved in preventing pathogenic contact with the mucosal surface, thus subsequently the pathogens undergo peristaltic clearance [4]. This mechanism might not be enough once pathogens get into the mucosa. On the other hand, systemic humoral immunity helps phagocytes to efficiently eliminate pathogens [36]. Because Trif^{LPS2} mice have impaired phagocytosis of Gram-negative bacteria, this might be the reason for their high mortality rate after enteric *Y. enterocolitica* infection [30]. Tfh cell generation is ultimately mediated by transcription factor Bcl6 which is induced by several cytokines including IL-6, IL-21, and type I IFNs [22,37,38]. We have shown that Trif^{LPS2} mice had increased generation of memory Th17 cells upon enteric *Y. enterocolitica* infection [30]. Because Th17 cells can be converted to Tfh in the presence of IL-6 and IL-21, these findings may be the underlying mechanism of the increased generation of CD4$^+$CXCR5$^+$ T cells in the PPs of Trif^{LPS2} mice [39-41].

We have succeeded in the *in vitro* generation of CD4$^+$CXCR5$^+$ T cells that confer protective immunity against enteric infection with *Y. enterocolitica*. As we could see an induction of systemic IgG response after transferring *in vivo* generated CD4$^+$CXCR5$^+$ T cells to mice, even if it did not increase fecal IgA secretion, this strategy may have the potential to induce systemic immunity. As it has been suggested, those transferred CD4$^+$CXCR5$^+$ T cells may not retain Tfh characteristics but can become memory T helper cells [15,42]. Nevertheless, those injected CD4$^+$CXCR5$^+$ T cells increased survival rate of the mice after enteric infection with *Y. enterocolitica*. The extent of the protective effect was similar between *in vitro* and *in vivo* generated CD4$^+$CXCR5$^+$ T cells, which highly encourage its clinical applicability. While MyD88 signaling is necessary to generate and maintain Tfh cells [25], neither their ability to induce B cell differentiation *in vitro* nor their protective effect against enteric *Y. enterocolitica* infection *in vivo* differed between WT and Trif^{LPS2} CD4$^+$CXCR5$^+$ T cells (data not shown). Therefore, unlike MyD88 signaling, T cell intrinsic TRIF signaling is not involved in Tfh function. Rather, TRIF signaling in antigen-presenting cells (APCs) especially in macrophages may have important roles in the regulation of Tfh cell differentiation. Because TRIF-deficient APCs have been shown their impaired antigen-presentation capacity [43], the accelerated generation of Tfh cells by Trif^{LPS2} macrophages may be due to their unique cytokine response to Gram-negative bacteria [30]. Although it will need more optimizations, *in vitro* generated pathogen-specific CD4$^+$CXCR5$^+$ T cells from naïve T cells especially by blocking TRIF signaling can be a potent additive to the current mucosal vaccines to increase their efficacy, induce systemic responses, and reduce adverse effects. Targeting TRIF-mediated regulation of CD4$^+$CXCR5$^+$ T cells may provide us with an opportunity to overcome current obstacles in developing effective mucosal vaccines.

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Disclosure

The authors have no conflicts of interest to disclose.

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