B and T cell crosstalk in anti-bacterial immune responses

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Human *Salmonella*-specific B cells solicit optimal T cell aid by IL-6 dependent induction of IL-21 in plastic CD4⁺ Th cells

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Abstract

B cells mediate humoral immunity against pathogens, but also direct CD4+ responses. In mice, *Salmonella*-infected dendritic cells (DCs) and B cells induce Th1 polarization, which aids the cytotoxic response against *Salmonella*. Here, we demonstrate that human *Salmonella*-infected B cells strongly induced IL-21 in naive and memory Th cells, leading to prominent formation of IFN-γ+/IL-21+ double positive cells that share lineage characteristics of both Th1 (T-bet) and follicular T helper (Tfh) cells. IL-21 strongly promoted antibody secretion and suppressed Th2 formation. Exogenous IL-4 inhibited IL-21 production, demonstrating mutual exclusive actions for IL-4 and IL-21 in *Salmonella*-specific immunity. B cells induced IL-21 via IL-6 in naive CD4+ cells, but IL-6 was not required for reactivation of IFN-γ+/IL-21+ CD4+ memory. Induction of IL-21 was B cell-specific, as *Salmonella*-infected DCs induced classical Th1 polarization. Thus, *Salmonella*-infected B cells direct an optimal pathogen-specific CD4+ T cell response by exploiting Th1/Tfh plasticity. Induction of a T cell subset coexpressing IL-21 and IFN-γ combines IL-21-mediated T cell aid for antibody production, while maintaining Th1 cytokine expression to support the cellular immune defenses against *Salmonella*.
Introduction

The immune response against pathogens consists of a multi-layered network that aims to combine pathogen-specific immunity with minimal tissue damage. Upon infection, immune specificity is orchestrated via regulation of the adaptive humoral and cellular immune responses that direct the innate immune response towards the most efficient elimination of the invaders. Central in the humoral response is activation and differentiation of antigen-specific B cells to generate pathogen-specific antibodies to neutralize the pathogen and/or enhance elimination by complement and Fc-receptor mediated routes. B cell differentiation mostly requires T cell help via CD40-CD154 (CD40L) interactions, whereby class switching to the correct antibody isotype is (in part) regulated by specific cytokines derived from defined effector CD4+ Th subsets. Classically, activated dendritic cells regulate which effector T helper (Th) subset is formed during infection and gives B cell aid. Recently, the picture is emerging that B-T cell interactions are not one-sided events. The clinical effects of B cell depletion with anti-CD20 monoclonal antibodies showed that B cells have immune functions beyond antibody formation. Specifically, B cells seem to regulate memory CD4+ T cell responses, which in turn may affect cellular immune responses including those by phagocytes and cytotoxic T lymphocytes (CTLs). This role of B cells seems most prominent during memory reactivation upon antigen recall.

We have demonstrated that antigen-specific B cells phagocytose pathogenic bacteria via their specific B cell receptor (BCR). This results in activation of B cells and CD4+ T helper cells, leading to specific antibody secretion. It is not known whether uptake of bacterial pathogens by B cells forms a means to direct the type of CD4+ T cell response. Th1 cells secreting IFN-γ and Th2 cell secreting IL-4 are well known to participate in B cell help and antibody secretion. Recently, follicular T helper cells (Tfh) have been put forward as the most important cell type that specializes in B cell help by virtue of its localization near or in the B cell follicles and its pronounced support of various B cell functions. Tfh cells are characterized by IL-21 as a hallmark cytokine and by specific surface markers, like CXCR5. IL-21 plays a major role in B cell help. IL-21 is more potent than IL-10 or IL-4 in inducing B cell proliferation both in mice and human and enhances antibody production of IgM, IgG and IgA. In human, the role of IL-21 in class switching of IgM+ B cells is less clear, but enhanced class switching of human naive B cells to IgG3 and IgA and stimulation of plasma cell differentiation has been described.

Although the transcription factor Bcl-6 is required for Tfh differentiation and has been put forward as the master regulator for Tfh cells, the origin of the Tfh cell and its relation to other Th subsets remain matter of intense ongoing research. Recent findings point to plasticity between Tfh cells and other effector Th cells and the concept that Tfh form a
functional distinct lineage has been challenged. Activation of mouse naive T cells in presence of IL-12, induced a temporarily Th1/Tfh mixed phenotype, expressing both Tfh (Bcl-6) and Th1 (T-bet) characteristics, before becoming full Th1 cells and suppressing the Tfh phenotype. Furthermore, in mice, several Tfh subset with IFN-γ and IL-4 secreting potential may exist in the T cell zones. Alternatively, these cells may be T cells with Th1 or Th2 characteristics that upon interaction with B cells differentiate into Tfh cells that migrate into the germinal center. In human, little is known about plasticity between Tfh and other Th subsets. Human blood CXCR5+CD4+ T cells were reported to contain specific subsets, with alteration of polarization in autoimmunity. Small IFN-γ+/IL-21+ double positive populations have been described in healthy CD4+ memory cells and in inflammatory bowel disease, but their function remained unclear.

Here we have studied B cell–T cell interactions that play a role in human antibody formation against a genuine pathogen: Salmonella typhimurium. During infection with facultative intracellular Salmonella, both B cells and T cells play a critical role in clearance of the pathogen: via antibodies and induction of Th1 and CTL responses. Although usually eliciting Th2 responses, B cells specifically reactivate Th1 cells upon Salmonella infection in mice. We show that Salmonella-infected human B cells induce both IFN-γ and IL-21 in CD4+ T cells, in part via polarization towards IFN-γ+/IL-21+ double positive population. Stable coexpression of T-bet and Bcl-6 showed that these IFN-γ+/IL-21+ cells form a plastic intermediate between the Th1 and Tfh subsets. IL-21 strongly promoted antibody secretion. Salmonella-infected B cells induced little IL-4 and IL-4 antagonized IL-21 expression. Salmonella-infected DCs did not induce IL-21, implying that IL-21 was specifically induced by B cells, as confirmed by identifying the B cell cytokine IL-6 as critical factor. These data show that human B can exploit CD4+ T cell plasticity to generate the cytokine cocktails for optimal support of immune responses against specific pathogens.

Materials and methods

Antibodies
mAb anti-human IgM (MH15, Sanquin, Amsterdam, The Netherlands) was mixed with rat anti-mouse IgG1 antibody (RM161.1, Sanquin) and mAb anti-S. typhimurium LPS (1E6, Bodesign International, Kennebunk, ME) to generate BCR-LPS tetrameric antibody complexes, used to coat bacteria as previously described. The following blocking antibodies were used: anti-IL-21 (Peprotech, Rocky Hill, NJ), anti-IL-4 (eBioscience, San Diego, CA), anti-IFN-γ (U-CyTech Biosciences, Utrecht, The Netherlands), anti-IL-12p35 (B-T21, Gen Probe, San Diego, CA), and anti-IL-6 (Sanquin). The following labeled anti-
human mAbs were obtained from BD Biosciences (San Jose, CA): anti-IFN-γ, anti-IL-4, anti-CD4. Anti-IL-21 and anti-T-bet were obtained from eBioscience, and anti-PD-1, anti-Bcl-6 and anti-CXCR5 were obtained from R&D Systems (Abingdon, UK). DAPI was obtained from Sigma-Aldrich (Steinheim, Germany) and CFSE (Invitrogen, Paisley, UK) labeling was used in proliferation assays.

**Bacterial growth conditions**

*S. typhimurium* SL1344 and GFP-*Salmonella* were grown in Luria-Bertani (LB) broth with carbenicillin (Sigma-Aldrich, St Louis, MO) to maintain GFP expression. Bacteria were cultured overnight at 37°C while shaking, subcultured at a dilution of 1:33 in fresh LB media, and incubated at 37°C while shaking for 3 hours to obtain exponentially growing bacteria. For coating, bacteria were washed twice with PBS and incubated with BCR-LPS tetrameric antibody complexes for 30 minutes at room temperature and washed twice with PBS to remove unbound antibodies.

**Lymphocyte isolation**

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS, Oslo, Norway) from a buffycoat obtained from healthy donors (Sanquin). All donors provided written informed consent in accordance with the protocol of the local institutional review board, the Medical Ethics Committee of Sanquin Blood Supply (Amsterdam, The Netherlands), and the Medical Ethics Committee of Sanquin approved the study. B and T cells were subsequently purified using anti-CD19 and anti-CD4 Dynabeads and DETACHaBEAD (Invitrogen), according to the manufacturer’s instructions. From CD4+ T cells, untouched naive CD4+ T cells (CD4+CD45RO-) were purified via MACS isolation kit using CD45RO-PE and anti-PE beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Untouched memory CD4+ T cells were isolated via MACS isolation using CD45RA-PE and anti-PE beads. Populations were >98% purified. Monocytes were isolated by positive selection using CD14 microbeads and a magnetic cell separator (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were cultured at a concentration of 1x10⁶ cells/ml in 20 ml Cellgro medium (CellGenix, Freiburg, Germany) supplemented with GM-CSF (1,000 IU/ml; Cellgenix) and IL-4 (800 IU/ml) in a 80 cm² cell culture flask (Nunc, Roskilde Denmark) to generate immature DCs. At day 7, the DCs were harvested and washed with antibiotic free medium.

**Salmonella infection**

B lymphocytes or dendritic cells were incubated for 45 minutes at 37°C with *Salmonella* without antibiotics. Next, cells were washed to remove unbound bacteria four times and
cultured for 1 hour in medium containing 100 µg/ml gentamycin (Invitrogen) to eliminate non-phagocytosed bacteria. Cells were washed and cultured in RPMI 1640 medium w/o phenol red (Lonza, Basel, Switzerland), supplemented with 5% FCS (Bodinco, Alkmaar, The Netherlands), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine (Invitrogen), 50 µM 2-ME, 20 µg/ml human apo-transferrin ((Sigma-Aldrich), depleted for human IgG with protein G sepharose (Amersham, Uppsala, Sweden)) and 10 µg/ml gentamycin. 1x10⁵ *Salmonella*-infected cells were cultured with 5x10⁴ CD4⁺ T cells. The following cytokines were added when described: IL-21 (50 ng/ml; Invitrogen), IFN-γ (Immukine; 1000 IU/ml; Boehringer Ingelheim, Ingelheim am Rhein, Germany), IL-4 (50 ng/ml; Janssen Biochemica, Beerse, Belgium), IL-13 (Sanquin). 20,000 events were acquired on a LSR II (BD) and analyzed with FlowJo (v7.6.5 Treestar Inc.).

**Flow cytometry**

Proliferation was measured after 6 days of culture of CFSE labeled B and T cells. DAPI was used to analyze living cells. To study T cell polarization, we used intracellular cytokine stainings. B cells and T cells were cultured for 11 days. Cytokine production was measured by intracellular staining after restimulation with 0.1 µg/ml PMA, 1 µg/ml ionomycin and 10 µg/ml brefeldin A (Sigma-Aldrich) for 5 hours. Cells were washed twice with PBS, fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes and after washing with PBS and PBS containing 1% BSA (Sigma-Aldrich), permeabilized with 0.5% saponin (Calbiochem, CA) in PBS containing 1% BSA and incubated with fluorescent antibodies for 30 minutes at room temperature. 20,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD).

**ELISA assays**

To determine IgM, IgG (subclasses) and IgA levels in culture supernatants, flat bottom MaxiSorb plates (Nunc, Roskilde, Denmark) were coated with polyclonal anti-IgM (SH15, Sanquin), anti-IgG (MH-16, Sanquin), anti-IgG1 (MH161-1, Sanquin), anti-IgG2 (MH162-1, Sanquin), anti-IgG3 (MH163-1, Sanquin), anti-IgG4 (MH164-1, Sanquin) or anti-IgA (Dako) in 100 µl PBS, overnight at room temperature. Plates were washed with PBS/0.02% Tween-20 (Mallinckrodt Baker, Deventer, the Netherlands). After washing, samples were incubated for 2 hours in high performance ELISA buffer (HPE, Sanquin). As a standard, pooled human serum was used. Plates were washed and incubated for 1 hour with 1 µg/ml mAb anti-IgM-HRP (MH15-HRP, Sanquin), anti-IgG (MH16-1-HRP, Sanquin), also for IgG subclasses, or anti-IgA (MH14, Sanquin). After washing, peroxidase activity was visualized by incubation with 100 µl 3,5,3′,5′-tetramethylbenzidine (Merck, Darmstadt, Germany), 100 µg/ml in 0.11 M Na-acetate, pH 5.5, containing 0.003% H₂O₂.
The reaction was stopped by addition of an equal volume of 2M \( \text{H}_2\text{SO}_4 \) (Merck) and the absorbance at 450 nm and 540 nm was measured immediately in a Titertek plate reader.

**RNA isolation, cDNA synthesis and real-time semi-quantitative RT-PCR**

RT-PCR has been described before.\(^{74}\) Briefly, RNA was reverse transcribed to cDNA using random hexamers in combination with Superscript II and a RNase H-reverse transcriptase kit. Primers for 18S rRNA, IL12p40 and IL6 were developed to span exon-intron junctions to prevent amplification of genomic DNA (primer sets in supplemental Table 1). Primers were validated on cDNA of total CD4\(^+\) T cells. Product specificity of each primer set was verified by agarose gel electrophoresis and sequence analysis of the amplified PCR product. Gene expression levels were measured in triplicate reactions for each sample in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR green method (Applied Biosystems). All results were normalized to the internal control 18S rRNA, and are expressed relative to the expression levels found in naive CD4\(^+\) T cells stimulated with non-infected B cells.

**Statistical analysis**

Statistical differences were determined by a paired Student’s t test, using GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA).

**Results**

*Salmonella*-infected B cells induce T cell polarization towards IFN-\(\gamma\) and IL-21

The humoral response against *Salmonella* is supported by CD4\(^+\) T cell help. To study the role of T cell help in antibody production, primary human B cells that have internalized *Salmonella* were cultured with autologous CD4\(^+\) T cells. Since a minor population of B cells (around 2-4\%, ref. \(^8\)) is specific for *Salmonella* antigens, bacteria were coated with anti-IgM antibodies, to enhance BCR-mediated uptake by IgM\(^+\) B cells. This increased the number of *Salmonella*-internalizing B cells up to 60\%, without affecting B cell function (ref. \(^8\) and data not shown). *Salmonella*-infected B cells induced T cell proliferation (Figure 1A and ref. \(^8\)) and cytokine secretion (Figure 1B). As observed in mice,\(^ {42}\) B cells induced IFN-\(\gamma\). Costaining with anti-IL21 mAb showed strong IL-21 expression, yielding IL-21\(^+\) single positive (SP) and IFN-\(\gamma\) SP cells, but also a prominent IFN-\(\gamma\)/IL-21\(^+\) double positive (DP) population (Figure 1B-D). As *Salmonella*-infected B cells induced little IL-4 in the T cells (Figure 1B-C), only few IL-4\(^+\)/IL-21\(^+\) DP were induced. IL-17 induction was very low
and IL-10 could not be detected (data not shown). Coculture with human B cells infected with non-coated *Salmonella* showed similar Th polarization, confirming that the coating procedure did not affect T cell polarization (data not shown).

**IL-21-induction is a B cell-specific process upon *Salmonella* infection**

Does induction of a Th21+ phenotype by *Salmonella*-infected APC depend on B cells? Internalization of *Salmonella* by monocyte-derived human DCs also induced CD4+ T cell proliferation (Figure 2A). In contrast to B cells (Figure 2B-C), *Salmonella*-infected DCs did not induce polarization towards IL-21+ (Figure 2D-E). Only IFN-γ polarization was induced. In line with the absence of IL-21, *Salmonella*-infected DCs induced less CXCR5 in the CD4+ T cells than *Salmonella*-infected B cells (Figure 2F). Thus, upon infection with *Salmonella*, B cells specifically induce Tfh-like characteristics in CD4+ T cells.

*Salmonella*-infected B cells induce Tfh-like cells upon naive T cell priming and reactivation of memory CD4+ T cells

The pathways of Tfh differentiation are still incompletely understood. In some models, B cells are proposed to mainly serve as a last step to stabilize the Tfh phenotype that has initially been induced by DCs.31 Therefore, we investigated if the observed induction of IL-21 only resulted from reactivation of memory CD4+ T cells or whether naive cells could also be primed towards IL-21. Similar to mice,42 human *Salmonella*-infected B cells activated both naive and memory CD4+ T cells (Figure 3A). Priming of naive CD4+ T cells induced
The B-T cell network for IL-6 induced IL-21 secretion

**Figure 2.** DCs skew CD4⁺ T cell polarization towards IFN-γ secreting cells. Dendritic cells or B cells were incubated with *Salmonella* and cocultured with autologous T cells. (A) Proliferation of CFSE labeled T cells was measured at day 6. T cell polarization induced by B cells (B and C) or DCs (D and E) was measured at day 11 after 5 hours of restimulation with PMA, ionomycin and BFA. Data shown are from one representative experiment (B and D) of four individual experiments with different donors in which *Salmonella*-infected DCs and *Salmonella*-infected B cells were directly compared (C and E) with mean + SEM. (F) CXCR5 expression of stimulated T cells was measured at day 11. Data are from one representative experiment of four individual experiments with different donors.

**Figure 3.** B cell induce IL-21 in both naive and memory CD4⁺ T cells. B cells were incubated with *Salmonella* and cocultured with either total CD4⁺ T cells, naive CD4⁺ T cells or memory CD4⁺ T cells. (A) Proliferation of T cells at day 6 was measured by CFSE. Data shown are from seven individual experiments with different donors. (B) T cell polarization was measured at day 11, after 5 hours stimulation with PMA, ionomycin and BFA. Data shown are from two individual experiments comparing polarization of total, naive and memory CD4⁺ T cells. (C-D) CXCR5 and PD-1 expression was measured at day 11 of naive (C) or memory (D) CD4⁺ T cells, cultured as described in (B). FACS plots are shown from one representative donor. Bars show number of positive cells (mean + SEM) from two experiments with different donors.
comparable T cell polarization as observed upon CD4+ memory reactivation and strongly skewed towards IFN-γ and IL-21 expression, and to a lesser extent to IL-4 (Figure 3B). We next analyzed if IL-21 expression correlated with expression of Tfh-markers. Salmonella-infected B cells induced expression of both CXCR5 and PD-1 in primed naive T cells, with a prominent population expressing both CXCR5 and PD-1 (Figure 3C). Reactivated memory T cells showed less CXCR5 expression and little PD-1 (Figure 3D). This indicates that, although Salmonella-infected human B cells skew both naive and memory T cells towards IL-21 expression, primed naive T cells shows characteristics of fully differentiated GC-Tfh cells (CXCR5+/PD-1+), whereas reactivated memory T cells show a stronger resemblance to pre-Tfh cells (CXCR5+/PD-1-).43, 44

**B cell-induced IFN-γ+/IL-21+ DP CD4+ T cells show features of both Th1 and Tfh**

Salmonella-infected B cells induced IFN-γ+/IL-21+ DP cells both through priming of naive CD4+ T cells and upon reactivation (Figure 4A-B). The phenotype of this unusual T cell subset was further analyzed in activated naive and memory T cells. Intracellular staining of primed naive T cells at day 11 showed higher expression of the Tfh specific transcription factor Bcl-6 in IFN-γ+/IL-21+ DP cells compared to the IFN-γ SP population (Figure 4A), albeit at lower levels than in IL-21+ SP cells. The IFN-γ+/IL-21+ population also strongly expressed the T-bet, even at higher levels than the IFN-γ SP population. Stimulation of memory T cells by Salmonella-infected B cells induced comparable Bcl-6 expression in IL-21+ SP and IFN-γ+/IL-21+ cells and more than in the IFN-γ SP population (Figure 4B). Similar to primed naive T cells, the IFN-γ+/IL-21+ population observed after antigen recall showed higher T-bet expression than the SP populations. The observed coexpression of Bcl-6 and T-bet shows that the IFN-γ+/IL-21+ population shares characteristics of the Th1 and Tfh transcription programs. This indicates that Salmonella-infected B cells induce a CD4+ T cell population that forms a Th1/Tfh intermediate. In mice a Th1/Tfh intermediate was described recently to be a form of early Th1 differentiation with gradual loss of Tfh characteristics after day 5.45 Our data showed stable Th1/Tfh formation at day 11 after B cell-induced T cell activation. To investigate if the stability of the Th1/Tfh phenotype required the continued presence of B cells, proliferating T cells that were activated by Salmonella-infected B cells were sorted after six days and further cultured either alone or in the presence of autologous Salmonella-infected B cells, infected DCs, or with anti-CD3 and anti-CD28 antibodies. In all conditions tested, the cytokine pattern secreted by the activated CD4+ T cells did not change (Figure 4C) and the IFN-γ+/IL-21+ DP population remained stable (Figure 4D).
Memory T cells are the main source of providing help in antibody secretion

Since *Salmonella*-infected B cells, but not *Salmonella*-infected DCs, specifically induce CD4+ T cells with Tfh characteristics in addition to Th1, we investigated if these cells were able to provide help for B cell proliferation and antibody secretion. *Salmonella*-infection of human primary B cells induced some B cell proliferation, but proliferation was strongly enhanced in the presence of autologous CD4+ T cells (Figure 5A-B). Besides enhancing B cell expansion, the activated T cells enhanced IgM, IgG and IgA secretion by *Salmonella*-infected B cells (Figure 5C). Analysis of IgG subclass distribution showed that secreted...
IgG mainly belonged to the IgG1 subclass (Figure 5D). Thus, the interaction between Salmonella B cells and T cells results in Tfh and Th1-prone T cell polarization, which in turn promotes both B cell expansion and B cell differentiation into antibody-secreting cells.

Cocultures of Salmonella-infected B cells with naive or memory T cells showed that primed naive T cells did support both IgM and IgG production, but that reactivated memory T cell were significantly superior in providing help to B cells for immunoglobulin

Figure 5. Autologous T cells enhance proliferation and immunoglobulin production of Salmonella-infected B cells. CFSE labeled B cells (either or not incubated with Salmonella) were cultured alone or in the presence of CD4+ T cells. Proliferation of B cells was measured at day 6. Data shown are from one representative experiment (A) of nine independent experiments using different donors (B), with mean + SEM. (C-D) Secreted antibodies were measured in the supernatant after 12 days of culture. Data shown are mean + SEM of twelve (IgM and IgG) or five (IgA, IgG1-4) individual experiments with different donors. (E) B cells were incubated with Salmonella and cocultured with either naive, memory or total CD4+ T cells. Immunoglobulin secretion in the supernatant was measured at day 12 of culture. Data shown are from eight individual experiments with different donors. (F) Cells were cultured as described, with or without addition of extra IL-21 (50 ng/ml). Immunoglobulin levels in the supernatant were measured at day 12. Data shown are from seven individual experiments with different donors.
secretion and were responsible for the main antibody promoting effect of total CD4+ T cells (Figure 5E). We tested whether this could be due to differences in the production of IL-21 in the different activated T cells, but could not detect this (Figure 3B). In fact, addition of exogenous IL-21 to cocultures containing either total CD4+ T cells or naive CD4+ T cells did not enhance antibody secretion in the B-Tnaive cocultures to the levels observed in total T cells (Figure 5F). This indicates that additional factors from memory T cells are essential for optimal stimulation of B cells for antibody production.

Different functions of IL-4 and IL-21 in stimulation and differentiation of human B cells
Salmonella infected B cells polarize T cells towards IFN-γ and IL-21, but minor induction of IL-4 (Figure 1). IFN-γ is important for the clearance of intracellular Salmonella by macrophages and cytotoxic T cells, but was also reported to be involved in B cell help. IL-4 was originally considered the classical cytokine for B cell help until the more recent discovery of IL-21 as mediator for B cell help. Which of these cytokines contribute to help for Salmonella-infected B cells? Blocking of IL-21 in Salmonella-containing B-T cocultures attenuated both IgM and IgG secretion, while blocking of IFN-γ or IL-4 did not affect IgM or IgG secretion (Figure 6A and B). This suggests that IL-21 is critical for an optimal T-cell mediated antibody response against Salmonella.

As the individual cytokines in the B-T cocultures may both affect B and T cell function. Therefore, cytokines were added to B cells that had been activated by anti-IgM-coated Salmonella in the absence of T cell help. IFN-γ had no effect on B cell proliferation (Figure 7A) or antibody secretion (Figure S1). IL-4 strongly induced proliferation of Salmonella-infected B cells and enhanced IgM secretion (Figure 7B). The Tfh cytokine IL-21 did not alter B cell proliferation, but secretion of both IgM and IgG were respectively 5-fold and 18-fold enhanced (Figure 7B). The combination of IL-4 and IL-21 did not further enhance
B cell proliferation, but showed an additive effect on IgM and IgG secretion. Thus, IL-4 induces proliferation of B cells, and might thereby enhance antibody levels. IL-21 on the other hand does not induce B cell proliferation, but is superior in the induction of the antibody secretion program in B cells.

In presence of T cells, extra IL-4 again enhanced B cell proliferation (Figure 7C-D). Surprisingly, while IL-4 enhanced B cell proliferation, secretion of both IgM and IgG were strongly reduced (Figure 7E). A similar reduction in immunoglobulin secretion was obtained by addition of IL-13, another Th2 cytokine (Figure S2). The addition of IL-21 to the human B-T cell cocultures reduced B cell proliferation (Figure 7C-D), but it significantly improved IgG secretion by *Salmonella*-infected B cells, while IgM secretion was not further enhanced (Figure 7E). Addition of IFN-γ had no effect on proliferation (Figure 7C-D), but did enhance IgM antibody secretion (Figure S2). Thus, although IL-4 has a direct positive effect on proliferation and antibody secretion of isolated B cells, in T cell mediated activation it strongly negatively affects antibody secretion. In contrast, IL-21 enhances both T cell dependent and independent-antibody secretion without.

Figure 7. IL-21 reduces T cell mediated B cell proliferation, but enhances IgG secretion. (A-B) CFSE labeled B cells with *Salmonella* were cultured in presence of IFN-γ (1000 IU/ml), IL-4 (50 ng/ml) or IL-21 (50 ng/ml). (A) Proliferation was measured at day 6. Data shown are mean ± SEM of at least four individual experiments with different donors. (B) Antibody secretion was measured at day 12. Data shown are mean ± SEM of eight individual experiments with different donors. (C-E) CFSE labeled B cells with *Salmonella* were cocultured with T cells, in the presence of different cytokines. (C-D) Proliferation of the B cells was measured at day 6. Data are shown as one representative experiment (C) of four individual experiments with different donors (D) with mean ± SEM. (E) Antibody secretion was measured at day 12. Data shown are from twelve individual experiments with different donors.
stimulating B cell proliferation.

**IL-21 and IL-4 show reciprocal regulation**

Since IL-21 stimulated antibody secretion, and IL-4 decreased antibody secretion only in the presence of CD4+ T cells, the effects of these cytokines on T cell polarization were further investigated. While addition of IL-4 had no significant effect on T cell proliferation in the B-T cocultures (Figure 8A), extra IL-4 enhanced differentiation towards IL-4+ T cells, and reduced differentiation towards IL-21+ T cells (Figure 8B-C). The negative effect of IL-4 on IL-21 induction in CD4+ T cells may explain why IL-4 is detrimental for T cell-dependent, but not for T cell-independent antibody production. Addition of a combination of IL-4 and IL-21 to the B-T cocultures partially restored IgM and completely restored IgG secretion (Figure 8D), demonstrating that IL-4 suppresses T cell aid to *Salmonella*-infected B cells via inhibition of CD4+ T cell polarization towards IL-21.

![Figure 8. IL-4 attenuates IL-21+ T cell polarization while IL-21 downmodulates IL-4+ T cell polarization. *Salmonella*-infected B cells were cocultured with autologous T cells in the presence of either IL-21 (50 ng/ml) or IL-4 (50 ng/ml). (A) Proliferation of CFSE labeled CD4+ T cells was measured at day 6. Data shown are mean ± SEM from thirteen different experiments with different donors. (B) T cell polarization was measured at day 11 after 5 hours restimulation with PMA, ionomycin and BFA. Data shown are from one representative experiment out of nine individual experiments with different donors, combined in (C) and (E), with mean ± SEM. (D) IgM and IgG levels were measured at day 12. Data shown are mean ± SEM of twelve individual experiments with different donors. (F) Proliferation of CFSE labeled B cells was measured at day 6. Data shown are mean ± SEM from at least four different experiments with different donors.](image-url)
Reciprocally, addition of extra IL-21 to the coculture of *Salmonella* B cells and T cells enhanced proliferation of the T cells (Figure 8A), but reduced the amount of IL-4+ cells (Figure 8B and E). Exogenous IL-21 also downmodulated induction of the amount of IL-21+ SP and IFN-γ/IL-21+ DP cells. Although IL-21+ T cell differentiation was attenuated, the extra IL-21 added provided direct help to B cells to secrete antibodies. Since IL-4 enhances B cell proliferation, the reduction of IL-4+ T cells caused by additional IL-21 might explain the decrease in B cell proliferation caused by exogenous IL-21 (Figure 7D-E). Indeed, addition of IL-4 next to IL-21 restored B cell proliferation (Figure 8F). In conclusion, IL-21 suppresses Th2 differentiation and thereby B cell proliferation. Yet, the strong induction of antibody secretion by IL-21 is still favorable for B cells and antibody responses in the defense against *Salmonella*.

**Tfh induction by B cells is mediated via IL-6**

Since IL-12 was reported to be involved in induction of Tfh differentiation, we investigated if *Salmonella*-infected B cells polarized CD4+ T cells towards IL-21 via IL-
12. *Salmonella*-infected B cells had enhanced levels of IL12p40 mRNA (Figure 9A). IL-12 protein however, could not be detected in the supernatant (data not shown). Moreover, blocking antibodies directed against IL12p35 had no effect on IL-21 induction in both naive and memory T cells (Figure 9B). In other cell systems in mice and human, IL-6, either or not in combination with IL-21, were described to mediate Tfh differentiation. Since enhanced IL6 mRNA levels were also detected in *Salmonella*-infected B cells (Figure 9C), we studied the effects of IL-6 and IL-21 in our system. Blocking IL-6 reduced differentiation towards IL-21+ in naive T cells (Figure 9D), while IFN-γ production was not affected (Figure 9E). Addition of blocking IL-21 antibodies had no obvious effect on Tfh cell differentiation, not by itself or in combination with IL-6 blockage. Memory T cell differentiation towards IL-21 or IFN-γ was not affected by either IL-6 or IL-21 (Figure 9D and E). In conclusion, while reactivation of memory IL-21+ T cells does not depend on IL-6, CD4+ T cell differentiation towards an IL-21+ phenotype upon naive T cell priming does require IL-6.

**Discussion**

The role of B cells in anti-bacterial immune responses is evident, since antibodies are required for good protection. Where for many years Th2 cytokines, like IL-4, were thought to be most important for B cell differentiation, now the Tfh cytokine IL-21 has emerged as new key player. DCs are required for naive CD4+ T cell differentiation into specific subsets and thus seem to determine the type of Th cell aid offered to B cells. Antigen-activated B cells may play a role as APC in naive Th priming and are essential regulators of CD4+ T cell memory. This implies that B-T cell interactions in fact lead to bidirectional signaling, where each cell type controls differentiation of the other. Little is known about reciprocal regulation of human B-T cell responses in antigen-specific systems. We used a physiological model using *Salmonella*-infected human primary B cells to study bidirectional regulation of B-T cell differentiation in a cognate setting and relate this to the efficiency of T cell aid for antibody production.

In *Salmonella* infection in mice B cells elicit Th1 polarization, in contrast to other systems were B cells mainly support Th2. Also in human, *Salmonella*-infected B cells yielded prominent IFN-γ expression in naive and memory CD4+ T cells. IL-21 was also strongly induced, giving rise to IFN-γ+ SP, IL-21 SP and IFN-γ+/IL-21+ DP populations. Control of *Salmonella* infection depends on IFN-γ Th cells aiding macrophages in bacterial clearance and supporting reactivation of *Salmonella*-specific CTLs. IL-21 was also implicated in help for chronic CD8+ T cell responses, indicating that concurrent IFN-γ
and IL-21 expression may optimally support CTL-driven immunity against *Salmonella*. The main function of IL-21 however, probably lies in its importance for the humoral response against *Salmonella*. While IFN-γ did not contribute to IgM and IgG secretion, IL-21 was crucial for antibody production, as observed before in non-cognate human B-T systems. Both IL-21 and IL-4 support B cells. We also observed a beneficial effect of IL-4 on isolated B cells, most significantly via support of B cell expansion. In presence of T cells, IL-4 strongly inhibited antibody production. Previously, IL-4 was demonstrated to be detrimental for IL-21-mediated antibody secretion during B-T interactions, but the underlying mechanism was not elucidated. Here we demonstrate that IL-21 and IL-4 exhibit strong reciprocal negative regulation. *Salmonella*-infected B cells induce superior levels of IL-21 compared to IL-4. As IL-21 most strongly supported antibody secretion, *Salmonella*-infected B cells thus polarize T cells to secrete those cytokines that are most optimal for B cell aid. In our system IL-4 and IL-21 have mutual exclusive functions. In vivo, it may well be that IL-21 and IL-4 are expressed consecutively, with each performing specific functions for B cells at specific times. In helminth infection in mice, cells coexpressing IL-21 and IL-4 were observed. Thus mutual interference of IL-4 and IL-21 expression may be alleviated by yet unidentified signals of specific pathogens.

Specific induction of IL-21 in CD4+ T cells by *Salmonella*-infected B cells, but not by DCs, correlated with enhanced expression of CXCR5 and Bcl-6, suggesting that B cells induced Tfh-like cells. Our data show that upon phagocytosis of *Salmonella*, human B cells differentiate naive Th cells and reactivate memory T cells in vitro to secrete a similar cytokine pallet. Whereas, primed naive T cells (CXCR5+/PD-1+) resembled GC-Tfh cells, reactivated memory T cells, not coexpressing PD-1, were more similar to pre-Tfh cells. Nevertheless, these memory cells are superior in help for antibody production. Whether the latter relates to the phenotypic differences observed remains to be elucidated.

In *Salmonella* infection in vivo, B cells play a role as APC in regulation of CD4+ T cell responses. In contrast to *Salmonella*-infected DCs, B cells confer specific signals to skew Th cell differentiation towards Tfh-like cells. The pathways regulating Tfh differentiation are under debate. ICOS has been postulated to induce Tfh differentiation, but seems not to play a role in our system (data not shown). Cytokine signaling via IL-21, IL-6 or IL-12 were all implicated. In mice, IL-12 induces transient expression of IL-21 during Th1 differentiation. Although this also involves coexpression of IL-21 and IFN-γ, we did not observe a role for IL-12. Instead, *Salmonella*-infected B cells induce IL-21 in naive T cells via IL-6. Nakayamada and coworkers postulate that IL-12 acting through STAT4 leads to transient IL-21 expression, as upregulation of T-bet attenuates IL-21 expression in the late phase of Th1 differentiation. In contrast, IL-6 acting through STAT3 did not induce T-bet, yielding a stable IL-21+ phenotype. Indeed, in our system, IL-21 expression was
sustained throughout the complete differentiation phase of naive Th cells. In contrast to mice,27 also the IFN-γ+/IL-21+ DP CD4+ T cell population is stable, in spite of high T-bet expression. Thus, in human T-bet is not detrimental for IL-21 induction. It is likely that IL-21 induction in our system is indeed mediated by pSTAT3.24, 71-73 This however, requires further investigation.

The IFN-γ+/IL-21+ DP population induced by Salmonella-infected B cells forms a plastic intermediate between Th1 and Tfh, as the Th1 (T-bet+/IFN-γ+) and Tfh differentiation (Bcl-6+/CXCR5+/IL-21+) programs are simultaneously executed. This concurs with the recent indications of plasticity between Tfh and other effector Th subsets.24-26 It also shows that the classical paradigm of pathogen-driven Th polarization to defined subsets with specific effector functions needs to be reevaluated. We have shown that specific APCs, like Salmonella-activated B cells can exploit plasticity between Tfh and Th1 to generate an optimal immune response for clearance of the bacteria. While the Th1 phenotype supports the cellular immune response, simultaneous induction of a Tfh-like phenotype provides optimal B cell aid for effective humoral immunity. Thus, the emerging concept of plastic intermediates between Th subsets adds another level in B cell mediated regulation of integrated and pathogen-optimized immune responses.

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Supplementals

Table S1

<table>
<thead>
<tr>
<th>Primers</th>
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<tr>
<td><strong>IL6</strong></td>
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</tr>
<tr>
<td>F: GTACATCCTCGACGGCATC</td>
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</tr>
<tr>
<td>R: CCAGGCAAGTCTCCTCATTG</td>
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<tr>
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</tr>
<tr>
<td>R: CTGCAGAGAGTGTAGCAGC</td>
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Chapter 3

**Figure S1.** *Salmonella*-infected B cells were cultured in the presence of IFN-γ (1000 IU/ml). The levels of secreted IgM (A) and IgG (B) were measured at day 12. Data shown are mean ± SEM of two experiments with different donors.

**Figure S2.** *Salmonella*-infected B cells were cocultured with autologous T cells in the presence of either IL-13 (50 ng/ml) or IFN-γ (1000 IU/ml). The levels of secreted IgM (A) and IgG (B) were measured at day 12. Data shown are mean ± SEM of ten (IL-13) or four (IFN-γ) experiments with different donors.
The B-T cell network for IL-6 induced IL-21 secretion

References


are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4+ T cell (Tfh) differentiation. *PLoS. One.* 6: e17739.


71. Wei, L., A. Laurence, K. M. Elias, and J. J. O’Shea. 2007. IL-21 is produced by Th17 cells and drives IL-17

