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The significance of Th17 cells and IL-17A signaling in host defense and disease development has been demonstrated in various infection and autoimmune models. Additionally, the generation of Th17 cells is highly influenced by microbes. However, the specific bacterial components capable of shaping Th17 responses have not been well defined. The goals of this study were to understand how a bacterial toxin, cholera toxin (CT), modulates Th17-dominated response in isolated human CD4⁺ T cells, and what are the mechanisms associated with this modulation. CD4⁺ cells isolated from human peripheral blood were treated with CT. The levels of cytokine production and specific Th cell responses were determined by ELISA, Luminex assay, and flow cytometry. Along with the decreased production of other proinflammatory cytokines (IFN-γ, TNF-α, and IL-2), we found that CT could directly enhance the IL-17A production through a cAMP-dependent pathway. This enhancement is specific for IL-17A but not for IL-17F, IL-22, and CCL20. Interestingly, CT could increase IL-17A production only from Th17-committed cells, such as CCR6⁺CD4⁺ T cells and in vitro-differentiated Th17 cells. Furthermore, we also demonstrated that this direct effect occurs at a transcriptional level because CT stimulates the reporter activity in Jurkat and primary CD4⁺ T cells transfected with the IL-17A promoter-reporter construct. This study shows that CT has the capacity to directly shape Th17 responses in the absence of APCs. Our findings highlight the potentials of bacterial toxins in the regulation of human Th17 responses. The Journal of Immunology, 2013, 191: 4095–4102.
Davis). PBMCs were purchased from AllCells. According to the manufacturer’s protocols, total CD4+ T cells were positively selected from PBMCs using magnetic CD4 MicroBeads (Miltenyi Biotec) or negatively selected using a human CD4+ T cell enrichment kit (StemCell Technologies). The purity of CD4+ T cells is 98–99% as confirmed by using the FACS system (BD Biosciences). Human naive CD4+ T cells were negatively selected using the naive CD4+ T cell isolation kit II (Miltenyi Biotec). The purity of isolated naive CD4+ T cells was confirmed as >95% on a FACSAria (BD Biosciences) with allophycocyanin-conjugated anti-CD25 (BioLegend), and PE-conjugated anti-CD4 (BioLegend), FITC-conjugated anti-CD45RA (BD Biosciences), stimulated with subunit B of CT (CT-B), CT, and PGE2 for 1 h under CD3/Primer3 or were previously described (1, 21) and are listed in Supplemental Table II.

**Cell culture and differentiation**

CD4+ T cells were cultured for 2–4 d in serum-free X-VIVO 15 medium (Lonza) along with beads coated with anti-CD3 and anti-CD28 Ab (CD3/CD28 beads; 10 cells/bead; Dynabeads human T-activator; Invitrogen). Th17-polarizing cytokines IL-1β (10 ng/ml), IL-6 (20 ng/ml), IL-10 (10 ng/ml), IL-12 (10 ng/ml), IFN-γ (100 ng/ml) and TNF-α (20 ng/ml), were added to the culture as indicated. Cytokines were purchased from R&D Systems. CT (List Biological Laboratories), forskolin (FK; Calbiochem), dithiothreitol (DTT), PGE2, and sodium ibuprofen (IBMX; Sigma-Aldrich) were added at various concentrations to the culture media as indicated. Naïve CD4+ T cells were activated with CD3/CD28 beads under Th0 (IL-2-) or Th17-polarizing media (IL-1β/IL-23) in the presence or absence of CT. After a 6-d culture, Dynabeads were removed with a magnet and cells were expanded in media supplemented with IL-2 for 3 d.

**Cytokine production assay and intracellular αMP assay**

IL-17A and IL-17F protein were detected by the human IL-17 and IL-17F DuoSet (R&D Systems), and other cytokines, including GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, and TNF-α, were detected by using an Invitrogen human cytokine 10-plex panel on the Bio-Plex 200 system (Bio-Rad) according to manufacturers’ protocols. CD4+ T cells were stimulated with subunit B of CT (CT-B), CT, and PGE2 for 1 h under CD3/CD28 activation. The αMP levels of cell lysate were measured by using the αMP complete ELISA kit (Enzo Life Sciences) according to the manufacturer’s protocol.

**Intracellular staining**

Treated CD4+ T cells were restimulated for 5 h with 12-O-tetradecanoylphorbol-13-acetate (TPA; 50 ng/ml; Cell Signaling Technology) and ionomycin (1 μM; Cell Signaling Technology) in the presence of GolgiPlug (BD Biosciences). Cells were then processed for intracellular staining with BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s protocol. Abs used in this experiment were Alexa Fluor 700–conjugated anti–IFN-γ (BioLegend), FITC-conjugated anti–IL-17A (eBioscience), PE–conjugated anti–IL-17F (eBioscience), and Alexa Fluor 647–conjugated anti–IL-22 (eBioscience) for 30 min at 4°C. Samples were assayed on a FACScan (BD Biosciences), and data analysis was performed using FlowJo software (Tree Star).

**RNA isolation and quantitative RT-PCR**

Total RNA was extracted from cells with TRIzol according to the manufacturer’s instructions. SYBR Green quantitative real-time PCR (Roche) was carried out to quantify the levels of cytokine expression after normalization with the housekeeping gene, GAPDH. The primers were designed by Primer3 or were previously described (1, 21) and are listed in Supplemental Table I.

**Generation and site-directed mutagenesis of IL-17A promoter-luciferase reporter plasmids**

A DNA fragment containing the proximal 229 bp of the IL-17A promoter region from the transcription start sites was amplified from genomic DNA of human primary CD4+ T cells by PCR. The amplified product was subcloned into a pGL3 vector (Promega) with the firefly luciferase reporter gene to generate pGL3-IL17A luciferase plasmid (IL-17p/WT). The two individual αMP response elements (CREs) in the IL-17A promoter construct, CRE1 (–183 to –178) and CRE2 (–111 to –104), were mutagenized by using the QuickChange II site-directed mutagenesis kit (Agilent Technologies). The resulting CRE constructs were termed IL-17p/CREmt (mutations on both CRE sites). The authenticity of the clone was confirmed by DNA sequencing. The primer sequences used in this experiment are listed in Supplemental Table II.

**Reporter gene transfection assay in activated CD4+ T cells and Jurkat cells**

CD4+ T cells were activated with plate-coated anti-CD3 Abs and soluble anti-CD28 Abs for 16–20 h. Activated cells were transfected with indicated plasmids together with Renilla luciferase expression vector pRL-CMV (Promega) using a Nucleofector kit for stimulated human T cells (Amaxa). Twenty-four hours after transfection, cells were further activated with CD3/CD28 beads and treated with CT as indicated. Jurkat cells were transfected with indicated plasmids and Renilla pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. Twenty-four hours after the transfection, cells were treated with 10 ng/ml CT together with TPA and ionomycin (Cell Signaling Technology) for 5 h. Cell extracts were lysed and luciferase activity was quantitated in triplicate using a Dual-Glo luciferase assay system (Promega) according to the manufacturer’s protocol. The relative IL-17A promoter activities were expressed as relative luciferase units after normalization to the internal control, Renilla luciferase activity. The results were averaged from triplicate wells of three separate experiments.

**Statistical analysis**

A p value was determined using the two-tailed Student t test, and multiple groups were analyzed using one-way ANOVA. A p value <0.05 was considered statistically significant.

**Results**

**Direct stimulation of IL-17A production in CD3/CD28-activated PB CD4+ T cells by CT**

CT is potent mucosal immunomodulator and is also known to induce a Th17 response in experimental animals (22, 23). However, it is unclear whether CT can directly regulate IL-17A production from human CD4+ T cells. Total PB CD4+ T cells were stimulated with CT for 3 d under CD3/CD28 activation. We found that CT enhanced IL-17A production by >2.5-fold but slightly decreased IL-17F production in stimulated CD4+ T cells cultured under the nonpolarizing condition (Fig. 1A). As for other cytokine expression, CT strongly inhibited IFN-γ, TNF-α, IL-2, and IL-10 production but not IL-4 and IL-5 (Fig. 1B–E). Interestingly, CT also induced GM-CSF production by >1.8-fold (Fig. 1D), which was consistent with prior reports that showed inhibition of Th1 cytokines like IL-2 and IFN-γ and increase of Th17 cytokines, such as IL-17A (4). Our results are consistent with prior reports that showed induction of Th1 cytokines like IL-2 and IFN-γ and increase of Th17 cytokines, such as IL-17A (4). Our results are described at mRNA levels, which were isolated from these cultures 48 h after CT treatment. As shown in Fig. 2A, CT treatment increased mRNA levels of IL17A by >2.5-fold, whereas CT treatment did not enhance the expression of other Th17 cell–associated cytokine genes, such as IL17F, IL22, and CCL20. Retinoic acid–related orphan receptor (ROR)γt and IFN regulatory factor (IRF)4 are two Th17 lineage–specific transcriptional factors. RORγt is the major transcription factor in Th17 cell differentiation to induce IL-17A production (24); IRF4 has been shown to induce both IL-4 and IL-17 production (25, 26). However, the mRNA levels of RORC and IRF4 in CD4+ T cells were not affected by the treatment of CT (Fig. 2C). Regarding other Th cell–associated transcriptional factor genes (Fig. 2C), CT had no effect on FOXp3 expression, but it decreased mRNA levels of TBX21 expression.

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but slightly increased \textit{GATA3} expression. These last two transcriptional factor expressions were consistent with levels of expression of their downstream cytokine genes, \textit{IFNG} and \textit{IL4}, respectively (Fig. 2B).

To characterize the change in cell population, especially with different cytokine expression, CT-treated cultures were analyzed by flow cytometry staining with anti–IFN-\(\gamma\), anti–IL-17A, anti–IL-17F, and anti–IL-22 Abs. As shown in Fig. 3, CT decreased the percentage of IFN-\(\gamma\)+IL-17A\(^+\) cells and did not change the percentages in IFN-\(\gamma\)+IL-17A\(^{+}\) populations, but it increased the IFN-\(\gamma\)-IL-17A\(^+\) population after a 4-d treatment (Fig. 3A). In contrast, there was no change in the percentage of total IL-17F\(^+\) or IL-22\(^+\) T cells (Fig. 3B). Therefore, CT selectively enhances IL-17A production but constrains IFN-\(\gamma\) in CD4\(^+\) T cells.

\textit{CT induces more IL-17A production from lineage-committed Th17 or Th1/17 cells but not from Th1 cells}

Because CT caused the decrease of IFN-\(\gamma\)- but the increase of IL-17A\(^+\) populations, we first examined whether CT converts non-Th17 cells to Th17 cells or whether CT directly acts on Th17 cells.

\textbf{FIGURE 2.} CT increases \textit{IL17A}, \textit{IL4}, and \textit{GATA3} mRNA expression but decreases \textit{IFNG} and \textit{TBX21} expression. CD4\(^+\) T cells were treated with or without CT (10 ng/ml) for 2 d under CD3/CD28 activation and total RNAs of cells were collected. The expression of (A) Th17-associated genes, (B) Th1 and Th2-associated genes, and (C) transcription factors were examined by a SYBR Green real-time PCR assay (\(n = 6\)). \(p < 0.05\), \(p < 0.01\).
Chemokine receptor CXCR3 is expressed primarily on activated T cells and NK cells, being especially preferentially expressed on Th1 cells (27, 28). It has been reported that CCR6 is predominately expressed on Th17 cells (29–31). Using Abs specific to these two receptors, we isolated unstimulated Th1, Th17, and Th1/Th17 hybrid populations (32). The sorted Th cell subsets separately carry CXCR3+CCR6− (enriched Th1), CXCR3−CCR6+ (enriched Th17), and CXCR3−CCR6− (enriched Th1/17) surface chemokine receptors (Fig. 4A, upper panels). Further flow cytometric analysis of these subtypes confirmed their specificity of cytokine expression for IFN-γ, IL-17A, and both cytokines (IFN-γ/IL-17A) as related to Th1, Th17, and Th1/Th17 subtypes, respectively (Fig. 4A, lower panels). Treatments of these isolated subtypes of cells with CD3/CD28 activation have shown that CT could further enhance IL-17A production from Th17 and Th1/Th17 cells, but not from Th1 cells (Fig. 4B). These results support the notion that CT could only enhance IL-17A production from T cells that are capable of secreting IL-17A.
CT promotes further IL-17A production from naive CD4+ T cells that were cultured under Th17-polarizing condition

In addition to enhancing the effector function of Th17 cells, CT may also promote Th17 cell differentiation to enhance the overall IL-17A production. To test this possibility, naive CD4+ T cells were differentiated or not to Th17 cells under IL-1β and IL-23 supplemented or unsupplemented culture conditions, respectively. As shown in Fig. 5A, flow cytometric analysis revealed a small increase in IL-17A–producing cells with a big drop in IFN-γ–producing cells by CT in nonpolarizing (Th0) naive CD4+ T cell culture. In the Th17-polarizing culture condition with IL-1β and IL-23 treatments, CT enhanced more IL-17A–producing cells, but not the IFN-γ–producing cell population. These results are consistent with the enhancement of IL-17A production in this polarized subset of cells activated by anti-CD3 treatment (Fig. 5B). These results suggested that CT alone has a minimal effect on Th17 cell differentiation from naive CD4+ T cells under nonpolarizing conditions. However, this CT effect was further synergized with the IL-1β/IL-23 polarized condition and resulted in more IL-17A–producing cells and IL-17A production from activated CD4+ T cells.

CT-induced IL-17A production is cAMP-dependent

The main biochemical consequence caused by CT is the sustained increase in intracellular cAMP owing to the constitutive activation of adenylate cyclase by subunit A of CT (CT-A), which relies on the binding of CT-B to the cell surface to exert its function (10). To elucidate which CT subunits are responsible for the IL-17A induction, we compared the ability of CT and CT-B in the induction of IL-17A. As expected, CT-B alone did not effectively increase IL-17A production (Fig. 6A). This result indicates that CT-A is required for the increase of IL-17A in T cells. We also measured the intracellular cAMP levels of CD4+ T cells after treatment with CT-B or CT. As shown in Fig. 6B, the intracellular cAMP levels were increased by CT but not by CT-B. These results suggest that CT-A, upon the translocation, exerts its effect on the elevation of both the cAMP levels and IL-17A production.

Next, we examined whether the IL-17A induction by CT is due to the elevation of intracellular cAMP levels. Total CD4+ T cells were treated with various cAMP-elevating agents for 3 d under CD3/CD28 activation. The treatment of FK, which directly activates adenylate cyclase to induce intracellular cAMP, increased IL-17A production (Fig. 6C). A similar result was observed when cells were treated with db-cAMP (cell-permeable cAMP). We also used a phosphodiesterase inhibitor, IBMX, to determine whether the treatment could increase intracellular cAMP levels by blocking its degradation, resulting in an enhancement of IL-17A production. The addition of IBMX slightly increased IL-17A production. We also found that CT and these cAMP-elevating agents synergized with IL-1β/IL-23-polarizing conditions to enhance more IL-17A production (Fig. 6D). In summary, all cAMP-elevating agents can increase IL-17A production either under nonpolarizing or Th17-polarizing condition.

PGE2 has dual roles in anti-inflammation and proinflammation. Several reports also suggest that PGE2 regulates mouse and human Th17 function through cAMP (33, 34). Therefore, we also examined the effect of PGE2 on CD4+ T cells in our system and the results showed that PGE2 also increased cAMP (Fig. 6B) and IL-17A production under nonpolarizing (Fig. 6C) or Th17-polarizing (Fig. 6D) conditions. Additionally, we have also looked into the similarity of the effects of CT and PGE2 treatments on various cytokine productions by CD4+ T cells. As shown in the Supplemental Fig. 1, both CT and PGE2 have a similar trend of effects on the production of GM-CSF, IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, and IL-10 in this CD4+ T cell system whereas this similarity was not seen in CT-B and control treatments. These results further confirm that both CT and PGE2 enhance IL-17A production and alter other cytokine production via a common cAMP-dependent pathway.

CT also stimulates IL-17A promoter activity in Jurkat cells and primary CD4+ T cells via cis-CRE elements

In addition to cAMP signaling, our data also indicate that CT regulates IL-17A production at the mRNA level (Fig. 2A). To examine whether the effect of CT on IL-17A expression is due to transcriptional activation, a study of the CT effect on the IL-17A promoter activity was conducted both in Jurkat cells and primary CD4+ T cells using transient transfection approach with a pGL3-IL-17A construct. As shown in Fig. 7, CT increased the luciferase activity of IL-17A promoter construct in both Jurkat cells (Fig. 7B) and stimulated CD4+ T cells (Fig. 7C).
Our data showed that CT inhibited IL-2, IL-10, IFN-γ. Exogenous cytokine effects on CT-induced IL-17A production were mediated via a cAMP-dependent pathway and occurs at the transcriptional level. Our data also indicated that CT exclusively activates the expression of IL-17A and its promoting cytokines IL-6 and GM-CSF. Because various cAMP analogs can have similar CT effects, these results suggest the importance of the cAMP signaling pathway in mediating Th17 cell differentiation.

Unlike IL-17A expression, CT did not increase the expression of other Th17-associated cytokine genes, such as IL-17F and IL-22. The difference in the regulation of Th17 cytokines has also been reported in other studies. For instance, TGF-β suppresses IL-22 expression but enhances IL-17A expression (39). Furthermore, it has been shown that Th17 cytokines act similarly on the induction of downstream genes and signaling; however, their physiological roles and functions in disease pathogenesis are different. (40). IL-17A and IL-17F knockout mice have different disease outcomes in experimental models of allergic encephalitis and allergic airway diseases (41). These studies suggest that in response to various environmental stimuli, Th17 cells secrete various combinations of cytokines to execute their function properly. Our study found that a combination of CT and IL-12 treatment resulted in increased IL-17A expression, whereas IL-12 alone did not significantly affect IL-17A expression.

Discussion

Th17 cell differentiation is mediated by the integration of signals from TCRs, costimulatory molecules, and cytokines. An increasing number of studies also showed that microbial components regulate Th17 differentiation directly or via the activation of APCs (6, 8). In this study, we demonstrated a novel function of CT to enhance IL-17A production from human PB CD4+ T cells and the generation of Th17 cells. The mechanism of CT-induced IL-17A production is mediated via a cAMP-dependent pathway and occurs at the transcriptional level. Our data also indicated that CT exclusively activates the expression of IL-17A and its promoting cytokines IL-6 and GM-CSF. Because various cAMP analogs can have similar CT effects, these results suggest the importance of the cAMP signaling pathway in mediating Th17 cell differentiation.
CT is one of the immunomodulators that selectively enhances IL-17A production from Th17 cells. Besides IL-17A, CT also induced GM-CSF production in T cells. GM-CSF is an activator for the development of DCs and has been used as a cytokine adjuvant in vaccine development (42, 43). Furthermore, GM-CSF is also involved in Th17-mediated autoimmune encephalomyelitis. IL-23 has been reported to drive GM-CSF expression by Th17 cells, and then GM-CSF activates DCs to secrete more IL-23. This IL-23/GM-CSF circuit enhances Th17 responses (4, 44, 45). Whether GM-CSF secreted from CT-treated T cells also has this IL-23/GM-CSF loop to enhance Th17 cell function in vivo needs to be clarified.

We also studied the underlying mechanism of CT-modulated IL-17A production in T cells. Our data suggest that CT-A plays the major role in the induction of IL-17A, the subunit of which activates adenylyl cyclase through ADP ribosylation. Additionally, we found that cAMP-eliminating agents, such as FK and db-cAMP, mimic the CT effect on IL-17A stimulation. This result suggests that CREM motifs on the IL-17A promoter are responsible for this CT-induced IL-17A promoter-reporter activity in these transfected studies in Jurkat cells and primary T cells. We observed a CT-induced IL-17A promoter-reporter activity in these transfected cells. This result supports an increased transcriptional activity as a mechanism for IL-17A production. We further demonstrated that CRE motifs on the IL-17A promoter are responsible for this CT-induced activity. However, the sequence around these CRE sites can be occupied with multiple transcription factors, such as RORγt, CREMζ, and BATF (47). The transcriptional factors RORγt (24, 48) and CREMζ (49) have been reported to bind to the region across the CRE2 (−111 to −104) sequence of the IL-17A promoter. RORγt is the major transcription factor of Th17 differentiation and induces IL-17A expression via direct binding to the promoter. In the present study, CT did not enhance mRNA levels of RORγt. Additionally, the small interfering RNA knockdown of RORγt and the pretreatment of RORα/γ inverse agonist SR1001 did not affect CT-induced IL-17A production (data not shown). This result indicates that CT may induce IL-17A production via transcriptional activation but an RORγt-independent pathway. However, whether CT-induced IL-17A expression is dependent on BATF, CREMζ, or other transcription factors needs to be explored.

PGE2 is one of the physiological molecules using cAMP as its secondary messenger. PGE2 is a negative regulatory molecule in Th17 immune activation. It has been thought to inhibit the function of Th1 cells (50). However, some evidence shows that PGE2 also has proinflammatory properties in inflammatory bowel disease (51) and collagen-induced arthritis (52). In vitro culture of CD4+ T cells with PGE2 showed that PGE2 enhances IFN-γ expression and Th17 expansion in certain conditions (33, 34). In our system, similar to CT, PGE2 inhibited IL-2, IL-10, and TNF-α production but its effect on IFN-γ was not consistent among samples. A variety of cytokines and other immunomodulators are expressed at the site of inflammation, and their crosstalk directs Th cells to preform specific effector functions. In this study, we found that IL-6 was increased but IFN-γ and IL-10 were decreased. These results indicate that the CT-induced response favors Th17 cells, although the addition of these cytokines into T cell culture media did not change the ability of CT on IL-17A induction. Furthermore, we also examined cytokines that favor Th17 development and the one that favors Th1 development. Our data showed that CT had a synergistic effect with IL-1β plus IL-23 to promote more IL-17A production. Additionally, we observed that IL-12 could abrogate the ability of CT to inhibit Th1 responses. However, we still detected more IL-17A production from CT-treated cells even when IL-12 was added. Interestingly, the increase in the percentage of IL-17A+ cells is constrained by IL-12. It is likely that IL-12 changes the proliferative ability of different Th cells or IL-12 directly constrains new Th17 cell generation. This finding emphasizes the invincible role of CT on IL-17A stimulation and also indicates that the crosstalk among different modulators is critical to determine the outcome of immune responses.

In conclusion, our findings suggest that CT is a powerful agent to determine the outcome of the T cell response. Therefore, more studies on other bacterial toxins in T cells can be executed to discover new modulators that can directly promote or suppress specific Th responses. The exploration of the mechanism triggered by toxins also provides an attractive target to develop new therapeutic strategies of immune-mediated diseases.

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Disclosures
The authors have no financial conflicts of interest.

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