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Cholera Toxin Induces cAMP-dependent Th17 Differentiation by Dendritic Cells

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TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE THESIS

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by

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Cholera toxin (CT), the causative factor responsible for the life-threatening acute diarrhea caused by *Vibrio cholerae*, is also well known as a potent mucosal vaccine adjuvant. However, little is known about the cellular and molecular mechanisms that mediate the mucosal adjuvant properties of CT. Recent studies have shown that Th17 cells discovered at mucosal sites have important role in generating strong immune responses that can be either protective (e.g. antimicrobial immunity) or destructive (e.g. autoimmune diseases EAE). Here, we showed that CT activates dendritic cells (DC) via cAMP-dependent mechanisms to drive the differentiation of naïve T cells into IL-17-

producing Th17 cells *in vitro* and *in vivo*. Importantly, we identified an alternative pathway for Th17 differentiation that depends on the CT-induced secretion of calcitonin gene-related peptide (CGRP) by DC but is independent of IL-6. CGRP, a neuropeptide, in turn activates cAMP-dependent pathways in T cells that contribute to the generation of Th17 cells. These findings implicate Th17 induction as a contributing factor to the adjuvant effects of CT and identify novel pathways involved in T cell differentiation at mucosal sites.

1. Introduction

1.1. Vaccination: Use in Clinical Practice

Cholera toxin (CT)-associated infectious diarrhea, resulting from *Vibrio cholerae* and transmitting mainly through the contamination of food and water, take more than 3 million children's lives in third world countries annually. In the US, diarrhea, surprisingly, also accounts for more than 10% of preventable childhood deaths. The incubation period is usually 2-5 days but may be only a few hours where approximately 10^8-10^{11} bacterial colonies have already found a comfortable niche in the small intestine to establish infection in people with normal gastric acidity. In 5-10% cases of those that are severely infected, dehydration and massive loss of electrolytes can result in metabolic acidosis and circulatory collapse. If left untreated, more than 50% of the cases would result in mortality within several hours, whereas prompt treatment would reduce the mortality to less than 1%. In mild cases, usually asymptomatic, treatment requires rehydration with oral intravenous fluids. In severe cases, antibiotic treatment can be given to reduce the volume of diarrhea and duration of excretion of *V. cholerae* (Hill, Ford et al. 2006; Burleigh and Banks 2007).

V. cholerae, like many other infectious pathogens, utilizes the mucosal surfaces as an entry site in which they adhere to, colonize, penetrate, replicate and from where they spread to the entire body. It is, therefore, important that immune defense mechanisms against these agents function sufficiently well at the mucosal level in order to prevent infection. Live, attenuated vaccines, administered parenterally, have been proven to

effectively induce mucosal immune responses. However, live, attenuated vaccine can potentially generate both local and more generalized systemic reactions, thus limiting the efficacy of vaccination (Stevceva and Ferrari 2005; Hill, Ford et al. 2006).

Non-living vaccines, although are much safer and manufactured more easily, are unable to generate comparable immunogenic effects. Recent efforts are moving towards developing oral vaccines that are cheaper to administer, eliminate the need of sterile syringes and needles, ensure high rate of complacency (painless administration) and are suitable for mass vaccination. Mucosal immunization offers the advantage of inducing antigen-specific responses at both the mucosal and systemic levels. It is also important to consider the potential of mucosal vaccines, when administered orally, can induce oral tolerance in the host (Stevceva and Ferrari 2005; Hill, Ford et al. 2006). CT has long been known as a potent mucosal adjuvant. Here, we are interested in determining the mechanism underlying the adjuvant property of the toxin to formulate a vaccine to induce immunity against the pathogenic *V. cholerae* and other pathogens in general.

1.2. Cholera Toxin: Underlying Biological Mechanisms

V. cholerae, a Gram-negative rod bacterium with a single unipolar flagellum, synthesizes and secretes CT in soluble forms that are responsible for the massive watery diarrhea (Burleigh and Banks 2007). CT, consisting of A and B components, belongs to the AB_5-subunit bacterial toxin category. The A-subunit, 28 kDa, is the enzymatically catalytic domain consisting of two chains: A1 and A2. The A1-peptide functions as an ADP-ribosyltransferase, whereas the A2-peptide, connected to A1-peptide by a disulfide-bonded peptide loop consisting of a serine protease cleavage site, is a linker that connects

A1-subunit to the B component of the toxin. The 55 kDa-B component, stabilized by hydrogen bonds, salt bridges and hydrophobic interactions, composes of five identical peptides arranged in a ring-like configuration to form a homopentameric subunit. Along the lumen of the gastrointestinal tract, when CT first encounters mammalian cells, in this case the enterocytes of the gut, the B-subunit binds to the monosialoganglioside GM1, a glycosphingolipid found ubiquitously on the surface of mammalian cells, such as epithelial cells, M cells and antigen-presenting cells, that contain a complex of oligosaccharide with one sialic acid. Each B subunit monomer can bind to the GM1 receptor making a cross-link of pentamer. The A2-peptide binds to the central pore of the B-subunit pentamer to link the B-subunit and the A1-peptide together non-covalently (Freytag and Clements 2005; Sanchez and Holmgren 2008).

Ligand-receptor interaction triggers the internalization of the toxin into the cell via one of the following models depending on the cell type: caveolin-coated vesicles, clathrin-coated vesicles, or Arf6 endocytic pathway (Sanchez and Holmgren 2008). No single pathway for endocytosis of CT is required as the inhibition of endocytosis by clathrin-, caveolin-, or ARF6-dependent pathways does not prevent CT movement into the cell or attenuate toxicity. Following endocytosis, CT enters the endoplasmic reticulum, ER, in retrograde manner, i.e. from plasma membrane to the early endosomes to the Golgi cisternae en route to the ER. In the ER, CT is dissociated into CT-A and CT-B subunits. The CT-A subunit is further processed by protein disulfide isomerase, PDI, proteolytically cleaving at Arg192 to separate and unfold A1 and A2 peptides (Freytag and Clements 2005).

The free A1 peptide is released into the cytosol via the Sec61p channel. The transport of A1 peptide to the cytosol involves the ER-associated degradation (ERAD) pathway, in which ER chaperones typically can recognize and retrieve misfolded proteins from the ER and transport them to the cytosol for proteasomal degradation. The A1 peptide manages to circumvent this pathway possibly due to their low lysine content, which signals for ubiquitinylation, thus avoiding ERAD (Sanchez and Holmgren 2008).

Once in the cytosol, the A1 peptide finds its way to the α-subunit of the heterotrimeric stimulatory GTP-binding protein, $G_s\alpha$, which is a component of a G-protein coupled receptor, GPCR. Typically at the cell surface, when a ligand binds to the extracellular domain of a stimulatory GPCR, it results in the activation and conformational change in the structure of the G protein, in particular the intracellular loops that release the bound GDP in exchange for GTP. This subsequently leads to the activation of $G_s\alpha$ resulting in its release from the surface membrane (Freytag and Clements 2005). Similarly, when A1 peptide binds to ARF6-GTP, where ARFs are essential, ubiquitous ADP-ribosylation factors of the G protein, the A1 peptide changes in its conformation, thus allowing nicotinamide adenine dinucleotide, NAD^+, substrate to bind to the active site of A1 peptide (Sanchez and Holmgren 2008). The A1 peptide then transfers the ADP-ribose component of NAD^+ to the $G_s\alpha$ when GTP is still bound. Under steady state condition, the $G_s\alpha$ subunit will hydrolyze the GTP molecule with the help of its partner GTPase activating protein, GAP, thus $G_s\alpha$ will become deactivated. However, due to the ADP-ribosylation, $G_s\alpha$ remains constitutively activated and in turn induces the activation in its target adenylate cyclase irreversibly, thus resulting in

elevated level of intracellular cyclic AMP, a common second messenger. cAMP can then activate protein kinase A, PKA, or exchange protein directly activated by cAMP, Epac further downstream (Freytag and Clements 2005).

The massive loss of fluid caused by CT can be explained by the PKA-induced phosphorylation and activation of the cystic fibrosis transmembrane conductance regulator, CFTR, Cl^- channel of the crypt epithelial cells. The activated Cl^- channels result in the efflux of salts into the lumen that is then followed with the osmotic movement of water manifesting the physiological diarrhea. Binding of the toxin to the jejunal villus epithelial cells also results in elevated cAMP production and consequently inhibits the absorption function of the small intestine by tampering with the Na^+H^+ exchanger. Fluid secretion, ranging from 500-1000 mL/hr to 30-40 L/day, localizes to specific regions of the intestinal tract with reduced secretion as it proceeds from the jejunum to colon. The fluid secretion is also mediated by the afferent sensory neurons that are relayed through both the submucosal plexus and myenteric plexus (Burleigh and Banks 2007).

Described previously, another downstream target of cAMP is Epac, also known as cAMP-GEF, a cyclic AMP-regulated guanine nucleotide exchange factor. Following the binding of cAMP, Epac activates Rap, which is a small molecular weight GTPases of the Ras family, that can subsequently mediate cell adhesion, vascular endothelial cell barier formation, cardiac gap junction formation and hormone gene expression. Epac also interacts with other Ras GTPases, microbutule-associated proteins for transporter activity, secretory granule-associated proteins for exocytosis, e.g. Rim2 and Piccolo, and

the sulphonylurea receptor-1 or SUR1, a subunit of ATP-sensitive K^+ channels (de Rooij, Zwartkruis et al. 1998).

Epac1, with only one binding site for cAMP, is ubiquitously expressed in the brain, heart, kidney, pancreas, spleen, ovary, thyroid and spinal cord. Epac2, with one of low affinity and one of high affinity binding site for cAMP, is more localized in certain parts of the brain, the adrenal glands, liver and pancreatic islets of Langerhans. Epac interacts directly with intracellular Ca^{2+}-release channels, e.g. IP3 receptor or ryanodine receptor, to promote the receptor opening in response to Ca^{2+} or various Ca^{2+}-mobilizing second messengers, e.g. IP3 and cADP-ribose. Epac can also act via Rap and ERK to promote the PKA-independent phosphorylation of Ca^{2+} channels. In addition, Epac also acts via Rap to stimulate phospholipase C-epsilon, PLC-ε, to induce the hydrolysis of PIP2 to IP3 (de Rooij, Zwartkruis et al. 1998).

1.3. Cholera Toxin: Neurological Aspects

The gut is innervated with a network of different types of neuron. The afferent neurons, also known as intrinsic primary afferent neurons or IPANs, can sense the intestinal luminal contents, e.g. pH, fat or glucose, or even enterotoxins, indirectly through the release of chemicals such as 5-hydroxytryptamine from enteroendocrine cells. Inter-neurons process the signals in the submucosa plexus and myenteric plexus. The motor neurons relay the information to the epithelial effector cells, e.g. secreto-motor neurons that terminate in the lamina propria stimulate the mucosal epithelial cells to transport chloride ions into the intestinal lumen in which sodium and water would soon follow (Burleigh and Banks 2007).

Studies with denervated small intestinal segments in feline and murine animals have demonstrated that CT stimulates the enterochromaffin cells to release serotonin that would promote the secretion of vasoactive intestinal peptide, VIP, from the neighboring neural networks (Sanchez and Holmgren 2008). The enteric nervous system, ENS, rich in neurons containing secretory VIP-granules, regulate electrolyte/fluid transport and subsequently blood supply. Under steady state condition, the tonic release of VIP is mediated via the action of acetylcholine on the postsynaptic nicotinic receptors stimulating the vagus nerve. VIP-receptors belong to the secretin family of GPCRs comprising of secretin, pituitary adenylate cyclase-activating polypeptide, or PACAP, and calcitonin gene-related peptide, or CGRP. In the gut, the receptor binding sites are predominantly localized in the mucosal layer, especially in the jejunum. The binding of VIP to its receptor activates $G_s\alpha$ subunit of GPCR that in turn activates adenylate cyclase and increases intracellular cAMP concentration in the large intestine. As described previously, cAMP-induced activation of PKA results in the opening of CFTR Cl^- channel and the subsequent Cl^- efflux with osmotic movement of water. As a negative feedback mechanism, the activation of VIP-receptor induces its phosphorylation and internalization, a process known as desensitization (Burleigh and Banks 2007).

1.4. The Biology of T Lymphocytes: Adaptive Immunity

The immune system, armed with both innate and adaptive components, has evolved to enable the host to combat infectious disease. The innate system, consisting of cells such as neutrophils, dendritic cells, macrophages, mast cells and natural killer (NK) cells, is continuously poised and ready to assault invading pathogens. However, this

rapidity is counterbalanced by a lack of both pathogen-specific recognition and memory. By contrast, the adaptive immune system needs priming to eradicate pathogens. Although it is slower, the adaptive immune system has greater precision to enable recognition of specific pathogens by virtue of its vast receptor diversity. Most importantly, it has memory and upon subsequent infection with the same pathogen, can rapidly respond and clear the invader (Zenewicz, Antov et al. 2009).

The adaptive immune system harbors B and T lymphocytes. T cells are further divided into (i) CD4 T cells, which express CD4 surface glycoprotein and which are also termed T helper (Th) cells owing to the help they provide to B cells and other T cells in directing B- and T-cell responses; and (ii) CD8 T cells, which express the CD8 surface glycoprotein, and which are also termed cytotoxic T cells owing to their high expression of IFN-γ and granzymes. Naïve T cells are inexperienced T cells that have yet to receive two principal signals to differentiate into effector cells. The first signal comes from their T cell receptor engaging with their cognate antigen presented on the major histocompatibility complex (MHC) molecules of professional antigen-presenting cells, including dendritic cells, macrophages and B cells. The second signal derives from the costimulatory molecules, indicators of danger, along with the specific set of cytokines produced by the innate immune cells following the encounter with foreign pathogens. T-cell receptor (TCR) engagement in an inflammatory cytokine milieu induces the cells to undergo numerous changes leading to the induction or repression of transcription factors in differentiated cell subsets. This in turn results in the differential expression of cell-surface receptors and cytokines. Th1 cells help combat intracellular bacteria and viruses,

Th2 cells aid in the clearance of parasites and helminthes, and regulatory T (Tregs) cells, through secretion of TGF-β and/or IL-10, suppress Th proliferation and effector functions, to maintain immune homeostasis or down-modulate immune responses after infection (Zenewicz, Antov et al. 2009).

Th1 cell differentiation can be induced *in vitro* by IL-12, an innate-system-derived cytokine that is highly expressed by activated macrophages and dendritic cells following the processing of microbial antigens. IL-12 activates STAT4 that in turn activates the genes that encode for IFN-γ, a signature cytokine for Th1 cells, and T-bet, which is a transcription factor. Transcription factors are downstream mediators of TCR and cytokine receptor activation that partake in inducing the expression of lineage-specific genes. IFN-γ works in a positive feedback loop to amplify the differentiation of more Th1 cells. Activation of IFN-γ receptor leads to the activation of STAT1 and more T-bet. T-bet, in turn, can bind to IFN-γ promoter and induces more expression of IFN-γ. T-bet also induces the expression of IL-12Rβ2 chain of the IL-12R making these cells even more responsive to IL-12 signaling to maintain Th1 responses. However, IL-12 is not the only pathway to induce Th1 differentiation as IL-$12^{-/-}$ mice are able to generate Th1 cells via the Notch pathway. The interaction of Delta ligand on DCs with Notch on T cells result in Th1 differentiation through the activation of the Notch intracellular activation domain (NICD) (Korn, Bettelli et al. 2009; Zenewicz, Antov et al. 2009).

Th2 cell differentiation can be triggered by IL-4, which signals through STAT6 that in turn activates the genes that encodes for the transcription factor GATA-3. GATA-3 commits naïve T cells to Th2 lineage and induces the expression of IL-4. IL-4 binds to

its receptor and activates STAT6 and more GATA-3 to increase the production of IL-4 and stabilize Th2 lineage. IL-4 also positively feeds back to the system to enhance the expression of other cytokines: IL-5, IL-9, IL-10, IL-13 and IL-25. Th2 cells can also signal through Notch pathway similarly to Th1 cells (Korn, Bettelli et al. 2009; Zenewicz, Antov et al. 2009).

Tregs are T cells that can suppress the effector functions of other T cell types. There are two major classes of Tregs: natural Tregs and induced Tregs. Natural Tregs are generated in the thymus during T-cell development, whereas induced Tregs continue their differentiation in the periphery. Natural Tregs produce TGF-β. Induced Tregs include Tr1, which produce IL-10, and Th3 cells, which produced TGF-β. TGF-β and retinoic acid or IL-2, a growth factor for Tregs binding to IL-2R consisting of IL-2Rα and IL-2Rβ, activate STAT5/Smad3 to up-regulate the expression of Foxp3 transcription factor promoting Treg differentiation (Korn, Bettelli et al. 2009; Zenewicz, Antov et al. 2009).

Recently, a new subset of Th cells, Th17 cells produced mainly IL-17A (also known as IL-17), IL-17F, Il-21, IL-22, IL-6 and IL-8, has been identified. IL-17A, IL-17F and IL-8 function to recruit neutrophils to the site of infection for pathogen clearance. Th17 cells have important role in targeting extracellular bacteria at mucosal sites that are beyond the scope of both Th1 and Th2 cells. Pathogens as diverse as the gram-positive *Propionibacterium ances*; the gram-negative *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Bacteroides* spp., and *Borrelia* spp.; the acid-fast *Mycobacterium tuberculosis*; and fungi-like *Pneunocystis carinii* and *Candida albicans* can all trigger a strong Th17 response. In addition, several lines of evidence have established that Th17

rather than Th1 cells are responsible for the development of autoimmune diseases, e.g. experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD), as well as allergic diseases such as contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) in mice. Skin lesions of psoriasis patients showed high amounts of CCL20/CCR6 that are associated with Th17 memory cells. Patients with rheumatoid arthritis have high levels of TNF, IL-1 and IL-17. Multiple sclerosis lesions express high amounts of IL-6, IL-8 (CXCL8) and IL-17 (Ishigame, Kakuta et al. 2009; Korn, Bettelli et al. 2009).

IL-17A and IL-17F are largely coexpressed in $CD4^+$ T cells. IL-17 family of cytokines includes IL-17, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25), and IL-17F. IL-17E is not produced by Th17 cells but by Th2 cells. NKT cells, NK cells, neutrophils, eosinophils, γδ T cells, and Th17 cells can all manufactured IL-17A and IL-17F. IL-17RA, highly expressed on hematopoietic cells, binds to both IL-17A and IL-17F with higher affinity towards IL-17A. IL-17A and IL-17F act on various cell types to induce the expression of cytokines, e.g. TNF, IL-1b, IL-6, GM-CSF, G-CSF, chemokines, e.g. CXCL1, CXCL8, CXCL10, and metalloproteinases (Korn, Bettelli et al. 2009).

Similar to the intrinsic property of Th1 and Th2 cells, Treg and Th17 cells are antagonizing each other. According to the current literatures, Th17 cell differentiation can be canonically driven by IL-6 and TGF-β. IL-21 amplifies the frequency of Th17 cells. IL-23 terminally differentiates and stabilizes the phenotype of Th17 cells (Ishigame, Kakuta et al. 2009; Korn, Bettelli et al. 2009).

IL-6 is produced by cells of the innate immune system such as DCs, monocytes, macrophages, mast cells, B cells, and subsets of activated T cells, but also by tumor cells, fibroblasts, endothelial cells, and keratinocytes. IL-1, TNF, platelet-derived growth factor (PDGF), IL-3, GM-CSF, and IL-17 can induce the expression of IL-6. Naïve T cells express a functional IL-6 receptor composed of IL-6Rα and the signaling subunit gp130, which is ubiquitously expressed. TGF-β induces the expression of IL-6Rα, TCR or IL-6 stimulation can down-regulate IL-6Rα and reduce the responsiveness to IL-6. TGF-β is necessary to maintain the responsiveness of T cells to IL-6 (Korn, Bettelli et al. 2009).

Stimulation of IL-6R results in STAT3 activation but not sufficient to induce steroid receptor-type nuclear receptor RORγt, the signature transcription factor for Th17 cell differentiation. However, STAT3 deficiency does lead to deficiency in Th17 development. IL-6-induced STAT3 activation also up-regulates the expression of IL-21. IL-21-induced STAT3 activation, in turn, up-regulates RORγt expression and drives the production of IL-17A and other cytokines. The full induction of RORγt is only achieved in the presence of TGF-β. Interestingly, TGF-β induces the expression of RORγt but represses its function. Only when IL-6 or IL-21 present is the repression of RORγt relieved and Th17 differentiation is promoted. The retinoid nuclear receptor RORα is also selectively expressed in Th17 cells. RORα plays a synergistic and somewhat redundant role with RORγt during Th17 cell polarization. Similar to RORγt, RORα is also strongly induced by IL-6 or IL-21 in the presence of low amounts of TGF-β via the

STAT3 activation. Over-expression of either RORγt or RORα can induce IL-17A production in T cells (Korn, Bettelli et al. 2009).

IL-21 belongs to the IL-2 family of cytokines and uses the common γ chain (γ_c). IL-21R consists of γ_c and IL-21R. IL-21 is produced by activated Th1 and Th2 and NKT cells in general, but not by APCs. However, the major source of IL-21 is from Th17 cells, where IL-6 is a strong inducer of IL-21. The expression of IL-21 is dependent upon STAT3 but not on RORγt. IL-21 helps to maintain and amplify the pool of Th17 precursors when the supply of IL-6 is limited. IL-21 becomes important in generating Th17 pool when the supply of IL-6 is depleted and Treg cells are deleted from the peripheral repertoire (Korn, Bettelli et al. 2009).

Traditionally TGF-β is essential in inducing the expression of Smad4 to upregulate the expression of Foxp3. In non-inflammatory environment, the immune system produces TGF-β, which induces the generation of iTregs that together with nTregs keep activated/effector memory cells in check. The source of TGF-β for Th17 development is from T cells and even Tregs cells. TGF-β are produced in latent form and needs to be activated either through proteolytic degradation or by conformational changes of latency-associated protein (LAP). DCs, not the primary producers of TGF-β, however, are essential for the generation of active form of TGF-β in the local environment. IL-6 or IL-21 inhibits TGF-β-driven induction of Foxp3 in naïve T cells, and IL-6/IL-21 with TGF-β drives the differentiation of Th17 cells. RORγt and RORα physically associated with Foxp3 to antagonize each other's function (Chung, Chang et al. 2009; Korn, Bettelli et al. 2009).

iTregs and nTregs could be reprogrammed to Th17 phenotype in the presence of IL-6/TGF-β up to five days after differentiation by down-regulating Foxp3 expression. However, in the presence of retinoic acid, IL-6 would not be able to down-regulate Foxp3 expression in differentiated iTregs or nTregs (Korn, Bettelli et al. 2009).

Interestingly, IL-6/IL-21 combined with low amount of TGF-β induces the expression of IL-23R, a marker of activated/memory T cells, via the up-regulation of STAT3 and RORγt. But high concentration of TGF-β can inhibit the expression of IL-23R. IL-23, a member of the IL-12 cytokine family, composes of a p19 and a p40 subunit. The major source of IL-23 is predominantly from the cells of the innate immune system, e.g. macrophages and DCs. Besides maintaining the survival of Th17 cells, IL-23 also induces other proinflammatory cytokines, e.g. IL-17, IL-1β, IL-6, IFN-γ and TNF-α from innate immune cells, and inhibits the production of anti-inflammatory cytokines, e.g. IL-10 in Th17 cells (Korn, Bettelli et al. 2009).

Described previously, Th17 cells also produce IL-22. IL-6 is known to induce IL-22 expression, whereas TGF-β inhibits the secretion of IL-22. IL-22 is also produced by activated T cells in general, as well as by IL-2- or IL-12-stimulated NK cells. When Th17 cells are induced in APC-free system by the of TGF-β/IL-6 stimulation, IL-22 production is essentially absent unless they have been exposed to IL-23 (Korn, Bettelli et al. 2009).

IL-22 belongs to the IL-10 family of cytokines that include IL-19, IL-20, IL-24, IL-26, IL-28, and IL-29. IL-22R consists of the IL-22R and IL-10R2 components. IL-10R2 component is ubiquitously expressed, whereas IL-22R is only expressed on non-immune cells. IL-22 signals through MAPK pathways and activates STAT3 to promote

anti-apoptosis and proliferation in tissues, induce antimicrobial protein production and maintain epithelial barrier function (Zenewicz, Yancopoulos et al. 2008; Korn, Bettelli et al. 2009).

1.5. Cholera Toxin: Mucosal Adjuvant Properties

Cholera toxin is one of the most potent mucosal adjuvants known. Vaccines containing recombinant CT-B subunit and inactivated whole-cell *V. cholera* have been registered in more than 50 countries worldwide where more than 10 million doses have already been given. Given orally in two or three doses, the vaccine induces intestinal IgA anti-toxin and anti-bacterial (e.g. anti-LPS) antibodies. Immunological memory in the intestinal mucosa can last more than 5 years. The immunogenic efficacy depends on the age group and the time period following immunization (Sanchez and Holmgren 2008).

In general, CT is delivered simultaneously with another antigen via the mucosal route to potentiate the immunogenicity to the co-administered antigen (Fernandez-Miyakawa, Brero et al. 2006). These enterotoxins are resistant to degradation by proteases, bile salts, or other compounds in the intestine. The major negative side of both CT is its toxicity in humans. As little as 5 μg of CT, a dose that is non-toxic in mice, could trigger massive fluid loss in a person. CT mutants that are non-toxic but retain their strong adjuvanticity at mucosal level have been shown to be particularly effective. However, these remain to be confirmed and fully explored (Stevceva and Ferrari 2005; Sanchez and Holmgren 2008).

The adjuvant property of CT, mainly mediated via the CT-A subunit, depends on several factors. Studies in murine models have shown that CT can increase intestinal

epithelium permeability to enhance the uptake of coadministered antigen, but only to low molecular weight peptides not to larger proteins. In humans, when intestinal epithelial cells are activated, via entero-invasive microorganisms, TNF-α or IL-1β, they increase the expression of CXC chemokines (e.g. IL-8, GROα, GROβ, GROγ, and ENA-78), CC chemokines (e.g. MCP-1, MIP-1β, MIP-1α, and RANTES) or even proinflammatory cytokines (e.g. TNF-α, GM-CSF, IL-1α and IL-1β) to recruit polymorphonuclear cells and lymphocytes to the site of infection. M cells in the follicle-associated epithelium can also reach out to the lumen and capture these toxins and deliver them to the underlying dendritic cells, B and T cells. Dendritic cells have the ability to open tight junctions between epithelial cells and send out their dendrites to sample antigens in the luminal gut site. Upon processing, these dendritic cells up-regulate their costimulatory molecules and becoming more mature possibly via the cAMP pathway (Freytag and Clements 2005)

Increasing antigen presentation in APC (dendritic cells, macrophages and B cells) is the most important contributor to the adjuvant property of CT. Measuring antigen presentation relies on the up-regulation of the following molecules: (a) MHC/HLA-DR molecules, (b) CD86/B7.2 costimulatory molecules, (c) chemokine receptors CCR7 and CXCR4 on both murine and human dendritic cells and other APCs (Freytag and Clements 2005; Sanchez and Holmgren 2008).

CT also promotes isotype switching in B cells to increase IgA production at mucosal surfaces (e.g nasal, oropharyngeal, respiratory, genitourinary and gastrointestinal tract). Secretory IgA at mucosal surfaces can block the attachment of bacteria and

viruses, neutralize bacterial toxins, or can even inactivate viruses within epithelial cells. Mucosal immunization would induce not only secretory IgA, but it would also generate more systemic antibody response and cell-mediated immunity against the particular protein antigen when co-administered with the potential protein antigens mentioned previously (Freytag and Clements 2005; Sanchez and Holmgren 2008).

CT has both stimulatory and inhibitory effects on cytokine production. It enhances the secretion of IL-6 in intestinal epithelial cells and IL-1β, IL-6, and IL-10 in dendritic cells. IL-1β in turn induces maturation of dendritic cells. IL-1β has been shown previously to work as a mucosal adjuvant when co-administered with other protein antigens. IL-12, TNF-α and NO productions are inhibited. CT can abrogate the oral tolerance induction by oral antigen administration thereby boosting the immunogenicity to that antigen (Freytag and Clements 2005; Sanchez and Holmgren 2008).

CT also induces depletion of $CD8^+$ intraepithelial lymphocytes and $CD4^+$ Th1 cells in cAMP-dependent induction of apoptosis. The induction of apoptosis seems to be a consequent of the binding of CT-B subunit to the GM1 on the cell surface of $CD4^+$ or $CD8^+$ T cells. In the APC-$CD4^+$ T cell interaction, it has been shown that the CT-B subunit inhibits the TCR-CD3 signaling complex resulting in inhibited proliferation and induced apoptosis of $CD4^+$ T cells via cAMP-dependent manner (Freytag and Clements 2005; Sanchez and Holmgren 2008).

In mouse models, CT can induce antigen-specific $CD4^+$ Th2 cell responses characterized by high production of IL-4, IL-5, IL-6 and IL-10 and suppress IL-12 and IFN-γ production. These cytokines enhance the production of antigen-specific secretory

IgA and serum IgG1 production. However, other studies had showed mixed results with both inductions of IFN-γ and IL-4 following nasal and intranasal immunization with CT. Lee et al. showed that CT delivered intranasally is able to induce Th17 differentiation where the CT-B subunit is responsible for the observed effect (Freytag and Clements 2005; Lee, Jang et al. 2009). Another group showed that CT also promotes the generation of Tregs. Oral administration of the antigen coupled to CT-B subunit efficiently induces peripheral tolerance through the generation of $CD25^{+}CD4^{+}$ Treg in the Peyer's patches, mesenteric lymph nodes, and, to a lesser extent, in spleen due to increased secretion of TGF-β. $CD25^{+}CD4^{+}$ Treg generated also sufficiently suppressed the effector T cell proliferation and IL-2 production in vitro (Sun, Raghavan et al. 2006). However, due to its toxicity in humans, its practice in clinical use is currently limited.

1.6. Hypothesis

A large proportion of $CD4^{+}$ IL-17-expressing cells, characterized STAT3-dependent expression of RORγt and production of IL-17A, IL-17F, IL-21 and IL-22, are resident of the intestinal lamina propria. In particular there is increasing number of Th17 cells in the colonic lamina propria corresponding to the increase in age (Atarashi, Nishimura et al. 2008). In light of the mucosal adjuvant activities of CT, and the role of Th17 cells at mucosal sites, we hypothesized that CT induces Th17 differentiation to partly mediate its adjuvant property. Since DC initiate antigen-specific T cell responses, we further hypothesized that CT acts on DC to drive differentiation of naïve T cells. Here we showed that CT activates dendritic cells (DCs) by cAMP-dependent mechanisms to drive the differentiation of naïve T cells into IL-17-producing Th17 cells

in vitro and *in vivo*. This differentiation depends on CT-induced secretion of β-calcitonin gene-related peptide (β-CGRP) by DC, but is independent of IL-6, previously shown to be required for differentiation of Th17 cells, a subset of $CD4^+$ T cells that play an important role in autoimmunity as well as in the control of certain extracellular pathogens. This IL-17/IL-22-producing Th17 subset may mediate its protective response at the mucosal site by stimulating the production of antimicrobial peptides and secretory IgA.

2. Methods

2.1. Reagents

HPLC-purified OVA was purchased from Worthington Biochemicals (Lakewood, New Jersey). CT and *Escherichia coli* heat-labile toxin were purchased from List Biological Laboratories (Campbell, California). Forskolin, 8-Br-cAMP, IBMX, 8-CPT-2'-O-Me-cAMP, and 6-Bnz-cAMP were purchased from EMD Biosciences (San Diego, California). H89 was purchased from Sigma-Aldrich (St. Louis, Missouri). Antibodies against mouse CD3 (clone 145 2C11) and CD28 (clone 37.51) were purchased from eBioscience (San Diego, California). Neutralizing antibodies against mouse IL-1 (clone B122) and IL-6 (clone MP5-20F3) were purchased from eBioscience. Neutralizing antibodies against mouse IL-4 (clone 11B11) and IFN-g (clone XMG1.2) were purchased from BioXCell (West Lebanon, New Hampshire). Recombinant mouse IL-6 was purchased from eBioscience, and recombinant human TGF-b1 was purchased from Peprotech (Rocky Hill, New Jersey). b-CGRP and $CGRP_{8-37}$ were purchased from Phoenix Pharmaceuticals (Burlingame, California).

2.2. Animals

C57BL/6 mice were purchased from Harlan-Sprague-Dawley (Indianapolis, Indiana). OT-II and *Il6*$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). OT-II *Rag1*$^{-/-}$ mice were purchased through the NIAID Exchange Program from Taconic Farms, Inc. (Hudson, New York). *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice were a gift from S. Akira (Osaka University, Osaka, Japan). *Nod2*$^{-/-}$ and *Caspase1*$^{-/-}$ mice were a gift

from R. Flavell (Yale University, Newhaven, Connecticut). All animal procedures followed University of California, San Diego and NIH/NIAID animal care guidelines.

2.3. ELISA

ELISA analysis was performed according to the manufacturer's instructions for the following molecules: cAMP (EMD Biosciences); IL-4, IL-5, IL-10, IFN-g (BD Biosciences); IL-17A (eBioscience); IL-21 (R&D Systems, Minneapolis, Minnesota); and IL-22 (Antigenix America, Huntington Station, New York).

2.4. Immunization

C57BL/6 mice (8/group) were immunized on day 0 and day 14 by gavage with PBS, OVA (200 μg), or CT (20 μg) + OVA (200 μg) in 0.1 M sodium bicarbonate. On day 39, the mice were challenged with OVA (200 μg i.g. + 50 μg i.v.), and sacrificed on day 42. Total cells from spleens and mesenteric lymph nodes were restimulated *ex vivo* with media or OVA (200 μg/ml) for three days, and cytokine content in the supernatants was analyzed by ELISA.

2.5. *In vitro* T cell differentiation

To induce T cell differentiation by DC, mouse bone marrow progenitor cells were cultured for six days in the presence of GM-CSF (BD Biosciences) to obtain myeloid DC as previously described (Datta, Okamoto et al. 2006). The DC were incubated overnight with OVA (10 μg/ml) and indicated stimuli as described in the text. Briefly, both CT and *Escherichia coli* heat-labile toxin (HLT) were given at 1 μg/ml. Total or $CD62L^{high}$ $CD4^{+}$ T cells from OT-II mouse spleens were then isolated using MACS beads and

columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, California). $CD62L^{high}$ selection typically yielded a population of $CD4^+$ cells that were 85-99% $CD62L^{high}$; approximately 97% of these cells were $CD44^{low}$ (data not shown). The T cells were then incubated with the DC for six days. In some instances, neutralizing antibodies (10 μg/ml) to indicated cytokines were added 30 minutes prior to addition of the T cells. After six days, the T cells were restimulated with anti-CD3 (10 μg/ml plate-bound) and anti-CD28 (1 μg/ml soluble). For intracellular cytokine analysis, GolgiStop (BD Biosciences) was added after two hours of restimulation. After four additional hours, the cells were stained for the indicated cytokines according to the manufacturer's instructions prior to data acquisition on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences). Data were analyzed with Flow Jo software (Treestar, Ashland, Oregon). For ELISA analysis, the cells were restimulated for 24 hours before supernatants were collected for cytokine analysis.

To show inhibition using chemical reagents, in some instances, DC were treated with or without H89 (10 μM) during their 3-hour incubation with media, OVA, and/or CT. The DC were then fixed with 2% buffered formalin without methanol (Sigma-Aldrich) and incubated in their original media with OT-II CD4+ T cells before analysis of the T cell supernatants as described previously.

To induce T cell differentiation in a DC-free system, $CD4^+$ T cells isolated from spleens of C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 as described above in the absence or presence of anti-IL-4 (10 μg/ml), anti-IFN-g (10 μg/ml), IL-6 (20

ng/ml), TGF-b1 (3 ng/ml), and/or CT-CM. After four days, the T cells were restimulated with anti-CD3 and anti-CD28 prior to assessing cytokine production as described above.

2.6. Microarray Analysis

DC were incubated in duplicate for two hours with media, CT (1 μg/ml), or forskolin (10 μM). Five micrograms of total RNA per treatment per sample were analyzed on Affymetrix mouse MU430 2.0 gene chips by the Microarray Core at the University of California, San Diego. The raw expression values were imported into VAMPIRE without prior normalization. This program uses a Bayesian approach to identify altered genes. Statistical analysis by VAMPIRE requires two distinct steps: modeling of the error structure of sample groups and significance testing with *a priori*-defined significance thresholds. VAMPIRE models the existing error structure to distinguish signal from noise and identify the coefficients of expression-dependent and expression-independent variance. These models are then used to identify microarray features that are differentially expressed between treatment groups. We compared cells treated with CT or forskolin against untreated controls to generate sets of genes that change with each treatment. Bonferroni multiple testing correction ($\alpha_{Bonf} < 0.05$) was applied to identify only those genes with the most robust changes. The overlap of these gene sets was used to define CT-sensitive genes that were also cAMP-dependent (Suppl. Fig. 7 and Suppl. Tables 1-2).

2.7. Isolation of RNA and Quantitative RT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. After isolation, RNA was treated with deoxyribonuclease I (DNase I) (Invitrogen, Carlsbad, CA) to digest contaminating DNA. One microgram of RNA was then used for reverse transcription and synthesis of cDNA using Superscript III First-Strand system (Invitrogen). Quantitative real-time PCR was performed on an AB7300 (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems). GAPDH expression was used as an internal reference in all the PCR experiments. Primers (Suppl. Table 3) for specific target genes were designed based on their reported sequences and synthesized by IDT Technologies (San Diego, CA).

2.8. Statistics

ANOVA with Bonferoni post-test analysis for comparison of means was performed with Prism 5 for Macintosh (GraphPad Software, San Diego, California).

This chapter, in full, is currently being prepared for submission for publication of the material. Sandip K. Datta is the primary investigator and author of this material. Other co-authors include Jose Gonzalez-Navajas, Ivan Mihajlov, Paul Insel, Nicholas Webster, and Eyal Raz.

3. Results

3.1. Cholera Toxin Induces Th17 cells *in vivo* After Mucosal Immunization

In light of the mucosal adjuvant activities of CT and the role of Th17 cells at mucosal sites, we hypothesized that CT induces Th17 differentiation. Since DC initiate antigen-specific T cell response, we further hypothesized that CT acts on DC to drive differentiation of naïve T cells. To test this hypothesis *in vivo*, we immunized mice by delivering OVA or CT+OVA intragastrically and OVA-specific cytokine responses in the spleens and mesenteric lymph nodes were assessed. Mice immunized with CT+OVA showed an OVA-specific IL-17 response in both the spleens and mesenteric lymph nodes but no significant enhancement in IL-5 or IFNγ production compared with mice immunized with OVA alone. Interestingly, mice immunized with CT+OVA also induced OVA-specific IL-22 response but not a detectable IL-21 response (Figure 3.1.1).

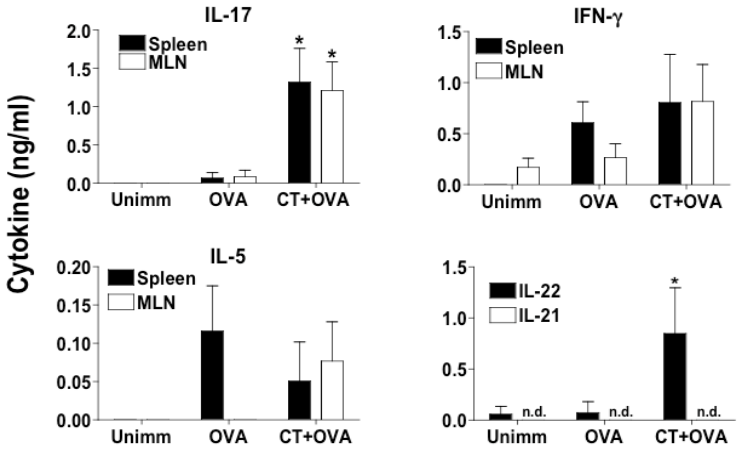


Figure 3.1.1: Cholera toxin induces Th17 cells *in vivo* after mucosal immunization.

3.2. Cholera Toxin-treated Dendritic Cells Induce Th17 Cells *in vitro*

We showed previously that CT induces OVA-specific Th17 response *in vivo*, here we wanted to confirm the effects of CT on T cell differentiation *in vitro*. We incubated C57BL/6 mouse bone marrow-derived DC with the antigen ovalbumin (van Beelen, Zelinkova et al.) with or without CT. After overnight incubation, we added OVA-specific splenic $CD4^+$ T cells from OT-II mice. After 6 days of co-culture, we restimulated the T cells with anti-CD3 and anti-CD28, and assessed the differentiation of these T cells by measuring their cytokine profile using flow cytometry.

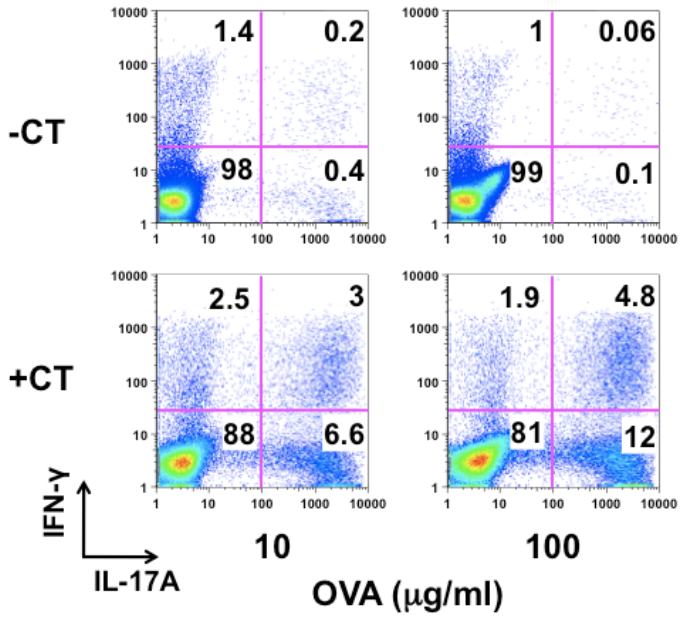


Figure 3.2.1: Cholera toxin-treated dendritic cells induce Th17 cells.

Compared with untreated DC, CT-treated DC induces a 120-fold increase in the frequency of IL-17-expressing CD4 T cells at optimal concentration of CT and OVA. In contrast, there is less than 2-fold increase in the frequency of cells producing IFN-γ. Interestingly, there is an 80-fold increase in frequency of cells that stained positive for both IFN-γ and IL-17, a subset whose function remains unclear (McGeachy and Cua 2008). There was no increase in the frequency of IL-4-, IL-5-, or IL-10-secreting cells (need pictures), but there was a 3-fold increase in the frequency of Foxp3^{+} regulatory T cells (Figure 3.2.1).

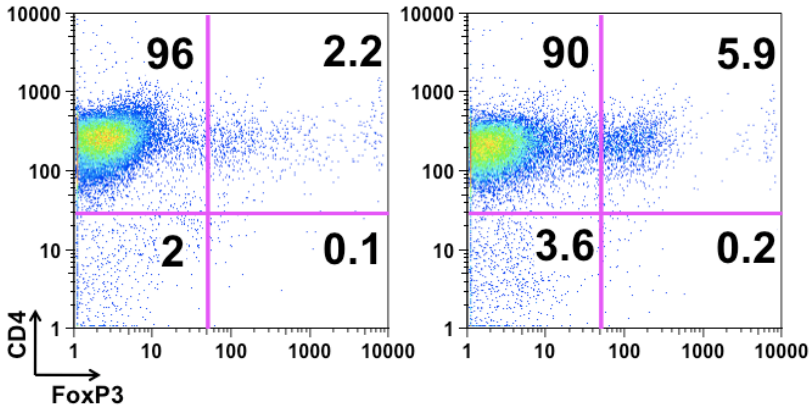


Figure 3.2.2: Cholera toxin-treated dendritic cells induce FoxP3^{+} cells.

The supernatants collected 24 hours after restimulation of similarly treated OT-II CD4^{+} T cells also showed that CT induced IL-17 and Th17-associated cytokine IL-22, but not IFN-γ, IL-5, IL-10, or IL-21 (Figure 3.2.1). Despite the presence of IL-17/IFN-γ double-positive cells by intracellular staining, secretion of IFN-γ into the supernatant was inhibited by CT, consistent with its known Th1-inhibitory effects (Freytag and Clements 2005).

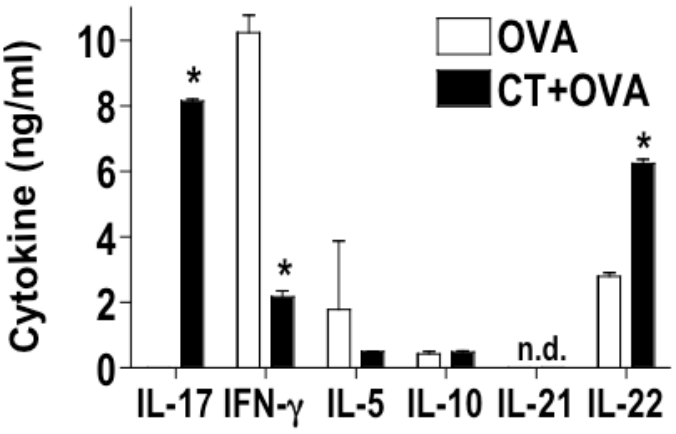


Figure 3.2.3: Cholera toxin induces Th17-associated cytokines.

3.3. CT-treated DC Induced Differentiation in Naïve T cells.

CT-treated DC also drove the differentiation of $CD62L^{high}$-selected naïve $CD4^+$ T cells into IL-17-secreting cells (Figure 3.2.1).

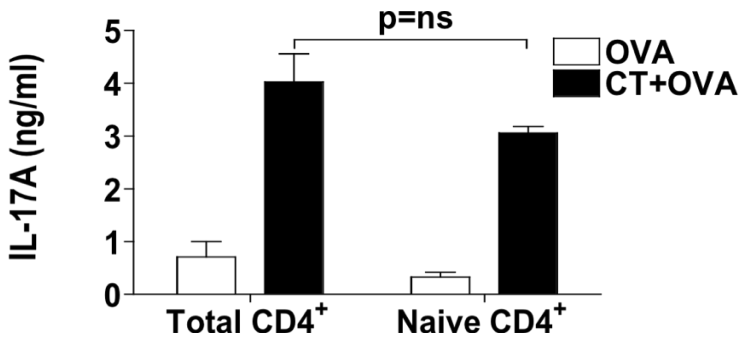


Figure 3.3.1: CT-treated DC Induced the Differentiation of Naïve CD4+ T cells.

Furthermore, $CD4^+$ T cells from OT-II *Rag1-/-* mice, composed exclusively of OVA-specific naïve T cells, differentiated into IL-17-secreting cells upon incubation with CT-treated DC (data not shown).

Since CT was present when T cells were added in the experiments above, we evaluated whether CT exerts its effects on T cell differentiation by acting via DC or directly on T cells. Treatment of T cells with CT during stimulation by formalin-fixed OVA-pulsed DC did not induce IL-17. In contrast, OVA-pulsed DC treated with CT prior to fixation, and then incubated in their original CT-conditioned media with T cells, induced IL-17. Thus, CT does not act directly on T cells but instead via DC to induce T cell differentiation (Figure 3.2.1).

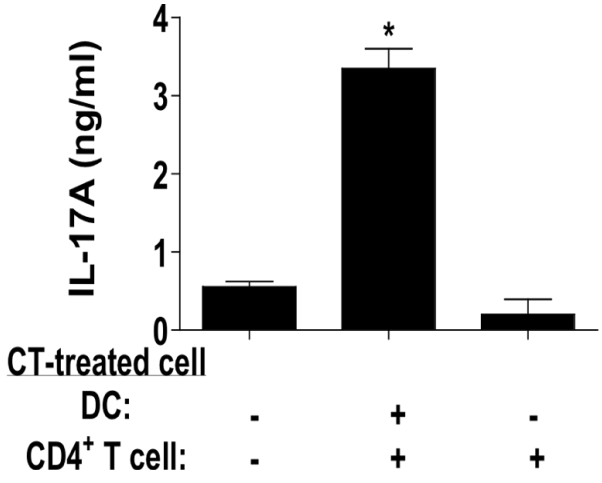


Figure 3.3.2: CT Acts Directly on DC to Induce the Th17 Cell Differentiation.

3.4. Cholera Toxin Functions Independent of TLR and NLR Pathways

Upon stimulation with various microbial agents (pathogen-associated molecular patterns), that activate TLRs or dectin receptors, DCs secrete IL-12p70, IL-23, or IL-27 to develop the type of immunity. TLR4 stimulation by LPS induces p19, p35, and p40. TLR2 by peptidoglycan induces IL-23 but not p35. $Pam_3CysSerLys_4$/TLR2 stimulation results in high level of IL-23. Peptidoglycan can also signal through NOD2 to induce the expression of IL-23. TLR3 stimulation by polyiosinic-polycytidylic acid (polyI:C) induces DCs to secrete both IL-12p70 and IL-27. IL-27 has STAT1-dependent inhibitory effects on the differentiation of Th17 cells (Korn, Bettelli et al. 2009).

Toll-like receptor (TLR)-4 and Nod2 have been shown to induce the IL-17 production in naïve and memory T cells, respectively. DC from Nod2-/- and MyD88-/- mice retained the ability to induce Th17 cells after exposure to CT, indicating that these pathways are not necessary for CT-induced Th17 differentiation (Figure 3.4.1). Intact Th17 induction by *Tlr2-/-* and *Tlr4-/-* DC further confirmed TLR-independence (data not shown).

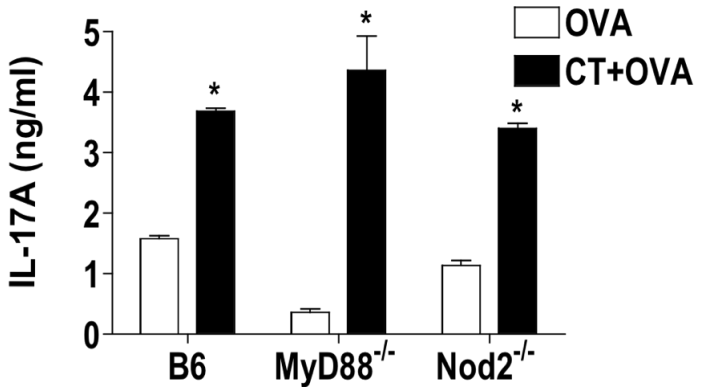


Figure 3.4.1: Cholera toxin induces Th17 differentiation independent of TLRs and NLRs

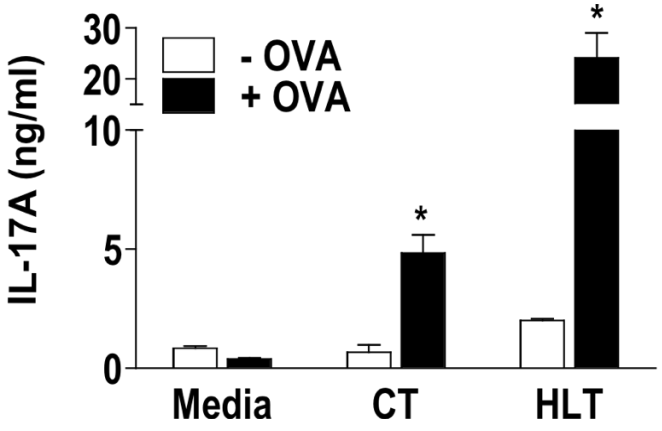


Figure 3.4.2: *E. coli* heat-labile toxin also induced Th17 cell differentiation

DC treated with *Escherichia coli* heat-labile toxin, a closely related adjuvant to CT that has similar structure and similar activities, also induced Th17 cells (Figure 3.4.1

3.5. Cholera Toxin Induces Th17 Cells via cAMP.

CT has been known to function mainly through the induction of cAMP. To determine if CT also employs similar cAMP mechanism to induce the differentiation of Th17, we first determined the level of cAMP induction in dendritic cells. ELISA of

cellular lysates from DC treated for 3 hours with either CT or forskolin (10 μM), an activator of adenylyl cyclase, confirmed that both agents increase cAMP levels in DC (Figure 3.5.1).

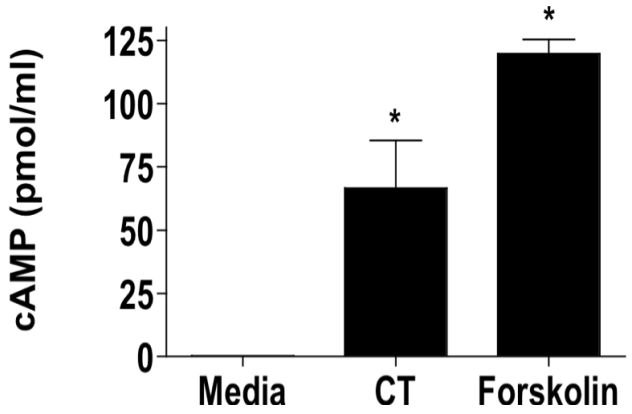


Figure 3.5.1: CT induces intracellular cAMP in DCs.

Treatment of DCs with a cell-permeable cAMP analogue (8-Br-cAMP at 500 μM), or with agents that increase cellular cAMP (e.g. forskolin at 10 μM or IBMX, a phosphodiesterase inhibitor at 100 μM), mimicked the ability of CT to induce Th17 cells (Figure 3.5.2).

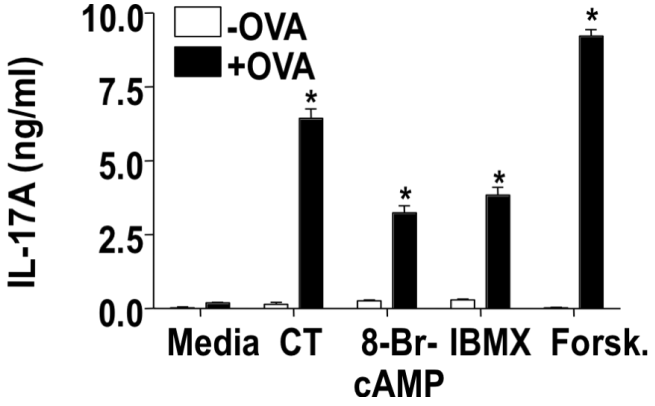


Figure 3.5.2: Cell-permeable cAMP analogue mimicked effects of CT.

N6-benzoyl-cAMP, an activator of cAMP-dependent protein kinase (PKA) at 500 μM, the major downstream effector of cAMP (Walsh, Perkins et al. 1968), also induced Th17 differentiation capabilities in DC. However, activation of Epac (Exchange protein activated by cAMP), at 500 μM, involved in an alternative cAMP signaling pathway (de Rooij, Zwartkruis et al. 1998); (Kawasaki, Springett et al. 1998), with 8-CPT-2'-O-Me-cAMP failed to generate Th17 cells.

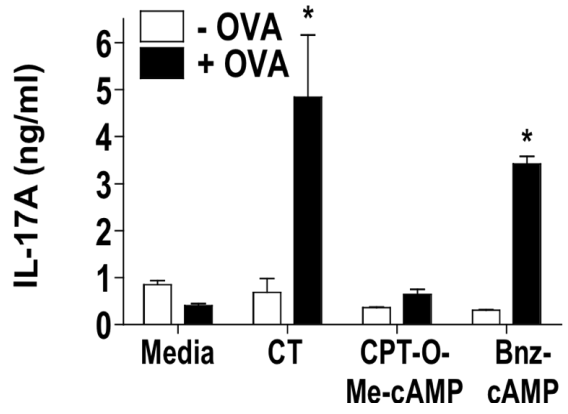


Figure 3.5.3: PKA mediates Th17 differentiation.

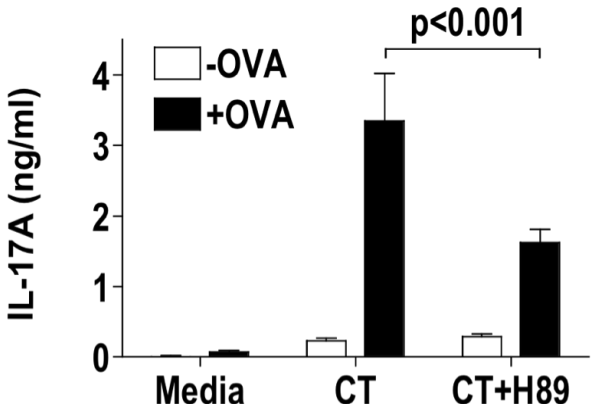


Figure 3.5.4: PKA mediates the differentiation of Th17 cells.

Treatment of DC with the PKA inhibitor H89 prior to incubation with CT blunted the ability of CT to induce Th17 cells. Taken together, these data imply that CT induces the Th17 differentiation program in DC by PKA-dependent pathways.

3.6. Cholera toxin-treated dendritic cells induce Th17 cells by an IL-6-independent pathway

We have determined that the action of CT is mainly mediated through dendritic cells. The question remains whether naïve T cells need the costimulatory molecules introduced by DC following stimulation, the various factors produced in the supernatant (or in more relevant physiological environment the factors or mediators secreted by DCs in the surrounding arena) or both to mediate the differentiation of Th17 cells. Fixation of DC after incubation with CT and OVA inhibited their ability to induce Th17 cells, indicating that cognate interaction was not sufficient. Adding back media from CT-treated DC (CT-conditioned media, CT-CM) to the fixed DC restored their ability to induce Th17 cells, implicating one or more secreted factors (Figure 3.6.1).

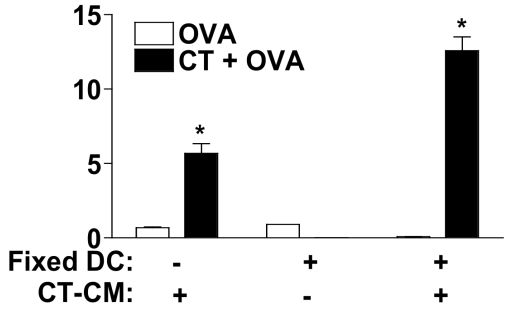


Figure 3.6.1: Co-stimulatory molecules are not sufficient to drive Th17 differentiation.

Addition of CT-CM to OVA-pulsed DC (data not shown), or directly to T cells stimulated with anti-CD3 and anti-CD28 in the presence of neutralizing antibodies to IL-4 and IFN-γ (DC-free system), showed that secreted factors were sufficient to induce Th17 cells (Figure 3.6.1). Consistent with prior reports (Bettelli, Carrier et al. 2006); (Mangan, Harrington et al. 2006); (Veldhoen, Hocking et al. 2006), addition of IL-6 and TGF-β to this DC-free system also generated IL-17.

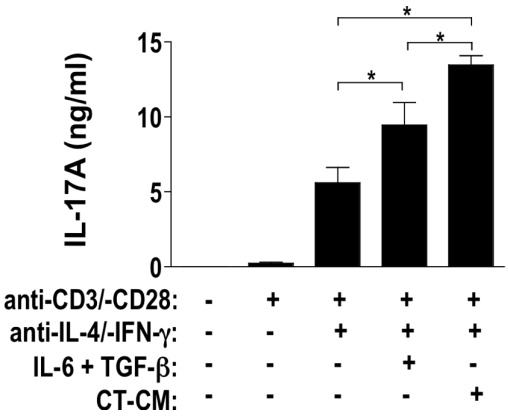


Figure 3.6.2: Secreting factors are sufficient to drive Th17 differentiation.

Analysis of CT-treated DC by RT-PCR and ELISA showed that among a panel of cytokines involved in T cell differentiation, only low levels of IL-6 and IL-1β were induced (Figure 3.6.1).

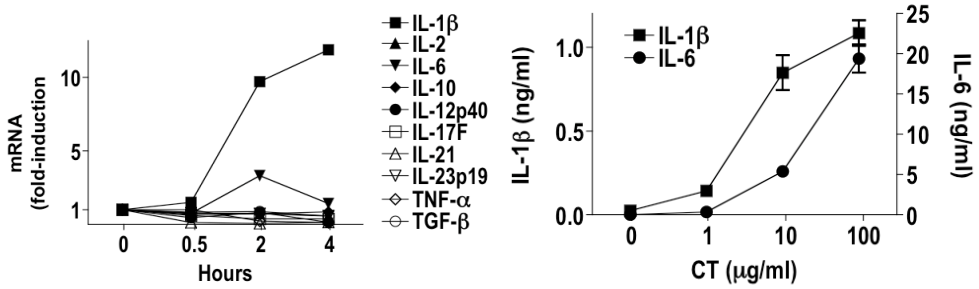


Figure 3.6.3: CT-treated DC expressed IL-6 and IL-1β.

However, DC from mice deficient in either IL-6 or Caspase-1, which is required for processing IL-1β into its active form, were fully able to induce Th17 cells (Figure 3.6.1).

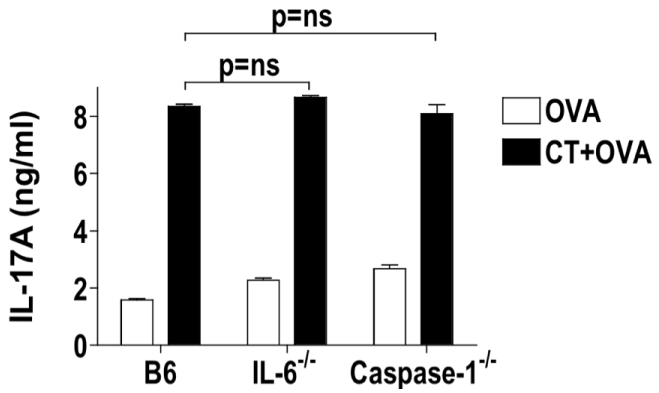


Figure 3.6.4: Th17 differentiation is independent of IL-6 and IL-1β.

Neutralizing antibodies against IL-6 and/or IL-1β did not inhibit CT-induced Th17 differentiation (Figure 3.6.1).

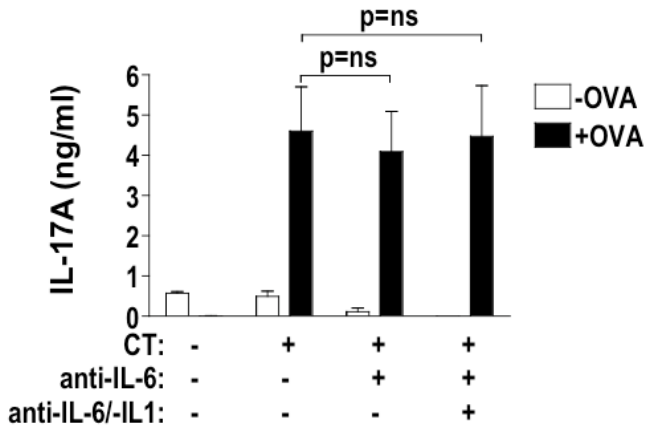
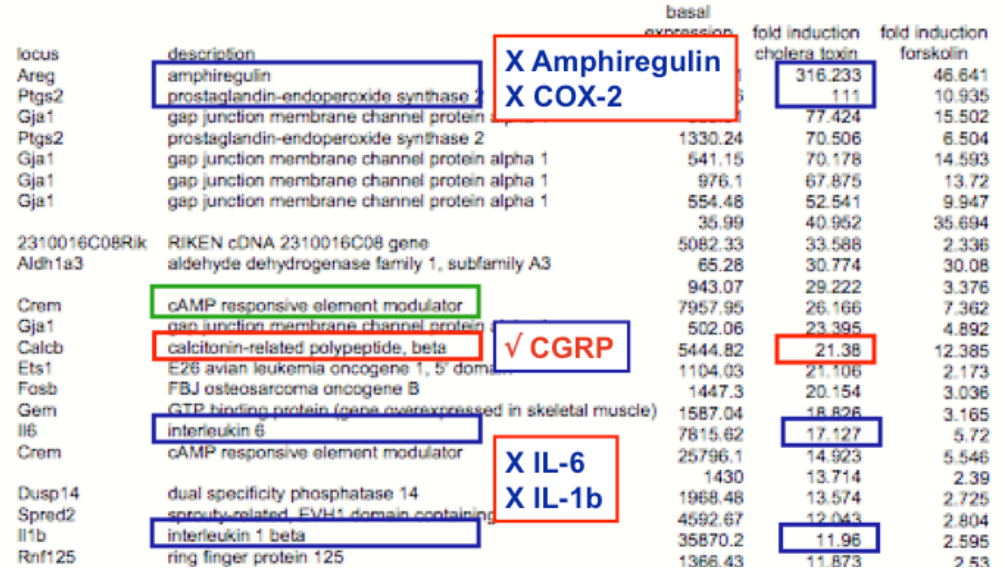


Figure 3.6.5: Th17 differentiation is independent of IL-6 and IL-1β using neutralizing antibodies.

To identify pathways involved in CT-induced Th17 differentiation, we performed a microarray-based gene expression analysis on DC to detect transcripts induced after 2 hours exposure to CT or forskolin, a time sufficient to induce Th17-differentiating capacity (data not shown). Based on our data invoking a cAMP-dependent mechanism, we focused on secreted gene products that were induced by both CT and forskolin (data not shown). We assayed Th17 differentiation by directly activating C57BL/6 CD4+ T cells in the DC-free system described above.



locus	description	basal expression	fold induction cholera toxin	fold induction forskolin
Areg	amphiregulin		316.233	46.641
Ptgs2	prostaglandin-endoperoxide synthase 2		111	10.935
Gja1	gap junction membrane channel protein		77.424	15.502
Ptgs2	prostaglandin-endoperoxide synthase 2	1330.24	70.506	6.504
Gja1	gap junction membrane channel protein alpha 1	541.15	70.178	14.593
Gja1	gap junction membrane channel protein alpha 1	976.1	67.875	13.72
Gja1	gap junction membrane channel protein alpha 1	554.48	52.541	9.947
		35.99	40.952	35.694
2310016C08Rik	RIKEN cDNA 2310016C08 gene	5082.33	33.588	2.336
Aldh1a3	aldehyde dehydrogenase family 1, subfamily A3	65.28	30.774	30.08
		943.07	29.222	3.376
Crem	cAMP responsive element modulator	7957.95	26.166	7.362
Gja1	gap junction membrane channel protein	502.06	23.395	4.892
Calcb	calcitonin-related polypeptide, beta	5444.82	21.38	12.385
Ets1	E26 avian leukemia oncogene 1, 5' dom	1104.03	21.106	2.173
Fosb	FBJ osteosarcoma oncogene B	1447.3	20.154	3.036
Gem	GTP binding protein (gene overexpressed in skeletal muscle)	1587.04	18.826	3.165
Il6	interleukin 6	7815.62	17.127	5.72
Crem	cAMP responsive element modulator	25796.1	14.923	5.546
		1430	13.714	2.39
Dusp14	dual specificity phosphatase 14	1968.48	13.574	2.725
Spred2	sprouty-related, EVH1 domain containing	4592.67	12.043	2.804
Il1b	interleukin 1 beta	35870.2	11.96	2.595
Rnf125	ring finger protein 125	1366.43	11.873	2.53

Figure 3.6.6: Genes regulated in CT- and Forskolin-stimulated DC.

Amphiregulin and prostaglandin endoperoxide synthase-2 (also known as cyclooxygenase-2, or COX-2) were highly induced on microarray analysis (Figure 3.6.1) (only part of the gene chip data is shown). Amphiregulin was detected in Th2 subset of T cells that aid in helminth parasite removal (Zaiss, Yang et al. 2006). However, in general, the role of amphiregulin on T cell differentiation remains largely unknown.

Amphiregulin receptor antibodies or downstream kinase inhibitors of amphiregulin signaling failed to inhibit Th17-differentiating activities of CT-CM (data not shown).

PGs are small lipid mediators that are generated from arachidonic acid by COX. Exposure to PGE2 has been shown to induce Foxp3 expression in $CD4^{+}CD25^{-}$ T cells and a suppressive regulatory phenotype (Bryn, Yaqub et al. 2008). PGE2 is produced by a variety of cells in the GI mucosa and is found throughout the gut. PGE2 involves in the regulation of gastric acid secretion, GI motility, bicarbonate secretion and mucus secretion. It is also implicated in the pathophysiology of inflammatory bowel disease and colorectal neoplasia (Dey, Lejeune et al. 2006).

PGE2 is a key mediator of pyrexia, hyperalgesia, and arterial dilation, which increases blood flow to inflamed tissues and, in combination with enhanced microvascular permeability, results in edema (Boniface, Bak-Jensen et al. 2009). Prostaglandins, particularly PGE2, which is found at high concentration inflammatory foci, play a complex role in inflammation. Although in vitro PGE2 down-regulates the expression of proinflammatory cytokines/chemokines from activated dendritic cells, in autoimmune diseases, PGE2 has been implicated as mostly a proinflammatory agent. Cyclooxygenase 2 (COX-2) and microsomal PGE synthase 1 (mPGES-1), both of which are enzymes involved in PGE2 generation, are highly expressed in patients with rheumatoid arthritis. Studies indicated that PGE2 enhances DC-derived IL-6 production and induces a shift in the IL-23/IL-12 balance in favor or IL-23, resulting in increased IL-17 production, presumably through the amplification of self-reactive Th17 cells. In addition, PGE2 also induces both IL-6 and IL-1β expression in DCs (Sheibanie,

Khayrullina et al. 2007). In another study, PGE2 was shown to act directly on both human and murine T cells to enhance Th17 development and effector cytokine production. In human T cells, PGE2 acts via the prostaglandin receptor EP2- and EP4-mediated signaling and cAMP pathways to up-regulate IL-23 and IL-1 receptor expression. PGE2 also synergizes with IL-1β and IL-23 to drive RORγt, IL-17, IL-17F, CCL20, and CCR6 expression, which is consistent with the previously reported Th17 phenotype. PGE2 down-modulates the expression of IFN-γ and inhibits the production of anti-inflammatory cytokine IL-10 in TH17 cells predominantly through EP4 (Boniface, Bak-Jensen et al. 2009).

However, CT-CM from *Cox-2-/-* DC, or DC treated with COX inhibitors prior to stimulation with CT, also promoted Th17 differentiation (data not shown). These data made amphiregulin and prostaglandins unlikely mediators of CT-induced Th17 differentiation.

Microarray analysis also revealed induction of calcitonin-related polypeptide beta (also known as β-calcitonin gene-related peptide, β-CGRP, a result confirmed by RT-PCR (Figure 3.6.1).

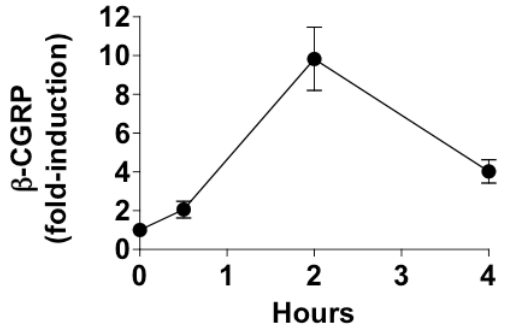


Figure 3.6.7: CT- and Forskolin-stimulated DC generated β-CGRP.

Addition of $CGRP_{8-37}$, a competitive antagonist of full-length β-CGRP, at 0.1 μM, inhibited the ability of CT-CM to induce Th17 cells but had no effect on generation of Th17 cells by IL-6 and TGF-β. Addition of full-length β-CGRP (1.0 μM) to T cells did not elicit Th17 differentiation, indicating that β-CGRP is necessary but not sufficient to induce Th17 differentiation by CT (Figure 3.6.1).

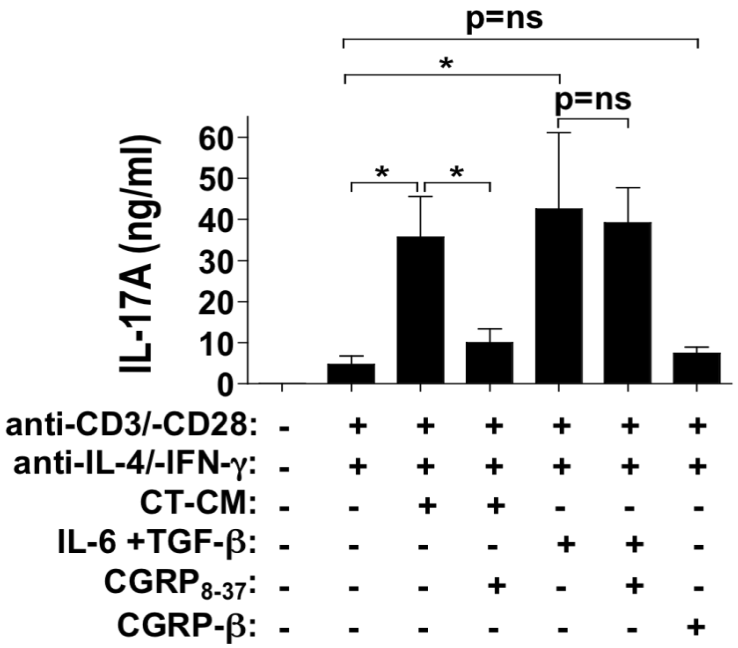


Figure 3.6.8: β-CGRP is necessary for the differentiation of Th17 cells.

Since β-CGRP acts via G protein-coupled receptors to induce cAMP (Conner, Simms et al. 2007), we assessed the effect of cAMP induction in T cells. Addition of 8-Br-cAMP, IBMX, forskolin, N6-benzoyl-cAMP, or CT directly to T cells did not generate Th17 cells (need slides). However, addition of the PKA inhibitor H89 (at 0.1 μM) to T cells inhibited the ability of CT-CM to generate Th17 cells, suggesting cAMP-dependent PKA activation in T cells is necessary but not sufficient to drive CT-mediated Th17 differentiation (Figure 3.6.1).

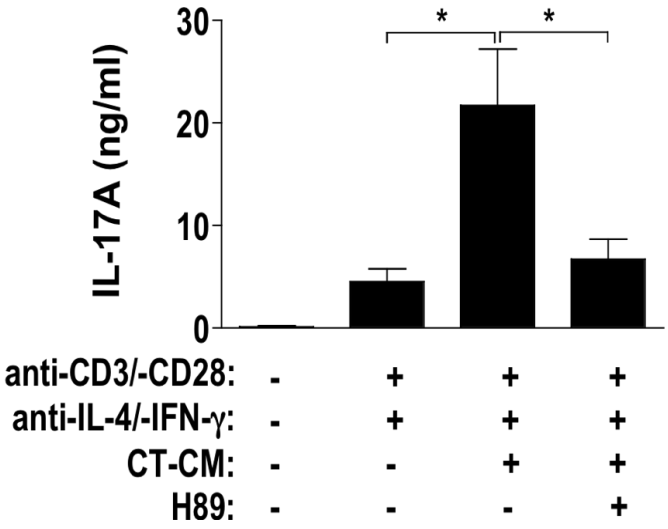


Figure 3.6.9: Th17 differentiation also requires cAMP-dependent pathways in T cells.

3.7. CT-induced Th17 cells delineate from canonical Th17 cells.

Similar to IL-6 and TGF-β, CT-CM induced T cells to express the Th17-defining transcription factor RORγt (Ivanov, McKenzie et al. 2006). However, CT-CM induced higher levels of IRF4, a transcription factor associated with Th2 and Th17 differentiation (Brustle, Heink et al. 2007); lower levels of IL-21, an autocrine cytokine produced by Th17 cells; and higher levels of IL-22, a Th17 cytokine involved in mucosal immune responses (Figure 3.6.1) (Liang, Tan et al. 2006); (Zheng, Danilenko et al. 2007) (Aujla, Chan et al. 2008; Zheng, Valdez et al. 2008).

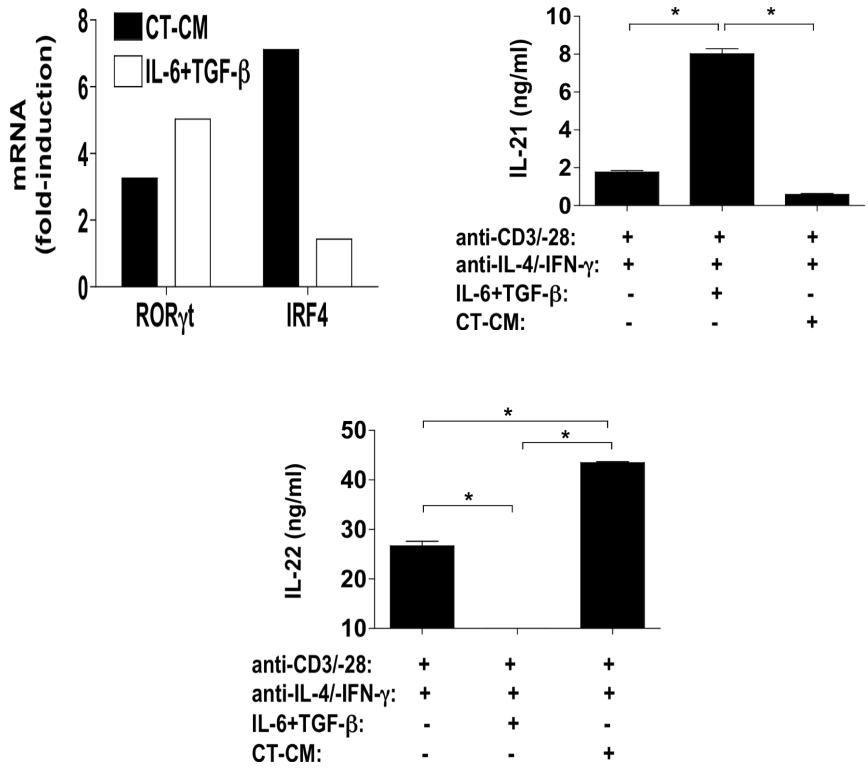


Figure 3.7.1: 1 CT-induced Th17 cells different from IL-6/TGFb-induced Th17 cells.

This chapter, in part, is currently being prepared for submission for publication of the material. Sandip K. Datta is the primary investigator and author of this material. Other co-authors include Jose Gonzalez-Navajas, Ivan Mihajlov, Paul Insel, Nicholas Webster, and Eyal Raz.

4. Discussion

Here we showed that CT activates dendritic cells by cAMP-dependent mechanisms to drive differentiation of naïve T cells into IL-17-producing Th17 cells *in vitro* and *in vivo.* This differentiation depends on CT-induced secretion of β-calcitonin gene-related peptide (β-CGRP) by dendritic cells, but is independent of IL-6, previously shown to be required for differentiation of Th17 cells, a subset of CD4+ T cells that play an important role in autoimmunity as well as in the control of certain extracellular pathogens. The involvement of cAMP and β-CGRP, a neuropeptide, identifies a potential role for these signaling pathways in determining T cell differentiation at nerve-rich mucosal sites, such as the gut, which are crucial for host defense. These insights into the mechanisms controlling differentiation of this important subset of T cells reveal new potential targets for the beneficial modulation of immune responses involved in infection and autoimmunity.

CT is often classified as a Th2 adjuvant because it induces an antibody response and inhibits a Th1 response. However, the effect of CT on Th2 cytokine production is less consistent (Holmgren, Adamsson et al. 2005). In three distinct experimental system involving *in vivo* immunization and both APC- and antibody-mediated TCR activation *in vitro*, we have identified Th17 induction as a potentially important component of mucosal adjuvant effects of CT.

The β-CGRP-/PKA-mediated events are alternative to the IL-6 and TGF-β paradigm of Th17 differentiation, and are a novel addition to the recent identification of IL-6-dependent and –independent mechanisms for Th17 differentiation that involve

molecules such as aryl hydrocarbon receptor (AHR), ATP, and vasoactive intestinal peptide (VIP) (Kimura, Naka et al. 2007) (Quintana, Basso et al. 2008) (Yadav, Rosenbaum et al. 2008). A recent report also notes that c-Kit can influence Th2 and Th17 differentiation by regulating the DC IL-6 response to CT and other agents. The dual upregulation of c-Kit and membrane-bound SCF, c-Kit ligand or stem cell factor, in DCs causing sustained activation of the c-Kit-PI3 kinase signaling axis, is crucial for promoting IL-6 production. Defective c-Kit signaling, associated with reduced IL-6 production, compromises the ability of DCs to mount a robust Th2 and Th17 response (Krishnamoorthy, Oriss et al. 2008).

The involvement of neuropeptides, such as β-CGRP and VIP, implies an intriguing role for these molecules in T cell physiology at richly innervated locations such as the gut mucosa, an important site for immune education and host defense. The recent identification of Th2-inducing activities of β-CGRP on skin Langerhans cells provides additional evidence for its role in T cell differentiation (Ding, Stohl et al. 2008). Furthermore, our results implicate cAMP signaling, previously recognized to inhibit T cell responses (Skalhegg, Funderud et al. 2005), in the regulation of T cell differentiation under homeostatic conditions and in the context of infection and autoimmune diseases. Since ATP is the substrate for cAMP generation, cAMP may also be involved in the recently identified ability of bacterially-derived ATP to drive Th17 responses in the gut (Quintana, Basso et al. 2008). The differences that we and others find in the cytokine profiles of cells generated under different Th17-inducing conditions suggest discrete effector functions of these cells depending on the context of differentiation.

The microenvironment during antigen presentation drives T cell differentiation. Interactions of antigen presenting cells with microbial compounds largely shape this microenvironment. Not surprisingly, TLR and NOD pathways of microbe recognition influence T cell differentiation and contribute to Th17 differentiation. Our findings with CT highlight the ability of a bacterial toxin to influence host immune responses beyond its contribution to bacterial virulence. Further studies will be needed to investigate if Th17 responses in the setting of cholera serve the host or the bacteria, and to uncover the factors that determine whether a Th17 response leads to appropriate control of infection, or precipitates an inflammatory cascade that leads to autoimmunity.

Emerging evidence suggests that Th17 cells also play key roles in mucosal host defense against pathogens. Studies done by Jaffar et al. demonstrated that $CD4^+$ Th17 cells, in addition to causing neutrophilic inflammation in mice, mediated a pronounced influx of $CD19^+$ B cells into the lungs following antigen inhalation. Coincident with this recruitment was a striking induction in pIgR expression by the bronchial epithelium and a subsequent increase in airway IgM and secretory IgA levels. IL-17 or IL-17F cooperates with IL-22 to enhance the production of antimicrobial peptides. Th17 cells can induce epithelial cells to express CXCL8 (IL-8) and CXCL5 (IL-5) to recruit neutrophils. Human Th17 cells produce CXCL13 to promote B cell migration. The majority of IgA that enters the mucosal secretions and the blood is produced at specifically adapted inductive sites in the intestine (Peyer's patch) and to a lesser extent in bronchus-associated lymphoid tissue of the respiratory tract. IL-17 was shown to up-regulate the

expression of basallateral pIgR in bronchial epithelial cells that mediate the transcytosis of IgA and IgM into the apical surface (Jaffar, Ferrini et al. 2009). Thus, CT induces Th17 differentiation. This IL-17/IL-22-producing Th17 subset can potentially mediate antimicrobial peptide secretion and secretory IgA production at the mucosal site to mediate its adjuvant effect.

This chapter, in part, is currently being prepared for submission for publication of the material. Sandip K. Datta is the primary investigator and author of this material. Other co-authors include Jose Gonzalez-Navajas, Ivan Mihajlov, Paul Insel, Nicholas Webster, and Eyal Raz.

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