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The *IL17A* and *IL17F* loci have divergent histone modifications and are differentially regulated by prostaglandin E2 in Th17 cells

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Abstract

Prostaglandin E2 (PGE2), IL-23 and IL-1 are implicated in inflammatory bowel disease susceptibility, likely in part by modulating IL-17 producing CD4⁺ T helper (Th17) cells. To better understand how these three mediators affect Th17 cell memory responses, we characterized the gene expression profiles of activated human peripheral CD4⁺ effector memory T cells and sorted Th17 memory cells from healthy donors concurrent with *IL17A* mRNA induction mediated by PGE2 and/or IL-23 plus IL-1. We discovered that PGE2 and IL-23 plus IL-1 differentially regulate Th17 cytokine expression and synergize to induce IL-17A, but not IL-17F. IL-23 plus IL-1 preferentially induce IL-17F expression. The addition of PGE2 to IL-23 plus IL-1 only enhances IL-17A expression as mediated by the PGE2 EP4 receptor, and promotes a switch from an IL-17F to an IL-17A predominant immune response. The human Th17 HuT-102 cell line was also found to constitutively express IL-17A, but not IL-17F. We went on to show that the *IL17A* and *IL17F* loci have divergent epigenetic architectures in unstimulated HuT-102 and primary Th17 cells and are poised for preferential expression of *IL17A*. We conclude that the chromatin for *IL17A* and *IL17F* are distinctly regulated, which may play an important role in mucosal health and disease.

Keywords

IL-17A; IL-17F; prostaglandin E2; Th17; chromatin

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Design of the study, acquisition, analysis and interpretation of data, drafting the article: J.A.; design of the study, acquisition, analysis and interpretation of data, critical review of the article: M.H.; analysis and interpretation of data, critical review of the article: A.R.; design of the study, analysis and interpretation of data, critical review of the article: P.E.A.; conception and design of the study, analysis and interpretation of data, critical review of the article: R.D.; conception and design of the study, analysis and interpretation of data, drafting the article: A.B.

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1. Introduction

IL-17 producing CD4⁺ T helper (Th17) cells have been implicated in inflammatory bowel disease (IBD) by a confluence of data derived from IBD mouse models and human genome wide association studies (GWAS) [1]. Th17 cells are normally involved in mucosal host defense against extracellular pathogens, but have been identified in a variety of immune-mediated diseases including IBD. Th17 cells drive T cell-mediated colitis in mice, and an excess of Th17 cells infiltrate chronically inflamed intestinal tissues in human IBD patients [2, 3].

Th17 immune responses are characterized by the production of the cytokines IL-17A, IL-17F, IL-21 and IL-22. Despite sharing 50% sequence identity and the same heterodimeric receptor complex (IL-17RA and IL-17RC) on target cells, IL-17A and IL-17F have distinct and sometimes seemingly opposing effects in inflammatory disease including in certain mouse models of IBD in which IL-17A is protective while IL-17F is pathogenic [4–7]. Accordingly, their unique gene regulation may be essential in modulating disease susceptibility, and multiple loci associated with IL-17 gene regulation have been identified in GWAS including the IL-23 receptor subunit encoding gene *IL23R* and the prostaglandin E2 (PGE2) EP4 receptor encoding gene *PTGER4*, two of the four most significant association identified in the Crohn's disease GWAS [8–10].

The *IL17A* and *IL17F* genes are located at the same chromosomal locus in both humans and mice and early studies suggested that IL-17F is expressed by almost all IL-17A-producing T cells [6], and that expression of *IL17A* and *IL17F* mRNA is likely coordinately regulated via chromatin remodeling at the *IL17A/IL17F* locus by various inflammatory mediators [11, 12]. More recent studies however have suggested that IL-17A and IL-17F expression is regulated differentially in disease states. For example, the transcription factor cAMP response element modulator alpha (CREM), which is overexpressed in the T cells of systemic lupus erythematosus (SLE) patients [13], induces epigenetic alterations in the *IL17A* promoter, thereby activating *IL17A* mRNA expression while concurrently repressing *IL17F* mRNA expression and creating an imbalance in the IL-17A/IL-17F ratio that may favor SLE [14, 15].

We and others have previously shown that three pro-inflammatory mediators found in excess in IBD [16–18], PGE2, IL-23 and IL-1, synergize to induce IL-17A production and expand Th17 cells [19–21]. In the current study, we defined the PGE2, IL-23 and IL-1 mediated mRNA expression of *IL17F*, *IL21*, and *IL22* concurrent with *IL17A* induction in human CD4⁺ T cells, and discovered that the three mediators differentially regulate *IL17A* and *IL17F* mRNA expression. We validated our findings by investigating *IL17A* and *IL17F* gene regulation in homogeneous human Th17 cells. Lastly, we characterized the distinct chromatin structure of the *IL17A* and *IL17F* loci in Th17 cells via extensive high resolution chromatin immunoprecipitation (ChIP) assays, suggesting possible mechanisms for differential gene regulation of the loci.

2. Material and methods

2.1. Cell isolation

Anonymous adult human blood samples were obtained from the Pittsburgh Central Blood Bank as approved by the University of Pittsburgh Institutional Review Board. PBMC were isolated by density gradient centrifugation on Ficoll-based Lymphocyte Separation Medium (MP Biochemicals, Solon, OH, USA). CD4⁺CD45RO⁺CCR7[−] T cells were enriched from PBMC using the human CD4⁺ effector memory T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). CD161⁺/CD161[−] and CCR6⁺/CCR6[−] Th memory cells were isolated

from the enriched cells in a four-way sort gated on viable CD4⁺CD45RO⁺ cells using a FACSaria (BD Biosciences, San Jose, CA, USA) and the following antibodies and reagents: fluorescein isothiocyanate (FITC)-labeled anti-CD45RO (clone UCHL1, BD Biosciences), R-Phycoerythrin (PE)-labeled anti-CD161 (clone DX12, BD Biosciences), peridinin chlorophyll protein (PerCP)-labeled anti-CD4 (clone RPA-T4, BioLegend, San Diego, CA, USA), allophycocyanin (APC)-labeled anti-CCR6 (clone 11A9, BD Biosciences), and fixable viability dye eFluor® 506 (eBioscience, San Diego, CA, USA).

2.2. Cell culture

Isolated T cells or HuT-102 cells were cultured at 1×10^6 cells/ml in X-VIVO-20 medium (Lonza BioWhittaker, Walkersville, MD, USA). The cells were stimulated with T cell activation beads (Miltenyi Biotec) coated with anti-CD2/-CD3/-CD28 antibodies (1 bead per 5 cells), PGE2 (1 μ M, Sigma-Aldrich Chemical, St. Louis, MO, USA), IL-23 plus IL-1 (50 ng/ml each, R&D Systems, Minneapolis, MN, USA), PGE2 and IL-23 plus IL-1, sulprostone (1 μ M, Cayman Chemical, Ann Arbor, MI, USA), butaprost (1 μ M, Cayman Chemical) or 1-hydroxy prostaglandin E1 (1-OH-PGE1, 1 μ M, Cayman Chemical) as described in the text and figure legends. The cells were incubated at 37°C, 5% CO₂, for either 6 hours for mRNA expression studies or 24 hours for protein expression studies unless otherwise stated.

2.3. Real-time quantitative PCR

Cultured T cells were harvested and lysed with Qiazol reagent, and total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using the high capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA), and *IL17A* and *IL17F* mRNA expression were measured relative to *HPRT* or *B2M* mRNA expression using predesigned TaqMan real-time PCR gene expression assays (Applied Biosystems) and the comparative threshold cycle (C_T) method.

For multigene expression profiling, cDNA was synthesized using the RT² First Strand Kit for BioMark (SABiosciences, Frederick, MD, USA). Pre-amplification of gene-specific cDNA targets was performed using the RT² Nano PreAMP cDNA Human Th17 for Autoimmunity and Inflammation Primer Mix (SABiosciences). cDNA samples were run in triplicate on a Fluidigm 96.96 Dynamic Array integrated fluidic circuit for gene expression (Fluidigm, South San Francisco, CA, USA) using the Human Th17 for Autoimmunity and Inflammation RT² Profiler PCR Array (SABiosciences). Real-time PCR was performed on a Fluidigm BioMark Reader (Fluidigm).

2.4. ELISA analysis

Secreted proteins were measured using individual ELISA Kits (human IL-17A homodimer, human IL-17F homodimer, human IL-17AF heterodimer, human IL-21 from eBioscience, San Diego, CA, USA; human IL-22).

2.5. Chromatin immunoprecipitation assays

ChIP was performed using a modification of the Millipore/Upstate protocol (MCPROTO407). Fold enrichment was calculated based on C_t as $2^{(C_t - C_{t_{input}})}$, where $C_t = (C_{t_{input}} - C_{t_{IP}})$. Final enrichment values were adjusted by subtraction of the nonspecific IgG antibody binding. A total of 1×10^7 cells were fixed in 1% formaldehyde (Fisher) for 10 min at room temperature. Cross-linking was inhibited by addition of glycine to a final concentration 0.125 M. Samples were sonicated (to generate DNA fragments of 250 base pairs (bp) average length) and chromatin from 5×10^6 cells was diluted 7-fold in ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl,

pH8.1, 167 mM NaCl), pre-cleared with protein Agarose/Salmon Sperm DNA beads (Protein G Agarose, 16–201 Millipore, Protein A Agarose 16–157 Millipore; IgM A4540 Sigma-Aldrich) for 30 min at 4°C, and centrifuged at 10,000 RPM for 2 min. Chromatin supernatants were incubated at 4°C overnight with respective antibodies against Pol II sc-899; H3K4me3 (a.motif 39915); H3K27me3 (a.motif 39156); H3K36me3 (ab9050); and H3K9ac (upstate 07-352). Aliquots for INPUT and non-specific IgG control samples were included with each experiment. Primer pairs specific for various regions of the human *IL17A* and *IL17F* genes were designed using PrimerQuest software available at the Integrated DNA technologies website (Supplementary Tables 1 and 2). The size of the PCR products ranges between 80 and 150 bp. Twenty microliter qPCR reactions containing 2x Maxima SYBR Green/ROX qPCR Master Mix (K0223, Fermentas), 250 nM of primers and 3 µl of precipitated DNA were set up in Fast 96-Well Reaction Plates (Applied Biosystems). qPCR reactions were carried out in a StepOnePlus Applied Biosystems Real Time Instrument.

2.6 Statistical analysis

Statistically significant differences between treatment groups were determined using the paired 2-tailed Wilcoxon signed-rank test. Differences were considered significant when *p* values were < 0.05.

3. Results

3.1. Th17 cytokine mRNA expression profiling of CD4⁺ effector memory T cells treated with PGE2 and/or IL-23 plus IL-1β

We profiled the mRNA expression of the Th17 cytokines *IL17A*, *IL17F*, *IL21*, and *IL22* in activated CD4⁺ effector memory T cells from 14 blood donors in response to PGE2 and/or IL-23 plus IL-1 utilizing a commercially available Th17 pathway quantitative PCR array. Cell cultures consisted of highly enriched CD4⁺ memory T cells, with an average purity of 96.28% (SD ± 2.33%) based on CD4 and CD45RO surface expression (Supplementary Figure 1). Our final PCR analyses included only validated mRNA expression assays defined by melting curves suggesting amplification of a single gene product and with amplification efficiencies that were approximately equal to that of 5 internal control genes.

PGE2, IL-23, and IL-1 synergistically increased the mRNA levels *IL17A*, while divergently regulating the mRNA levels of the other Th17 cytokines (Table 1). PGE2 repressed the mRNA expression of the Th17 cytokines *IL17F*, *IL21*, and *IL22*. IL-23 plus IL-1 significantly enhanced *IL17F* expression, remarkably to a greater extent than *IL17A*, and moderately induced *IL21* and *IL22* expression. In combination with PGE2, IL-23 plus IL-1 dominated the regulation of *IL17F* and *IL22* expression, but not *IL21*, which continued to be repressed by PGE2.

3.2. Th17 cytokine protein expression profiling of CD4⁺ effector memory T cells treated with PGE2 and/or IL-23 plus IL-1β

To verify our mRNA expression findings, we determined whether PGE2, IL-23 and IL-1 also modulate significant changes in related protein production of activated CD4⁺ effector memory T cells from 9 additional blood donors. We measured the secreted levels of IL-21, IL-22, as well as the IL-17A and IL-17F homodimers and the IL-17AF heterodimer, and found that the combination of PGE2 and IL-23 plus IL-1 synergistically increased the secreted level of IL-17A, as well as the heterodimer IL-17AF (Table 2). In contrast, as in the mRNA expression study, we found that PGE2 and IL-23 plus IL-1 differentially regulate the production IL-17F. IL-23 plus IL-1 induced IL-17F production, while PGE2 had no statistically significant effect on IL-17F secretion. Comparative analyses of IL-17A and

IL-17F levels in response to the different treatments further demonstrate that IL-23 plus IL-1 preferentially enhance IL-17F production compared to IL-17A, but that the addition of PGE2 to IL-23 plus IL-1 treatment promotes a switch from a predominant IL-17F to a predominant IL-17A immune response due to their synergistic effects on IL-17A expression alone (Figure 1).

PGE2 repressed the production of IL-22, while IL-23 plus IL-1 enhanced IL-22 production, and in combination, IL-23 plus IL-1 dominated the regulation of IL-22 protein expression. This is consistent with the *IL22* mRNA expression results. The remaining Th17 cytokine IL-21 was undetectable in cell culture supernatants (data not shown).

3.3. The PGE2 EP4 receptor mediates the differential regulation of *IL17A* and *IL17F* mRNA expression by PGE2

PGE2 exerts its effects on cells by binding E type prostanoid (EP) cellular membrane receptors, of which there are four different subtypes, EP1, EP2, EP3, and EP4. To determine which of the four EP receptors mediates the differential regulation of *IL17A* and *IL17F* mRNA by PGE2, we measured *IL17A* and *IL17F* expression in activated T cells from five different donors treated with various EP receptor selective agonists including sulprostone, an EP1 and EP3 receptor selective agonist, butaprost, a highly selective EP2 agonist, and 1-hydroxy prostaglandin E1 (1-OH-PGE1), a moderately selective EP4 agonist. We found that sulprostone had no significant effect on the expression of either *IL17A* or *IL17F*, while butaprost only moderately increased *IL17A* mRNA expression (Figure 2). In contrast, 1-OH-PGE1 induced *IL17A* mRNA and repressed *IL17F* expression in a manner comparable to PGE2. These results suggest that PGE2 regulates *IL17A* and *IL17F* mRNA expression primarily via the EP4 receptor.

3.4. PGE2-EP4 receptor-cAMP signaling pathway amplifies IL-17A expression in HuT-102 cells

To verify that PGE2, IL-23 and IL-1 differentially regulate IL-17A and IL-17F expression in homogenous Th17 cells, we chose to initially study the human T cell lymphotropic virus 1 (HTLV) transformed human CD4⁺ T cell line HuT-102. HuT-102 cells have many features of primary Th17 cells including the constitutive expression of IL-17A and the hallmark Th17 transcription factor *RORC* [22]. We cultured HuT-102 cells in serum free medium alone or in combination with T cell activation beads, PGE2, and/or IL-23 plus IL-1, and confirmed that unstimulated HuT-102 cells constitutively express *IL17A* mRNA and secrete IL-17A protein (Figure 3A and 3B). Interestingly, it was discovered that the cell line does not produce *IL17F* mRNA or IL-17F protein under any condition, including IL-23 plus IL-1 treatment (data not shown).

Despite the abundant constitutive expression of IL-17A, PGE2 was capable of moderately increasing *IL17A* mRNA and IL-17A protein expression in unactivated and activated HuT-102 cells. IL-23 plus IL-1 tended to increase *IL17A* mRNA expression in activated HuT-102 cells only, but did not confer increased IL-17A protein production. The addition of IL-23 plus IL-1 to PGE2 only modestly increased IL-17A expression compared to PGE2 treatment alone, with no synergistic induction of IL-17A expression by the three mediators as seen in primary CD4⁺ effector memory T cells.

To determine whether the PGE2 EP4 receptor also drives the PGE-mediated induction of IL-17A expression in HuT-102 cells, we measured *IL17A* mRNA and IL-17A protein expression upon treatment with various EP receptor selective agonists, as well as forskolin, which increases intracellular cAMP, a downstream signaling mediator of the EP2 and EP4 receptors. We found that sulprostone and butaprost had no significant effect on *IL17A*

mRNA and IL-17A protein expression in HuT-102 cells (Figure 3C and 3D). In contrast, 1-OH-PGE1 and forskolin induced IL-17A expression in a manner comparable to PGE2. These results suggest that PGE2 amplifies IL-17A expression in HuT-102 cells predominantly via the EP4 receptor-cAMP signaling pathway.

3.5. PGE2 and IL-23 plus IL-1 β differentially regulate *IL17A* and *IL17F* mRNA expression in primary Th17 cells

We next aimed to study the differential gene regulation of *IL17A* and *IL17F* by PGE2, IL-23, and IL-1 in primary human Th17 cells. We purified CD4⁺CD45RO⁺ T cells from enriched peripheral Th effector memory cells and then sorted cells based on the cell surface expression of the C-type lectin-like receptor CD161 and the chemokine receptor CCR6 (Supplementary Figure 2), both of which have been identified as distinctive surface markers of human Th17 cells, including Th17 cells that infiltrate the gastrointestinal tract in IBD [23–25]. We then examined early *IL17A* and *IL17F* mRNA expression in response to T cell activation alone or in combination with PGE2 and/or IL-23 plus IL-1 in the four different sorted Th cell populations based on CD161 and CCR6 expression. In response to T cell activation alone, we found that early *IL17A* mRNA expression was more robust than *IL17F* in all cell types (Table 3), which is consistent with our prior work and the known differential regulation of IL-17A and IL-17F by T cell receptor signaling [26]. *IL17A* and *IL17F* expression was most abundant in CD161⁺CCR6⁺ Th cells followed by CD161⁺CCR6[−] Th cells, with minimally detectable mRNA levels of either cytokine in CD161[−]CCR6[−] and CD161[−]CCR6⁺ Th cells.

PGE2 and IL-23 plus IL-1 synergistically increased *IL17A* but not *IL17F* mRNA expression in CD161⁺CCR6⁺ Th17 cells and to a lesser extent in CD161[−]CCR6⁺ Th17 cells (Figure 4A and 4B). Conversely, PGE2 alone repressed while IL-23 plus IL-1 induced *IL17F* mRNA expression in both Th17 cell types, particularly CD161⁺ Th17 cells. These results validate our previous findings in heterogeneous CD4⁺ effector memory T cells, and show that the three mediators differentially regulate IL-17A and IL-17F expression in primary Th17 cells. These findings also demonstrate that CCR6 is superior to CD161 as a solo marker of Th17 cells, but that the combination of CCR6 and CD161 enriches for Th17 cells with a greater potential for *IL17A* and *IL17F* mRNA expression.

3.6. ChIP studies of the *IL17A* and *IL17F* loci in HuT-102 cells

Prior reports have demonstrated that most Th17 cells express IL-17F, and that IL-17A and IL-17F are coordinately regulated via chromatin remodeling of the *IL17A/IL17F* chromosomal locus [6, 11, 12]. To further investigate the divergent constitutive expression profiles of IL-17A and IL-17F in HuT-102 cells, we sought to define the transcriptional and native chromatin modifier status of the individual *IL17A* and *IL17F* loci in unstimulated cells. Positional analysis of RNA Polymerase II (Pol II) along the length of the genes revealed significantly higher levels of constitutive transcription at the promoter and throughout *IL17A*, as compared to the *IL17F* locus (Figure 5A). Consistent with this, we observed a significant enrichment profile of H3K36me3 increasing toward the 3' end of *IL17A*, a histone modification that marks the footprint of active transcription [27].

The differences in positional distribution of the expression-permissive chromatin marks H3K4me3 and H3K9ac further delineated the transcriptional competency of *IL17A* and *IL17F* in HuT-102 cells. While *IL17A* revealed a classical robust promoter-proximal enrichment of H3K4me3 and H3K9ac, these active modifications [28, 29] were minimal on *IL17F* (Figure 5A). The lack of transcriptional activity or promoter competency for *IL17F* prompted examination of the enrichment profile for H3K27me3, a histone modification associated with programmable developmental repression of euchromatic promoters [30]. As

expected, a significant enrichment for H3K27me3 was detected throughout *IL17F*. The epigenetic analysis of HuT-102 cells argues that *IL17A* and *IL17F* have distinct chromatin architectures. While *IL17A* is extensively marked with active chromatin modifications and elongating Pol II, the quiescent *IL17F* locus displays repressively methylated silenced chromatin.

3.7. ChIP studies of the *IL17A* and *IL17F* loci in primary Th17 cells

Given the prominent differences in chromatin modification of the *IL17A* and *IL17F* loci in HuT-102 cells, we sought to characterize the chromatin state of the loci in unstimulated sorted primary Th17 cells from 6 blood donors. ChIP analysis revealed relatively high Pol II levels at the *IL17A* promoter, and to a lesser extent throughout the gene, in CD161⁺CCR6⁺ Th17 cells (Figure 5A). Pol II was detected at significantly lower levels at *IL17A* in CD161⁻CCR6⁺ and CD161⁻CCR6⁻ Th17 cells. Similar to HuT-102 cells, only low levels of Pol II were present at *IL17F* in CD161⁺CCR6⁺ Th17 cells, with even lower detectable Pol II levels found at *IL17F* in CD161⁻CCR6⁺ and CD161⁻CCR6⁻ Th17 cells.

Due to the lack of available chromatin samples, we focused our subsequent analysis on the classical H3K4me3 mark associated with both active and poised promoters [31] in order to distinguish the transcriptional competency of *IL17A* and *IL17F* in primary Th17 cells. As expected, the H3K4me3 levels on *IL17A* correlated with the presence of Pol II, with the highest signal in CD161⁺CCR6⁺ Th17 cells. H3K4me3 was relatively absent at *IL17F* in CD161⁺CCR6⁺ and CD161⁻CCR6⁺ Th17 cells, further validating the relative transcriptional repression of *IL17F* compared to *IL17A* in Th17 cells. It is noteworthy that upon closer examination of *IL17F* epigenetic architecture among the sorted Th17 cell populations, it appears as though *IL17F* may be slightly more poised for activation in CD161⁺CCR6⁺ than in CD161⁻CCR6⁺ Th17 cells consistent with the observed cell-type specific *IL17F* mRNA expression profile.

Discussion

We and others have previously found that PGE2 synergizes with IL-23 plus IL-1 to induce CD4⁺ T cell expression of IL-17A. In contrast, we now show for the first time that PGE2, IL-23 and IL-1 differentially regulate the CD4⁺ T cell expression of all other Th17 cytokines including IL-17F, and that PGE2, via the PGE2 EP4 receptor, promotes an IL-17A predominant Th17 immune response. We also discovered that the human Th17 HuT-102 cell line constitutively expresses IL-17A, but not IL-17F, and that the *IL17A* and *IL17F* loci have clear differences in histone modifications favoring *IL17A* preferential expression in HuT-102 cells. We went on to determine that the *IL17A* and *IL17F* loci also have divergent epigenetic architectures in primary human Th17 cells and are distinctly poised for subsequent activation.

IL-17A and IL-17F are both directly implicated in mucosal defense as well as the pathogenesis of multiple immune-mediated diseases including IBD [5, 6]. Though the two proteins have significant homology and share the same receptor interactions, IL-17A and IL-17F have specialized functions in inflammatory disease, such that an imbalance between the levels of IL-17A and IL-17F may predispose disease. We now describe that IL-23 plus IL-1 preferentially induce IL-17F expression, while PGE2 enhances only IL-17A production. Consequently, the addition of PGE2 to IL-23 plus IL-1 treatment causes a switch from an IL-17F to an IL-17A predominant immune response. Such changes in the balance between the levels of IL-17A and IL-17F may be important in IBD pathogenesis, as IL-17A is protective while IL-17F is pathogenic in certain mouse models, and IL-17F is the predominant IL-17 family member detectable in the serum of Crohn's disease patients [5, 6, 32].

PGE2 has previously been shown to induce IL-17A expression in human CD4⁺ T cells by activating the PGE2 EP2 and EP4 receptors [20, 21]. Via a similar experimental approach utilizing selective EP receptor agonists, we found that the EP4 receptor is primarily responsible for the PGE2-mediated regulation of *IL17A* and *IL17F* mRNA expression. This finding further highlights the potential disease significance of the EP4 receptor and its encoding gene *PTGER4*, which protects mice from colitis and is implicated as one of the four most significant association identified in the Crohn's disease GWAS [8, 33]. The observed discrepancy pertaining to the requirement of the EP2 receptor may be related to the timing of cytokine expression and/or CD4⁺ cell type under investigation, as we measured early mRNA expression specifically in effector memory T cells and HuT-102 cells while the prior studies measured the levels of secreted cytokine protein from differentiating naïve T cells or from all memory T cells after extended stimulation.

IL-17F was previously found to be expressed by almost all Th17 cells, and it was originally proposed that the *IL17A* and *IL17F* genes, which are immediately adjacent to each other on human chromosome 6 and mouse chromosome 1, are coordinately regulated via chromatin remodeling [6, 11]. However, more recent studies have found that *IL17A* and *IL17F* are differentially regulated, including by the T cell receptor and several transcription factors such as the retinoic acid receptor-related orphan receptor alpha, and the cAMP response element modulator alpha (CREM) [14, 15, 26, 34]. CREM in particular has been shown to bind both the *IL17A* and *IL17F* gene promoters in human CD4⁺ T cells and induce epigenetic alterations specifically in the *IL17A* promoter thereby activating *IL17A* mRNA expression, while concurrently repressing *IL17F* mRNA expression.

Our ChIP study of the *IL17* gene locus is the most extensive to date based on gene regions and histone modifications analyzed, and the first study to examine both the *IL17A* and *IL17F* individual loci in primary human Th17 cells. In contrast to previous studies [11, 35], we used a ChIP-scan method in order to more precisely characterize the positional distribution of Pol II and chromatin marks along *IL17A* and *IL17F*. Figure 5B illustrates the spatial positioning of the qPCR amplicons spanning *IL17A* and *IL17F*. Our epigenetic analysis focused on the gene coding regions, which encompass the conserved non-coding sequences (CNS) 2, 3, and 5 [14]. Since CNS 2 specifically co-localizes with the human *IL17A* promoter, our data suggest that this is an important site for *IL17A* transcriptional regulation, marked by high levels of permissive chromatin marks H3K4me3 and H3K9ac (Figure 5A).

We observed epigenetic differences between the *IL17A* and *IL17F* loci that were consistent with their distinct expression profiles in HuT-102 and primary Th17 cells. In HuT-102 cells, the dormant *IL17F* is extensively H3K27 tri-methylated and depleted of activation-specific chromatin marks. This transcriptional repression is likely maintained by Polycomb Repressive Complex 2 (PRC2), which has been shown to facilitate this methylation pattern [36]. The constitutively expressed *IL17A* reveals strong promoter proximal H3K4me3 and H3K9ac patterns and high levels of transcribing Pol II. The increased enrichment of promoter-bound Pol II relative to the body of *IL17A* suggests the presence of a rate-limiting step mediating the release of a paused Pol II into the elongation state [37]. These observations suggest the presence of additional distinctions relating to the transcriptional control of *IL17A* and *IL17F*. While the *IL17A* is actively transcribed, the quiescent *IL17F* is under a state of methylation-induced repression.

Analysis of the primary Th17 cells also showed epigenetic variations between *IL17A* and *IL17F*, as well the *IL17A* gene among the sorted Th17 cell populations from healthy donors. The highest levels of Pol II and the H3K4me3 mark at the *IL17A* locus were observed in CD161⁺CCR6⁺ Th17 cells. Cells lacking one (CD161⁺CCR6⁻) or both (CD161⁻CCR6⁻) of

the Th17 specific surface markers exhibited significant reduction in these indicators of active transcription. Similar to HuT-102 cells, *IL17F* exhibited less H3K4me3 at the gene promoter and only low levels of Pol II, indicative of repressed transcription. Collectively, our epigenetic analyses reveal that these two genes are not coordinately regulated in both HuT-102 and primary Th17 cells.

In conclusion, our study enhances the understanding of *IL17A* and *IL17F* gene regulation and highlights that the related genes have divergent histone modifications and are differentially regulated by pro-inflammatory mediators linked to Th17 biology and IBD susceptibility. These findings further suggest that IL-17A and IL-17F have distinct roles in mucosal health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations

ChIP	chromatin immunoprecipitation
C_T	threshold cycle
EP	E prostanoid
GWAS	genome wide association studies
IBD	inflammatory bowel disease
PGE₂	prostaglandin E ₂
1-OH-PGE₁	1-hydroxy prostaglandin E ₁
Th	T helper

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Highlights

- PGE2 and IL-23 plus IL-1 differentially regulate Th17 cytokine expression.
- PGE2 and IL-23 plus IL-1 synergize to induce IL-17A, but not IL-17F.
- IL-23 plus IL-1 preferentially induce IL-17F expression.
- PGE2 only enhances IL-17A expression as mediated by the PGE2 EP4 receptor.
- The *IL17A* and *IL17F* loci have divergent epigenetic architectures in Th17 cells.

Figure 1. PGE2 promotes an IL-17A predominant immune response

Human peripheral CD4⁺ effector memory T cells from healthy donors were cultured for 24 hours in serum free medium with T cell activation beads alone or in combination with PGE2 and/or IL-23 plus IL-1 β . IL-17A, IL-17F, IL-17AF were measured in the culture supernatants via ELISA. The box plots depict the median, lower quartile, and upper quartile of 9 donors, as indicated by the horizontal line in the box, and the lower and upper edges of the box. The lower and upper whiskers represent the minimum and maximum of the data within 1.5 $\times$ IQR. Outliers are displayed by dots. Statistically significant differences between treatment groups are denoted by *, $P < 0.05$.

Figure 2. The PGE2 EP4 receptor mediates the differential regulation of *IL17A* and *IL17F* mRNA expression by PGE2

Human peripheral CD4⁺ effector memory T cells from healthy donors were cultured for 6 hours in serum free medium with T cell activation beads alone or in combination with PGE2, sulprostone (EP1/EP3 receptor agonist), butaprost (EP2 receptor agonist), or 1-OH-PGE1 (EP4 receptor agonist). *IL17A* and *IL17F* mRNA expression were measured compared to the *B2M* internal control gene by real-time qPCR using the comparative C_T method. The plot depicts individual donor fold change in *IL17A* and *IL17F* mRNA expression compared to T cell activation alone of five donors.

A **B**

Figure 3. PGE2-EP4 receptor-cAMP signaling pathway amplifies IL-17A expression in HuT-102 cells

(A and B) HuT-102 cells were cultured in serum free medium alone (unstimulated) or in combination with T cell activation beads, PGE2, and/or IL-23 plus IL-1. (A) *IL17A* mRNA expression was measured at 6 hours and compared to the *HPRT* internal control gene by real-time qPCR using the comparative C_T method. The graph depicts the mean levels of *IL17A* mRNA, as defined by 2^{-C_T} , $\pm$ SEM of three independent experiments. (B) IL-17A was measured in cell culture supernatants at 24 hours via ELISA. The graph depicts the mean protein levels $\pm$ SEM of three independent experiments. (C and D) HuT-102 cells were cultured in serum free medium alone (unstimulated) or in combination with either PGE2, sulprostone (EP1/EP3 receptor agonist), butaprost (EP2 receptor agonist), 1-OH-

PGE1 (EP4 receptor agonist), or forskolin (cAMP activator). (A) *IL17A* mRNA expression was measured at 6 hours and compared to the *HPRT*. The graph depicts the mean fold change in mRNA expression compared to unstimulated cells $\pm$ SEM of three independent experiments. (D) IL-17A was measured in cell culture supernatants at 24 hours. The graph depicts the mean protein levels $\pm$ SEM of three independent experiments.

Figure 4. PGE2 and IL-23 plus IL-1 differentially regulate *IL17A* and *IL17F* mRNA expression in primary human Th17 cells

Human CD4⁺CD45RO⁺ T cells were purified from enriched peripheral Th effector memory cells and sorted based on the cell surface expression of CD161 and CCR6. The cells were cultured in serum free medium with T cell activation beads alone or in combination with PGE2 and/or IL-23 plus IL-1. *IL17A* (A) and *IL17F* (B) mRNA expression were measured at 6 hours and compared to the *B2M* internal control gene by real-time qPCR using the comparative C_T method. The graphs depict the mean mRNA levels, as defined by 2^{-C_T} , in the four cell populations of two representative healthy blood donors.

Figure 5. ChIP analysis of *IL17A* and *IL17F* in HuT-102 and primary human Th17 cells
 (A) Positional enrichment profiles along the length of the *IL17A* and *IL17F* genes for Pol II, H3K4me3, H3K9ac, H3K36me3, and H3K27me3 are presented for cell samples, as indicated. Histograms correspond to the relative antibody enrichment for specific qPCR amplicon. Several amplicons spanning the length of each gene were designed, in order to generate the positional enrichment profile for Pol II and modified nucleosomes. For comparison purposes, gray bars locate promoter positions within each gene. (B) Schematic representation of the ChIP-qPCR amplicons (black squares) spanning the human *IL17A* and *IL17F* loci relative to locations of the previously reported conserved non-coding sequence (CNS) sites (gray columns). Arrows above *IL17A* and *IL17F* denote the direction of transcription.

Table 1
Treatment-mediated fold change in mRNA expression compared to T cell activation alone.

Treatment	PGE2		IL-23+IL-1		PGE2+IL-23+IL-1	
	Median fold change (IQR)	p-value	Median fold change (IQR)	p-value	Median fold change (IQR)	p-value
<i>IL17A</i>	1.34 (1.05, 1.71)	3.53E-02	1.89 (1.53, 2.09)	2.44E-04	3.87 (2.35, 5.45)	1.22E-04
<i>IL17F</i>	0.48 (0.34, 0.65)	1.22E-04	3.67 (3.23, 4.74)	2.44E-04	3.57 (3.26, 3.96)	1.22E-04
<i>IL21</i>	0.43 (0.275, 0.47)	1.22E-04	1.21 (1.03, 1.34)	4.64E-03	0.69 (0.41, 0.75)	6.10E-04
<i>IL22</i>	0.42 (0.21, 0.89)	2.32E-03	1.68 (1.27, 1.96)	1.22E-03	1.27 (1.06, 1.92)	2.45E-02

IQR, interquartile range
n = 14 donors

Statistically significant differences between treatment groups were determined using the paired 2-tailed Wilcoxon signed-rank test. Differences were considered significant when *p* values were < 0.05.

Table 2

Treatment-mediated fold change in protein expression compared to T cell activation alone.

Treatment	PGE2		IL-23+IL-1		PGE2+IL-23+IL-1	
	Median fold change (IQR)	p-value	Median fold change (IQR)	p-value	Median fold change (IQR)	p-value
IL-17A	3.39 (3.18, 3.89)	3.91E-03	2.25 (2.15, 2.70)	3.91E-03	10.42 (9.70, 11.42)	3.91E-03
IL-17F	0.91 (0.47, 1.01)	NS	22.85 (13.58, 131.25)	7.81E-03	21.44 (14.29, 120.69)	7.81E-03
IL-17AF	1 (1, 1)	NS	8.34 (1, 80.5)	NS	137 (67.56, 213.75)	2.25E-02
IL-22	0.60 (0.51, 0.64)	3.91E-03	1.94 (1.87, 2.21)	3.91E-03	1.84 (1.68, 1.96)	3.91E-03

IQR, interquartile range

n =9 donors

Statistically significant differences between treatment groups were determined using the paired 2-tailed Wilcoxon signed-rank test. Differences were considered significant when *p* values were < 0.05.

Table 3

Mean *IL17A* and *IL17F* mRNA levels in primary Th cell populations stimulated by T cell activation alone.

	CD161 ⁺ CCR6 ⁻	CD161 ⁺ CCR6 ⁺	CD161 ⁻ CCR6 ⁺	CD161 ⁻ CCR6 ⁻
<i>IL17A</i>	1.02×10 ⁻⁴	0.018	0.015	1.31×10 ⁻⁴
<i>IL17F</i>	8.89×10 ⁻⁷	1.97×10 ⁻³	7.92×10 ⁻⁴	1.58×10 ⁻⁶

mRNA levels as defined by 2^{-C_T}

n = 2 representative donors