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Cutting Edge: AhR Is a Molecular Target of Calcitriol in Human T Cells

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The immunoregulatory functions of vitamin D have been well documented in various immunological disorders, including multiple sclerosis, arthritis, and asthma. IL-10 is considered a chief effector molecule that promotes the vitamin D–induced immunosuppressive states of T cells and accessory cells. In this article, we demonstrate that the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (calcitriol), has a profound inhibitory effect on the development of human Th9, a CD4 T cell subset that is highly associated with asthma, in an IL-10–independent manner. Our data show that calcitriol represses the expression of BATF, a transcription factor essential for Th9, via suppressing the expression of aryl hydrocarbon receptor, without an increase in IL-10. The data show a novel link between vitamin D and two key transcription factors involved in T cell differentiation. The Journal of Immunology, 2015, 195: 000–000.

Vitamin D plays a significant role in immunity, and its deficiency is associated with multiple inflammatory disorders (1). An active metabolite of vitamin D, calcitriol (2), exerts its influence through the vitamin D receptor (VDR), which regulates >200 genes. VDR is present in circulating mononuclear cells, dendritic cells, and thymus and peripheral T and B cells. Calcitriol decreases the maturation of APCs and their ability to activate T lymphocytes (3). IL-10 was shown to mediate calcitriol’s effects on immune modulation (4). Other than IL-10, factors that are targeted by calcitriol in primary T cells have not been delineated.

In this study, we determined the effect of calcitriol on Th9 cells. Th9 cells are defined as a subset of CD4 T cells that produce IL-9, a pleiotropic cytokine that supports mast cell growth, and stimulates mucin transcription in respiratory epithelial cells (4). The immunosuppressive effect of calcitriol on mouse Th9 responses requires IL-10 (5); however, the effect of calcitriol on human Th9 cells is unknown. In this article, we demonstrate that calcitriol suppresses human Th9 differentiation in vitro in a manner independent from IL-10 and show the link between calcitriol and two transcription factors essential for Th9: aryl hydrocarbon receptor (AhR) and BATF.

Materials and Methods

Cell preparation and culture

Naive CD4 T cells were sorted from PBMCs using an EasySep Human Naive CD4+ T Cell Isolation Kit (STEMCELL TECHNOLOGIES, Vancouver, BC, Canada). Nonretreated CytomOne 24/48-well plates (USA SCIENTIFIC, Ocala, FL) coated with anti-CD3 (OKT3; 5 μg/ml) and anti-CD28 (28.2; 5 μg/ml; both from BioLegend, San Diego, CA) were used for cell stimulation. For induction of Th9 cells, naive CD4 T cells were stimulated in the presence of IL-4 (20 ng/ml) and IL-1β (20 ng/ml; both from PeproTech, Rocky Hill, NJ) for 3 d (RNA isolation and Western blot) or 5 d (ELISA). Anti–IL-10R Ab (10 μg/ml; BioLegend) was used for inhibition of IL-10. Calcitriol, AhR antagonist (CH223191) (both from Sigma-Aldrich, St. Louis, MO), or FICZ (Enzo Life Sciences, Farmingdale, NY) was added where indicated.

Small interfering RNA/DNA transfection

Predesigned small interfering RNA (siRNA) for ahb and its negative-control siRNA were obtained from Life Technologies (Grand Island, NY). Human BATF cDNA (GeneScript, Piscataway, NJ) was inserted into the pmMAX plasmid (Lonza, Allendale, NJ). Transfection was performed by electroporation using a Human T Cell Nucleofector Kit (Lonza).

ELISA and cytokine staining

Culture supernatant (day 5) was used to determine IL-9 or IL-10 concentrations with a Human IL-9/IL-10 ELISA MAX set (BioLegend). For cytokine staining, cells harvested at day 5 were restimulated with 50 ng/ml PMA and 1 μM ionomycin in the presence of 2 μM monensin for 4 h and then stained with anti–IL-4 and anti–IL-9 Abs (BioLegend). Data were analyzed using Flowjo software (TreeStar, Ashland, OR).

RNA isolation and real-time PCR

Total RNA was isolated using a ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI), followed by cDNA synthesis using an oligo (deoxynucleotidyl) and SuperScript III First-Strand Synthesis System (Life Technologies). PCR was performed using SoxAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA). The following primers were used: IL-9 forward 5′-TGGCATGGCGTGCTACAGGGA-3′; Ahb forward 5′-GACTGGACCAAAAGTCCATCG-3′, Ahb reverse 5′-TTGGTTGTGATGCCAAAGGA-3′; Batf forward 5′-AATCGGAGCGGCCATGACGC-3′; Batf reverse 5′-CTCCACAGGCACTGACGC-3′; and Gapdh forward 5′-GCAGCTGCTACATTCTGCA-3′; Gapdh reverse 5′-GGTGGTCTCTTCCATACAGC3′; and Batf forward 5′-AAATCGGAGCGGCCATCAGC-3′, Batf reverse 5′-TCAGGGCGCAGGCTACCAAGC-3′. The fold change was calculated based on ΔΔCt. GAPDH was used to normalize the quantity of RNA in each sample.

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Abbreviations used in this article: AhR, aryl hydrocarbon receptor; siRNA, small interfering RNA; T cell, IL-10–secreting type 1 regulatory T cells; VDR, vitamin D receptor.

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Western blot

Equal numbers of cells (1.0 × 10^6 cells/100 μl) were lysed in SDS sample buffer (2% SDS, 125 mM DTT, 10% glycerol, 62.5 mM Tris-HCl [pH 6.8]), and proteins were subjected to Western blot analysis using the following Abs: anti-AhR Ab and anti-GATA3 Ab (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Pu.1 Ab and anti–IRF-4 Ab (Cell Signaling Technology, Danvers, MA); anti–BATF Ab (BioLegend); anti–β-actin Ab (Sigma-Aldrich); and anti–E-cadherin Ab (Thermo Fisher Scientific, West Palm Beach, FL). Signals were detected with the ECL system (GE Healthcare, Piscataway, NJ). The relative intensity of each band (shown below each lane) was determined by Image software (National Institutes of Health) after normalization using β-actin or histone deacetylase as the control.

Network analysis of transcription factors and genes highly expressed by Th9 cells

Genes specifically enriched >2-fold in Th9 cells compared with Th2 cells or inducible regulatory T cells (9) were analyzed using a list of transcription factors, with their potential target genes based on databases and published articles (10–18). Transcription factors and their predicted target genes were combined without allowing redundancy to generate the integrated data set. A total of 1,503 transcription factors and their 18,523 putative target genes was obtained from above data integration. The network analysis on this integrated dataset and 560 Th9-enriched genes was performed using a software package, Gephi (19). Results were converted into a schematic diagram using Python program package NetworkX (20). The shape of the network was determined by Force Atlas layout in Gephi.

Statistical analysis

Statistical analyses were performed using the two-tailed Student t test.

Study approval

This study was approved by Loyola University Chicago’s Institutional Review Board.

Results and Discussion

To address whether vitamin D inhibits human Th9 cell development, we cultured naive CD4 T cells under Th9-inducing conditions in the presence or absence of calcitriol. Calcitriol abrogated IL-9 protein and mRNA production by human T cells in a dose-dependent manner (Fig. 1A). Although a high dose of calcitriol is known to have an effect on T cell proliferation (21), we did not observe any significant inhibition of T cell growth (data not shown).

A previous study using murine CD4 T cells showed that calcitriol abrogated IL-9 production in an IL-10–dependent manner (8). Contrary to the mouse T cell observations, calcitriol decreased IL-10 production by human T cells cultured under Th9 conditions (Fig. 1B). Moreover, blockade of IL-10 signaling by an anti–IL-10R Ab did not enhance IL-9 production (Fig. 1C). These data suggest that calcitriol suppresses human Th9 differentiation in an IL-10–independent manner. The difference between human and mouse Th9 cells may be due to the species-specific requirement for factors involved in differentiation. Alternatively, this may be caused by the differences in culture conditions. Although our study used purified T cells only, the mouse T cell study (8) was performed in the presence of APCs that could produce IL-10 in response to calcitriol.

GATA3, PU.1, IRF-4, and BATF are known transcription factors involved in Th9 differentiation (5, 8). BATF was shown to be a critical transcription factor for mouse and human Th9 development (9). Under Th9-inducing conditions, BATF expression was highly upregulated compared with T cells stimulated without exogenous cytokines (“neutral conditions”) (Fig. 1D–F). Calcitriol significantly reduced the level of BATF protein and mRNA expression, but it did not cause a significant change in the expression of GATA3, IRF-4, or PU.1. These finding suggested that inhibition of Th9 differentiation by calcitriol is due, at least in part, to a reduction in BATF expression.

To decipher the detailed mechanisms by which calcitriol controls human Th9 development, we carried out transcription factor network analysis on genes highly expressed by Th9, based on previously published data (9). We focused on 560 genes expressed >2-fold in Th9 cells compared with Th2...
or inducible regulatory T cells (termed differentially expressed genes [DEGs]) (10–13) (Supplemental Table I). As described above, 1,503 transcription factors and their 18,523 putative target genes were obtained. Among the 560 Th9 DEGs, 458 genes (81.8%) were found in the integrated transcription factor–target gene dataset, and we analyzed these 458 genes for their possible transcription factor–target relationship (Fig. 2A). The results showed that a significant majority of DEGs may be controlled by two transcription factors: AhR and ERG. AhR is required in the differentiation pathways of various T cell subsets, including Foxp3+ regulatory T cells, IL-10–secreting type 1 regulatory T cells (Tr1 cells), Th17 cells, and Th22 cells (22). ERG is an ETS family transcription factor known for its role in thymocyte development, but its function in effector T cell differentiation was not clearly identified (23).

Upon stimulation under both Th9 and neutral conditions, AhR and ERG expression was substantially increased (Fig. 2B). When calcitriol was present, AhR, but not ERG, expression was significantly reduced (Fig. 2B, 2C). The reduction in AhR by calcitriol also was observed at the transcriptional level under both neutral and Th9-polarizing conditions (Fig. 2D).

Suppression of AhR may be mediated by direct binding of VDR in the ahr gene. Indeed, we identified ≥10 potential binding sites of VDR in the ahr gene based on the consensus-binding motif (24). Reduced AhR expression may be a cause of the calcitriol-induced decrease in IL-10, because AhR is required for Tr1 cell differentiation (25). Based on these data, we hypothesized that calcitriol inhibits Th9 development by reduction of AhR expression, and we tested whether inhibition of AhR function by an antagonist (CH223191) blocked Th9 development. When AhR antagonist was added to the culture, IL-9 production and IL-9* cell frequency were decreased significantly (Fig. 3A, 3B). We also used siRNA-based knockdown against ahr (Fig. 3C). As observed with the antagonist, siRNA against ahr markedly reduced il-9 mRNA and IL-9 protein expression (Fig. 3D). Together, the data demonstrate that AhR is essential for Th9 development.

To determine how AhR functions in Th9 development, we hypothesized that AhR promotes BATF expression and tested whether inhibition of AhR signaling reduces BATF expression. Indeed, siRNA against ahr mRNA reduced batf mRNA expression (Fig. 3D). Moreover, AhR antagonist caused a significant reduction in BATF protein expression by T cells cultured under Th9 conditions (Fig. 3E). These data demonstrate that AhR signaling is required for BATF expression during Th9 differentiation and that calcitriol abrogates Th9 differentiation, in part by inhibiting the AhR–BATF

![FIGURE 2. AhR expression is suppressed by calcitriol. (A) Potential regulatory network of transcription factors and their possible targets among the Th9-enriched genes. Each node represents a Th9-enriched gene, and nodes are connected by directed edges if regulator–target relationships are predicted. The sizes of the nodes reflect eccentricity of the genes in this network. Colored circles represent AhR (red) and ERG (green)-related genes. (B and C) Suppression of AhR expression by calcitriol. Naive CD4 T cells were stimulated under neutral or Th9-inducing conditions in the presence or absence of calcitriol. (B) Total cell lysates were subjected to Western blot analysis with Abs specific for the proteins listed on the right. Data represent one of three independent experiments. (C) The relative intensity of each band was determined as in Fig. 1, and an average from three samples is shown. (D) mRNA was analyzed by real-time PCR for ahr expression. Samples were normalized using gapdh transcripts as a control. Data represent one of three independent experiments. *p < 0.005, **p < 0.001.

![FIGURE 3. AhR is required for BATF expression and Th9 differentiation. (A and B) IL-9 expression by AhR inhibited T cells. Naive CD4 T cells were stimulated under neutral (n) or Th9 culture conditions in the presence of AhR antagonist (10 μM) or carrier control. (A) IL-9 concentrations determined by ELISA. Data represent one of six independent experiments. (B) Cytokine staining for IL-9 and IL-4. (C and D) Expression of IL-9 or BATF by naive CD4 T cells transfected with siRNAs against ahr. (C) Western blot analysis of AhR protein expression by T cells transfected with siRNA against ahr (siA) or nonspecific target control (siC). Data represent one of three independent experiments. (D) Secreted IL-9 concentrations (left panel) and relative IL-9* (middle panel) and relative batf (right panel) mRNA levels. Normalization of real-time PCR was performed using gapdh transcripts as a control. Data represent one of three independent experiments. (D) Secreted IL-9 concentrations (left panel) and relative IL-9* (middle panel) and relative batf (right panel) mRNA levels. Normalization of real-time PCR was performed using gapdh transcripts as a control. Data represent one of three independent experiments. (F) Average band intensity from three independent experiments under Th9-polarizing conditions. *p < 0.001.
CD4 T cell differentiation under pathophysiological conditions. It has been shown that the balance between vitamin D and the AhR ligand may influence T cell differentiation. For example, vitamin D can promote the differentiation of regulatory T cells (Tregs) and Th1 cells, whereas AhR ligands can suppress these processes. Together, these data suggest that, in addition to AhR, there are Th9-related molecules controlled by calcitriol in T cells. AhR is required for the development of multiple CD4 T cell subsets, and it may serve as a potential molecular link between vitamin D and immune homeostasis.

This study shows that calcitriol suppresses AhR and BATF expression in human T cells. AhR is required for the development of multiple CD4 T cell subsets, and it may serve as a potential molecular link between vitamin D and immune homeostasis. The data also reveal that perturbation of AhR inhibits BATF. The link between AhR and BATF had not been demonstrated previously. The data indicate the potential mechanism by which exposure to AhR ligands could increase the expression of BATF and promote effector T cell differentiation, including Th9.

Because calcitriol suppresses AhR and Th9 differentiation, vitamin D deficiency could lead to enhanced differentiation of Th9 cells and promote the activation and expansion of mast cells. Th9-associated diseases, like asthma, may be one of such conditions in which vitamin D plays a protective role via the reduction of Th9 cells, whereas AhR ligand could exacerbate the disease (27). Vitamin D deficiency also is linked to atopic dermatitis (1). Vitamin D and AhR ligands are both generated from UV light exposure and have effects on lymphoid and nonlymphoid tissues (28). A recent study showed that human Th9 cells preferentially localize in skin (29). Although the role that Th9 plays in skin inflammation is not clearly defined, it is conceivable that the vitamin D and/or AhR local ligand levels alter the differentiation process of Ag-activated T cells in the skin. In addition, AhR is involved in the differentiation of proinflammatory Th17 cells and anti-inflammatory regulatory T cells and Tregs (22, 25). Together, these data suggest that the balance between vitamin D and the AhR ligand may be a critical factor that determines the overall outcomes of CD4 T cell differentiation under pathophysiological conditions.

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Disclosures

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