T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism

Kristina Edfeldt[a,1], Philip T. Liu[a,b,1], Rene Chun[b], Mario Fabri[a], Mirjam Schenk[a], Matthew Wheelwright[c], Caroline Keegan[c], Stephan R. Krutzik[a], John S. Adams[b], Martin Hewison[b], and Robert L. Modlin[a,c,2]

[a]Division of Dermatology, Department of Medicine; [b]Orthopaedic Hospital, Department of Orthopaedic Surgery; and [c]Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine at University of California, Los Angeles, CA 90095

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We investigated the mechanisms by which T-cell cytokines are able to influence the Toll-like receptor (TLR)-induced, vitamin D-dependent antimicrobial pathway in human monocytes. T-cell cytokines differentially influenced TLR2/1-induced expression of the antimicrobial peptides cathelicidin and DEFB4, being up-regulated by IFN-γ, down-regulated by IL-4, and unaffected by IL-17. The Th1 cytokine IFN-γ up-regulated TLR2/1 induction of 25-hydroxyvitamin D-1α-hydroxylase (i.e., CYP27B1), leading to enhanced bioconversion of 25-hydroxyvitamin D_3 ($25D_3$) to its active metabolite $1,25D_3$. In contrast, the Th2 cytokine IL-4, by itself and in combination with the TLR2/1 ligand, induced catabolism of $25D_3$ to the inactive metabolite $24,25D_3$, and was dependent on expression of vitamin D-24-hydroxylase (i.e., CYP24A1). Therefore, the ability of T-cell cytokines to differentially control monocyte vitamin D metabolism represents a mechanism by which cell-mediated immune responses can regulate innate immune mechanisms to defend against microbial pathogens.

innate immune response | interferon-γ | interleukin-4 | *Mycobacterium tuberculosis*

The ability of Toll-like receptors (TLRs) to trigger a direct antimicrobial activity is a key aspect of their role in innate immunity. In mouse monocytes, activation of the TLR2/1 heterodimer by microbial lipoproteins (1–3), induces an antimicrobial activity against *Mycobacterium tuberculosis* that is nitric oxide (NO)-dependent, but in human monocytes is NO-independent (4). Instead, a key antimicrobial mechanism for TLR-activated human monocytes involves induction of the 25-hydroxyvitamin D-1α-hydroxylase (i.e., CYP27B1), which enzymatically converts the major circulating form of vitamin D, 25-hydroxyvitamin D3 ($25D_3$) into the active form of vitamin D, $1,25D_3$. Parallel TLR-mediated up-regulation of the vitamin D receptor (VDR) and activation of this receptor by $1,25D_3$ leads to downstream induction of the genes encoding the antimicrobial peptides cathelicidin and DEFB4 (5–10). Here, we tested the hypothesis that adaptive T-cell cytokines, including key cytokines of the Th1, Th2, and Th17 pattern, regulate the TLR2/1-induced, vitamin D-dependent antimicrobial pathway.

Results

Effect of T-Cell Cytokines on TLR2/1 Induction of Cathelicidin and DEFB4. To determine the role of individual cytokines on the TLR-triggered vitamin D-dependent induction of antimicrobial peptides, monocytes were treated with TLR2/1L with or without a specific T-cell cytokine, and cathelicidin and DEFB4 mRNAs measured at 24 h. IFN-γ by itself up-regulated cathelicidin and DEFB4 mRNA levels by twofold (Fig. 1*A*; $P < 0.05$ and $P < 0.001$). Consistent with previous findings, TLR2/1L induced both cathelicidin and DEFB4 mRNAs (8, 10). However, whereas IFN-γ augmented TLR2/1L-triggered induction of cathelicidin by 4.1-fold ($P < 0.01$), it had no effect on TLR2/1L-mediated induction of DEFB4 (Fig. 1*A*). The addition of IL-17 had no effect on induction of antimicrobial peptide gene expression in the presence or absence of TLR2/1L (Fig. 1*B*).

Whereas IFN-γ augmented TLR2/1 induction of cathelicidin, IL-4 had the opposite effect. IL-4 inhibited TLR2/1 induction of both cathelicidin and DEFB4 mRNA by greater than 90% (Fig. 1*C*; $P < 0.05$). IL-4 also affected baseline expression of both cathelicidin and DEFB4 in the absence of TLR2/1 induction, reducing mRNA levels by 20% to 40% (Fig. 1*C*; $P < 0.001$ and $P < 0.05$). Together, these data indicate that IFN-γ and IL-4 differentially modulate TLR2/1-induced expression of cathelicidin and DEFB4.

Effect of T-Cell Cytokines on TLR2/1 Induction of CYP27B1 and the VDR. To explore the mechanism by which the T-cell cytokines IFN-γ and IL-4 differentially regulated TLR2/1-induced antimicrobial peptide gene expression, we investigated the mRNA levels for CYP27B1 and the VDR. TLR2/1 activation of human monocytes is known to up-regulate both CYP27B1 and the VDR, the activity of both being required for induction of cathelicidin expression (8). IFN-γ induced by 2.4-fold the expression of CYP27B1 in human monocytes, but synergized with TLR2/1L to induce CYP27B1 mRNA levels to 6.9-fold over media control ($P < 0.01$) and 2.5-fold over cells treated with TLR2/1L alone ($P < 0.05$; Fig. 2*A*). In addition, IFN-γ increased TLR2/1L up-regulation of VDR expression, although the effect was less pronounced (2.1-fold; $P < 0.05$).

Surprisingly, the effect of IL-4 on CYP27B1 and VDR expression was similar to that of IFN-γ. IL-4 augmented TLR2/1-induced CYP27B1 (sixfold; $P < 0.05$) and VDR (threefold; $P < 0.05$) mRNA expression in human monocytes (Fig. 2*B*). Therefore, although IFN-γ and IL-4 differentially regulated TLR2/1L induction of antimicrobial peptide gene expression, they had identical effects on TLR2/1-induced expression of key vitamin D pathway genes, CYP27B1, and the VDR.

Effect of IL-4 on Monocyte Vitamin D Response. To explore the possible mechanisms by which IL-4 inhibited TLR2/1L-induced antimicrobial peptide expression despite increasing CYP27B1 and VDR, the effect of IL-4 on direct VDR activation was in-

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[1]K.E. and P.T.L. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: rmodlin@mednet.ucla.edu.

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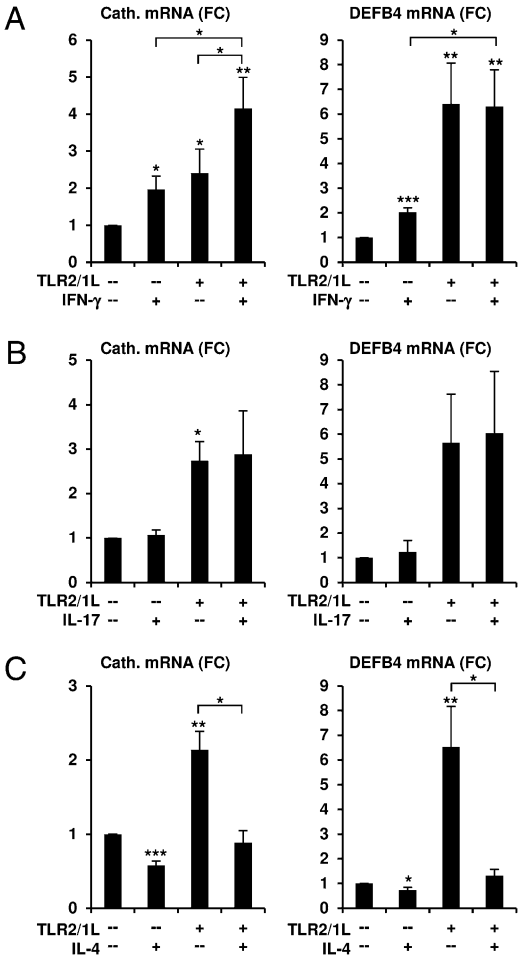

Fig. 1. T-cell cytokines differentially influence TLR2/1-induced expression of the antimicrobial peptides cathelicidin and DEFB4. Primary human monocytes were stimulated with TLR2/1L (10 μg/mL) with or without the T-cell cytokines (*A*) IFN-γ (1 ng/mL), (*B*) IL-17A (10 μg/mL), or (*C*) IL-4 (10^3 U/mL) for 24 h in vitamin D sufficient serum. mRNA levels of cathelicidin and DEFB4 were determined by qPCR and fold change (FC) was calculated. Data represent mean values ± SEM from three to eight independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

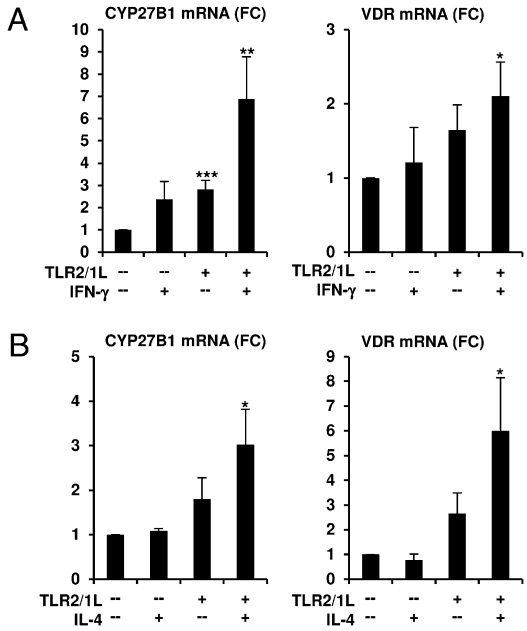

Fig. 2. IFN-γ and IL-4 up-regulate vitamin D pathway genes in TLR2/1-activated monocytes. Primary monocytes were stimulated with TLR2/1L (10 μg/mL) with or without the T-cell cytokines (*A*) IFN-γ (1 ng/mL) or (*B*) IL-4 (10^3 U/mL) for 24 h. mRNA levels of CYP27B1 and the VDR were subsequently determined by qPCR and fold change (FC) was calculated. Data represent mean values ± SEM from four to seven independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

vestigated. The simultaneous addition of IL-4 with $1,25D_3$ to monocytes inhibited cathelicidin expression (Fig. 3*A*). Similarly, when monocytes were pretreated for 6 h with IL-4, there was inhibition of $1,25D_3$-induced cathelicidin expression (Fig. 3*B*). In these experiments, the addition of exogenous $1,25D_3$ was limited to 10^{-8} M, after which the amount of $1,25D_3$ affected cellular viability. However, in both cases, increasing $1,25D_3$ to concentrations higher than 10^{-8} M did not overcome the IL-4 inhibition of cathelicidin expression. This is most likely a result of the well recognized ability of $1,25D_3$ to self-induce expression and activity of the catabolic enzyme CYP24A1, leading to the rapid inactivation of bioactive vitamin D (11).

The vitamin D host defense against mycobacteria requires the up-regulation of cathelicidin as well as the induction of autophagy, a critical cellular process for inducing phagosome maturation (7, 12). Given that IL-4 has been shown to inhibit starvation and IFN-γ–induced autophagy (13), we next examined whether IL-4 could inhibit $1,25D_3$-induced autophagy. Primary human monocytes incubated with $1,25D_3$ showed a 3.8-fold enhancement of autophagy ($P < 0.01$) as measured by an increase in the percentage of LC3 punctate cells (Fig. 3 *C* and *D*). Preincubation of monocytes with IL-4 for 6 h before the addition of $1,25D_3$ resulted in a marked decrease in LC3 punctate cells, reaching levels lower than media-treated background levels ($P < 0.001$). Therefore, IL-4 inhibits vitamin D-induced autophagy in primary human monocytes.

Effect of T-Cell Cytokines on Monocyte Vitamin D Metabolism. The differential ability of IFN-γ and IL-4 to affect TLR2/1-induced cathelicidin expression, as well as the effects of IL-4 on $1,25D_3$-induced host responses, suggested that these cytokines achieve at least some of their effects by regulating monocyte vitamin D metabolism. Therefore, we next examined the bioconversion of $25D_3$ to its active metabolite $1,25D_3$ as well as the inactive metabolite $24,25D_3$. This was accomplished by adding ^{3}H-$25D_3$ to TLR2/1L and/or T-cell cytokine treated monocytes and measuring conversion to the resulting ^{3}H-vitamin D metabolites by HPLC. TLR2/1L-treated monocytes converted $25D_3$ to $1,25D_3$ at a slightly higher rate (9.5 fmol/h per million cells) compared with media control cells (8.7 fmol/h per million cells), both shown as a summary of five separate experiments (Fig. 4*A*; $P < 0.05$). Treatment of monocytes with IFN-γ alone induced a similar conversion of $25D_3$ to $1,25D_3$ as observed in cells treated with TLR2/1L (11.4 fmol/h per million cells; $P < 0.05$; Fig. 4*A*). However, IFN-γ in combination with TLR2/1L strongly induced bioconversion of $25D_3$ to active $1,25D_3$ (45.4 fmol/h per million cells), to levels sevenfold higher than media control cells (Fig. 4*A*; $P < 0.05$). The Th2 cytokine IL-4 alone had no effect on CYP27B1 activity (6.4 fmols/h per million cells; Fig. 4*A*). However, in combination with TLR2/1L, IL-4 induced bioconversion of $25D_3$ to $1,25D_3$ (14.6 fmol/h per million cells; $P < 0.05$; Fig. 4*A*). Together, these data indicated that IFN-γ potentiation of TLR2/1-induced antimicrobial peptides is associated with the synergistic effects of IFN-γ plus TLR2/1 on CYP27B1 activity leading to enhanced bioconversion of $25D_3$ to $1,25D_3$.

Given that IL-4 inhibited TLR2/1-induced antimicrobial peptide expression but also enhanced CYP27B1 activity, we next examined the effects of IFN-γ and IL-4 on vitamin D catabolism,

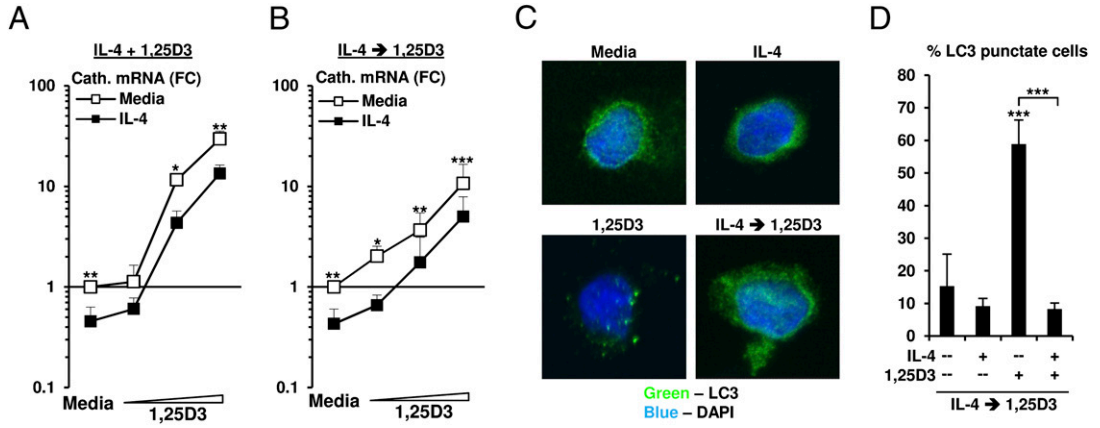

Fig. 3. Effects on IL-4 on $1,25D_3$ responsiveness. (*A*) Primary monocytes were cotreated with IL-4 (10^3 U/mL) and $1,25D_3$ (10^{-10} to 10^{-8} M) for 18 h or (*B*) pretreated with IL-4 (10^3 U/mL) for 6 h and then stimulated with $1,25D_3$ (2.5×10^{-10} to 10^{-8} M) for 18 h. mRNA levels of cathelicidin were subsequently determined by qPCR and fold change (FC) was calculated as ratio to media control cells. Data represent mean values ± SEM from four to seven independent experiments. Primary monocytes were treated as in *B* and immunolabeled for intracellular LC3 expression (green) and cellular nuclei (blue). Cells were visualized using confocal microscopy (*C*) and enumerated (*D*) for the percentage of cells positive for LC3-punctate formation. Data displayed are the average percentage of cells positive for LC3-punctate formation per field of view from three independent donors ± SEM ($n > 7$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

measuring conversion of $25D_3$ to the inactivate metabolite $24,25D_3$. TLR2/1 activation had no statistically significant effect on vitamin D catabolism in monocytes (Fig. 4*B*). Similarly, IFN-γ alone or together with TLR2/1L had no effect on the conversion of $25D_3$ to $24,25D_3$, which was similar to media control monocytes. Strikingly, treatment of monocytes with the Th2 cytokine IL-4 alone strongly induced 24-hydroxlyase activity, increasing the production of $24,25D_3$ by fivefold over media control monocytes to a rate of 51.0 fmol/h per million cells compared with 10.6 ± fmol/h per million cells in media control cells (Fig. 4*B*; $P < 0.05$). IL-4 had a comparable effect in TLR2/1L-treated monocytes, inducing catabolism of $25D_3$ to $24,25D_3$ at a rate of 40.7 fmol/h per million cells compared with 10.6 fmol/h per million cells in control-treated cells (Fig. 4*B*; $P < 0.05$). These data suggest that up-regulation of vitamin D catabolism (i.e., conversion of $25D_3$ to $24,25D_3$) provides a mechanism to explain the IL-4–mediated inhibition of TLR2/1-induced antimicrobial protein expression. Although IFN-γ and IL-4 both up-regulated TLR-induced CYP27B1 mRNA expression by approximately twofold, the TLR-induced conversion of $25D_3$ to $1,25D_3$ was threefold greater in the presence of IFN-γ versus IL-4. Interestingly, treatment of monocytes with 1,25D3 alone (10^{-8} M) induced 24-hydroxylase activity to a level (65.8 fmol/h per million cells) similar to that observed for treatment with IL-4, providing a potential explanation for the inability of exogenously added $1,25D_3$ to overcome the cathelicidin-suppressive effects of IL-4 (Fig. S1).

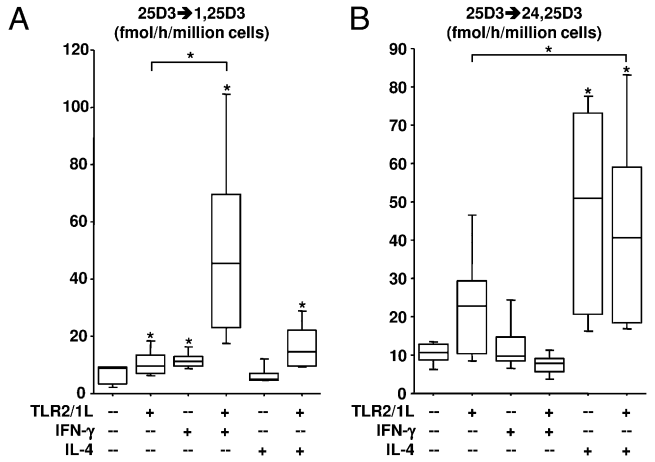

Fig. 4. IFN-γ and IL-4 differentially regulate vitamin D metabolism. Primary monocytes treated with TLR2/1L with or without IFN-γ (100 ng/mL) or IL-4 (10^3 U/mL) for 48 h and the ability to convert $[^3H]$-25D3 to $[^3H]$-$1,25D_3$ and $[^3H]$-$24,25D_3$ was measured by HPLC. The rate of conversion of (*A*) $[^3H]$-$25D_3$ to $[^3H]$-$1,25D_3$ and (*B*) $[^3H]$-$24,25D_3$ was calculated ($n = 5$) and presented in box-and-whisker plots depicting minimum value, lower quartile, median, upper quartile, and maximum value (*$P < 0.05$).

IL-4–Induced Catabolism of Vitamin D Is Dependent on CYP24. To investigate the mechanism by which IL-4 up-regulates 24-hydroxylation of $25D_3$, further studies were carried out to characterize expression of CYP24A1, the primary enzyme involved in catalyzing 24-hydroxylation of vitamin D metabolites (11). Intriguingly, IL-4 inhibited the baseline expression of CYP24A1 mRNA (35%; $P < 0.01$), and also inhibited the TLR2/1-induced up-regulation of CYP24A1 expression (84%; $P < 0.05$; Fig. 5*A*). We were also unable to detect any significant changes in CYP24A1 protein expression or alterations in mRNA encoding the CYP24A1 splice variant (14) (Fig. 5*B*). A number of other cytochrome p450 enzymes (CYP3A1, CYP2J2, CYP27A1) have been suggested to exhibit potential 24-hydroxylase activity; however, none of these were found to be up-regulated in monocytes following treatment with IL-4 (Fig. S2).

To directly measure the contribution of CYP24A1 to the 24-hydroxylase activity in monocytes treated with IL-4, monocytes were transfected with siRNA oligos specific for human CYP24A1 or a nonspecific control (siCTRL) and then treated with IL-4. Knockdown of CYP24A1 expression inhibited the conversion of $25D_3$ to $24,25D_3$ by 58% relative to the control siRNA ($P < 0.05$; Fig. 5*C*), whereas conversion of $25D_3$ to $1,25D_3$ increased by 235% ($P = 0.059$; Fig. 5*D*). The effect of CYP24A1 knockdown on IL-4 suppression of TLR2/1 responses was next investigated. In siCTRL transfected monocytes, IL-4 significantly inhibited TLR2/1L-induced cathelicidin ($P < 0.05$; Fig. 5*E*) and DEFB4 mRNA expression ($P < 0.05$; Fig. 5*F*). The effect of IL-4 was reversed in siCYP24A1 transfected monocytes, restoring TLR2/1L-induced responses. It was also noted that TLR2/1L-induced cathelicidin trended to be increased in CYP24A1 knockdown monocytes, suggesting a regulatory role for CYP24A1 even in the absence of IL-4.

Given that IL-4 inhibited $1,25D_3$-induced autophagy, we next determined whether the effect of IL-4 was dependent on CYP24A1 expression. In monocytes transfected with siCYP24A1,

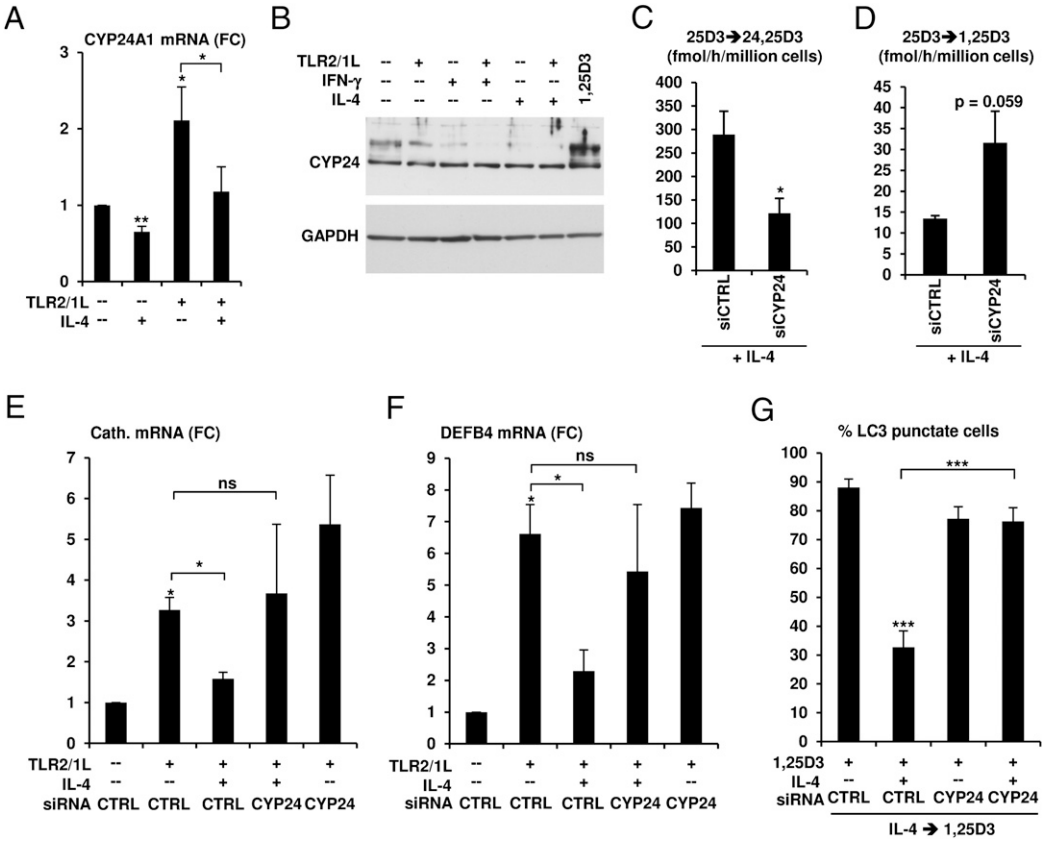

Fig. 5. Regulation of 24-hydroxlase activity by IL-4. (*A*) Primary human monocytes were stimulated with TLR2/1L (10 μg/mL) with or without IL-4 (10^3 U/mL) for 24 h in vitamin D sufficient serum. mRNA levels of CYP24A1 were subsequently determined by qPCR and fold change (FC) was calculated. Data represent mean values ± SEM from five independent experiments. (*B*) Western blot was performed on total cell lysates from primary human monocytes stimulated with TLR2/1L (10 μg/mL) with or without IFN-γ (100 ng/mL) or IL-4 (10^3 U/mL) for 48 h. 1,25D_3 (10^{-8} M) was included as a positive control. (*C*) Primary human monocytes were transfected with siRNA oligos specific for CYP24A1 (siCYP24) or nonspecific (siCTRL), then treated with IL-4 (10^3 U/mL) for 40 h, followed by 25D_3 bioconversion as measured by HPLC. The rate of conversion of [^{3}H]-25D_3 to [^{3}H]-24,25D_3 and [^{3}H]-1,25D_3 was calculated (n = 3). Primary human monocytes transfected with siCTRL or siCYP24 were stimulated with TLR2/1L and IL-4 as detailed in *A*. (*E*) Cathelicidin and (*F*) DEFB4 mRNA levels were measured by using qPCR. Data represent mean values ± SEM from five independent donors. (G) Primary monocytes transfected with siCYP24 or siCTRL, then treated with IL-4 (10^3 U/mL) for 6 h followed by the addition of 1,25D_3 (10^{-8} M) for 18 h. The cells were immunolabeled for intracellular LC3 expression and cellular nuclei. Cells were visualized by using confocal microscopy and enumerated for the percentage of cells positive for LC3-punctate formation. Data displayed are the average percentage of cells positive for LC3-punctate formation per field of view from two independent donors ± SEM (n > 23; *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant).

IL-4 treatment had no effect on 1,25D_3-induced autophagy (Fig. 5*G*). In contrast, in monocytes transfected with siCTRL, IL-4 inhibited 1,25D_3-induced autophagy by 62% (P < 0.001; Fig. 5*G*). These observations confirmed that although IL-4 had no effect on monocyte expression or activity of CYP24A1, the enzyme mediated, at least in part, IL-4–induced 24-hydroxylase activity as well as IL-4 inhibition of TLR2/1-induced antimicrobial peptide expression and autophagy.

Discussion

Although a key function of the innate immune response is to instruct the acquired T-cell response, it has become increasingly clear that T-cell cytokines regulate the innate immune response. We therefore investigated whether specific T-cell cytokines regulate the TLR2/1-induced, vitamin D-dependent antimicrobial pathway in human monocytes. The striking result was that IFN-γ and IL-4 differentially affected TLR2/1 induction of the antimicrobial peptides cathelicidin and DEFB4 by different mechanisms. TLR2/1 and IFN-γ together up-regulated expression of CYP27B1 and monocyte conversion of 25D_3 to the active metabolite 1,25D_3, resulting in activation of the VDR and downstream induction of cathelicidin and DEFB4. In contrast, IL-4 induced CYP24A1-dependent conversion of 25D_3 to the inactive metabolite 24,25D_3, inhibiting TLR2/1 induction of antimicrobial peptide expression and autophagy. Therefore, Th1 and Th2 cytokine responses differentially affect innate antimicrobial pathways through regulation of opposing facets of monocyte vitamin D metabolism.

A key finding of the present study was that IL-4 was found to have a previously unrecognized and dramatic effect on monocyte vitamin D metabolism. IL-4, alone or in the presence of TLR2/1L, enhanced conversion of 25D_3 to the inactive catabolic product 24,25D_3. However, there was no detectable up-regulation, as measured by quantitative PCR (qPCR) or Western blot, of cytochrome P450 enzymes with known or potential 24-hydroxylase activity in IL-4–treated monocytes. Nevertheless, we were able to demonstrate by siRNA knockdown a role for the established 24-hyroxylase CYP24A1 in mediating the conversion of 25D_3 to 24,25D_3. Knockdown of CYP24A1 also reversed the ability of IL-4 to inhibit TLR2/1-induced antimicrobial peptide expression as well as 1,25D_3-mediated autophagy. There is evidence to suggest that increases in 24-hyrdoxylase activity can occur in the absence of elevated enzyme levels, but may instead be a result of preferential delivery of the substrate to a mitochondrial microenvironment containing the enzyme (15). Although our data indicate that one mechanism by which IL-4 inhibits TLR2/1-

induced and $1,25D_3$-triggered responses is via up-regulation of 24-hydroxylase activity, we cannot exclude that IL-4 inhibits $1,25D_3$ induction of antimicrobial peptide expression and autophagy via other mechanisms. In addition, it will be of interest to determine the effect of IL-4 on vitamin D metabolism in other cell types, including macrophages. Nevertheless, inhibition of CYP24A1 expression by siRNA also led to increased conversion of $25D_3$ to $1,25D_3$, presumably by (*i*) maintaining the pool of substrate $25D_3$ available to the CYP27B1-hydroxylase and (*ii*) inhibiting the conversion of active metabolite $1,25D_3$ to inactive, 24-hydroxylated metabolites. The present findings are clinically relevant, as increased IL-4 responses have been observed in patients with tuberculosis in developing countries (16–18) and associated with the development of tuberculosis in health care workers in a developed country (19).

In contrast to the action of IL-4 on vitamin D metabolism, IFN-γ enhanced TLR2/1L induction of the 1-α hydroxylase CYP27B1, potentiating the conversion of $25D_3$ to $1,25D_3$. IFN-γ has been shown to up-regulate conversion of $25D_3$ to $1,25D_3$ in activated macrophages from patients with tuberculosis and sarcoidosis (20, 21) and was later shown to induce CYP27B1 directly (22, 23). In addition, local IFN-γ production was seen in disease lesions of patients with localized mycobacterial infection, including tuberculosis pleuritis (24).

We previously reported that Th1 and Th2 cytokines differentially affect TLR2/1-induced proinflammatory cytokine release, specifically IL-12p40 and TNF-α (25). This was related in part to the ability of IFN-γ to up-regulate TLR1 expression and IL-4 to inhibit TLR2 expression. However, both of these cytokines up-regulated expression of the mRNAs encoding for CYP27B1 and CYP24A1, yet differentially affected monocyte vitamin D metabolism and subsequent expression of antimicrobial peptide mRNAs, providing a unique mechanism by which these cytokines affect host immune responses. These cytokines influence a number of other key host defense pathways, for example autophagy, which facilitates endosomal maturation, required for inhibiting the growth of intracellular *M. tuberculosis* (12, 26). IFN-γ has been shown to induce autophagy (27), whereas IL-4 can block starvation-induced or IFN-γ–induced autophagy and autophagic control of intracellular *M. tuberculosis* growth (13). In data presented here, vitamin D-induced autophagy (12) was also blocked by treatment with IL-4. More recently, the TLR2/1L-induced, vitamin D-dependent antimicrobial pathway has been shown to involve autophagy (28). It is therefore reasonable to hypothesize that the ability of IFN-γ and IL-4 to differentially regulate monocyte vitamin D metabolism controls the monocyte autophagy program.

In summary, our data point to a key and differential role for Th1 versus Th2 cytokines in regulating TLR-induced antimicrobial responses through their ability to trigger distinct monocyte vitamin D metabolic pathways, providing a unique mechanism by which IL-4 regulates innate immune responses.

Materials and Methods

Reagents. TLR2/1L is a triacylated lipopeptide of the *M. tuberculosis* 19-kDa antigen (EMC Microcollections). Recombinant human cytokines used were IFN-γ (BD Biosciences), IL-4 (PeproTech), and IL-17A (eBioscience). Predesigned siRNA oligos were purchased (Thermo Scientific) and used as recommended by the manufacturer. Radiolabeled ^3H-$25D_3$ (specific activity, 155 Ci/mmol; Perkin-Elmer) and unlabeled $1,25D_3$ (Biomol) were purchased. Antibodies used for Western blot were rabbit anti-human CYP24 (Santa Cruz Biotechnology), mouse anti-human GAPDH (BioVision), and HRP-conjugated goat anti-mouse and goat anti-rabbit (Thermo Scientific). Antibodies used for confocal were mouse anti-human LC3 antibodies (MBL International) and Alexa 488-labeled goat anti-mouse IgG1 secondary antibody (Invitrogen).

Cell Culture. All donors were healthy and provided written informed consent, with approval by the institutional review board of the University of California, Los Angeles, for the collection of peripheral blood and subsequent analysis. Human monocytes were purified from peripheral blood mononuclear cells through plastic adherence, and cultured in 10% FCS or 10% vitamin D sufficient human serum as reported (8). For transfections, Percoll-enriched monocytes were transfected with 100 pmol of siRNA oligos by using the Amaxa Nucleofection System and the Human Monocyte Kit (Lonza) as previously described (10). For measurement of autophagy, monocytes were adhered onto glass chamber slides and then treated as noted. The cells were then immunolabeled for LC3 as previously described (12) and visualized by using confocal microscopy.

Real-Time qPCR. Following stimulation, RNA was isolated, cDNA synthesized, and qPCR performed as previously described (8). Reactions use SYBR Green PCR Master Mix and were run on a DNA Engine Opticon II (Bio-Rad). The relative quantities of the gene tested per sample were calculated against 36B4 using the delta delta cycle threshold formula as previously described (29). The data were normalized by fold change to media control samples.

Measurement of $25D_3$ Bioconversion. Cells were treated for 48 h, followed by incubation with radiolabeled ^3H-$25D_3$ for 5 h in serum-free media. Measurement of $25D_3$ bioconversion to $1,25D_3$ or $24,25D_3$ was carried out as previously described (9).

Western Blot Analysis. Monocytes were treated with for 48 h and total cell extracts prepared using M-PER reagent with protease inhibitors (Thermo Scientific) according to the manufacturer's recommendations. Equal amount of protein were loaded onto 12% Bis-Tris NuPage minigels (Invitrogen) and electrophoresed for 1 h at 125 V. Western blotting was performed by standard methods. Briefly, resolved proteins were transblotted onto nitrocellulose membrane for 1 h at 95 V, then blocked for 1 h followed by overnight incubation at 4 °C with primary antibody. The appropriate secondary antibody was incubated for 1 h at room temperature and specific protein detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

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