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Research Article **Immunology**

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Ligation of TLR9 induced on human IL-10–secreting Tregs by 1α,25-dihydroxyvitamin D3 abrogates regulatory function

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Introduction

The TLRs represent a family of evolutionarily conserved receptors, which recognize pathogen-associated molecular patterns (PAMPs) and certain host molecules. Ten TLRs (TLR1–10) have been identified in humans to date. These are proposed to play central roles in the induction of innate immune responses and in triggering host immunity to infection (1–3). The capacity of TLRs to control adaptive immunity was thought to critically involve APCs, which activate naive T cells or modulate effector T cells following ligation of one or more TLRs (4).

Recent evidence has highlighted a role for direct stimulation of T cells by PAMPs. mRNA specific for TLRs in human T cell populations has been reported (5, 6). Signaling through TLR2, TLR5, and TLR7/8 has been shown to be a costimulator of highly purified human T cells, enhancing cytokine production, survival, and proliferation in the absence of APCs (7–10).

A significant indication that TLR signaling may play a role in the maintenance and/or function of Tregs was an observed reduction in the frequency of natural $CD4^+CD25^+$Tregs, but not $CD25^-$ T cells, in mice lacking MyD88, a key adaptor molecule involved in signaling through the majority of TLRs (11). mRNA for a range of TLRs has now been detected in rat, mouse, and human $CD4^+CD25^+$ Tregs (9, 11–15). An early study reported that the TLR agonist LPS potently enhanced proliferation and regulatory activity of murine $CD4^+CD25^+$ Tregs (12). However, more recent studies have indicated that ligation of other TLRs, for example TLR2, on effector T cells or $CD4^+CD25^+$ Tregs may actually alleviate suppression by enhancing effector T cell proliferation and, in some cases, diminishing FoxP3 expression in Tregs (9, 11, 13–15).

The binding of PAMP to TLRs on APCs results in the production of an array of proinflammatory cytokines, including IL-12, IFN-α, IL-6, IFN-γ, and IL-8, in order to mount an effective innate response. TLR signal transduction can, under certain circumstances, also elicit a counterregulatory response in the form of IL-10 secretion, as demonstrated in *Tlr2*$^{-/-}$ mice, which have an impaired capacity to synthesize IL-10 (16). Human natural $CD4^+CD25^+$ Tregs have also been shown to secrete IL-10 in response to TLR2 stimulation and subsequently to induce IL-10 synthesis in cocultured $CD25^-$ T cells (17). However, the presence of TLRs on IL-10–secreting T cells themselves and the functional consequences of TLR signaling in these Tregs have not been reported.

IL-10 is a potent antiinflammatory cytokine and inhibits Th1 and Th2 immune responses, which has led to considerable interest in its therapeutic potential to treat a wide range of immune-mediated pathologies, including allergy, transplantation, and autoimmune disease (18, 19). We have shown that human IL-10–secreting Tregs (IL-10–Tregs), which express low levels of the $CD4^+CD25^+$ Treg-associated transcription factor FoxP3, can be induced following activation, through either polyclonal stimuli or antigen presented by APCs in the presence of the glucocorticoid dexamethasone (Dex) and the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α25VitD3) (20, 21). In our search

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: Dex, dexamethasone; IL-10–Treg, IL-10–secreting Treg; CpG-ODN, CpG oligonucleotide; PAMP, pathogen-associated molecular pattern; VDR, vitamin D receptor; 1α25VitD3, 1α,25-dihydroxyvitamin D3.

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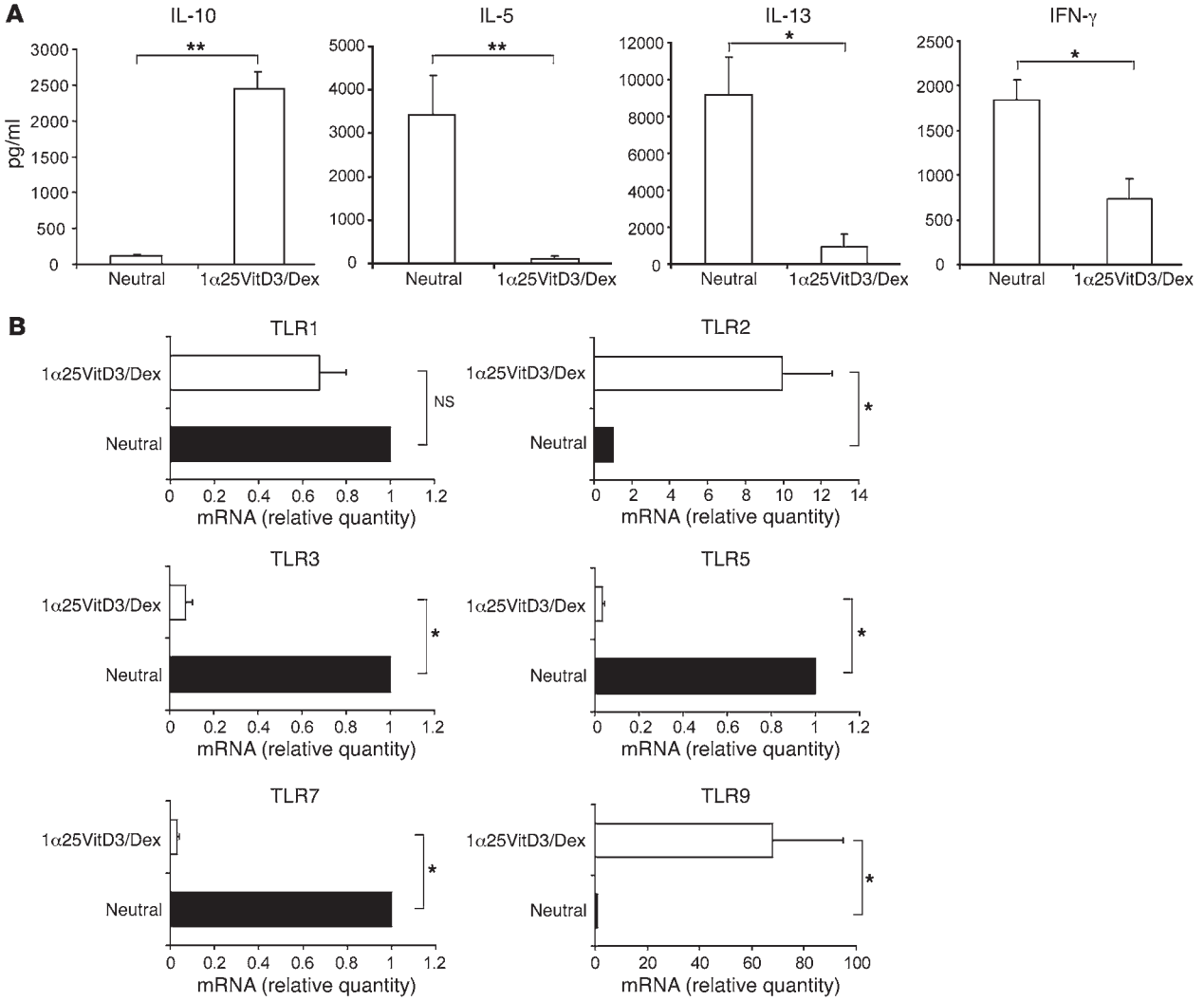

Figure 1
Profile of TLR gene expression by human drug-induced IL-10–Tregs. $CD4^+$ T cells were cultured for two 7-day cycles with anti-CD3, IL-2, and IL-4 (neutral) or additionally with 1α25VitD3 and Dex to generate IL-10–Tregs. (**A**) Cultured supernatants from IL-10–Tregs (1α25VitD3/Dex) or neutral cell lines were generated by restimulation with anti-CD3 and IL-2 for 48 hours. IL-10, IL-5, IL-13, and IFN-γ in supernatants was measured by ELISA. Mean data ± SEM from 10 healthy donors are shown. (**B**) Analysis of TLR gene expression profile was determined by real-time RT-PCR, in neutral versus VitD3/Dex T cell lines at day 14. Data are shown normalized to an endogenous control (18S rRNA) and expressed relative to neutral cells. Mean mRNA levels ± SEM from 5 independent experiments from different healthy donors are depicted. $^*P < 0.05$, $^{**}P < 0.001$ as assessed by Mann-Whitney rank sum test.

to identify molecules uniquely expressed by this population, the profile of TLR expression on 1α25VitD3 and Dex-induced IL-10–Tregs was examined and compared with other relevant peripheral cell populations. High TLR9 transcript abundance was detected in human drug-induced IL-10–Tregs but not in other human effector cell or Treg populations. The functional consequences of signaling via TLR9 on IL-10–Tregs were therefore examined and shown to impair regulatory function. These findings have implications for the use of TLR9 ligands in cancer therapy and as adjuvants in vaccine design.

Results

TLR9 expression is increased in human drug-induced IL-10–Tregs. Human peripheral blood derived $CD3^+CD4^+$ T cells were stimulated with CD3-specific antibody, IL-2, and IL-4 in the absence or presence of 10^{-7} M 1α25VitD3 and 10^{-7} M Dex for 7 days and then restimulated for a further 7 days under identical conditions. This protocol induces T cells producing high levels of IL-10 but low levels of Th1- and Th2-associated cytokine mRNA and protein, referred to as "drug-induced IL-10–Tregs" (20, 21) (Figure 1A). The profile of TLR gene expression by drug-induced IL-10–Tregs was compared with that of cells in control cultures lacking any drugs ("neutral"). Expression of *TLR2* and *TLR9* mRNA was clearly and significantly elevated in drug-induced IL-10–Tregs in comparison with cells from control cultures and freshly isolated $CD4^+$ T cells (Figure 1B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32354). In contrast, TLR1 transcript abundance was not significantly different,

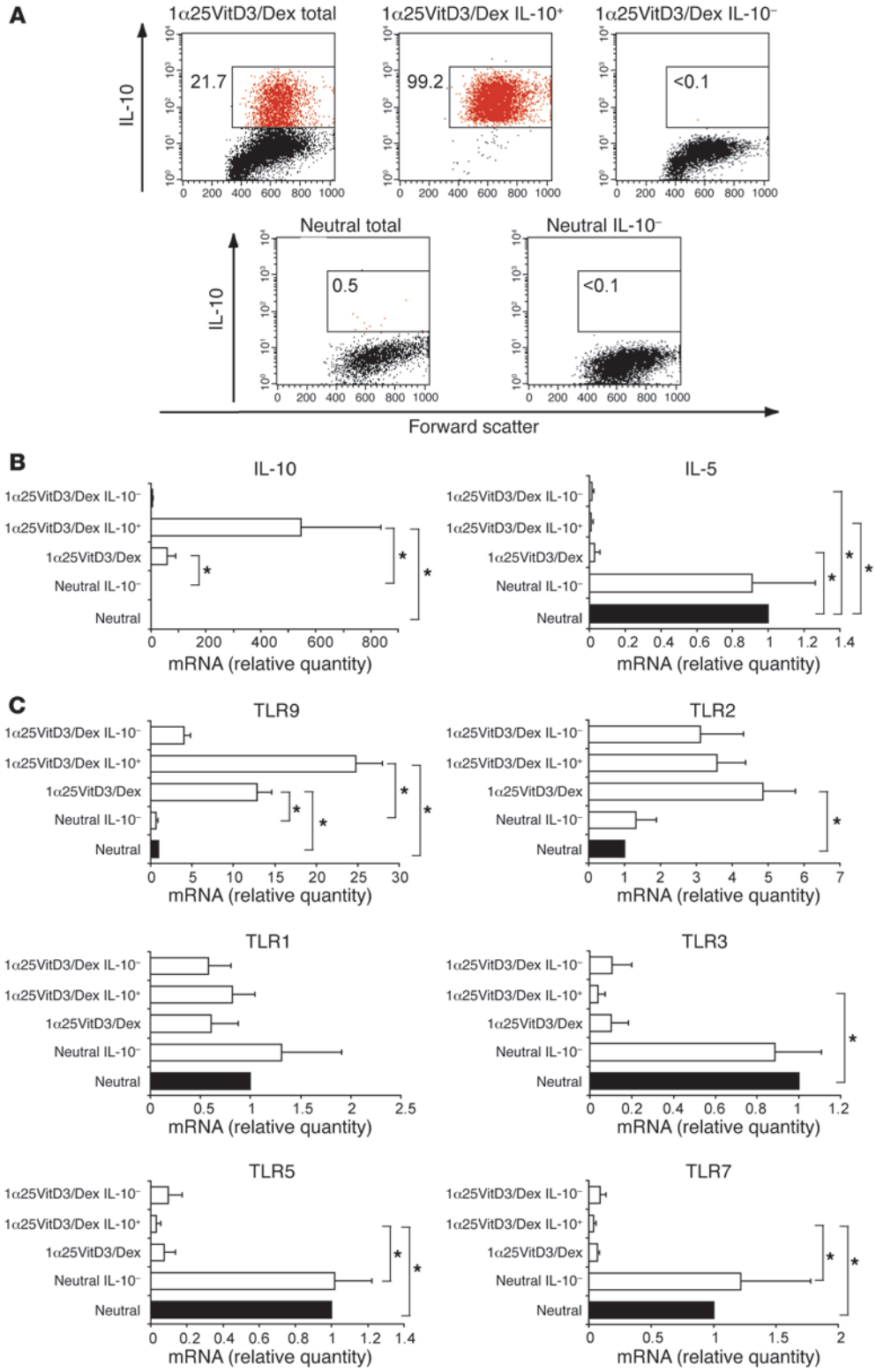

Figure 2
TLR9 expression is selectively enhanced in human drug-induced IL-10–Tregs. (**A**) At day 14, neutral and 1α25VitD3/Dex-treated cells were restimulated for 16 hours with anti-CD3 and IL-2. IL-10$^+$ and IL-10$^-$ cells were detected and isolated using a commercially available IL-10 secretion assay and a cell sorter. FACS profiles of IL-10 expression by control T cell lines (neutral total), drug-induced IL-10–Tregs (1α25VitD3/Dex total) and the isolated IL-10$^+$ (1α25VitD3/Dex IL-10$^+$) and IL-10$^-$ (1α25VitD3/Dex IL-10$^-$ and neutral IL-10$^-$) T cell fractions are shown. Values represent the percentage of gated IL-10$^+$ cells. Data are representative of 4 independent experiments. (**B**) Cytokine and (**C**) TLR gene expression of T cell populations shown in **A**, as assessed by real-time RT-PCR. Data are shown normalized to an endogenous control (18S rRNA) and expressed relative to neutral cells. Mean mRNA levels ± SEM from 4 independent experiments from different donors are depicted. *P < 0.05 as determined using 1-way ANOVA on Ranks.

while *TLR3*, *TLR5*, and *TLR7* mRNA were significantly decreased in IL-10–Tregs compared with control cultures (Figure 1B). *TLR6*, *TLR8*, and *TLR10* mRNA expression was undetectable on both T cell populations, while that of *TLR4* was barely detectable (Supplemental Figure 1 and data not shown).

TLR9 expression correlates with that of IL-10. To examine whether the expression of TLR2 and TLR9 correlated with IL-10 expression, live IL-10–Tregs were enriched from the 1α25VitD3/Dex-treated cultures using an established antibody capture technique and cell sorting. This resulted in an enrichment in bulk drug-treated cultures

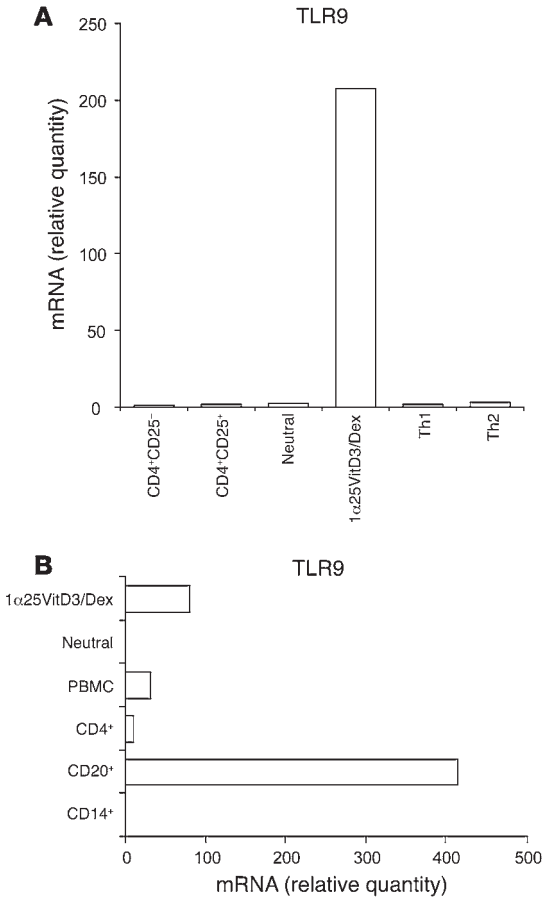

Figure 3
Comparison of TLR9 expression by IL-10–Tregs with other human peripheral blood–derived populations. (**A** and **B**) TLR9 transcript abundance as assessed by real-time RT-PCR in day 14 drug-induced IL-10–Tregs (1α25VitD3/Dex) and control cultures (neutral) was compared with expression in other human T cell populations including naive ($CD4^+CD25^-$), $CD4^+CD25^{hi}$, and day 28 highly differentiated Th1 and Th2 cell lines (**A**) and in non–T cell populations (**B**). $CD4^+CD25^+$ and $CD4^+CD25^-$ populations were isolated by cell sorting and were routinely more than 99% pure. Th1 and Th2 cells were generated from naive T cells according to a previously published protocol (22). B cells were isolated on the basis of CD20 antigen expression and were more than 99% $CD19^+$. Monocytes ($CD14^+$) were more than 96% pure. Data are shown normalized to an endogenous control (18S rRNA) and expressed relative to $CD25^-$ T cells. One representative experiment of 3 performed is depicted.

routinely containing 15%–30% $IL\text{-}10^+$ T cells, to greater than 98% viable $IL\text{-}10^+$ T cells (Figure 2A). TLR profiles were compared with those of the IL-10–depleted cell fraction (<0.1% $IL\text{-}10^+$ T cells) and control (neutral) cultures (<1% $IL\text{-}10^+$ T cells). The predicted profile of cytokine mRNA expression in these populations was confirmed by real-time RT-PCR (Figure 2B). TLR9 transcript abundance was elevated in the $IL\text{-}10^+$ enriched cell fraction in comparison with both the bulk 1α25VitD3/Dex and IL-10–depleted cultures (Figure 2C). However, no difference in TLR2 expression was observed between the $IL\text{-}10^+$ and $IL\text{-}10^-$ fractions, suggesting TLR2 expression did not directly correlate with that of IL-10, and therefore subsequent studies focused exclusively on TLR9. TLR1, TLR3, TLR5, and TLR7 gene expression were also examined for comparative purposes and were not increased in $IL\text{-}10^+$ T cell fractions, compared with the $IL\text{-}10^-$ T cell population (Figure 2C). These data confirm elevated expression of *TLR9* mRNA by drug-induced IL-10–Tregs.

In order to identify whether TLR9 reflects a specific marker for IL-10–Tregs, expression was measured in other human peripheral blood–derived $CD3^+CD4^+$ Treg and effector T cell populations. Expression of *TLR9* mRNA was detectable in Th1 and Th2 effector cell lines differentiated using previously described methodology (22), as well as in naturally occurring Tregs isolated on the basis of high levels of expression of the CD25 antigen by flow cytometry (Figure 3A). However, in all cases, TLR9 was expressed at much lower levels than in the drug-induced IL-10–Tregs. For comparative purposes, the complete TLR profile of $CD4^+CD25^+$ Tregs is shown in Supplemental Figure 2.

Human B lymphocytes are reported to express high levels of TLR9 (23). Expression of TLR9 in bulk 1α25VitD3/Dex-treated cells, which routinely contain 15%–30% $IL\text{-}10^+$ T cells, was therefore compared with that of highly purified B cell populations (>99% $CD19^+$). 1α25VitD3/Dex cultures expressed approximately 25% of *TLR9* mRNA levels detected in the B cell population (Figure 3B), implying comparable levels of expression between $IL\text{-}10^+$ T cells and B cells.

TLR9 is expressed on both human peripheral blood– and respiratory tissue–derived IL-10–Tregs. The clinical symptoms of disease occur at tissue sites. We therefore addressed whether IL-10–Tregs could be induced from human respiratory tissue–derived T cells and whether TLR9 represents a marker of these cells. As seen in peripheral blood–derived cultures, $CD3^+CD4^+$ T cells derived from lung-draining lymph nodes and $CD3^+$ T cells from nasal polyps, stimulated with anti-CD3 in the presence of 1α25VitD3 and Dex for 14 days, expressed elevated levels of IL-10 but low amounts of Th1- and Th2-associated cytokines in comparison with cells stimulated under neutral conditions (Supplemental Figure 3 and data not shown). Drug-induced IL-10–Tregs derived from both lung-draining lymph nodes and from nasal polyps expressed elevated levels of TLR9 compared with the same cells cultured in the absence of drugs (Supplemental Figure 3).

TLR9 expression is induced by 1α25VitD3 in vitro. To investigate whether TLR9 expression was primarily regulated by 1α25VitD3 or Dex, peripheral blood–derived $CD3^+CD4^+$ T cells were stimulated in the presence of either drug alone or in combination. TLR9 induction was predominantly regulated by 1α25VitD3, with little or no effect of Dex alone. TLR9 was maintained or slightly enhanced in cultures containing both drugs, although this did not reach statistical significance (Figure 4A). In contrast to effects on TLR9, 1α25VitD3 profoundly downregulated TLR3, TLR5, and TLR7 but had little effect on TLR1 (Supplemental Figure 4). The effect of 1α25VitD3 upon TLR and cytokine transcript abundance was dose dependent, and a highly comparable concentration dependency for the induction of both IL-10 and TLR9 was observed (Figure 4B). Concentrations of 10^{-9}–10^{-7} M 1α25VitD3 enhanced *TLR9* and *IL10* mRNA but inhibited the expression of the Th1- and Th2-associated cytokines IL-13 and IFN-γ. However, the highest concentration of 1α25VitD3 (10^{-6} M), likely to represent supraphysiological levels (24), failed to significantly induce IL-10 or TLR9 and resulted in less profound reduction in the expression of the effector cytokines. These data highlight the close association and regulation of IL-10 and TLR9 expression.

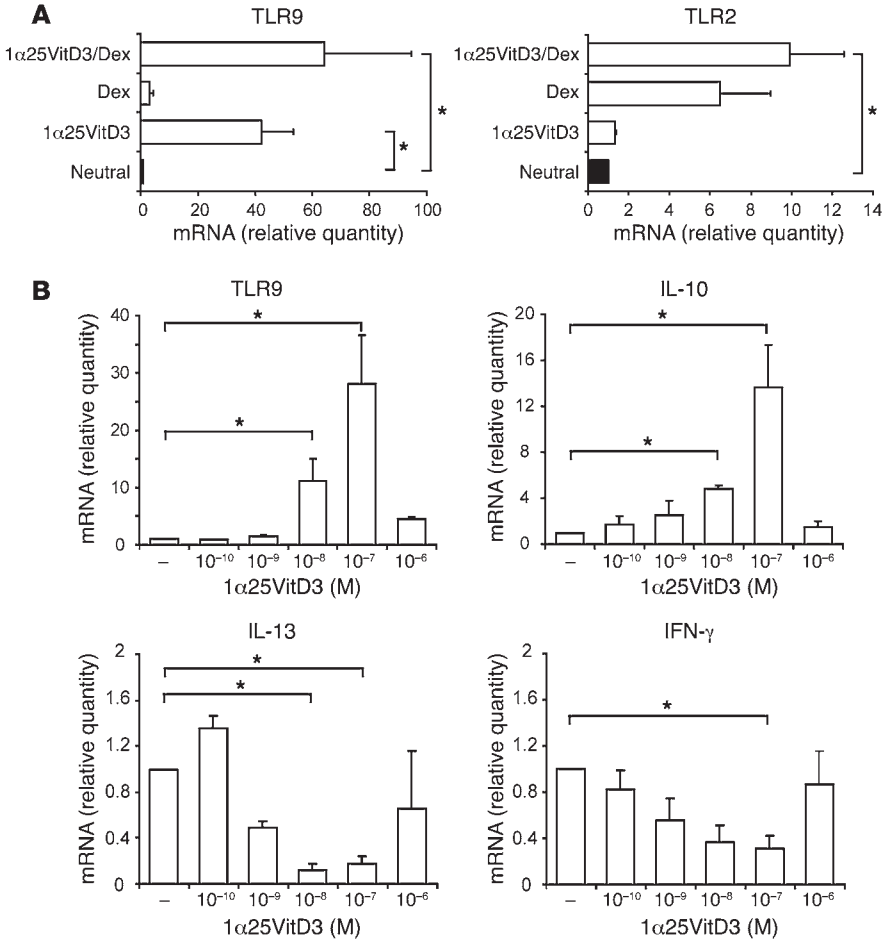

Figure 4
TLR9 expression is primarily induced by 1α25VitD3 in vitro. (**A**) The effects of 2 rounds of 7-day cultures with 1α25VitD3 and Dex, singly or in combination, on the expression of *TLR2* and *TLR9* mRNA by $CD4^+$ T cells were examined by real-time RT-PCR. (**B**) IL-10, IL-13, IFN-γ, and TLR9 transcript abundance was analyzed by real-time RT-PCR following 2 rounds of stimulation with anti-CD3, IL-2, and IL-4 alone (–) or with increasing concentrations of 1α25VitD3. Data are shown normalized to an endogenous control (18S rRNA) and expressed relative to neutral cells. Mean data ± SEM from 5 (**A**) or 4 (**B**) independent experiments from different healthy donors are depicted. *$P < 0.05$ as assessed by 1-way ANOVA on Ranks.

Ingestion of calcitriol by humans induces a parallel increase of IL-10 and TLR9 in $CD3^+CD4^+$ T cells. We previously demonstrated that administration of oral calcitriol (1α25VitD3) to 3 steroid-insensitive asthma patients restores IL-10 synthesis by their T cells (21). To determine whether TLR9 represents a potential marker of drug-induced IL-10–Tregs in vivo, we assessed how ingestion of calcitriol for 1 week by these individuals influenced expression of TLR9 and IL-10. $CD3^+CD4^+$ T cells were purified from freshly derived peripheral blood before and after calcitriol ingestion and analyzed directly ex vivo in the absence of any in vitro manipulation. In all individuals tested, IL-10 and TLR9 expression were increased following calcitriol ingestion, with the greatest increase being observed on day 3 (Figure 5). No increase or reduction in the Th2 cytokines IL-5 or IL-13 and Th1 cytokine IFN-γ or TLR1, TLR2, TLR3, or TLR5 was observed in the same T cell population.

Induction of IL-10 and TLR9 by 1α25VitD3 is IL-10 dependent and requires continued presence of 1α25VitD3 to maintain expression. The relationship between TLR9 and IL-10 expression was examined further. Kinetic studies indicated that the induction of TLR9 and IL-10 expression by 1α25VitD3 alone was not significantly increased above neutral cultures at day 7 (data not shown) and was most marked following 14 days or more of culture (Figure 6A). Removal of 1α25VitD3 at day 14 resulted in a gradual decline of both *IL10* and *TLR9* mRNA levels. TLR9 transcript abundance was comparable with neutral cultures 7 days after 1α25VitD3 withdrawal, while IL-10 showed a more gradual but steady loss of gene expression (Figure 6A), suggesting that the continued presence of 1α25VitD3 is required to maintain optimal expression of both molecules.

As shown in Figure 6B, blocking of IL-10 action throughout the 14-day culture period inhibited the 1α25VitD3-mediated increase in *TLR9* and *IL10* gene expression, indicating that the capacity of 1α25VitD3 to modulate both molecules was IL-10 dependent ($n = 4$; $P < 0.05$ for both *TLR9* and *IL10*). However, addition of exogenous IL-10 to the control cultures did not result in significant induction of either molecule, demonstrating that IL-10 was necessary but not sufficient to increase TLR9 and IL-10 expression. Supplementing 1α25VitD3 cultures with recombinant IL-10 increased TLR9 and IL-10 expression in 2 of the 4 donors tested, but overall this did not reach statistical significance (Figure 6B and data not shown).

In an attempt to dissect the association between TLR9 and IL-10 expression, knockdown of TLR9 was performed using 3 gene-specific lentiviral shRNA constructs containing 3 different sequences of TLR9 siRNA. $CD4^+$ T cells were cultured for 7 days with 1α25VitD3 and then transduced with either a non-targeting control siRNA or each of the 3 TLR9 shRNA lentiviral constructs (siRNA A, B, or C; Figure 6C). Knockdown of TLR9 of 85% or more compared with the control siRNA was achieved with all 3 lentiviral constructs in the 2 experiments performed. This effect appeared to be specific for TLR9, since mRNA for control TLRs (TLR2, TLR5 and TLR7) were not reduced by this treatment. TLR9 knockdown was associated with a slight reduction (maximally 20%) of *IL10* mRNA, but no decline in IL-13 or IFN-γ effector cytokine transcript

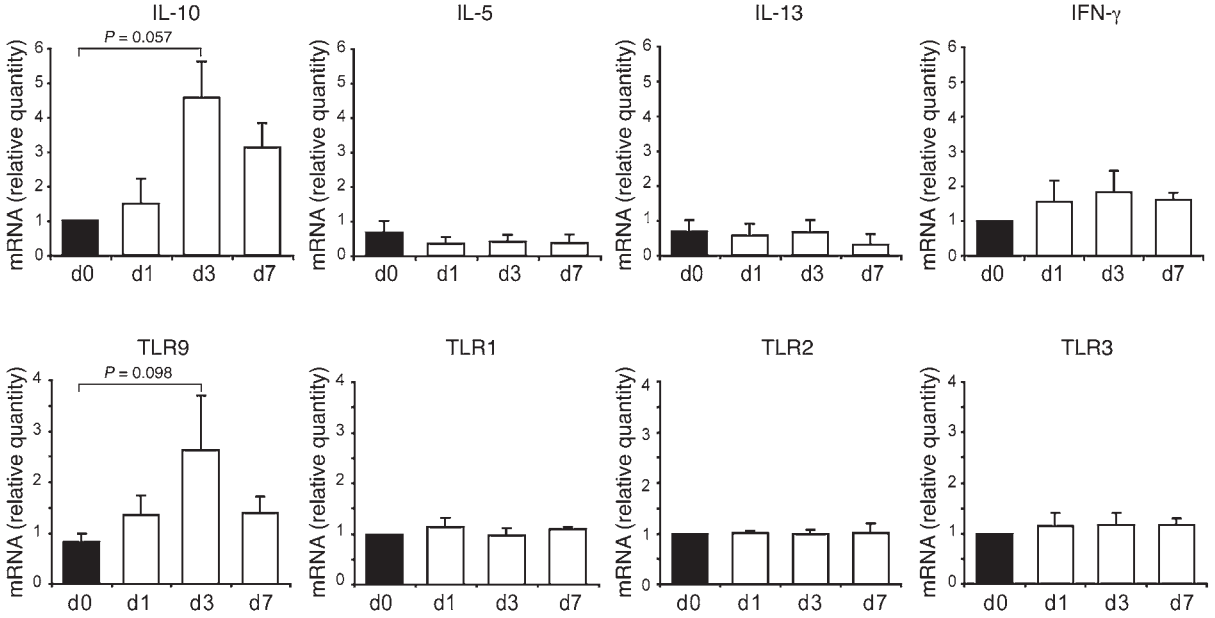

Figure 5

1α25VitD3 elevates IL-10 and TLR9 expression in vivo. $CD3^+CD4^+$ T cells were isolated from 3 steroid-insensitive asthma patients before treatment (d0) and 1, 3, or 7 after treatment with oral calcitriol (1α25VitD3). TLR and cytokine gene expression was examined ex vivo by real-time RT-PCR. Data are shown normalized to an endogenous control (18S rRNA) and expressed relative to day 0 cells. Mean mRNA levels ± SEM from 3 donors are depicted.

abundance was observed (Figure 6C). At this level, it seems probable that the loss of IL-10 is due to the non-specific loss of $IL\text{-}10^+$ T cells rather than specific downregulation of the *IL10* gene.

Pretreatment of drug-induced IL-10–Tregs with CpG oligonucleotides leads to loss of regulatory activity and reduced IL-10 synthesis. The functional relevance of TLR9 expression upon the suppressive activity of IL-10–Tregs was examined using the TLR9 agonist CpG oligonucleotide (CpG-ODN) 2006. $CD4^+$ T cells that had been stimulated for 14 days in the presence of 1α25VitD3 were pretreated overnight in medium or with CpG-ODNs and then washed extensively. The capacity of these cells to inhibit the proliferative response of freshly isolated, autologous CFSE-labeled naive $CD4^+CD45RA^+$ T cells was then assessed. The CFSE-labeled naive T cells proliferated following stimulation with anti-CD3 in culture, with around 42% of cells entering cell cycle at day 5 (Figure 7). Addition of the control (neutral) cell line did not alter their proliferative response. In contrast, the cell line generated in the presence of 1α25VitD3 inhibited naive T cell proliferation to a level comparable with that of unstimulated cells. Pretreatment of IL-10–Tregs with CpG-ODNs prevented their capacity to block the proliferation of the naive T cells, implying that ligation of TLR9 on drug-induced IL-10–Tregs impairs regulatory function.

Parallel experiments demonstrate that when $CD4^+$ T cells stimulated for 14 days in the presence of 1α25VitD3 were restimulated in the presence of CpG-ODNs for 48 hours, downregulation of IL-10 synthesis was observed in response to 2 separate CpG-ODN sequences 2216 and 2006 (Figure 8A). Inhibition of IL-10 synthesis by CpG-ODNs was concentration dependent, with statistically significant effects seen with concentrations of 5 μM or higher. An unexpected and reproducible observation was the dose-dependent upregulation of the Th2 cytokine IL-4 with CpG-ODN 2006, and to a lesser extent with CpG-ODN 2216. Although cytokine analysis by FACS demonstrated a 50% or greater reduction in IL-10 positivity upon CpG exposure, staining for IL-4 was consistently at less than 1%, and therefore the change in frequency of these cells could not be assessed (data not shown). Regulation of the Th2 cytokine gene cluster is generally thought to occur in parallel. However, the low levels of synthesis of the Th2 cytokines IL-5 and IL-13 were unaffected by either class of CpG-ODNs (IL-5 data not shown). CpG-ODN 2006, but not CpG-ODN 2216, inhibited IFN-γ synthesis. No evidence for the induction of either cell death or expansion of the CpG-ODNs exposed IL-10–Tregs was observed (data not shown). These data imply that CpG-ODNs actively modulate the function of IL-10–Tregs.

In order to confirm the specificity of TLR9 agonist effects on IL-10–Treg cytokine production, control TLR ligands specific for TLR3 (poly [I:C]), TLR4 (LPS), and TLR7 (imiquimod) were also assessed in an identical manner to CpG-ODNs (Figure 8B). All of these control TLR ligands failed to inhibit IL-10 or increase IL-4 expression.

Discussion

The present study demonstrates that 1α25VitD3 (calcitriol) increased IL-10 and TLR9 expression by human $CD3^+CD4^+$ T cells both in vitro and following ingestion by patients. Ligation of TLR9 by specific agonist CpG-ODNs inhibited IL-10 synthesis and the regulatory activity of 1α25VitD3-induced IL-10–Tregs. These data suggest that modulation of IL-10–Treg function by TLR9 ligands may occur during infection and natural exposure to ligands and might also influence the outcome of clinical strategies using TLR9 agonists for immune intervention. We propose a model whereby 1α25VitD3 plays a role in maintaining IL-10–Tregs in the host. Coexpression of TLR9 with IL-10 provides a mechanism whereby inappropriate actions of Tregs can be temporarily disabled to enhance the host immune response

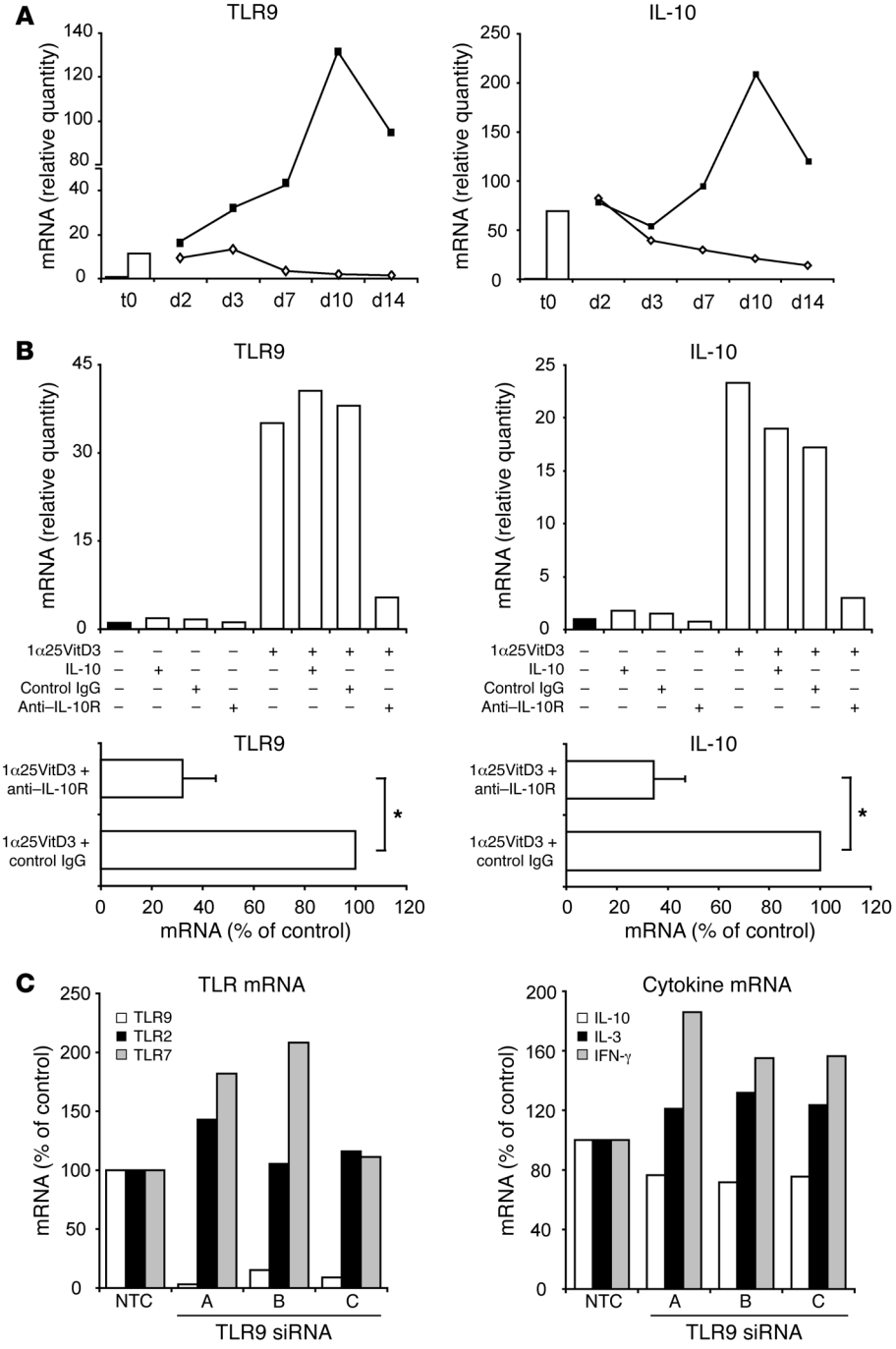

Figure 6

1α25VitD3-induced IL-10 and TLR9 are IL-10 dependent and require continued presence of 1α25VitD3 to maintain expression. (**A**) $CD4^+$ T cells were cultured in neutral conditions or with 1α25VitD3 (open bar) until day 14 (t0). Cells cultured with 1α25VitD3 for 14 days were then restimulated with (filled squares) or without (open diamonds) 1α25VitD3 for up 2 weeks. *IL10* and *TLR9* mRNA was assessed at the indicated time points. A representative experiment from 2 different healthy donors is shown. (**B**) Top: $CD4^+$ T cells were stimulated under neutral conditions or with 1α25VitD3 for 14 days. Recombinant IL-10 (5 ng/ml), anti–IL-10 receptor (5 μg/ml), or control IgG2a (5 μg/ml) was added as indicated from day 0. Results from 1 representative experiment of 4 performed is shown. Bottom: Mean mRNA levels ± SEM (from 4 donors) from cultures treated with 1α25VitD3 and control IgG or anti–IL-10 receptor are depicted. $^*P < 0.05$ as assessed by Mann-Whitney rank-sum test. (**C**) Following 7 days of culture with 1α25VitD3, cells were restimulated (with anti-CD3, IL-2, and 1α25VitD3) and transduced with either a non-targeting control siRNA or 3 TLR9 siRNAs (siRNAs A–C). Puromycin was added after 72 hours, and RNA was extracted 3 days later. TLR mRNA (white bar, TLR9; black bar, TLR2; gray bar, TLR7) and cytokine mRNA (white bar, IL-10; black bar, IL-13; gray bar, IFN-γ) were assessed. One representative experiment from 2 healthy donors is depicted.

to infection. IL-10 is known to impair the clearance of both viral and bacterial pathogens (25, 26).

There is increasing evidence to support the role of the vitamin D pathway in the regulation of immune function (27–29), in addition to its well-established role in the homeostatic control of calcium and bone metabolism. The prevalence of vitamin D insufficiency (defined by serum 25-hydroxyvitamin D3 levels of less than 75 nmol/l) is remarkably common. A recent study in the white British population suggested that up to 87% of individuals exhibit vitamin D insufficiency/deficiency in winter and spring (30). Epidemiological studies have assessed the effects of vitamin D availability on a range of immune pathologies, concluding that vitamin D sufficiency is associated with reduced risk of numerous cancers (31), while deficiency is linked with a higher risk of autoimmune conditions (32). In respiratory disease, a recent study showed a positive correlation between serum 25-hydroxyvitamin D3 levels and predicted lung function in a large sample of the USA population (approximately 14,000 subjects) (33, 34). In parallel, a high rate of vitamin D deficiency (<50 nmol/l serum 25-hydroxyvitamin D3) was reported among individuals of South Asian ethnic origin in London, a population that also exhibited high levels of severe and poorly controlled asthma (35). 25-hydroxyvitamin D3

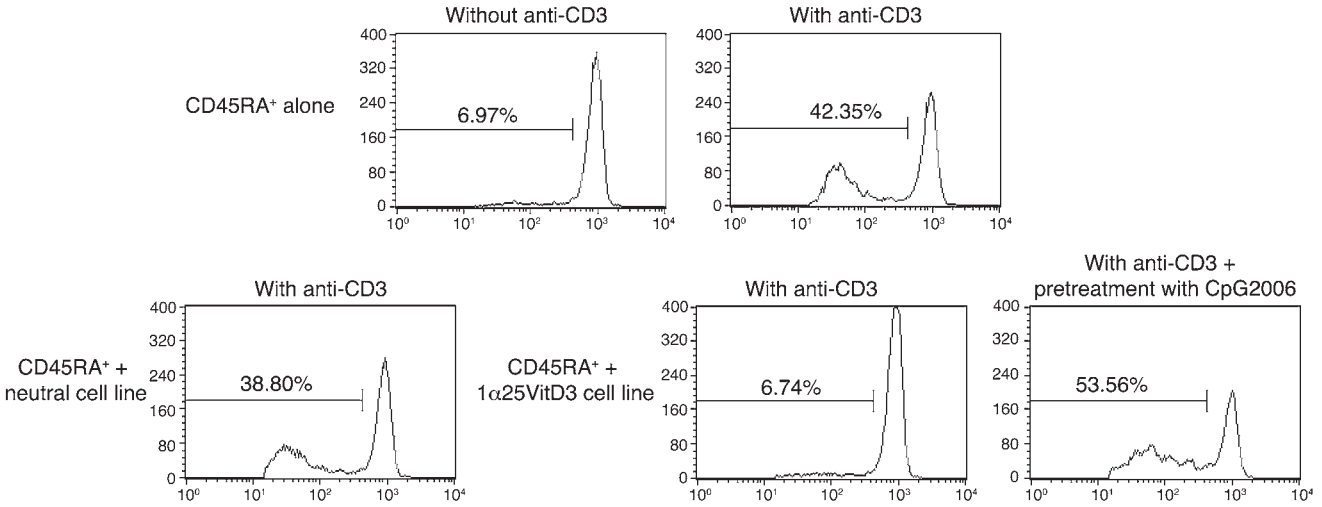

Figure 7

CpG-ODNs abrogate regulatory activity of drug-induced IL-10–Tregs. $CD4^+$ T cells were cultured for two 7-day cycles with anti-CD3, IL-2, IL-4 alone (neutral) or with 1α25VitD3, then harvested, washed, and pretreated for 24 hours with anti-CD3 and IL-2 alone or together with 10 μM CpG-ODN 2006. Autologous $CD45RA^+$ T cells were isolated, CFSE labeled, and cocultured with the cell lines at a ratio of 2:1 responder/cell line for 5 days with suboptimal anti-CD3 (0.1 μg/ml) and CD28 (2 μg/ml). Values within the histograms represent the percentage of proliferating, viable CFSE-labeled responders as assessed by FACS. Data from 1 representative healthy donor of 2 studied are depicted.

levels have also been positively associated with protective immune responses to infection in human populations, including infection with mycobacteria and influenza (reviewed in refs. 28 and 36). Indeed, one of the most striking effects of the vitamin D pathway is on the innate immune system. 1α25VitD3 and its analogs induce antimicrobial gene expression, including the human cathelicidin antimicrobial peptide and defensin β2 genes, in human keratinocytes, monocytes, epithelial cells, and neutrophils (36–40).

Our study was originally designed to identify biomarkers of IL-10–Tregs. However, our data support the conclusion that TLR9 is not specific to all IL-10–secreting T cells per se (e.g., steroid-induced $IL\text{-}10^+$ T cells; CD46-induced $IL\text{-}10^+$ T cells; our unpublished observations), which represent cells that are rapidly but transiently induced to express IL-10 (41). In contrast, induction by 1α25VitD3 occurs more slowly but is stable in the presence of 1α25VitD3, and under these conditions TLR9 and IL-10 expression is tightly linked. We therefore propose that vitamin D status of the host controls IL-10 (and TLR9) expression. There is emerging literature on the high prevalence of vitamin D deficiency and its association with immune disorders (e.g., Crohn disease, type 1 diabetes mellitus, rheumatoid arthritis) and, as we have emphasized, poor respiratory health (33, 42). We and others (e.g., ref. 43) propose a model whereby vitamin D sufficiency is essential to maintain appropriate regulatory pathways, specifically IL-10, to help prevent immune disorders and to maintain respiratory health.

A close association between the capacity of 1α25VitD3 to increase the expression of IL-10 and TLR9 on human T cells was observed not only in vitro, but also in vivo. We previously reported that ingestion of calcitriol by steroid-insensitive asthmatic patients, who respond poorly to steroids for the induction of IL-10 in vitro, restored their capacity to synthesize IL-10 in response to steroids, suggesting a potential role for calcitriol as a steroid-enhancing agent in chronic inflammation (18). Here we show the direct capacity of ingestion of calcitriol to increase IL-10 and that this correlates with increased TLR9 expression by their $CD3^+CD4^+$ T cells analyzed directly ex vivo. In vitro, the kinetics and 1α25VitD3 concentration dependency of increased TLR9 and IL-10 expression by human T cells were highly comparable. The sustained expression of both IL-10 and TLR9 by human T cells was dependent on the continuing presence of 1α25VitD3 in culture, suggesting that the expression of both of these molecules is likely to be influenced by vitamin D status. Inhibition of IL-10 signaling profoundly downregulated (by 75%–90%) both IL-10 and TLR9, implying that induction of both molecules is IL-10 dependent. In contrast, knockdown of TLR9 is highly effective, but only a slight (<20%) reduction in *IL10* mRNA is observed, likely due to the nonspecific loss of $IL\text{-}10^+$ T cells rather than specific regulation of the *IL10* gene. Furthermore, the 1α25VitD3 withdrawal experiments demonstrate that TLR9 expression is lost more rapidly than IL-10. Together these data imply that expression of IL-10 is more stable and can occur independently of TLR9, but that TLR9 (and IL-10) expression is highly IL-10 dependent.

The effects of 1α25VitD3 to increase TLR9 expression may be cell specific, as an independent study has shown that short-term culture of murine islet cells with a vitamin D receptor (VDR) agonist does not alter TLR transcript abundance, including TLR9 (44). However, its capacity to regulate IL-10 synthesis and tolerance appears to be more widely applicable. In vitro studies indicate that 1α25VitD3 enhances IL-10 secretion by human dendritic cells (45), in addition to the effects on $CD4^+$ T cells described here, while in vivo 1α25VitD3 has been shown to promote tolerance, presumably at least in part via its effects on APCs (46–48). In support of the present study in asthma patients, a double-blind, randomized placebo-controlled trial demonstrated that vitamin D supplementation in patients with congestive heart failure improved cytokine profiles by enhancing IL-10 and improving TNF/IL-10 ratios (49). Furthermore, in an animal model of allergic experimental encephalomyelitis, IL-10 signaling was essential for 1α25VitD3-mediated inhibition of dis-

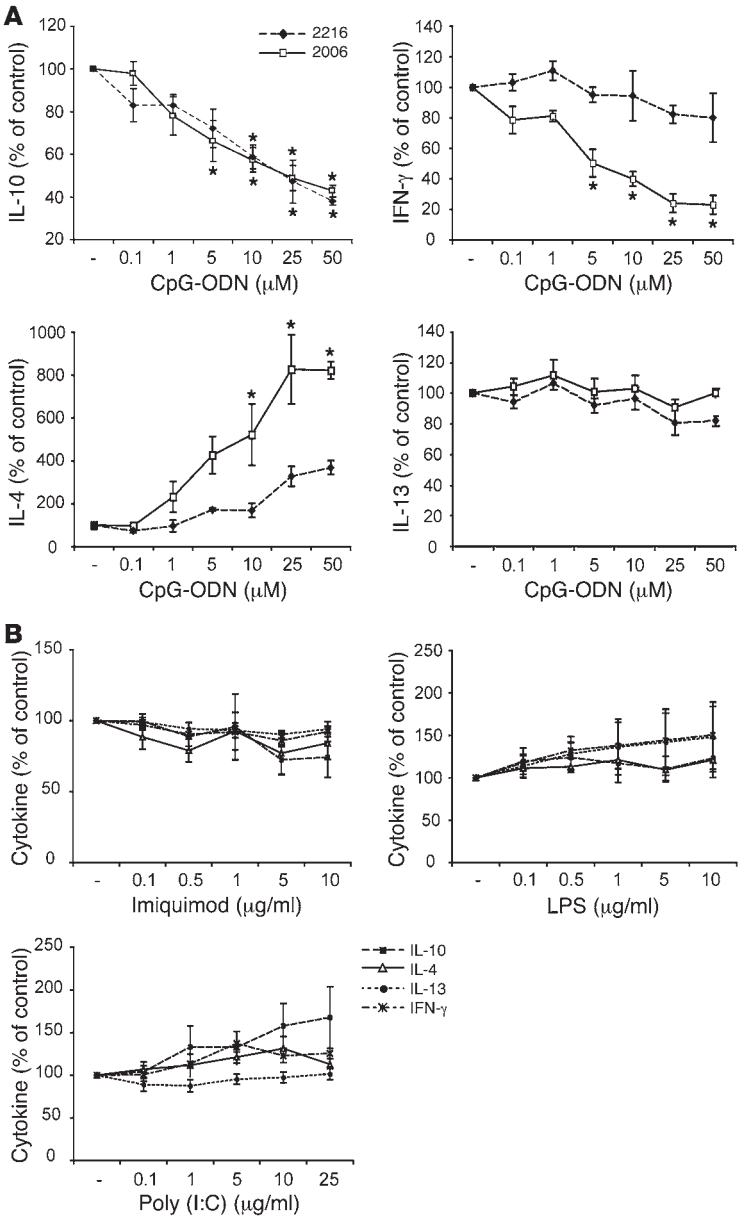

Figure 8
The TLR9 agonist CpG-ODN inhibit IL-10 production by drug-induced IL-10–Tregs. (**A**) $CD4^+$ T cells were cultured for two 7-day cycles with 1α25VitD3. Cells were restimulated for a further 48 hours with anti-CD3, IL-2, and the indicated concentrations of CpG-ODN 2006 (open squares) or CpG-ODN 2216 (filled diamonds). Cytokine production was analyzed in cultured supernatants. (**B**) Cells were cultured to day 14 as described in **A**, then restimulated with anti-CD3, IL-2, and the indicated concentrations of imiquimod, LPS, or poly (I:C) for 48 hours. Cytokine content (IL-10, filled squares; IL-4, open triangle; IL-13, filled circle; IFN-γ, asterisk) in cultured supernatants was then assessed. Data are shown as a percentage of the cytokine secretion in the absence of TLR stimulation (–). Note that basal cytokine concentrations were 3,246 ± 1,355 pg/ml, 512 ± 145 pg/ml, 56 ± 19 pg/ml, and 1,181 ± 711 pg/ml for IL-10, IFN-γ, IL-4, and IL-13, respectively. Mean data ± SEM from 5 (**A**) or 4 (**B**) healthy donors are depicted. *$P < 0.05$, as assessed by 1-way ANOVA.

ease (50). Together these data further suggest a role for vitamin D in controlling IL-10 production as well as playing a protective role in the host response to infection and inflammation.

Interactions between other TLRs and the vitamin D pathway on other cell lineages have been reported. For example, ligation of TLR2/1 on human monocytes or macrophages enhances their gene expression of VDR and Cyp27B1 (1α-hydroxylase; the enzyme that catalyses conversion of 25-hydroxyvitamin D3 into the active form 1α25VitD3), thereby increasing 1α25VitD3 synthesis, leading to activation of downstream VDR target genes (40). In keratinocytes, a similar pathway of TLR2-induced enhancement of 1α25VitD3 synthesis has been observed, but with an additional positive feedback loop whereby 1α25VitD3-VDR interaction then upregulates TLR2 expression (39). These studies, together with those of infection, support a link between TLRs and the vitamin D pathway and their capacity to control innate immunity.

The profile of TLR expression on dendritic cell populations is widely acknowledged to differ between mice and humans, and our data suggest differences also exist on T cells (10). TLR9 is expressed on murine effector T cell populations, and ligation with CpG-ODNs directly enhanced IL-2 production, proliferation, and survival via MyD88 and PI3K-dependent pathways (51). In rodent T cells, CpG-ODNs increased proliferation of $CD4^+CD25^-$ T cells, enabling these cells to overcome suppression mediated by natural $CD4^+CD25^+$ Tregs (14, 15). However, the effects of CpG-ODNs on human $CD4^+$ T cell function are conflicting. One study describing the costimulatory capacity of TLR2 ligands also briefly mentioned that human memory and naive T cells failed to respond to CpG-ODNs (8). Conversely, type A CpG-ODNs (such as CpG-ODN 2216), but not type B CpG-ODNs, has been shown to inhibit the suppressive activity of human natural $CD4^+CD25^+$ Tregs through direct stimulation of TLR8 (13). It is unlikely that CpG-ODNs acted through TLR8 in the current study, considering the complete absence of *TLR8* mRNA in 1α25VitD3-induced IL-10–Tregs. In a series of experiments designed to demonstrate the specificity of CpG-ODNs on IL-10–Tregs, we first demonstrated a loss of IL-10 synthesis when CpG-ODNs were added to cultures of IL-10–Tregs. Only the TLR9 agonist CpG-ODNs, and not control TLR ligands (LPS, poly [I:C], and imiquimod), modulated cytokine production by drug-induced IL-10–Tregs. Secondly, in functional inhibition assays, loss of regulatory function was observed when IL-10–Tregs were pre-pulsed with CpG-ODNs and then washed thoroughly prior to the addition to CFSE-labeled naive responder T cells. Based on these data, we conclude that the inhibition seen here is most likely due to the direct action of CpG-ODNs on human IL-10–Tregs.

Natural exposure to CpG motifs is likely to occur following infection with bacteria or viruses, which are known to exacerbate established asthmatic disease. We propose that our data, which show that stimulation of IL-10–Tregs with TLR9 agonist leads to a reduction in IL-10 synthesis and loss of regulatory activity, support a model in which loss of Treg function during infection promotes resolution of infection by cells of the innate and adaptive immune

system. Our demonstration that T cells derived directly from human lung-draining lymph nodes and nasal tissue respond to 1α25VitD3 by increased expression of IL-10 and TLR9 highlights that this pathway is present and likely to function at the active site of disease. TLR2 is expressed at increased levels by both human and murine naturally occurring (Foxp3$^+$) CD4$^+$CD25$^+$ Tregs. In most but not all studies, ligation of TLR2 results in expansion but temporary loss of suppressive activity (11, 17, 52, 53). These data have been used to support a similar model in which, during early inflammatory responses, Treg activity is impaired directly and/or indirectly through TLR-induced signals from dendritic cells and/or by enhanced IL-2 secretion by effector cells, allowing effector T cell populations to resolve the inflammatory insult. Upon resolution of the inflammation, the expanded Treg population would regain its functional competence in order to maintain immune homeostasis and prevent host damage and autoimmunity that might arise from overzealous effector cells (52). The evidence that TLR5-stimulated CD4$^+$ effector T cells are refractory to suppression (9), and that TLR8 ligation abrogates CD25$^+$ Treg function (13), suggests that the direct actions of other TLRs on both effector and regulatory populations fit this model. The data reported here suggest that adaptive IL-10–Tregs equally lose suppressive function during active infection as a result of TLR9 stimulation. Interestingly, TLR9 ligation on non–T cell populations has recently been shown to impair conversion of naive T cells into FoxP3$^+$ Tregs in the periphery (54). We have also become aware of studies demonstrating associations of polymorphisms in the *TLR9* gene with asthma (55, 56) and Crohn disease (57). Both conditions are influenced by vitamin D deficiency (42), and it would be of interest to determine whether control of Treg function by TLR9 is implicated in these associations.

Synthetic CpG-ODNs are currently of interest as immunomodulatory agents. Preclinical studies and human clinical trials document their capacity to improve vaccines and treat cancer, infectious disease, and allergy and/or asthma (58). One important mechanism of action proposed for CpG-ODNs is to activate endogenous plasmacytoid dendritic cells to enhance protective host T cell responses (58). For example, signaling via TLR9 enhances the antigen-presenting capacity of plasmacytoid dendritic cells isolated from the lung-draining lymph nodes of patients with non–small cell lung carcinoma for both CD4 and CD8 type I immune responses (59). Additional add-on strategies being considered include depletion of Treg populations that are proposed to impair the development of protective antitumor immune responses. The evidence in the present study that CpG-ODN signaling of inducible IL-10–Tregs leads to loss of regulatory activity is likely to be beneficial and desirable in conditions such as cancer or infection in which the aim is to boost immunological responses and competence.

Our findings that drug-induced IL-10–Tregs are susceptible to modulation by CpG-ODNs also has implications for the treatment of allergy. Conjugation of CpG-ODNs to specific allergens has been tested in human clinical trials and been shown to improve the safety of allergen immunotherapy by reducing allergenicity (60–63). A proposed mechanism is the deviation of the disease-promoting Th2 responses toward a Th1 response not associated with the allergic phenotype (60, 61, 64). We previously observed that drug-induced IL-10–Tregs are as effective, if not more so, in inhibiting Th1 responses than Th2 responses (21), suggesting that IL-10–Tregs might impair immune deviation. On this basis, possible recognition of CpG-allergen conjugates by IL-10–Tregs would result in loss of Treg inhibitory function and might facilitate deviation. Alternatively the pathway described here may limit the effectiveness of CpG-allergen immunotherapy, providing the opportunity for further optimization of CpG conjugate design to maximize benefit in treating allergic disease.

In summary, we have observed the expression of TLR9 by human adaptive IL-10–Treg populations is regulated by 1α25VitD3, and that ligation results in loss of Treg function, further highlighting the role of the vitamin D pathway in regulating immune function. Vitamin D deficiency is surprisingly widespread in populations within the northern hemisphere. It will therefore be important to fully identify the impact of the vitamin D pathway on immune function, including effects of endogenous and therapeutic vitamin D on IL-10, Tregs, and TLRs and more widely with respect to their influence in both allergy and immunity to infection.

Methods

Patient details. With the exception of calcitriol ingestion experiments, PBMCs were obtained from normal healthy individuals and used in all experiments. For calcitriol ingestion experiments, PBMCs were obtained from patients attending the Asthma Clinic at Guy's Hospital, London, United Kingdom. "Asthma" was defined by American Thoracic Society criteria as reversible obstruction (>15%) of the airways (65). "Glucocorticoid resistance" was defined as the failure of forced expired volume in 1 second (FEV_1) to improve by greater than 15% from a baseline of less than 75% after 14 days of 40 mg/day oral prednisolone. PBMCs were obtained from 3 glucocorticoid-resistant patients (all male), mean (SD) age 54 (15) years, mean (SD) basal FEV_1 55% (20%) predicted, and range of prednisolone reversibility 0% to 14% as previously described (18). Human lung-draining lymph nodes were obtained following resection from patients with early stage non–small cell lung cancer. Only non-involved lung-draining lymph nodes, as determined by histopathology, were used for this study (59). Nasal polyps were acquired from patients undergoing surgery for nasal polyposis. All donors signed a consent form, and all studies were fully approved by the Ethics Committee at Guy's Hospital, London, United Kingdom.

Cell purification and culture. PBMCs were isolated as previously described (21). Human lung-draining lymph nodes and nasal polyps were dissected and digested in HBSS with endotoxin-free collagenase (2 mg/ml; Liberase C1; Roche) for 1 hour at 37°C (59). CD4$^+$ T cells or CD3$^+$ T cells were purified by positive selection using Dynabeads (Dynal; typical purity 98.5%) or cell sorting (typical purity 99.5%) (21). CD4$^+$CD25^{hi} (purity >99%) and CD4$^+$CD25$^-$ (purity >99.5%) T cells were isolated by cell sorting from Buffy coats obtained from the National Blood Service. CD20$^+$ B cells (purity >99%) and CD14$^+$ monocytes (purity >98%) were also purified from PBMCs by cell sorting. Highly differentiated human Th1 and Th2 cells were generated from naive T cells as previously described (22). Isolation of live IL-10–secreting cells (purity >98%) was performed using an IL-10 Secretion Assay Detection Kit (Miltenyi Biotec).

CD4$^+$ T cells (1×10^6 cells/ml) were stimulated with 1 μg/ml plate-bound anti-CD3 (OKT-3), 50 U/ml IL-2 (Eurocetus), 10 ng/ml IL-4 (NBS), Dex (Sigma-Aldrich), and/or calcitriol (1α25VitD3; BIOMOL Research Labs), for 7 day cycles. In some experiments, 5 ng/ml recombinant human IL-10 (R&D Systems), 5 μg/ml anti–IL-10 receptor (clone 3F9-2; BD Biosciences — Pharmingen), or isotype control rat IgG2a (clone R35-9S; BD Biosciences — Pharmingen) was added. At the end of each cycle, cells were recultured with cross-linked anti-CD3 and IL-2 alone, and supernatants were harvested at 48 hours for cytokine analysis.

For analysis of the functional consequence of TLR expression, 2 categories of the TLR9 agonists unmethylated CpG-ODNs were obtained from Invivogen: CpG-ODN type B 2006 (5′-TCGTCGTTTTGTCGTTTTGTC-

GTT) and CpG-ODN type A 2216 (5′-GGGGGACGATCGTCGGGGGG). LPS was purchased from Sigma-Aldrich, and the remaining TLR agonists imiquimod and poly (I:C) were from Invivogen. On day 14, following 2 rounds of culture with anti-CD3, IL-2, IL-4, and 1α25VitD3, cells were harvested, washed, and stimulated for a further 48 hours with anti-CD3, IL-2, and various concentrations of the relevant TLR agonist as indicated in Figure 8. Supernatants were harvested for cytokine analysis.

Functional assays of regulatory function. Cell lines were generated by culture of isolated $CD4^+$ T cells under neutral conditions (anti-CD3, IL-2, and IL-4) or additionally with 1α25VitD3. On day 14, cell lines were harvested, washed, and pretreated for 24 hours with anti-CD3 and IL-2 alone or with 10 μM CpG-ODN 2006. Fresh autologous $CD4^+CD45RA^+$ naive T cells were purified and labeled with 2 μM CFSE (Invitrogen), and 2×10^5 cells were cocultured with 1×10^5 cells of the relevant line as indicated in Figure 7. Cultures were stimulated with 0.1 μg/ml plate-bound anti-CD3 and 2 μg/ml anti-CD28 (clone 15E8; Sanquin) for 5 days. Propidium iodide (Sigma-Aldrich) was then added to exclude dead cells, and 30,000 CFSE-positive responder cells were analyzed by FACS.

Cytokine analysis. IL-5, IL-10, IL-13, and IFN-γ were measured using ELISA and matched antibody pairs (BD Biosciences — Pharmingen), with reference to commercial standards (R&D Systems). The lower limits of detection were 50 pg/ml for IFN-γ and IL-10, 100 pg/ml for IL-5, and 100 pg/ml for IL-13. When less than 200 μl of supernatant was available, the Luminex (Luminex Corp.) or Meso Scale Discovery systems were used.

1α25VitD3 ingestion by asthmatic volunteers. This study was approved by the Research Ethics Committee of Guy's Hospital, and informed consent was obtained from volunteers. Three glucocorticoid-resistant asthma patients were given 0.5 μg/day (2 × 0.25 μg) oral calcitriol for 7 days, and PBMCs were obtained before treatment and on days 1, 3, and 7 after ingestion of calcitriol as previously described (21). $CD3^+CD4^+$ T lymphocytes were isolated by cell sorting and cell pellets kept for ex vivo mRNA extraction.

Real time RT-PCR. RNA was extracted from cell pellets using the RNeasy Mini kit (Qiagen) and quantified using Ribogreen RNA quantification kit (Eugene). RNA (250 ng) was reverse-transcribed in a total volume of 30 μl using random hexamer primers (Fermantas Life Sciences). Real-time RT-PCR was performed in triplicate using FAM-labeled Assay-on-Demand reagent sets (Applied Biosystems). Reactions were multiplexed using VIC-labeled 18S as an endogenous control and analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Knockdown of TLR9 using lentiviral shRNA constructs. Lentiviral shRNA constructs were purchased from the Mission shRNA collection (Sigma-Aldrich). TLR9 shRNA constructs (catalog SHDNA-NM_017442; Sigma-Aldrich) and a nontargeting negative control vector (catalog SHC002; Sigma-Aldrich) were used in this study. We transfected 90%–95% confluent 293FT cells with 6.48 μg of lentiviral plasmid DNA and 19.44 μg of ViraPower packing Mix DNA (pLP1, pLP2, and pLP/VSVG) using Lipofectamine 2000 (Invitrogen). After the addition of fresh medium the following day, the cells were cultured for an additional 48 hours. Viral supernatants were harvested, passed through a 0.45-μM filter, and concentrated by ultracentrifugation at 50,000 *g* at 4°C for 90 minutes. Virus pellets were resuspended in less than 1.5 ml of T cell medium, and viral titers were assessed by transducing A549 cells with serially diluted concentrations of virus, adding the selective antibiotic (puromycin; 10 μg/ml) and counting the puromycin-resistant colonies 12 days after infection. Human $CD4^+$ T cells were cultured for 7 days with 1 μg/ml anti-CD3, 50 U/ml IL-2, 10 ng/ml IL-4, and 10^{-7} M 1α25VitD3. Cells were then reactivated on anti-CD3–coated plates with IL-2, IL-4, and 1α25VitD3 (as described above) and the concentrated lentiviral supernatants with a MOI of 3, in a total volume of 0.5 ml T cell medium. After 72 hours of incubation, 0.25 ml of medium was removed and replaced with fresh medium containing 20 U/ml IL-2 and 1 μg/ml puromycin (Sigma-Aldrich). Following an additional 3 days of culture, cells were pelleted, RNA extracted, and cDNA generated, and the level of cytokine and TLR transcripts was assessed by real-time RT-PCR.

Statistics. Results are presented as mean ± SEM. Data were assessed for normality and equal variation, after which the appropriate parametric or nonparametric test was performed, as indicated in the figure legends. Differences were considered significant at the 95% confidence level.

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