Failure of itraconazole to prevent T-helper type 2 cell immune deviation: Implications for chronic rhinosinusitis

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ABSTRACT

Background: T-helper (Th) type 2 cell inflammation is the hallmark of several disease processes, including asthma, atopic dermatitis, and some forms of chronic rhinosinusitis. Itraconazole has been used as both an antifungal and an anti-inflammatory agent, with some success in many of these diseases, in part, by altering Th2 cytokine expression by T cells. It is not known whether this merely reflects inhibition of established Th2-like cells or the inhibition of differentiation of naive T cells into Th2-like cells.

Objective: To evaluate the role of itraconazole in the differentiation of naive T cells during activation.

Methods: Naive CD45RA⁺ T cells were isolated from peripheral blood mononuclear cells from healthy volunteers. Th1 and Th2 type cells were differentiated in the presence of varying concentrations of itraconazole. After stimulation with anti-CD3 and anti-CD28 beads, carboxyfluorescein succinimidyl ester dilution was performed to evaluate proliferation and intracellular cytokine staining for interleukin (IL) 4 and interferon (IFN) gamma within proliferating T cells was measured along with enzyme-linked immunosorbent assay for secreted IL-5, IL-13, and IFN gamma.

Results: Itraconazole had no effect on proliferation of unbiased, Th1, or Th2 cells. Similarly, there was no effect of itraconazole on either intracellular cytokine staining of IL-4 and IFN gamma or secreted cytokine expression of IFN gamma, IL-5, and IL-13 in any of the cell populations.

Conclusion: Itraconazole did not alter the ability of naive T cells to proliferate or secrete cytokines under Th1 or Th2 deviating conditions in vitro. As such, reported inhibition of Th2-like lymphocyte function by itraconazole reflected action on mature effector cells and may have underscored why antifungal treatment failed in many clinical trials of eosinophilic chronic rhinosinusitis.

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There are spectra of diseases associated with T-helper (Th) type 2 cell inflammation, including asthma, chronic eosinophilic rhinosinusitis, allergic rhinitis, and atopic dermatitis.^{1–4} Results of studies support the use of azole antifungals in the treatment of some of these disorders^{5–11} Although itraconazole and other azole antifungals are effective for fungi-associated hypersensitivity disorders, such as allergic bronchopulmonary aspergillosis^{10,12,13} and allergic fungal rhinosinusitis,⁷ their efficacy in non-fungal-mediated presentations of these Th2-associated disorders indicated “off target” effects that specifically ameliorate allergic inflammation.

However, results of other studies indicated more targeted (and less potentially toxic) effects of these agents in blocking Th2-associated hypersensitivity disorders. For example, ketoconazole has been reported to inhibit thromboxane synthetase, an important mediator of bronchospasm^{14,15} and thereby may provide therapeutic benefit in asthma. More importantly, these agents have been indicated to have direct immune suppressive effects on T lymphocyte-mediated adaptive immune responses. For example, Pawelec *et al.*,^{16,17} investigated the immunosuppressive effects of itraconazole, fluconazole, ketoconazole, and miconazole on proliferative responses in human mixed lymphocyte cultures and reported that, under certain conditions, itraconazole was as suppressive to T-cell function as cyclosporine A.

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Studies of antifungal agents as a generalized treatment for chronic rhinosinusitis (CRS) proved largely ineffective, as reported in a recent Cochrane review.¹⁸

Results of recent studies indicated a more-specific role of azole antifungals in modulating Th2 inflammation. Ward *et al.*,⁹ in a placebo-controlled study, demonstrated marked improvement in asthma after treatment with fluconazole. The investigators postulated that much of this effect was secondary to the antifungal effects of the fluconazole. However, although the patients in this study had evidence of sensitization to trichophyton (positive intradermal skin tests), none had evidence of either fungal colonization in their lungs or an inflammatory response in their lungs directed against trichophyton, which indicated that the benefit, at least in large part, may have been mediated by nonspecific targeting of Th2-mediated inflammation.⁹

The study by Kanda *et al.*¹⁹ was performed by using effector memory T cells, so the effect of itraconazole on the differentiation of naive T cells and, as such, the potential role of itraconazole as a disease-modifier remain unexplored. More importantly, it is not certain if their results are secondary to a reduction in cell number as opposed to directly influencing the cytokine repertoire (immune deviation). By determining the effect of itraconazole on naive T-cell differentiation and subsequent cytokine production, it will be possible to more accurately interpret the mechanism of therapeutic itraconazole administration. Given the reported therapeutic improvement in patient symptoms and radiographic findings reported with these agents,^{6,8–10} establishing their anti-inflammatory mechanism may broaden their applications in the treatment of Th2 diseases. The current studies, therefore, were designed to investigate the ability of itraconazole to prevent immune deviation of naive T cells to a Th2 phenotype.

METHODS

Subjects

Heparinized venous blood was obtained with informed consent from healthy human volunteers (18–55 years old) by using a protocol approved by the human investigation committee at the University of Virginia.

Cell Culture and Proliferation

Peripheral blood mononuclear cells were isolated by using Ficoll-Hypaque (Sigma, St. Louis, MO) density centrifugation. CD45RA⁺ T cells (95% pure) were enriched from peripheral blood mononuclear cells by using positive magnetic affinity column purification (CD45RA⁺; Miltenyi Biotec, Auburn, CA). The cells were resuspended in complete RPMI-1640 medium that contained 0.01 mol/L HEPES (Invitrogen, Carlsbad, CA) and 10,000 U/mL of penicillin and 10 μ g/mL of streptomycin supplemented with 10% autologous serum and maintained at 37°C in 5% CO₂. CD45RA⁺ cells were considered unbiased, with no addition of exogenous factors, Th1 cells were generated by using 10 ng/mL of interleukin (IL) 12 and anti-IL-4 (10 μ g/mL), and Th2 cells were generated with 10 ng/mL of IL-4 and anti-IL-12 (10 μ g/mL). IL-4 and IL-12 were from BD Pharmingen (San Diego, CA) and anti-IL-4 and anti-IL-12 were from R&D Systems (Minneapolis, MN). Itraconazole was added to cultures on day 0 at pharmacologically relevant concentrations (0, 0.01, and 0.1 μ M). The cells were cultured with anti-CD3 and anti-CD28 beads (Miltenyi Biotec) to stimulate cell activation. To measure cell proliferation, carboxyfluorescein succinimidyl ester dye (Invitrogen) was added to the cells on day 0. Time points of T-cell activation were taken at 7 and 14 days (time points were based on previous studies^{20–23}) and processed as described below for the various outcomes.

Intracellular Cytokine Staining

On days 7 and 14, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (2 μ g/mL; Sigma-Aldrich) for 5 hours with Brefeldin A (0.7 μ L/mL; BD Pharmingen) added for the final 4 hours. The cells were incubated with mouse or rat immunoglobulin G (20 μ g) for 5 minutes to block nonspecific binding²⁴ and then were incubated with either anti-IL-4 allophycocyanin or anti-interferon (IFN) γ allophycocyanin for 30 minutes (BD Biosciences, San Diego, CA). Intracellular staining was performed by using the Invitrogen Fix & Perm kit according to the manufacturer's instructions, with the addition of 5 μ L of appropriate antibody (IL-4 or IFN- γ). Cells were incubated for 10 minutes, after which flow cytometry was performed by using a Becton Dickinson FACSCalibur machine equipped with CellQuest software version 5.2 (BD Biosciences). Data were analyzed by using FlowJo version 6.4.1 (Tree Star Inc, Ashland, OR).

Cytokine and/or Growth Factor Determination

Supernatants from cells collected on days 7 and 14 were analyzed for Th1 and Th2 cytokines. IFN- γ , IL-5, and IL-13 levels were determined by using a Bio-Plex bead-suspension assay (Bio-Rad, Hercules, CA). The sensitivities for these assays were <20 pg/mL for IFN- γ , <10 pg/mL for IL-5, and <6 pg/mL for IL-13.

Quantitative Real-Time Polymerase Chain Reaction Detection of Transcripts

Quantitative real-time polymerase chain reaction (PCR) was performed for IL-4 and IFN- γ . Total RNA was extracted from cells by using TRI reagent (Sigma, St Louis, MO). Conversion of messenger RNA to complementary DNA was performed by using a Taqman Reverse Transcription kit (Roche, Branchburg, NJ). Briefly, 200 ng of RNA were added to each reaction, along with oligo dT primers, 5.5 mM MgCl₂, 2 mM dNTPs, RNasin, and reverse transcriptase. Reactions went through 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C in an iCycler thermocycler (Bio-Rad). PCR mix consisted of iQ SYBR-green supermix (Bio-Rad), complementary DNA, and 200 μ M of each primer. Quantification of changes in receptor expression induced by cytokines was performed by using the comparative C_T method. Briefly, the amount of target, normalized to an endogenous reference and relative to a calibrator was calculated by $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ = (threshold cycle unstimulated gene of interest –

threshold cycle unstimulated housekeeping gene) – (threshold cycle stimulated gene of interest – threshold cycle stimulated housekeeping gene). The comparative C_T method was validated by showing that the efficiencies of target and reference amplification were equal. Primers for β -actin were as described^{25,27} and for IL-4 and IFN- γ were as described.^{20–22}

Statistical Analyses

Data were contrasted between undifferentiated samples and those that underwent either Th1 or Th2 immune deviation by independent *t*-tests with or without equal variances when appropriate by using SPSS 17.0 software (Chicago, IL).

RESULTS

Immune Deviation

Th1 and Th2 cells were generated *via* established reported protocols^{20–23} and confirmed by expression of IFN- γ , IL-4, IL-5, and IL-13. Data for quantitative real-time PCR expression of IFN- γ and IL-4 under neutral, Th1, and Th2 immune deviating conditions at day 7 and 14 are displayed in Fig. 1. Under Th1 immune deviating conditions, a robust increase in the number of IFN- γ transcripts was observed at both days 7 and 14, along with significant inhibition of IL-4 at day 7. In contrast, under Th2 immune deviating conditions, significantly enhanced IL-4 expression was observed at day 7. Th1 and Th2 immune deviation was further established *via* intracellular cytokine staining and as secreted cytokines (see sections Intracellular Cytokine Staining and Enzyme Linked Immunoassays).

Proliferation

We tested whether itraconazole altered T-cell proliferation of unbiased CD45RA⁺ cells or cells that were being converted to Th1 or Th2 subtypes. Increasing doses of itraconazole had no effect on unbiased CD45RA⁺ cells, Th1, or Th2 proliferation (Fig. 2 and Table 1).

Intracellular Cytokine Staining

Although proliferation was not altered, we posited that its primary influence would be on immune deviation. However, itraconazole did

Figure 1. Confirmation of immune deviation by messenger RNA analysis. Naive T cells were either cultured alone, under T-helper (Th) type 1 deviating conditions (10 ng/mL of interleukin [IL] 12 and anti-IL-4), or Th2 deviating conditions (10 ng/mL of IL-4 and anti-IL-12) for 7 or 14 days. RNA was isolated at the indicated time points and analyzed for interferon γ or IL-4 expression in comparison with the housekeeping gene β -actin. Data are presented graphically as the change in C_T value in comparison with the naive T-helper lymphocytes (Th0) cells. *p < 0.002, **p < 0.03 in comparison with Th0 cells at the same time point.

A. CSFE proliferation representative Th1 7 days

B. CSFE proliferation representative Th2 7 days

Figure 2. Proliferation of naïve T cells stimulated with itraconazole under immune deviating conditions. Naïve T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured under T-helper (Th) 1 or Th2 deviating conditions for 7 days with anti-CD3 and anti-CD28 beads with increasing doses of itraconazole. Proliferation was determined by using flow cytometry to measure dilution of the CFSE dye after 7 days of culture. Representative data from one of six subjects are presented.

Table 1 Summary of Th subset proliferation in response to itraconazole*				
Day	Th Cell Subtype	Itraconazole (0 μM)	Itraconazole (0.01 μM)	Itraconazole (0.1 μM)
7	0	94.1 ± 2.0	92.8 ± 3.4	91.6 ± 4.2
	1	92.5 ± 3.3	92.5 ± 3.1	91.0 ± 4.1
	2	95.2 ± 1.2	93.8 ± 2.3	92.9 ± 3.2
14	0	99.7 ± 0.1	99.6 ± 0.2	99.7 ± 0.1
	1	99.7 ± 0.1	99.7 ± 0.1	99.7 ± 0.1
	2	99.2 ± 0.5	98.8 ± 0.9	99.3 ± 0.4

Th = T helper.
*Data (mean ± SEM) represent percentage of original cells in culture that proliferated (n = 6).

not change either the intracellular Th1 (Fig. 3 A) or Th2 (Fig. 3 B) cytokine profile at any dose.

Enzyme Immunoassays

To confirm the results observed with intracellular cytokine staining, we next analyzed secreted cytokine expression in the culture

supernatants. Specific enzyme-linked immunosorbent assays were performed for IFN-γ, IL-5, and IL-13 (Fig. 4); no changes were observed with any dose of itraconazole.

Quantitative Real-Time PCR

Finally, cell pellets were collected and RNA was extracted to evaluate gene expression. Consistent with the data from the intracellular cytokine analysis and culture supernatants, there were no changes in Th1 or Th2 cytokine gene expression (data not shown).

DISCUSSION

Antifungal agents have been used in the treatment of many disorders with proven efficacy in allergic bronchopulmonary aspergillosis¹⁰ but with a lack of efficacy as a general treatment for CRS.¹⁸ It should be appreciated that none of the studies demonstrating clinical efficacy of azole antifungal agents in allergic bronchopulmonary aspergillosis have causally linked the observed clinical benefit to antifungal effects, and it remains quite plausible that observed benefits reflect the putative Th2-targeting influences of these agents. Early studies that examined the effects of antifungal azoles in human lymphoid cells demonstrated that high doses (10 μg/mL) of itraconazole and miconazole were sufficient to suppress messenger RNA

Figure 3. Intracellular cytokine analysis of naive T cells stimulated with itraconazole under immune deviating conditions. Cells were collected and analyzed for intracellular cytokine expression by using flow cytometry. Data are presented as the percentage of positive cells for interferon (IFN) γ (A) and interleukin (IL) 4 (B) ($n = 6$).

expression of IL-2, IL-4, IL-9, granulocyte macrophage colony-stimulating factor, tumor necrosis factor α , and IFN- γ .¹¹

By extending this work, Kanda *et al.*¹⁹ showed that azole antimycotics, at concentrations close to what is obtained by the oral administration of these medications (0.01–1 μ M), inhibited Th2 cytokine production from effector T cells derived from subjects with atopic dermatitis. Specifically, they demonstrated that exposure of established effector T cells to itraconazole significantly reduced the con-

centration of IL-4 and IL-5 in the supernatant of cultures from subjects with atopic dermatitis when compared with controls.¹⁹ Although not statistically significant, there was an increase of IFN- γ secretion, which further indicated Th1 skewing.¹⁹ However, with these study designs, it is impossible to determine if the effects of itraconazole were limited to effector T cells or if they also impacted immune deviation of naive T cells. This has obvious importance because an influence on the immune deviation of naive T cells would indicate

Figure 4. Secreted cytokine analysis of naive T cells stimulated with itraconazole under immune deviating conditions. Supernatants were collected and analyzed for cytokine expression by enzyme-linked immunosorbent assay. Data are presented in pg/mL for interferon (IFN) γ (A), interleukin (IL) 5 (B), and IL-13 (C) ($n = 6$).

long-term disease-modifying effects for these agents in addition to their putative immediate therapeutic influences.

Therefore, we postulated that the effect of itraconazole on effector T cell function might derive in part from modulation of naive T-cell differentiation and that, with the addition of itraconazole, we could prevent or alter development of a Th2 cellular response. Our results, however, did not support this hypothesis. Differentiating naive T-helper lymphocytes cells into the Th1 or Th2 phenotype in the presence of escalating concentrations of itraconazole did not alter cellular proliferation (Fig. 2), intracellular cytokine staining for IL-4 or IFN- γ (Fig. 3), secreted cytokine protein levels (IFN- γ , IL-5, and IL-13) in supernatants (Fig. 4), or cytokine messenger RNA (not shown) of any T-cell subset.

Despite our findings, the fact remains that individuals in other studies identified the presence of itraconazole responsive T-cell pop-

ulations that improved Th2 disease.⁶⁻¹⁰ Azole antifungals target cytochrome P450 pathways, and, *via* this mechanism, decrease corticosteroid metabolism and thereby may have therapeutic benefits by synergizing with endogenous and pharmacologically administered corticosteroids.²⁸ The effect of itraconazole on T cells *in vivo* may simply be the alteration of expression of particular genes expressed only in T-memory effector cells. Although our findings do not allow for prevention of disease or disease modification, itraconazole may still be useful for treatment of some Th2 diseases through these or other mechanisms. Caution is needed, however, due to the lack of efficacy in trials of CRS.¹⁸

Although it is accepted that, in allergic fungal rhinosinusitis, colonizing fungi are a central mechanism that drive disease, what has been controversial is whether there is a role for endogenous fungi in all cases of CRS. Support for this concept is based on the observation

that fungi are universally present in the sinus cavity,²⁹ with the suggestion that CRS could develop in subjects in whom the usual state of immune nonresponsiveness breaks down, which allows a Th2 response to develop. These investigators demonstrated that subjects with eosinophilic CRS (and not the healthy controls) had circulating T-effector lymphocytes in response to *Alternaria*-secreted IL-5.³⁰ Subsequently, enthusiasm for this line of research has been diminished due to overwhelming evidence that sinus irrigation with antifungal agents fail to produce clinical benefit.^{31–33}

Our assumption going into this study was that the actions of azole antifungals would be to prevent *de novo* differentiation of naive T cells. Because this demonstrably is not its mechanism of action, the alternative explanation, in regard to potential actions on effector T cells, would be to inhibit the function, proliferation, or survival of established effector T cells. However, more and more, it is being appreciated that the persistent inflammation of CRS is likely driven by an innate immune response and that this may not require ongoing exposure to antigen and engagement of effector T cells. Persistent expression of the type 2 cytokine signature is increasingly recognized as a result of activation of mast cells, basophils, and innate lymphoid type 2 cells (thus, the current preference for the term “type 2 cytokine signature” instead of “Th2 signature”). As such, given our negative results and this newer understanding of eosinophilic CRS, actions of azole antifungals on these innate cells (and especially on innate lymphoid type 2 cells) invites exploration.

CONCLUSION

Itraconazole did not influence the ability of naive T cells from healthy subjects to differentiate into either Th1 or Th2 cells, which indicated that itraconazole did not act as an immunomodulator of naive cells and that the proven effects of itraconazole in improving Th2 disease has only been demonstrated for effector T cells. Further research is necessary to determine the exact mechanisms by which itraconazole affects memory effector T cell function and the exact circumstances under which that modulation can produce clinical benefit in established Th2-mediated diseases, *e.g.*, eosinophilic CRS.

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