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Randomized Trial of Adjunctive Interleukin-2 in Adults with Pulmonary Tuberculosis

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ABSTRACT

Interleukin (IL)-2 has a central role in regulating T cell responses to *M. tuberculosis*. Adjunctive immunotherapy with recombinant human IL-2 was studied in a randomized, placebo-controlled, double-blinded trial in 110 HIV-seronegative adults with newly diagnosed, smear-positive, drug-susceptible pulmonary tuberculosis. Patients were randomly assigned to receive twice daily injections of 225, 000 IU of IL-2 or placebo for the first 30 days of treatment in addition to standard chemotherapy. Subjects were followed for one year. The primary endpoint was the proportion of patients with sputum culture conversion after 1 and 2 months of treatment. After 1 month, the proportion of patients who converted their sputum culture to negative was 17% for the IL-2 group compared to 30% in the control group ($p= 0.14$; χ^2). After 2 months, 77% in the IL-2 group were culture negative compared to 85% of those receiving placebo ($p=0.29$, χ^2). Results were similar when patients with isoniazid monoresistance were included in the analysis. There were no differences in weight gain, and improvement in fever, cough and chest pain between groups. One patient in each arm relapsed. IL-2 did not enhance bacillary clearance or improvement in symptoms in HIV-seronegative adults with drug-susceptible tuberculosis.

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INTRODUCTION

Tuberculosis (TB) is a major global health problem. Up to one-third of the world's population is infected with *M. tuberculosis* (MTB*)*. The World Health Organization estimates that 8 million new TB cases and 1.9 million deaths due to tuberculosis occurred worldwide in 1997 (1). In addition drug resistance to standard antituberculosis drugs has increased in many areas during the past decade (2). New modalities for the prevention and treatment of tuberculosis are clearly needed.

Recovery from tuberculosis depends, in part, on the generation of an effective cell mediated immune response against the pathogen. Effective T cell function is key in controlling MTB infection. Interleukin (IL)-2, a cytokine produced by activated T lymphocytes, has a central role in the activation and expansion of T cells. In murine models of *M. lepraemurium*, *M. avium* and *M. bovis* BCG infection, IL-2 has been shown to limit mycobacterial replication, possibly by macrophage activation via interferon-mediated pathways or directly by the development of cytotoxic T lymphocytes recognizing mycobacterial antigens (3-5). Patients with tuberculosis frequently have deficient IL-2-induced cell proliferation and decreased IL-2 receptor generation (6). These observations form the basis for studies of recombinant IL-2 as adjunctive immunotherapy against mycobacterial diseases in humans.

Early clinical trials with IL-2 in patients with leprosy, leishmaniasis, tuberculosis and other serious infections due to intracellular pathogens, demonstrated that IL-2 immunotherapy may be useful in controlling these infections (7-14). In leprosy patients, IL-2 administration led to enhanced local cell mediated immune responses and resulted in more rapid and extensive reduction in bacilli compared to multidrug chemotherapy alone (11, 12).

In a pilot study from Bangladesh and South Africa, treatment of patients with newly diagnosed drug-sensitive and chronic multidrug resistant (MDR) TB with 12.5 μg (225, 000 IU) of intradermal IL-2 twice daily during the first month of TB therapy resulted in rapid sputum conversion (13). A later randomized trial in South Africa comparing daily and pulsed IL-2 with placebo in MDR TB found improved sputum clearance with daily treatment (14). These results suggested a potential role for IL-2 in tuberculosis treatment.

To further study this issue, we conducted a randomized, double-blinded, placebo-controlled phase II clinical trial to evaluate the safety and microbiologic and immunologic effects of IL-2 in HIV-seronegative adults with initial episodes of smear positive, drug-susceptible pulmonary tuberculosis. We hypothesized that adjunctive treatment with IL-2 would enhance cell-mediated immune responses in tuberculosis and increase the rate of killing of tubercle bacilli and that these effects may be most evident during early treatment in patients with drug-susceptible tuberculosis.

Some of the results of this trial have been previously reported in the form of an abstract (15).

METHODS

Patients

Ambulatory patients, aged 18 to 50 years, with suspected pulmonary tuberculosis were referred from the main outpatient clinic of the National TB Treatment Centre, Mulago Hospital, Kampala, Uganda for possible study participation. HIV-seronegative patients with initial episodes of newly-diagnosed smear positive, culture-confirmed TB who had moderately advanced or far advanced tuberculosis on chest X-ray (16), a Karnofsky performance scale score

greater than 50% (17), and were not pregnant or lactating were eligible for the study. Persons previously treated for TB, patients with asthma, untreated thyroid disease, or other serious medical conditions, those with a hemoglobin less than 80 g/L, total white blood cell count less than 3000/mm^3, serum aspartate aminotransferase greater than 100 IU/L or serum creatinine more than 177 μM/L or limited respiratory reserve, and persons on chronic corticosteroid or immunosuppressive drugs were excluded. Patients found to have initial drug resistance to isoniazid (INH), rifampin, ethambutol or pyrazinamide were excluded from the study when susceptibility testing results became available and their TB treatment was adjusted accordingly.

The study was approved by the institutional review boards at University Hospitals of Cleveland and Case Western Reserve University and the Ugandan National AIDS Research Subcommittee. All participants gave informed consent.

Treatment allocation and masking

After screening, eligible subjects were admitted to hospital for the first month of anti-TB treatment. Patients were randomly assigned to treatment with standard short course chemotherapy (2 months of daily INH, rifampin, pyrazinamide and ethambutol followed by 4 months of daily INH and rifampin) plus twice daily intradermal injections of 225,000 IU recombinant human IL-2 (Proleukin®, aldesleukin – kindly donated by Chiron, Emeryville, CA) or sterile 5% dextrose for injection, USP (placebo). Injections were administered intradermally twice daily into the skin of the back during the first 30 days of anti-TB treatment. The dosage, route, and schedule for IL-2 administration were identical to the earlier trial done in South Africa (14).

A computer-generated randomization sequence with a block size of 10 was used to assign

subjects to study treatment.

Clinical and laboratory staff were masked to treatment assignment. Separate clinical assessors were used to assess local and systemic adverse experiences. Treatment assignments were not revealed to any investigator or subject during the trial. Additional details are available in the online data supplement.

Baseline measurements

Purified protein derivative skin testing

Skin testing was performed with 5 tuberculin units of purified protein derivative (PPD) (Tubersol®, Aventis Pasteur) by the Mantoux method (18).

Bacteriology

Early morning sputum specimens were collected for qualitative AFB smears and culture in BACTEC liquid media and Middlebrook 7H-10 agar plates. Quantitative cultures were performed after homogenization with N-acetyl L-cysteine/sodium citrate and decontamination with 2% sodium hydroxide using previously published methods (19). Susceptibility testing against INH, rifampin, ethambutol, and pyrazinamide was performed using standard BACTEC methods (20).

Immunologic Measurements

Immunologic measurements including PPD skin test responses, expression of IL-2 receptor by CD4 and CD8 T-cells, determination of frequencies of natural killer cells (exhibiting the surface marker profile $CD3^-/CD16^+/CD56^+$), and assessment of serum IL-2 receptor (sIL-2R) immunoreactivity in serum were performed at baseline and during study drug treatment to demonstrate systemic activity of the IL-2 study drug. The proportion of peripheral blood T-cells expressing the activation marker CD25+ (IL-2 receptor) and frequencies of NK cells were assessed by 3-color flow cytometry (FACScan, Becton-Dickinson, San Jose, CA) using commercial reagents. Serum sIL-2R was measured using a commercially available enzyme linked immunosorbent assay kit (BioSource, Camarillo, CA) according to the manufacturer's instruction. The sensitivity of the kit was less than 0.016 ng/ml.

Sera were stored before beginning treatment and after 2, 4 and 6 weeks of anti-TB treatment for measurement of anti-IL-2 antibodies. Detailed procedures for the microbiological and immunologic assays are included in the online data supplement.

Anti-tuberculosis chemotherapy

Tuberculosis treatment was administered under direct supervision during the initial 30 day hospitalization and was then self-administered on an ambulatory basis. The dosage of standard anti-TB drugs was adjusted for body weight. After the first 30 days, drugs were dispensed monthly during the remainder of treatment. Adherence was measured by self-report, dispensing records and at least monthly urine INH metabolite testing.

Follow-up measurements

History, physical examination and adverse event surveys were performed thrice weekly during study drug treatment. Injection sites were inspected twice daily. Complete blood counts, blood chemistries and urinalysis were repeated weekly during study drug treatment and at 12 months. Serum thyroid stimulating hormone and thyroxine levels were checked after 1 month. Chest X-rays were repeated after 1, 2, 6 and 12 months and graded as normal, minimal, moderately advanced or far advanced disease using a standardized scheme (16). PPD skin testing was repeated after 4 weeks.

Sputum was collected for AFB smear and culture after 2 and 4 days of treatment, then weekly from weeks 1 through 4, after 6 weeks and then monthly during the remainder of treatment as long as the subject was able to produce sputum. Immunologic measurements were repeated after 2 weeks and 6 weeks of anti-TB treatment.

Subjects were followed for one year after the onset of anti-TB treatment.

Statistical analysis

The primary study endpoints were the rate of sputum culture conversion on solid media after 1 and 2 months of anti-TB treatment and the safety and tolerability of intradermal IL-2 as measured by the proportion of subjects with local and systemic reactions. Sputum culture conversion after one and two months was defined as having all sputum cultures negative at that time point and no positive cultures subsequently during treatment. Subjects who were unable to produce sputum at a follow-up time point and subjects for whom all sputum cultures overgrown by bacteria or yeast at a follow-up time point were considered unevaluable at that time point. Secondary clinical endpoints included improvement in cough and chest pain, defervescence,

weight gain and Karnofsky performance scale score. Time on treatment until culture conversion was a secondary microbiological endpoint. Immunologic endpoints included changes in PPD skin test responses, the proportions of circulating CD25+ CD4 and CD8 T-cells, frequencies of NK cells, and levels of immunoreactive IL-2R in serum.

The total sample size (110) was calculated to have 80% power [$\alpha = .05$ (1-tailed)] to detect a minimum difference of 19% in sputum culture conversion rates after 1 and 2 months of anti-TB treatment comparing the IL-2 and placebo groups, assuming a 75% culture conversion rate after 2 months of treatment with standard chemotherapy alone (21, 22). A treatment effect of this magnitude on 2 month culture conversion is similar to the effect adding rifampin to anti-TB treatment (23-25). The total sample size was adjusted for an estimated 5% mortality, 8% prevalence of primary drug resistance, and 10% loss-to-follow-up.

All statistical analyses were performed using SAS software (SAS, version 6.12, SAS Institute, Cary, NC). Significant univariate differences between the two study arms were determined using χ^2 contingency tables and t-tests for means of continuous data. Equivalent non-parametric tests were used when data were not normally distributed. The date of sputum culture conversion was defined as the first date of continuous culture negativity. Analyses were performed on an intention to treat basis.

Studies of the British Medical Research Council with rifampicin-containing short course chemotherapy regimens suggested that INH monoresistance or resistance to INH plus streptomycin had little effect on sputum culture conversion after 2 months of treatment and relapse (26). We therefore also analyzed our data combining those subjects with INH monoresistance with those with fully drug susceptible tuberculosis.

RESULTS

Study Population

Five hundred and fifty-four adult volunteers with suspected initial episodes of pulmonary tuberculosis were evaluated for study participation. One hundred and ten patients were enrolled and randomized to study treatment (Figure 1). Fifty-five patients received IL-2 immunotherapy and 55 patients received placebo.

Table 1 shows the characteristics of all subjects at the time of randomization. Patients randomized to IL-2 were less likely to have a BCG scar and report chest pain or night sweats. These variables were explored for potential confounding and interaction; no strong evidence for either was found. Twenty-five per cent of all subjects had moderately severe tuberculosis on chest X-ray and 75% cent had far advanced disease. Ninety-six per cent had cavitary disease. Ninety-two per cent of all subjects had grade 3+ or 4+ sputum AFB smears at entry.

One hundred and nine subjects received all 60 injections of study drug treatment. Injections were stopped early (after 32 injections) in one subject in the placebo arm who was found to have diabetes mellitus and was transferred from the TB ward to the medical ward for diabetic control.

Fifteen enrolled subjects were later terminated from the study or declared ineligible after review of initial drug susceptibility testing results – 12 due to INH monoresistance (7 subjects randomized to the IL-2 arm and 5 in the placebo arm), 2 subjects in the placebo arm with INH and rifampicin resistance, and 1 subject in the placebo arm who was found to be HIV-seropositive after enrollment. As specified in the study protocol, these subjects were followed but were excluded from the primary clinical, radiographic, microbiologic, and immunologic analyses. None of these reasons for exclusion differed significantly between treatment groups.

All enrolled subjects were included in the safety analysis. The number of patients completing each phase of the study is shown in Figure 1.

Compliance with Standard Short Course Chemotherapy

Patient compliance with standard anti-TB chemotherapy was excellent and comparable in both treatment arms at each time point. Ninety five percent of 384 urine INH metabolite tests performed during TB treatment were positive in the IL-2 group compared to 94% of 377 tests done in the placebo arm. Additional data on compliance are included in Table E2 in the on-line data supplement.

Microbiologic Outcomes

The primary study endpoint was the proportion of subjects in each treatment arm who had converted their sputum cultures to negative on solid media after 1 and 2 months of anti-TB treatment (Figure 2). After month 1, 8 (17%) of the subjects in the IL-2 arm had converted their sputum culture to negative compared to 14 (30%) of the subjects in the placebo group ($p = 0.14$, χ^2). After 2 months of treatment, 36 (77%) and 40 (85%) of the subjects in the IL-2 and control arms, respectively, were sputum culture negative ($p = 0.29$, $\chi^{2)}$. When considering both subjects with drug susceptible and INH-monoresistant tuberculosis ($n = 107$), the proportion of subjects who had converted their sputum cultures to negative after 1 month of treatment was 17% for the IL-2 arm and 28% for the placebo arm ($p = 0.15$; χ^2). After 2 months, the sputum culture conversion rate was 76% and 85% in the IL-2 and placebo arms, respectively ($p = 0.24$, χ^2). In a Kaplan-Meier analysis, time until sputum culture conversion after the onset of anti-TB treatment was longer among subjects in the IL-2 arm ($p = 0.05$, Wilcoxon, data not shown).

Quantitative sputum colony counts also were performed during anti-TB treatment. At several time intervals following initiation of treatment, the sputum bacillary load was lower in subjects receiving placebo compared to IL-2 ($p = 0.04$ after 4 days, $p = 0.02$ after 3 weeks and $p = 0.03$ after 4 weeks; t-test, Figure 3).

Clinical and Radiographic Outcomes

No treatment failures or deaths occurred. One bacteriologically confirmed relapse occurred in each treatment group. For both subjects who relapsed the sputum *M. tuberculosis* isolate obtained at the time of relapse was identical to the patient's baseline isolate when compared by IS6110 DNA genotyping (27). One subject who relapsed had initial resistance to INH. Neither subject who relapsed had acquired drug resistance.

There were no significant differences in the rate of weight gain, rate of defervescence, improvement in Karnofsky performance scale score and improvement in self-reported cough and chest pain during TB treatment between groups (data not shown). Radiographic improvement in the extent of disease by one or more severity grades (16) comparing baseline and follow-up chest X-rays after 1, 2, 6 and 12 months of TB treatment did not differ between treatment arms (data not shown). These results were unchanged when patients with INH monoresistance were included in the analysis. Data describing radiographic changes during treatment are included in Table E3 in the on-line data supplement.

Immunologic Changes during Anti-TB Treatment

There was no difference in the mean PPD size comparing study arms after 1 month of treatment 19.6 ± 2 mm for the IL-2 group and 19.5 ± 3 mm for the placebo arm ($p = 0.49$, t-test).

The mean change in PPD response from baseline to month 1 was 2.1 ± 6 mm in the IL-2 arm compared to 4.0 ± 6 mm in the placebo arm, p = 0.18, t-test). These results were comparable when subjects with INH resistance were included in the analysis.

Flow cytometric analysis of peripheral blood cells stained with a combination of monoclonal antibodies to CD3, CD4 or CD8 and CD25 (to assess activation of T cell subsets) or CD3, CD16 and CD56 (to determine frequencies of NK cells) were performed at baseline and after 2 and 6 weeks of anti-TB treatment. The 6 week time point was chosen to assess ongoing immune activation 2 weeks after the end of study drug treatment. The median percentage of CD4+/CD25+ T lymphocytes was greater after 2 and 6 weeks of anti-TB treatment in subjects receiving IL-2 than placebo (p = 0.05 and p = 0.08, Mann-Whitney U, Figure 4). By contrast, the percentage of CD8+/CD25+ cells was not increased with IL-2 treatment (data not shown). Systemic activity of IL-2 at week 2 of study was further corroborated by increased levels of sIL-2R (by ELISA) in serum from patients in the IL-2 compared to the placebo arm (p = 0.004, Mann-Whitney U, Figure 4). In contrast to previously published results (14), the percentage of $CD3^-$/CD16+/CD56+ NK cells was not increased among PBMC from subjects in the IL-2 arm compared to the placebo arm (data not shown).

The results for the above immunological measurements were comparable when patients with initial INH resistance were included in the analysis.

Safety

Immunotherapy with rhIL-2 was safe and generally well tolerated. Adverse events were usually mild to moderate in severity and of limited duration. No subject required dose reduction or discontinuation of IL-2 immunotherapy due to study drug-related side effects. Local pain, tenderness on palpation, erythema, ecchymoses and temporary hyperpigmentation at the injection site occurred more frequently in subjects receiving IL-2 (Table 2). Systemic adverse events did not differ between the treatment arms except transient mild lymphadenopathy that occurred more frequently in patients treated with IL-2. Thirteen subjects in the IL-2 group had transient mild lymphadenopathy compared to 2 patients in the placebo group. Additional information on systemic adverse events is included in Table E4 in the on-line data supplement. Hypothyroidism or hyperthyroidism did not occur in any subject. Changes in serum chemical and hematologic parameters were similar between the two treatment arms. The proportion of subjects with detectable serum anti-IL-2 antibodies after 4 weeks of study drug treatment was greater among subjects in the IL-2 arm than those receiving placebo (35% IL-2 arm vs. 6% placebo arm; $p < 0.001$, Fisher's exact test); however, there was no difference between groups at 6 weeks.

DISCUSSION

In this randomized placebo-controlled clinical trial, we found that adjunctive immunotherapy with recombinant human IL-2 did not enhance sputum bacillary clearance or improvement in important clinical symptoms in HIV-seronegative adults with drug-susceptible pulmonary tuberculosis. Intradermal therapy with IL-2 was generally safe and well tolerated. The study population included primarily patients with advanced cavitary tuberculosis and high

sputum bacillary loads where an effect of adjunctive immunotherapy would most likely be evident.

Two earlier trials of adjunctive IL-2 in pulmonary tuberculosis have been reported (13, 14). In Bangladesh and South Africa 20 patients with newly diagnosed, partially treated, or MDR tuberculosis received 30 days of twice daily intradermal injections of 225,000 IU (12.5 μg) of IL-2 in addition to chemotherapy (13). All patients with newly diagnosed TB and 5 of 7 patients with MDR TB converted their sputum smears to negative. An increase in PPD skin test size and enhanced T cell responses were seen in patients with drug susceptible TB.

A clinical trial in 35 patients with MDR TB from South Africa compared daily or pulsed IL-2 therapy with placebo (14). Patients received susceptibility directed chemotherapy and were randomized to receive daily [225,000 IU IL-2 intradermally twice daily] or pulsed [3 cycles of 450,000 IU IL-2 twice daily for 5 days, followed by nine days off IL-2] or placebo during the first 30 days of TB treatment. Among smear positive patients, 5 of 8 patients receiving daily IL-2 had reduced or negative sputum smears compared to 2 of 7 subjects receiving pulsed IL-2 and 3 of 9 subjects in the placebo group. The numbers of IL-2 receptor positive T cells and of NK cells were increased in patients receiving daily IL-2 but not in the pulsed IL-2 or placebo arms. Chest X-ray improvement after 6 weeks of TB treatment also was more frequent in patients receiving daily IL-2. No significant side effects of IL-2 treatment were observed.

We were unable to confirm these earlier reports of a positive effect of adjunctive immunotherapy with IL-2 on clinical, bacteriologic and radiographic responses in patients with newly diagnosed, partially treated, and MDR tuberculosis. The IL-2 treatment regimen used in our study, 225,000 IU intradermally twice daily for the first 30 days of tuberculosis treatment, was identical to that used in the randomized clinical trial performed in patients with MDR TB in

South Africa by Johnson and colleagues where IL-2 enhanced sputum smear clearance and radiographic improvement(14). In that study, IL-2 treatment was associated with an increase in peripheral blood of IL-2 receptor bearing T-cells and NK cells. Like Johnson and colleagues, we were able to demonstrate a modest systemic effect of IL-2 as evidenced by an increase in the percentage of CD25-positive CD4 T-cells and an increase of levels of sIL-2R and IL-2 in serum in our trial; however, the effect was transient. Thus, the lack of a clinical response to IL-2 therapy may be the result of the transient nature of its effect on immune parameters. However, recent evidence from the literature indicates that a successful anti-MTB immune response involves both CD4 and CD8 T-cells (28-30). Interestingly, IL-2 therapy resulted in expansion and excess activation of CD4, but not CD8 T-cells in the current study. Therefore, it is possible that the lack of a positive effect of adjunctive immunotherapy with IL-2 on clinical, bacteriologic and radiographic responses is due, at least in part, to its inability to elicit CD8 T-cell responses necessary for effective host defense against MTB.

The reasons underlying the differing results between our study and the earlier South African and Bangladesh trials are unclear but may be due to differences in the populations studied and the bacteriological methods. All of the studies involved patients with severe forms of pulmonary tuberculosis. The earlier South African and Bangladesh studies were small and only the South African trial in patients with MDR TB was a randomized trial. The South African and Bangladesh studies relied on sputum smear results whereas the current trial assessed changes in sputum bacillary load by both qualitative and quantitative cultures performed at frequent intervals. The most notable difference between the earlier and current studies is the inclusion of patients with MDR tuberculosis in the South African and Bangladesh trials. MDR TB is more difficult to treat with currently available chemotherapy and the effect of immunotherapy might

be more evident in patients with drug-resistant disease. Nonetheless, despite careful quantitative microbiologic surveillance, we were unable to demonstrate any positive impact of IL-2 immunotherapy on bacillary clearance in patients with advanced cavitary tuberculosis.

Our study has several important limitations. First, the intradermal IL-2 injections produce recognizable stigmata, such as warmth and pruritus, compared to placebo. Patients and examiners may have been able to determine treatment assignment by inspecting injection site, thus introducing ascertainment bias. Observation bias was minimized during the study by the use of dedicated nurse-injectors, who were not responsible for other patient assessments, to administer the test article. In addition, sputum smears and cultures, immunologic assessments, and chest x-ray interpretations were performed without knowledge of treatment assignment. Second, the trial was a phase II study focusing on preliminary evidence of microbiologic and immunological activity of IL-2 immunotherapy in patients with newly-diagnosed drug-susceptible tuberculosis. Our sample size estimate was calculated to have 80% power to detect a 19% improvement in sputum culture conversion after 1 and 2 months of anti-TB therapy, a difference similar to that of adding rifampin to combination chemotherapy. Our study had lower power to detect smaller improvements in bacteriological responses to treatment that might be beneficial for some patients. The trial also was not powered to detect significant differences in final tuberculosis treatment outcomes such as relapse between the immunotherapy and control arms. Finally, we analyzed patients with drug susceptible and INH monoresistant TB and cannot, therefore, exclude the possibility of an effect in patients with highly drug resistant TB. We studied only one dosing schedule of adjunctive IL-2, however, the regimen used in the current trial was selected based on positive results with this regimen in published studies and the best information available at the time the trial protocol was designed.

Our data from a double-blind, placebo-controlled clinical trial in patients with advanced, drug-susceptible pulmonary tuberculosis showed that, despite evidence of a transient systemic effect of adjunctive IL-2, immunotherapy with 450,000 IU of intradermal IL-2 daily during the first month of TB treatment did not enhance bacillary clearance or improvement in symptoms in HIV-seronegative adults with drug susceptible tuberculosis. Although IL-2 might potentially be of benefit in patients with MDR TB where drug treatment options and responses are sub optimal, our data suggest that adjunctive IL-2 immunotherapy is unlikely to improve results with current rifampicin-containing short course chemotherapy regimens in drug sensitive TB.

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FIGURE LEGENDS

Figure 1. Profile of the randomized clinical trial. AFB, acid-fast bacilli; HIV, human immunodeficiency virus; INH, isoniazid; TB, tuberculosis

Figure 2. Sputum culture conversion after 1 and 2 months of anti-TB treatment for subjects with drug-susceptible tuberculosis.

Figure 3. Sputum bacillary load during anti-TB treatment for subjects with drug-susceptible tuberculosis. Data are expressed as mean ± s.d. $\log_{10}$ colony forming units per ml of sputum. Filled circles (•) represent the IL-2 arm and open circles (o) represent the placebo arm. * Values significantly different ($p < 0.05$, t-test) between treatment groups. The number of subjects in each treatment arm with evaluable sputum specimens at each time point is: (a) baseline – IL-2 47 subjects, placebo 46 subjects; (b) at day 2 – IL-2 47 subjects, placebo 46 subjects; (c) at day 4 – IL-2 46 subjects, placebo 44 subjects; (d) at day 7 – IL-2 47 subjects, placebo 45 subjects; (e) at day 14 – IL-2 47 subjects, placebo 45 subjects; (f) at day 21 – IL-2 47 subjects, placebo 43 subjects; (g) at day 28 – IL-2 48 subjects, placebo 44 subjects; and (h) at day 60 – IL-2 43 subjects, placebo 42 subjects.

Figure 4. Expression of peripheral blood CD4+/CD25+ surface markers (A) and concentration of serum sIL-2R (B) during anti-TB treatment. Data are expressed as the median ± IQR. Filled circles (•) represent the IL-2 arm (n = 48) and open circles (o) represent the placebo arm (n = 47). * Values significantly different ($p < 0.05$, Mann-Whitney U) between treatment groups.

Table 1. Baseline characteristics of study subjects in each treatment group

Characteristic	IL-2 (n = 55)	Placebo (n = 55)	p value*
Age (yrs; mean ± s.d.)	26.7 ± 7	27.4 ± 7	0.60
Male n (%)	34 (61%)	41 (75%)	0.15
Weight (kg; mean ± s.d.)	52.7 ± 7	51.8 ± 7	0.47
BCG scar – n (%)	12 (22%)	26 (47%)	0.01
Fever – n (%)	14 (26%)	20 (36%)	0.22
Cough – n (%)	55 (100%)	55 (100%)	-
Hemoptysis – n (%)	2 (4%)	3 (6%)	1.0
Chest pain - n (%)	19 (39%)	38 (69%)	0.003
Night sweats – n (%)	22 (44%)	38 (69%)	0.01
Body temperature (°C; mean ± s.d.)	37.4 ± 0.8	37.2 ± 0.8	0.17
Sputum smear grade – n (%)			
1+ and 2+	3 (5%)	7 (13%)	0.33
3+	10 (18%)	12 (22%)	
4+	42 (76%)	36 (65%)	
White blood cell count (x $10^3/mm^3$; mean ± s.d.)	8.7 ± 3	9.0 ± 3	0.56

Characteristic	IL-2 (n = 55)	Placebo (n = 55)	p value*
Hemoglobin (gm/dL; mean ± s.d.)	12.1 ± 2	11.9 ± 2	0.67
PPD (mm induration; mean ± s.d.)	17 ± 5	16 ± 6	0.18
Extent of disease on chest X-ray - n (%)			
Moderately advanced	17 (31%)	10 (18%)	0.12
Far advanced	38 (69%)	45 (82%)	
Cavitary disease n (%)	54 (98%)	52 (95%)	0.62

*t-test, χ^2, or χ^2 for trend comparing IL-2 and placebo arms

Table 2. Number of local adverse events by treatment arm

Local adverse event description	IL-2 (N=55) n (%)	Placebo (N=55) n (%)	P-value*
Pain at injection site	27 (49)	1 (2)	<0.0001
Tenderness on palpation	48 (87)	2 (4)	<0.0001
Erythema	53 (96)	1 (2)	<0.0001
Ecchymosis	10 (18)	1 (2)	0.008
Hyperpigmentation	55 (100)	1 (2)	<0.0001
Axillary pain or lymphadenopathy	1 (2)	0	1.0

* Fisher's exact test comparing IL-2 and placebo arms

Figure 1.

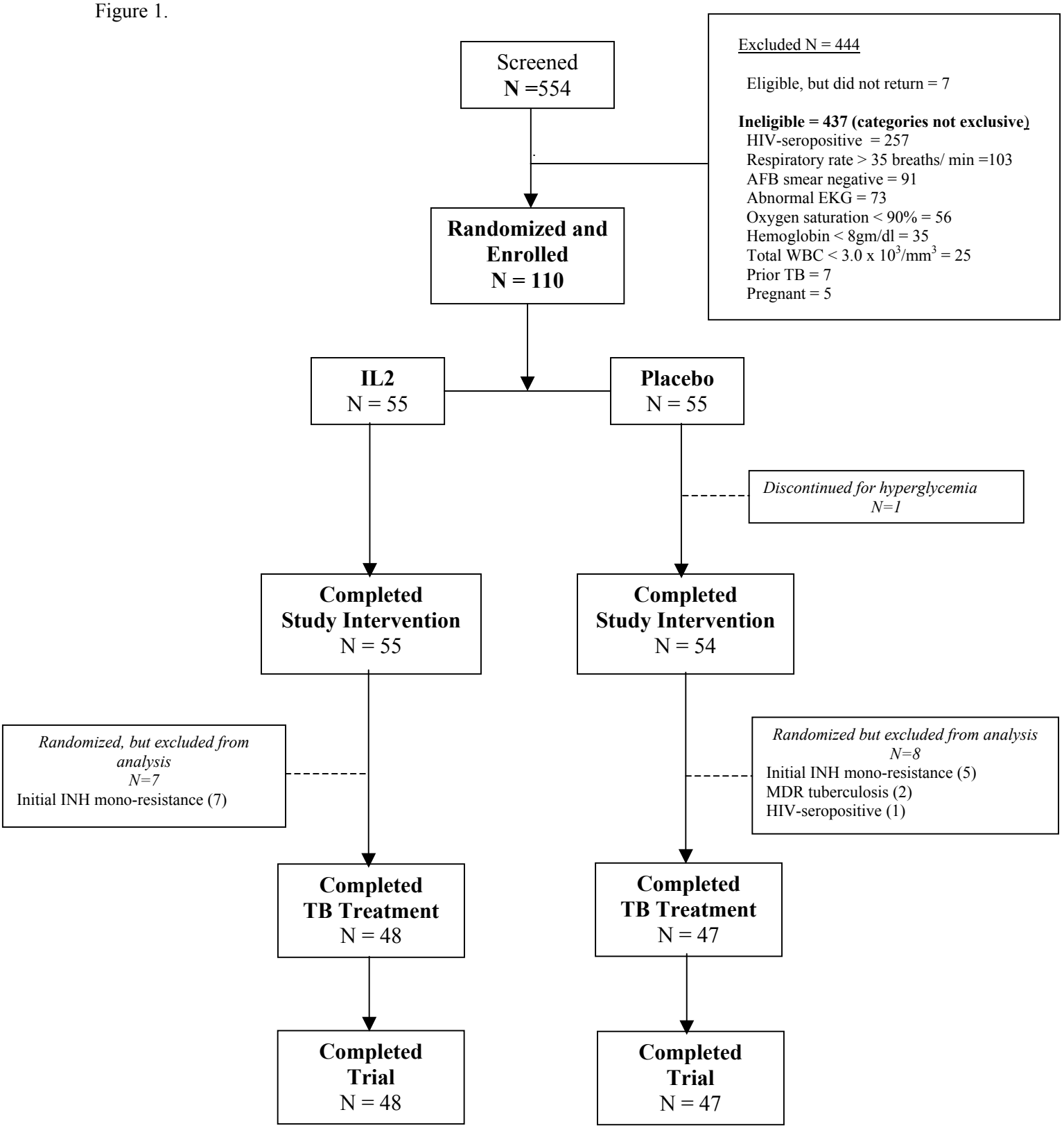

Screened
N =554
Excluded N = 444
Eligible, but did not return = 7
Ineligible = 437 (categories not exclusive)
HIV-seropositive = 257
Respiratory rate > 35 breaths/ min =103
AFB smear negative = 91
Abnormal EKG = 73
Oxygen saturation < 90% = 56
Hemoglobin < 8gm/dl = 35
Total WBC < 3.0 x 10^3/mm^3 = 25
Prior TB = 7
Pregnant = 5
Randomized and Enrolled
N = 110
IL2
N = 55
Placebo
N = 55
Discontinued for hyperglycemia
N=1
Completed Study Intervention
N = 55
Completed Study Intervention
N = 54
Randomized, but excluded from analysis
N=7
Initial INH mono-resistance (7)
Randomized but excluded from analysis
N=8
Initial INH mono-resistance (5)
MDR tuberculosis (2)
HIV-seropositive (1)
Completed TB Treatment
N = 48
Completed TB Treatment
N = 47
Completed Trial
N = 48
Completed Trial
N = 47

Figure 2.

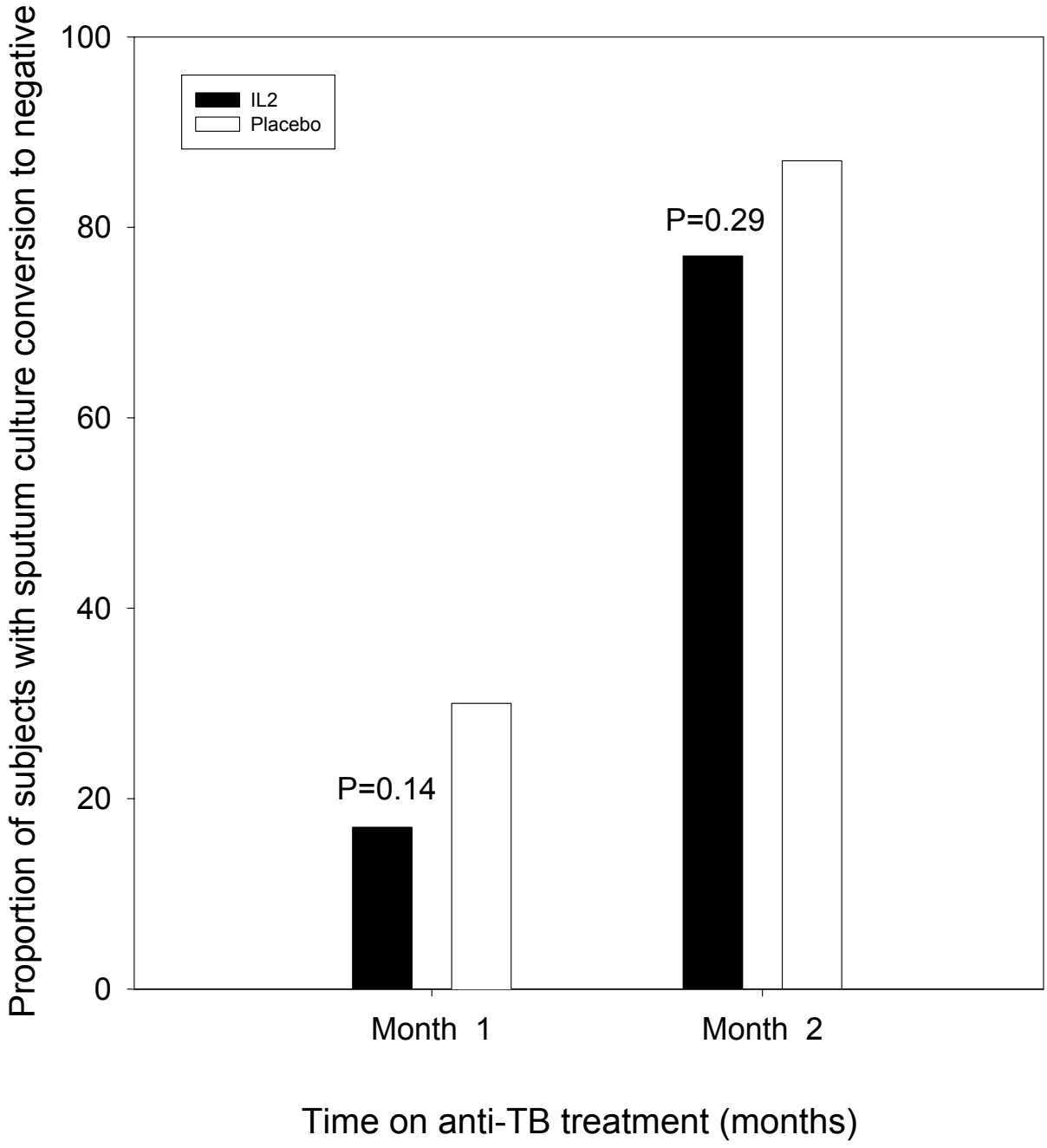

Proportion of subjects with sputum culture conversion to negative
100
80
60
40
20
0
IL2
Placebo
P=0.14
P=0.29
Month 1
Month 2
Time on anti-TB treatment (months)

Figure 3.

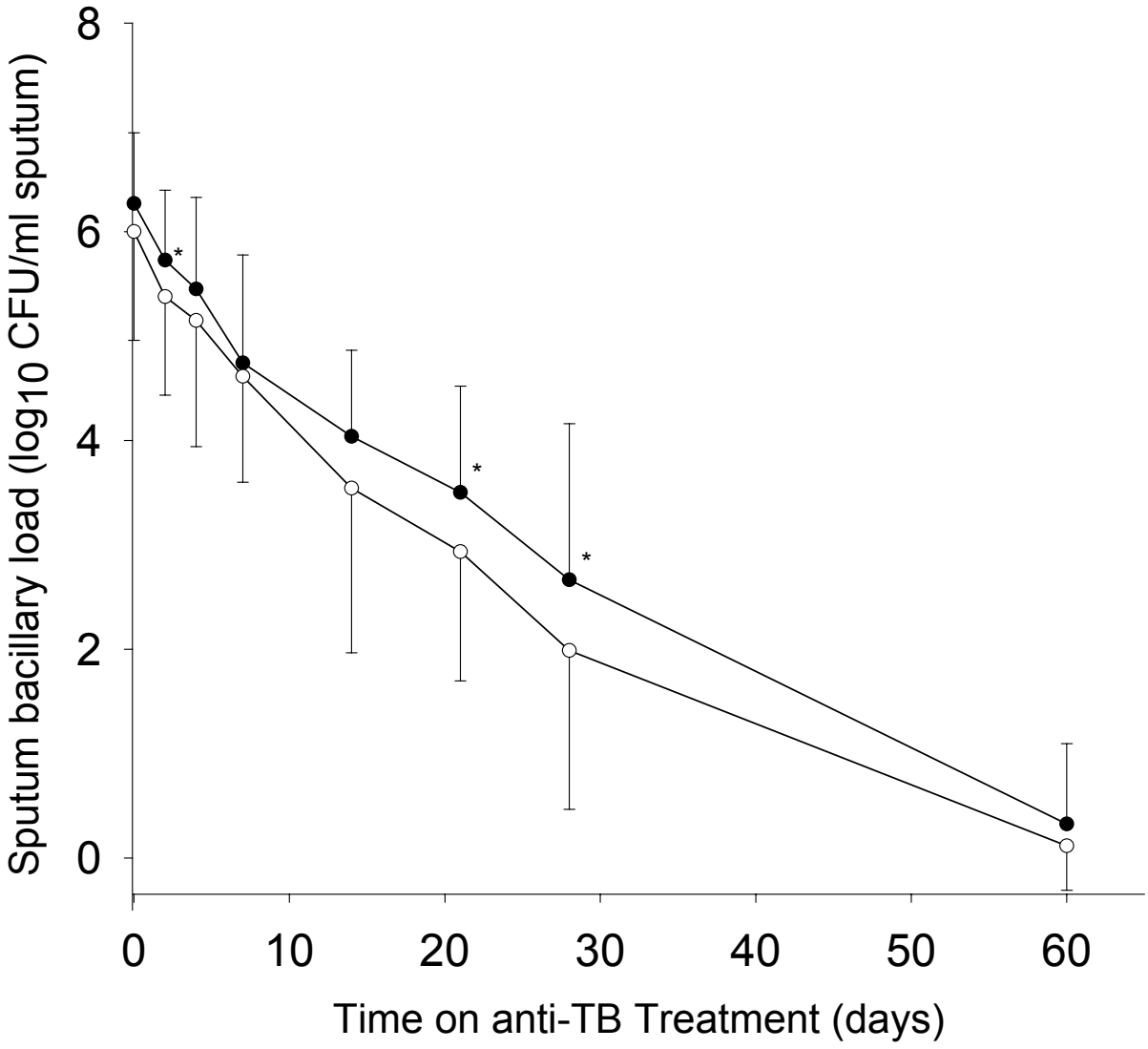
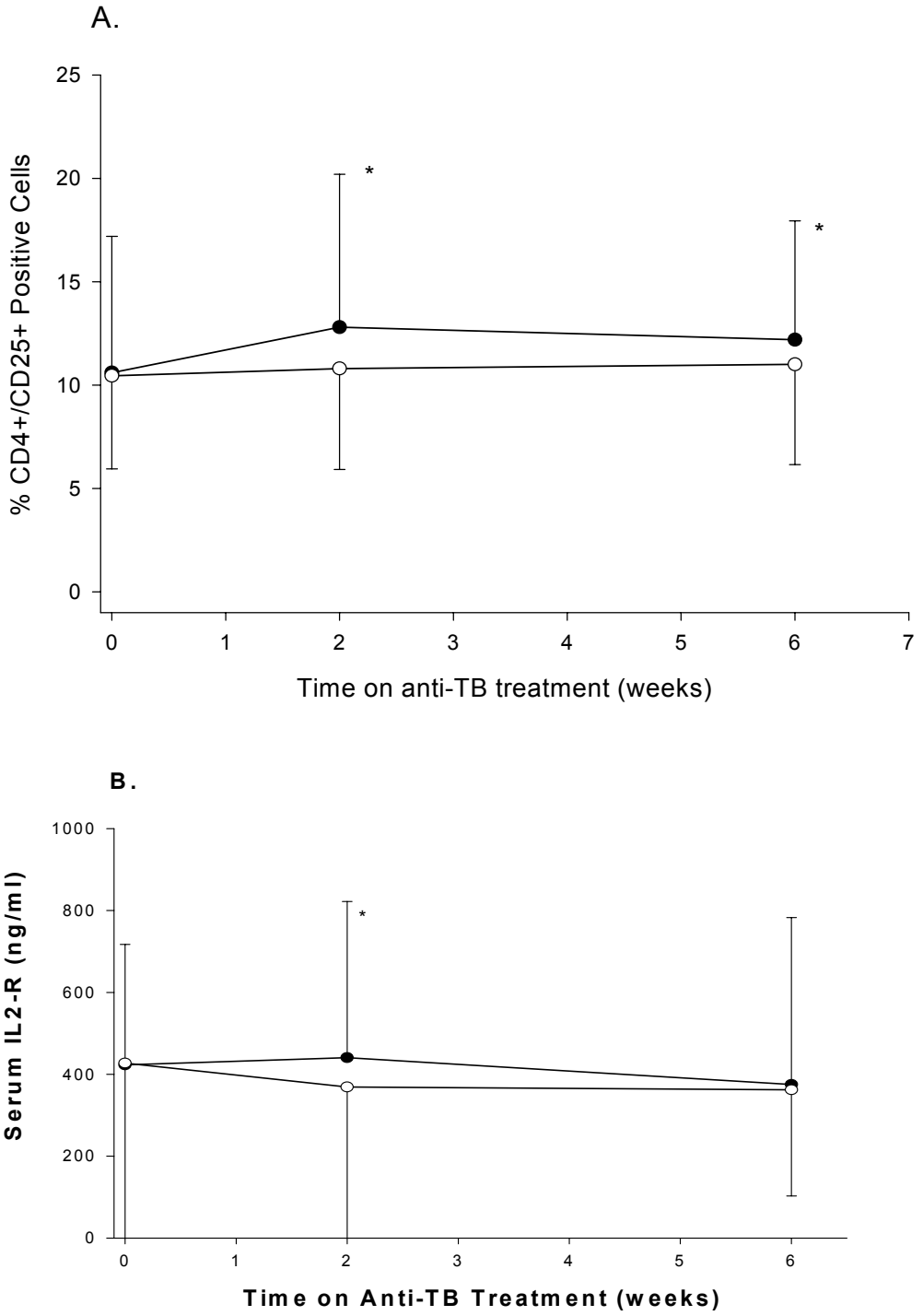

Sputum bacillary load (log10 CFU/ml sputum)
8
6
4
2
0
*
*
*
0
10
20
30
40
50
60
Time on anti-TB Treatment (days)

Figure 4.


A.
% CD4+/CD25+ Positive Cells
25
20
15
10
5
0
*
*
0
1
2
3
4
5
6
7
Time on anti-TB treatment (weeks)
B.
Serum IL2-R (ng/ml)
1000
800
600
400
200
0
*
0
1
2
3
4
5
6
Time on Anti-TB Treatment (weeks)

Randomized Trial of Adjunctive Interleukin-2 in Adults with Pulmonary Tuberculosis

John L. Johnson, Emmanuel Ssekasanvu, Alphonse Okwera, Harriet Mayanja, Christina S. Hirsch, Joseph G. Nakibali, Dana Drzayich Jankus, Kathleen D. Eisenach, W. Henry Boom, Jerrold J. Ellner, Roy D. Mugerwa for the Uganda-Case Western Reserve University Research Collaboration

Online Data Supplement

METHODS

Screening and Enrollment

From October 1998 to November 1999, 18 to 50 year old ambulatory adults with suspected pulmonary tuberculosis were referred from the outpatient clinics of the National Tuberculosis Treatment Center at Mulago Hospital, Kampala, Uganda for possible study participation. After giving informed consent, volunteers underwent sputum examination, chest X-ray, pulse oximetry, 12-lead electrocardiogram, complete blood count, renal, liver and thyroid function tests [serum thyroid stimulating hormone (TSH) and thyroxine], urinalysis, urine β-human chorionic gonadotropin pregnancy testing (women only) and Human Immunodeficiency Virus (HIV) enzyme immunoassay testing. The study protocol was approved by the institutional review boards at University Hospitals of Cleveland and Case Western Reserve University, and the Ugandan National AIDS Research Subcommittee. All subjects gave written informed consent for study participation and HIV testing and received pre- and post-HIV test counseling.

Subjects were eligible if they were sputum acid fast bacilli (AFB) smear positive (grade 1+ or greater), had moderately advanced or far advanced disease on chest X-ray (E1), had a normal EKG, were not pregnant or lactating and had a Karnofsky Performance Scale Score of greater than 50% (E2). Individuals previously treated for tuberculosis, HIV-seropositive individuals, patients with asthma or untreated thyroid disease, patients with renal (serum creatinine greater than 177 μM per liter) or hepatic (serum aspartate aminotransferase (AST) greater than 100 IU/L) dysfunction or severe anemia (hemoglobin less than 8 gm/L) or leukopenia (total white blood cell count less than $3 \times 10^3/mm^3$) were ineligible. Patients receiving chronic steroid or immunosuppressive therapy, patients with limited respiratory reserve

(respiratory rate greater than 35 per minute or arterial oxygen saturation less than 90% while breathing ambient air), and patients on treatment for cardiovascular disease also were excluded. Persons with initial drug resistance to isoniazid (INH), rifampicin, ethambutol or pyrazinamide were excluded from the study after susceptibility testing results were available and their anti-TB treatment regimen adjusted accordingly. Purified protein derivative (PPD) skin testing was performed at the time of screening with 0.1 ml (5 tuberculin units) of purified protein derivative (Tubersol®, Aventis Pasteur) by the Mantoux method using standardized procedures (E3).

After screening, eligible subjects were admitted to the hospital and baseline samples were collected. Serum was stored for measurement of anti-IL-2 antibodies. The proportion of peripheral blood lymphocytes subsets expressing CD25+ (IL-2 receptor) and CD 56+ [natural killer (NK) cell] surface markers was measured by flow cytometry. The concentration of serum IL-2 receptors (sIL-2R) also was measured using a commercially available enzyme linked immunosorbent assay (BioSource, Camarillo, CA) according to the manufacturer's instructions. The sensitivity of this assay was less than 0.016 ng/ml.

At least two additional sputum samples were collected for baseline qualitative smears, quantitative culture on Middlebrook 7H-10 medium, and BACTEC radiometric culture. After baseline samples were collected, patients were started on standard short course anti-TB chemotherapy [2 months of daily INH, rifampin, pyrazinamide and ethambutol followed by 4 months of daily INH and rifampin with doses adjusted for body weight] and study drug treatment [twice daily intradermal injections of 225, 000 IU of recombinant human IL-2 (Proleukin®; aldesleukin – kindly donated by Chiron, Emeryville, CA) or sterile 5% dextrose for injection, USP (placebo)]. IL-2 or placebo was administered twice daily by intradermal injection into the skin of the back during the first 30 days of anti-TB treatment.

Tuberculosis treatment

Standard short course combination chemotherapy was be administered to all study subjects with fully-drug susceptible MTB in accordance with established US and Ugandan national TB treatment guidelines. Treatment for tuberculosis was administered under direct supervision during the initial one-month hospitalization and then self-administered on an ambulatory basis. TB drugs were obtained from the International Dispensary Association (Amsterdam, Netherlands) and manufactured under Good Manufacturing Practices. Drugs were prepackaged into individual daily doses. Adherence with TB treatment was assessed by self-report, review of dispensing records and at least monthly urine INH metabolite testing during TB treatment.

Dosages of standard anti-TB drugs were modified by body weight (Table E1). Pyridoxine 50 mg was administered daily to all subjects during anti-TB treatment to prevent INH neurotoxicity.

Follow-up measurements

During the first 30 days of inpatient treatment body temperature was recorded twice daily; and weight, Karnofsky performance scale score and oxygen saturation by pulse oximetry was measured thrice weekly. History and physical examination and systemic adverse events surveys were done at least weekly throughout during study drug treatment. Injection sites were inspected twice daily and any local induration or reactions recorded. Sputum was collected for AFB smear after 2 and 4 days of treatment, then weekly from weeks 1 to 4, at 6 weeks and then monthly at 2, 3, 4, 5, 6, 9 and 12 months after beginning therapy as long as the patient was able to spontaneously produce sputum. PPD skin testing was repeated after 4 weeks (end of study

drug treatment). Serum was stored for measurement of serum sIL-2R and anti-IL-2 antibodies at weeks 2 and 6. Flow cytometric analysis for CD25 and CD56 surface markers was performed at weeks 2 and 6. Complete blood counts, liver function tests and urinalysis was done weekly during the first month of treatment and at the 12 month visit. Serum TSH and T4 measurements were repeated at the week 4 visit.

Clinical chemistry panels and thyroid function testing were performed using a Roche Cobas analytic system. HIV testing was done using two HIV-1 EIA assays (Abbott Murex HIV-1/2, Abbott Laboratories, Abbott Park, IL and Vironostika HIV-1 Microelisa System, Organon Teknika, Durham, NC) for all subjects. Serum TSH and T4 were measured by EIA using commercially available kits (Chiron) according to the manufacturer's instructions. A Coulter T-540 automated system (Coulter Electronics, Hialeah, FL) was used to perform complete blood counts.

Bacteriology

Early morning specimens were collected in sterile disposable 50 mL polypropylene centrifuge tubes and stored at 2 to 8 °C prior to processing. Samples were homogenized by incubation with N-acetyl L-cysteine/sodium citrate (50 mg/mL NALC in 2.9% sodium citrate) for 4 min and vortexing with several 4 mm glass beads for 30 seconds (E4). The homogenate was then decontaminated by incubation for 15 min with an equal volume of 2% sodium hydroxide and 1.5% sodium citrate and concentrated by centrifugation at 4000 x g at 8 to 10 °C for 15 min. The sediment was reconstituted to 2.5 mL volume with phosphate buffer and the resulting suspension was used to prepare smears and cultures on solid and BACTEC media. Sputum smears were examined by fluorescent microscopy and graded negative to 4+ (E5).

Quantitative culture (colony forming unit assay)

Serial 10-fold dilutions were prepared by adding 0.5 mL of the sediment to 4.5 ml of 0.25% Tween-80 (Sigma P1754) in 0.9% saline (E4). From each dilution (10^0 to 10^{-5}), 60 µL were inoculated on selective and non-selective sides of Middlebrook 7H10 agar biplates supplemented with oleic acid albumin-dextrose-catalase. The medium was made selective by the addition of final concentrations of 200 U/ml of polymyxin B, 50 mg/ml of carbenicillin, 20 mg/ml of trimethoprim, and 10 mg/ml of amphotericin B. Plates were sealed, incubated at 37°C in 5 to 10% CO_2 and examined after 2, 3, 4, and 6 weeks. Colonies were counted on plates with dilutions yielding 10 to 50 visible colonies and expressed as $\log_{10}$ CFU/ml of undiluted sputum.

Speciation of isolates and drug susceptibility testing

Pretreatment sputum isolates from each patient were confirmed as *M. tuberculosis* using the BACTEC® para-nitro-acetyl amino-hydroxy-propiophenone (NAP) susceptibility method (E6). Susceptibility testing against INH, rifampin, ethambutol, and pyrazinamide was performed on pretreatment sputum isolates from each patient using standard BACTEC® methods (E7). The critical concentrations used were isoniazid, 0.1 µg/mL; rifampin, 2.0 g/mL; ethambutol, 2.5µg/mL; and pyrazinamide, 100µg/mL.

Cell subset analysis

Immunostaining and 3-color flow cytometry were performed to assess relative frequencies of T-cell subsets, NK cells, and of mononuclear cells expressing CD25 and CD56.

Briefly 100 μL aliquots of whole blood were incubated with combinations of commercially available fluorochrome-conjugated antibodies (Becton-Dickinson, San Jose, CA) to CD3/CD4/CD8, CD3/CD16/56/CD19, CD4/CD8/CD25 and CD4/CD8/IgG_1 isotype control and incubated for 20 min at 4 °C in the dark. Following lysis of RBC, cells were fixed and stored at 4 °C until use. Samples were acquired (10,000 events per sample) using a 3-color flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed using WINMDI software (Scripps Institute, La Jolla, CA). To assure consistency in analysis, the same regions and quadrants were used for all samples.

Anti-IL-2 Antibody Assays

Stored sera were assayed for human anti-IL-2 IgG and IgM antibodies to Proleukin® at Chiron Analytical Operations, Emeryville, CA. Samples were initially screened at 1:10 and 1:100 dilutions. Bound antibodies were detected using HRP-labeled goat anti-human IgG or IgM conjugates. If the sample OD at the 1:10 dilution exceeds 40% of the OD in the IgG screen or if the sample OD at the 1:100 dilution exceeded the reference OD for the IgM screen, the samples were titered by serial two-fold dilution. The reference control for the IgM assay is anti-human anti-IL-2 that has been calibrated against 40 ng/ml of human IgM coated on the plate. The reference control for the IgG titering assay is commercial IgG coated on the plate. Samples with an OD less than or equal to the reference in the IgM screen at reported as less than 100. Samples with an OD less than or equal to 0.4 OD in the IgG screen were reported as less than 10.

Treatment Assignment

The Division of Microbiology and Infectious Diseases, National Institutes of Health, USA prepared the randomization sequence. Two code letters were assigned to designate either the IL-2 or placebo treatment arms. A computer-generated randomization sequence with a block size of 10 was used to prepare a list of treatment assignments allocating subjects to one of the four treatment code letters. Treatment code letters were placed on a sticker that was sealed in an opaque envelope and shipped to the trial site. The sealed envelopes were numbered sequentially and the injector nurses enrolled patients by opening the next envelope in the sequence. The stickers with the treatment assignment code letters were placed on the patient's injection record case report form. These forms were stored in a locked file cabinet to which only the injector nurse had access.

Masking

Allocation of treatment arm assignment was concealed by using dedicated nurse-injectors, who made no other subject assessments, to administer the test article. Other clinical staff and the study volunteers were masked to treatment assignment. Due to the previously reported local side effects of rhIL-2 (mild tenderness, erythema and induration), we sought to mask treatment assignment by using separate clinical assessors for local and systemic adverse experiences. Specimens sent to the laboratory and chest X-rays were identified only by subject identification numbers. Laboratory staff and chest X-ray readers were blinded to treatment assignment. Treatment assignments were not revealed to any investigators or subjects during the trial. Subjects were followed for one year after the onset of anti-TB treatment.

ADDITIONAL RESULTS

Adherence with anti-TB treatment

Adherence with anti-TB treatment was excellent and comparable between study groups at each study time point (Table E2).

Effect of IL-2 treatment on radiographic responses to anti-TB treatment

To assess whether IL-2 treatment affected radiographic response to anti-TB treatment, we compared pretreatment chest X-ray findings with findings on follow-up X-rays performed after 1 month (end of study drug treatment), 2 months (end of intensive phase of chemotherapy), 6 months (end of chemotherapy), and 12 months (end of follow-up). No difference in the proportion of subjects in each treatment group whose extent of disease improved by one or more severity grades (E1) was seen at any time point (Table E3). No improvement in the resolution of cavitary disease was evident at the end of study drug treatment or end of follow-up.

The results were comparable when subjects with INH monoresistance were included in these analyses.

Systemic adverse events

The number of subjects experiencing systemic adverse events categorized by body systems was similar between the IL-2 and placebo treatment groups except for transient lymphadenopathy that occurred more frequently in subjects receiving IL-2 (Table E4).

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Table E1. Dosage of anti-TB drugs

Drug	Body weight less than 50 kg	Body weight from 50 to 69 kg	Body weight 70 kg or greater
INH	300 mg/d	300 mg/d	300 mg/d
Rifampicin	450 mg/d	600 mg/d	600 mg/d
Ethambutol	800 mg/d	1000 mg/d	1200 mg/d
Pyrazinamide	1.5 gm/d	2 gm/d	2 gm/d

Table E2. Urine INH metabolite test results during anti-TB treatment

Time after beginning anti-TB treatment	IL-2 Group		Placebo Group	
	No. of urine INH metabolite tests done	No. positive tests	No. urine INH metabolite tests done	No. positive tests
Week 1	55	54	55	54
Week 4	55	52	54	53
Month 2	55	55	55	53
Month 3	54	50	53	50
Month 4	55	53	53	49
Month 5	55	53	55	50
Month 6	55	47	52	46
Total	384	364 (95%)	377	355 (94%)

Table E3. Improvement in extent of disease on chest X-ray by one or more severity grades during anti-TB treatment

Comparison	IL-2 Group (n = 48)	Placebo Group (n = 47)	p-value
Baseline to month 1	12 (26%)	9 (19%)	0.42[a]
Baseline to month 2	19 (44%)	15 (33%)	0.30[a]
Baseline to month 6 (end of TB treatment)	34 (71%)	35 (74%)	0.69[a]
Baseline to month 12 (end of follow-up)	43 (91%)	41 (91%)	1.0[b]

[a] χ^2 comparing IL-2 and placebo groups

[b] Fisher's exact test comparing IL-2 and placebo groups

Table E4. Numbers of systemic adverse events in each treatment group by body system

	IL-2 (n=55)				Placebo (N=55)			
	Grade				Grade			
	1	2	3	4	1	2	3	4
General	5	0	28	10	7	2	21	11
Dermatologic	26	7	0	0	17	10	0	0
Endocrine	1	0	0	0	1	0	0	0
Gastrointestinal	12	0	0	0	18	2	0	0
Genitourinary	7	0	1	0	3	1	0	0
Lymphatic[a]	13	0	0	0	2	0	0	0
Musculoskeletal	23	9	1	0	27	13	0	0
Neurological	0	1	0	0	1	0	0	0
Parasitic & infectious	9	2	1	0	10	0	0	0
Respiratory	35	6	1	0	35	8	1	0
Total	131	25	32	10	121	36	22	11

[a] $p < 0.01$ comparing IL-2 and placebo arms by occurrence of adverse events, regardless of grade. The highest grade per event per subject was analyzed.