Interleukin-10, Polymorphism in \textit{SLC11A1} (formerly \textit{NRAMP1}), and Susceptibility to Tuberculosis

Agnes A. Awomoyi,\textsuperscript{1,\textasteriskcentered} Arnaud Marchant,\textsuperscript{1,\textasteriskcentered} Joanna M. M. Howson,\textsuperscript{3} Keith P. W. J. McAdam,\textsuperscript{1} Jenefer M. Blackwell,\textsuperscript{1} and Melanie J. Newport\textsuperscript{1,3}

Host genetic factors are major determinants of susceptibility to tuberculosis, and an understanding of the molecular basis of this observation has major implications for the development of novel therapies and vaccines. \textit{Slc11a1} (formerly \textit{Rnamp1}), the first murine infection susceptibility locus identified, regulates early innate responses to intracellular pathogens. Variation in the human homologue \textit{SLC11A1} is associated with and linked to tuberculosis in genetically different populations. In a case-control study of 329 tuberculosis case patients and 324 control subjects, the association between allele 2 of a functional \textit{SLC11A1} polymorphism and tuberculosis has been reproduced. This variant is associated with higher lipopolysaccharide-induced production of the macrophage-deactivating cytokine interleukin-10. Furthermore, monocytes from persons who develop tuberculosis innately produce more interleukin-10 than do monocytes from healthy control subjects. These data therefore confirm the importance of \textit{SLC11A1} in tuberculosis susceptibility in humans and suggest that \textit{SLC11A1} influences tuberculosis susceptibility by regulation of interleukin-10.

Tuberculosis is a major public health problem globally [1], and bacille Calmette-Guérin vaccination does not afford protection in many populations in areas where tuberculosis is endemic [2]. Multidrug-resistant \textit{Mycobacterium tuberculosis} has been reported worldwide [3], further impeding the prospect of tuberculosis eradication. It is estimated that more than one-third of the world’s population is infected with \textit{M. tuberculosis}, yet only 10% develop clinical disease. Understanding the molecular mechanisms underlying protective immunity is a prerequisite for the development of improved therapies and vaccines for tuberculosis.

Host genetic factors are important determinants of susceptibility to tuberculosis [4]. Of all the genes associated with tuberculosis to date, \textit{Slc11a1} (solute carrier family 11 member 1, formerly known as natural resistance-associated macrophage protein 1 [\textit{NRAMP1}]) is the best characterized. Initially identified as a murine mycobacteria susceptibility gene [5], \textit{Slc11a1} was positioned cloned, and a point mutation, resulting in the substitution of glycine to aspartic acid at position 169, was identified in the susceptibility allele [6]. The human homologue, \textit{SLC11A1}, was cloned, and variation in this gene has been studied in relation to tuberculosis susceptibility in a number of genetically different human populations. The first study to demonstrate an association between tuberculosis susceptibility and \textit{SLC11A1} was a case-control study conducted in The Gambia, in which 4 different \textit{SLC11A1} polymorphisms (5' CA, INT4, D543N, and 3' UTR, as described elsewhere [7]) were found to be associated with tuberculosis, with odds ratios (ORs) of 1.13–1.87 [8]. Heterozygosity for both the INT4 and 3' UTR alleles led to a 4-fold increased risk of tuberculosis. This finding was followed up by the same group, who reported a significant association (\textit{P} < .04) between the INT4 polymorphism in a family-based association study in Guinea-Conakry [9]. The 3' UTR polymorphism appears to be associated with tuberculosis in the Korean population (OR, 1.85) [10], and the 5' CA repeat is associated with tuberculosis in Japan (OR, 2.07) [11]. Significant linkage (\textit{P} < 2 \times 10^{-5}) between \textit{SLC11A1} and tuberculosis was identified in an large Aboriginal Canadian family [12].

The 5' CA polymorphism is a complex repeat [13] that is functional and influences \textit{SLC11A1} expression in a luciferase reporter system [14]. There are 4 variants at this site: alleles 1 and 4 are rare (frequencies of <0.01), whereas alleles 2 and 3 occur at frequencies of ~0.25 and ~0.75, respectively. Allele 3 drives high expression relative to the other alleles, which is enhanced by lipopolysaccharide (LPS), and this allele has been associated with resistance to tuberculosis [8]. Conversely, allele

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Informed consent was obtained from all subjects recruited for the study. The study was approved by the Gambian Government/Medical Research Council Joint Ethical Committee.

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2 is associated with lower promoter activity and susceptibility to tuberculosis.

Skl1a1 plays a critical role in early innate macrophage responses to intracellular infection. Its pleiotropic effects include the activation of microbicidal responses, including the production of reactive oxygen and nitrogen intermediates and the proinflammatory cytokines tumor necrosis factor (TNF-α) and interleukin (IL)-1β, increased expression of inducible nitric oxide synthase, and up-regulation of major histocompatibility complex (MHC) class II molecules [15]. Macrophage activation is greatly enhanced by interferon (IFN)-γ, and IFN-γ response elements are present in the human SLC11A1 promoter region [14]. In humans, mutations in the genes encoding the IFN-γ receptor chains [16–18] or treatment with TNF-α blockers [19] lead to disseminated mycobacterial disease, highlighting the importance of these cytokines in mycobacterial immunity. The proinflammatory effects of TNF-α are counterbalanced by down-regulatory cytokines, including IL-10, which also is produced by the macrophage in response to infection [20]. Although Skl1a1’s enhancement of proinflammatory responses that are important for containment of mycobacterial infection are well defined, the role of Skl1a1 in regulating down-regulatory responses has not been as extensively evaluated.

In this study, we aimed to confirm the genetic association between polymorphism in SLC11A1 and susceptibility to tuberculosis and to investigate the role of this polymorphism in the regulation of innate macrophage responses required for control of early mycobacterial infection, a potentially important stage in disease pathogenesis at which susceptibility or resistance to the development of disease is expressed [21].

Subjects and Methods

Study Populations

Case-control genetic study. Three hundred forty men with pulmonary tuberculosis (mean age, 36 years; range, 19–58 years) were recruited from the tuberculosis clinic based at Serrekunda Health Centre, The Gambia. All case patients had microscopically proven smear-positive tuberculosis in the context of clinical and radiologic findings consistent with pulmonary tuberculosis, in the absence of other chronic illness, human immunodeficiency virus (HIV) infection, or treatment with corticosteroids. Three hundred forty healthy male HIV-negative blood donors (mean age, 32 years; range, 18–50 years), matched for ethnicity and age to the case patients with tuberculosis, were simultaneously recruited at the Royal Victoria Hospital, Banjul. These groups were used to confirm the association between allele 2 of the SLC11A1 promoter polymorphism and susceptibility to tuberculosis. Monocyte responses to LPS were assessed in the healthy control population by use of the whole-blood assay described below.

Study of monocyte responses in tuberculosis-susceptible and -resistant persons. Forty men (mean age, 35 years; range, 18–56 years) with a history of tuberculosis were recruited from tuberculosis records kept at Serrekunda Health Centre. These “ex-case patients” had all completed a 6-month course of chemotherapy (rifampicin, isoniazid, ethambutol, and pyrazinamide for 2 months, followed by 4 months of treatment with rifampicin and isoniazid only) at least 2 months before enrollment and had no symptoms or signs of active disease at the time of enrollment. This eliminated the possibility confounding immunomodulatory effects of active infection or antibiotics on immune responses. For each ex-case patient enrolled, a healthy, unrelated, tuberculin skin test–positive person matched for age, sex, and ethnic group was identified within the same household (mean age, 33 years; range, 18–59 years). All subjects were healthy at the time of the study and were HIV-seronegative. Monocyte responses to LPS were assessed by means of a whole-blood assay described below and compared between the 2 groups.

Nine milliliters of venous blood was collected aseptically from all subjects and mixed with 1 mL of 3.8% sodium citrate. This blood was used for both whole blood assays and DNA isolation.

Whole-Blood Assays

Venipuncture and transport of samples was done uniformly, and samples to be used for whole-blood assays were collected between 8:00 and 10:00 A.M. and processed in the laboratory within 2 h. One milliliter of blood was diluted 1:1 with RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco Life Technologies). One milliliter was incubated at 37°C with IFN-γ (final concentration, 200 IU/mL; Genzyme) for 2 h, and the other milliliter was incubated with an equivalent volume of medium. Next, 180 µL of blood was aliquoted into triplicate wells of sterile pyrogen-free microplates containing 20 µL of medium containing LPS (Escherichia coli; final concentration, 1 ng/mL; Sigma) or medium as the negative control. The blood was cultured at 37°C with 5% CO₂ for 18 h. Supernatants were stored at −70°C and analyzed in 1 batch. TNF-α, IL-1β, and IL-10 concentrations were measured in triplicate with commercially available ELISA kits (Biosource Europe), following the manufacturer’s instructions. Optical densities were read at 460 nm on a plate reader (Dynex Technologies). All plates included standard dilutions of the appropriate recombinant cytokine supplied in the kit, from which a standard curve was derived to calculate cytokine concentrations in each well. The lower limit of detection was 8 pg/mL.

SLC11A1 Genotyping

DNA was extracted by use of a standard proteinase K, phenol-chloroform protocol [22]. The SLC11A1 5’ promoter region (GT), polymorphism was amplified by polymerase chain reaction with a fluorescence-labeled forward primer, and fragments were sized by electrophoresis with an ABI 377 sequencer and Genescan/Genotyper software (Applied Biosystems), as described elsewhere [23].

Statistical Analysis

For the case-control genetic studies, 300 individuals in each group gives 80% power (P < .05) to detect an OR of 3 when the relevant allele has a frequency of >0.1. The χ² test was used to compare allele and genotype frequencies in the case-control tuberculosis study. The
sample size of 40 ex–case patients and 40 unaffected control subjects was determined to have 80% power to detect a 2-fold difference in mean cytokine production between the 2 groups at \( P < .05 \). Monokine responses in control wells were subtracted from responses to LPS or LPS plus IFN-\( \gamma \). Because data were normally distributed after logarithmic transformation, parametric tests for statistical significance were used. The mean natural logarithms of cytokine responses between persons with or without allele 2 of the \( SLC11A1 \) promoter polymorphism were compared by use of the unpaired Student’s \( t \) test, and data were expressed as geometric means and 95% confidence intervals (CIs). A paired Student’s \( t \) test was used for study of ex–case patients and control subjects, in which numbers were evenly matched. Graphpad Prism (Graphpad) and S-plus (MathSoft) software was used.

Results

\( SLC11A1 \) promoter genotype and tuberculosis susceptibility. Genotyping was completed successfully for 329 case patients and 324 control subjects. Allele frequencies in our population were similar to those reported elsewhere (table 1) and were in Hardy-Weinberg equilibrium. A significant association was observed between the presence of allele 2 of the \( SLC11A1 \) promoter polymorphism and susceptibility to tuberculosis in this study population (allele frequency, 0.184 in case patients, compared with 0.137 in control subjects; \( P = .024 \)). Allele 3 occurred less frequently in case patients (allele frequency, 0.78 in case patients, compared with 0.84 in control subjects; \( P = .0068 \)). When analyzed by genotype, 33% of case patients were homozygous or heterozygous for allele 2, compared with 25% of control subjects (\( P = .022 \)), whereas the absence of allele 2 was observed in 67% of case patients, compared with 75% of control subjects (\( P = .022 \)). There appears to be a codominant effect in that both homozygotes and heterozygotes for this allele are over-represented in the case patients. These data are consistent with allele 2 being a susceptibility factor (or allele 3 being a resistance factor) and confirm the findings of Bellamy et al. [8] in the same Gambian population.

Polymorphism in \( SLC11A1 \) and monokine production. Having demonstrated a significant association between a functional variant of the \( SLC11A1 \) gene promoter and tuberculosis, we investigated whether this polymorphism influenced monocyte responses in a large population of healthy adults. Monokine responses were measured in a total of 276 samples from the 340 blood donors recruited for the case-control study. (Samples collected at times other than 8–10 A.M. and those whose delivery to the laboratory was delayed were excluded from this part of the study). Table 2 summarizes the TNF-\( \alpha \), IL-1\( \beta \), and IL-10 data for the whole population. Statistically significant up-regulation of IL-1\( \beta \) and TNF-\( \alpha \) and down-regulation of IL-10 was observed when blood was primed with IFN-\( \gamma \) (\( P < .0001 \) for all cytokines).

Of the 276 subjects for whom cytokine responses were available, 66 were heterozygous (3/2), and 7 homozygous for allele 2, the allele associated with tuberculosis. Of the remaining 203, 191 were 3/3 homozygotes, and 12 were 1/3 heterozygotes. Subjects were grouped according to presence (heterozygous or homozygous) or absence of allele 2 for further analysis. There was no significant difference in the geometric mean for TNF-\( \alpha \) responses between persons who carried allele 2 of the \( SLC11A1 \) promoter polymorphism and those who did not (574 pg/mL [95% CI, 438–749 pg/mL] in persons carrying allele 2 and 508 pg/mL [95% CI, 409–570 pg/mL] in those not carrying allele 2; \( P = .69 \)). This was also observed for IL-1\( \beta \) (478 pg/mL [95% CI, 380–563 pg/mL] in persons carrying allele 2 and 408 pg/mL [95% CI, 354–450 pg/mL] in those not carrying allele 2; \( P = .95 \)) (figure 1A and 1B). Preincubation with IFN-\( \gamma \) significantly up-regulated production of these 2 cytokines (\( P < .0001 \) for both cytokines), but this effect was not influenced by \( SLC11A1 \) genotype (TNF-\( \alpha \): 1255 pg/mL [95% CI, 1011–1585 pg/mL] in persons carrying allele 2 and 1038 pg/mL [95% CI, 918–1170 pg/mL] in those not carrying allele 2; \( P = .5 \)); IL-1\( \beta \): 953 pg/mL [95% CI, 802–1352 pg/mL] in persons carrying allele 2 and 915 pg/mL [95% CI, 835–1245 pg/mL] in those not carrying allele 2; \( P = .97 \)).

As presented in figure 1C, a significant difference in mean IL-10 production was observed between persons who carried allele 2 and those who did not (167 pg/mL [95% CI, 139–193 pg/mL] in persons carrying allele 2 and 118 pg/mL [95% CI, 102–135 pg/mL] in those not carrying allele 2; \( P = .014 \)). This difference was also observed for IL-10 production after IFN-\( \gamma \) priming (99 pg/mL [95% CI, 85–123 pg/mL] in persons carrying allele 2 and 76 pg/mL [95% CI, 60–82 pg/mL] in those not carrying allele 2; \( P < .05 \)).

Table 1. Allele and genotype frequencies of \( SLC11A1 \) promoter polymorphism in patients with tuberculosis and unaffected control subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case patients (n = 329)</th>
<th>Control subjects (n = 324)</th>
<th>( \chi^2 )</th>
<th>( P )</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( SLC11A1 ) (GT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele 1 (124 bp)</td>
<td>20 (0.030)</td>
<td>15 (0.023)</td>
<td>0.64</td>
<td>.42</td>
<td>1.31 (0.67–2.56)</td>
</tr>
<tr>
<td>Allele 2 (122 bp)</td>
<td>121 (0.184)</td>
<td>89 (0.137)</td>
<td>5.09</td>
<td>.024</td>
<td>1.40 (1.04–1.88)</td>
</tr>
<tr>
<td>Allele 3 (120 bp)</td>
<td>514 (0.781)</td>
<td>544 (0.84)</td>
<td>7.31</td>
<td>.0068</td>
<td>0.68 (0.51–0.90)</td>
</tr>
<tr>
<td>Allele 4 (110 bp)</td>
<td>3 (0.005)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/2 or 2/other</td>
<td>109 (0.33)</td>
<td>81 (0.25)</td>
<td>5.25</td>
<td>.022</td>
<td>1.49 (1.06–2.09)</td>
</tr>
<tr>
<td>Other/other</td>
<td>220 (0.67)</td>
<td>243 (0.75)</td>
<td>5.25</td>
<td>.022</td>
<td>1.00 (reference)</td>
</tr>
</tbody>
</table>

Note: Data are no. of subjects with allele (frequency). CI, confidence interval; OR, odds ratio.
Discussion

**SLC11A1** has been extensively investigated as a candidate gene for genetic susceptibility to diseases caused by intracellular pathogens [24]. However, although many studies, including the present one, have found association or linkage with tuberculosis [4] and other mycobacterial infections [25, 26], the biological mechanism underlying these findings is not understood. Mice homozygous for the susceptibility **Slc11a1** allele show increased susceptibility to mycobacterial infection and predominantly decreased anti-inflammatory activity, rather than reduced proinflammatory activity (although both result in reduced capacity to control infection, highlighting the complex interactions allowing cross-regulation by pro- and anti-inflammatory cytokines). Although the influence of variation in **SLC11A1** on proinflammatory responses appears to be absent in humans, unlike mice, the anti-inflammatory effect appears in both species: macrophages from mice carrying the **Slc11a1** susceptibility allele express high levels of IL-10 mRNA in response to infection with *Salmonella typhimurium* [27], and the percentage of IL-10–secreting macrophages is higher in susceptible mice after in vitro stimulation with purified protein derivative, *M. tuberculosis*, or lipoarabinomannan than in wild-type resistant mice [28]. Furthermore, in mice, *M. tuberculosis* activates both TNF-α− and IL-10–dependent signals in the induction of apoptosis. The balance between apoptosis and survival of infected macrophages is determined by the TNF:IL-10 ratio, which, in turn, appears to be controlled by the **Slc11a1** genotype [28].

There are important differences between the murine mutation and the human polymorphism. First, the mouse mutation occurs in the coding region and results in an amino acid substitution and complete abrogation of protein function, such that the mutant mice mimic **Slc11a1** gene–disrupted mice [29]. The human promoter polymorphism is more subtle and influences gene expression, rather than protein structure. Second, murine studies have been conducted in highly inbred or congenic strains, and variation outside the **SLC11A1** locus is much lower than in the outbred human population studied here. These factors might also explain why the mutation in mice is recessive, whereas a codominant effect of allele 2, both in tuberculosis susceptibility and IL-10 responses, was observed in this study.

**Slc11a1** is a proton/bivalent cation antiporter that localizes to the membrane of late endosomes or lysosomes suggesting that it might alter the microenvironment of the phagosome to affect microbial killing [30, 31]. How this relates to its well-documented influence on macrophage activation, which involves functional up-regulation of several proinflammatory response genes, is not clear. One possibility is that **Slc11a1** regulates cytoplasmic cation levels, especially iron [32]. A variety of mRNA species, including **Slc11a1**, TNF-α, and inducible nitric oxide synthase mRNAs, are more stable in IFN-γ–activated macrophages from resistant mice than in macrophages derived from susceptible mice, and that the expression of **Slc11a1** controls their stability, rather than the expression of TNF-α.

### Table 2. Tumor necrosis factor (TNF)–α, interleukin (IL)–1β, and IL-10 responses to lipopolysaccharide (LPS; 1 ng/mL) or LPS (1 ng/mL) plus interferon (IFN)–γ (200 IU/mL) in 276 healthy blood bank donors.

<table>
<thead>
<tr>
<th>Cytokine level, pg/mL</th>
<th>Geometric mean</th>
<th>95% CI</th>
<th>Range</th>
<th>Geometric mean</th>
<th>95% CI</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>499</td>
<td>426–570</td>
<td>25–10,040</td>
<td>1175</td>
<td>935–1425</td>
<td>30–10,520</td>
</tr>
<tr>
<td>IL-1β</td>
<td>463</td>
<td>365–556</td>
<td>10–1360</td>
<td>770</td>
<td>629–894</td>
<td>10–17,720</td>
</tr>
<tr>
<td>IL-10</td>
<td>139</td>
<td>112–166</td>
<td>85–560</td>
<td>84</td>
<td>67–103</td>
<td>13–3495</td>
</tr>
</tbody>
</table>

NOTE: CI, confidence interval. *P* < .0001 for all comparisons of LPS-induced cytokines with and without IFN-γ priming (Student’s *t* test).
this stability might lead to increased protein translation [33]. mRNA stability is regulated by sequences in untranslated regions that enhance binding to proteins affording protection from nucleases, and in some mRNAs protein binding is controlled by iron levels [34]. Iron, in turn, is required for the generation of reactive oxygen and nitrogen intermediates, and increased Slc11a1 mRNA stability in resistant cells can be inhibited by antioxidants and protein kinase C inhibitors [35]. In humans, mutations resulting in amino acid substitution equivalent to the murine susceptibility allele have not been described. Although the promoter polymorphism investigated here differentially affects gene expression, a normal protein is produced, and secondary effects such as mRNA stability are difficult to detect in a relatively unsophisticated whole-blood assay. There was no difference between the 2 groups for TNF-α or IL-1β production, but IL-10 was significantly higher in persons carrying the promoter allele associated with both tuberculosis and lower activity in the luciferase reporter system. Further studies are required to investigate mRNA stability in relation to SLC11A1 genotype in humans, and IL-10 mRNA levels would be of particular interest.

It is also possible that the association between SLC11A1 allele 2 and IL-10 production in humans occurs as a result of linkage disequilibrium between SLC11A1 and another gene that influences IL-10 production and susceptibility to tuberculosis. However, the clear phenotype in Slc11a1-deficient mice, both in vitro and in vivo (in terms of increased susceptibility to mycobacterial infection), and lack of other convincing candidates in the 2q35 chromosomal region do not support this. IL-10 produced primarily by monocytes and, to a lesser extent,
Our data suggest that does not account for all variation in IL-10 production [39]. Variation in IL-10 production is genetic in origin [38], and polymorphism in the IL-10 gene promoter region influences, but does not account for all variation in IL-10 production [39]. Our data suggest that SLC11A1 contributes to this variation.

In the present study, we have also demonstrated that persons susceptible to the development of disease after infection with M. tuberculosis innately produce more IL-10 in response to LPS than do persons who are resistant. Increased IL-10 could lead to disease development via at least 2 different mechanisms. First, initial control of infection is dependent on macrophage up-regulation and production of microbicidal compounds, including reactive oxygen and nitrogen intermediates, and TNF-α. IL-10 counteracts this process, leading to enhanced survival and replication of the mycobacteria. Second, IL-10 has profound influence of the development of secondary immune responses in tuberculosis. IL-10 down-regulates MHC class II expression on monocytes, thereby preventing antigen-specific T cell proliferation [40]. It also down-regulates M. tuberculosis–induced IL-12 responses and CTLA4 expression [41, 42]. Antibodies to IL-10 enhance IFN-γ production by peripheral blood mononuclear cells in response to M. tuberculosis antigens [43]. It is noteworthy that M. tuberculosis can elicit IL-10 production by monocytes [44, 45], allowing survival and replication within macrophages, which suggests that this is yet another means by which mycobacteria have evolved to evade the host immune system. Induction of IL-10 by mycobacteria may be differentially affected by host genotype. However, not all persons who carry the SLC11A1 allele 2 are high innate IL-10 producers, nor do they all develop tuberculosis, which suggests that other genes contribute to the overall phenotype.

Current therapy for tuberculosis involves prolonged chemotherapy with consequent poor compliance and emergence of multidrug-resistant strains. IFN-γ has been used as therapy for mycobacterial infection but in doses that can be toxic [46]. A therapeutic alternative to enhancing antimycobacterial immune responses would be to reduce down-regulatory responses to achieve a more natural enhancement of IFN-γ production. Immunotherapy involving manipulation of the IL-10 pathway may therefore be a novel alternative to IFN-γ therapy for the treatment of mycobacterial infection.

Acknowledgments

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In an article in the 15 December 2002 issue of the Journal (Awomoyi AA, Marchant A, Howson JM, et al. Interleukin-10, Polymorphism in SLC11A1 (formerly NRAMP1), and Susceptibility to Tuberculosis) Infect Dis 2002; 186:1808–14), there was an error in table 2. The LPS range listed for IL-10 (i.e., the entry in the bottom row of the fourth column) should be 8–5600 (not 85–560). The authors regret this error.