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In-vitro characterization of novel and functional regulatory SNPs in the promoter region of *IL2* and *IL2R alpha* in a Gabonese population

Xiangsheng Huang[1†], Vera Kühne[1†], Jürgen F J Kun[1], Peter T Soboslay[1], Bertrand Lell[1,2] and Velavan TP[1*]

Abstract

Background: The selection pressure imposed by the parasite has a functional consequence on the immune genes, leading to altered immune function in which regulatory T cells (Tregs) induced by parasites during infectious challenges modulate or thwart T effector cell mechanism.

Methods: We identified and investigated regulatory polymorphisms in the immune gene *IL2* and its receptor *IL2R alpha* (also known as *CD25*) in Gabonese individuals exposed to plentiful parasitic infections.

Results: We identified two reported variants each for *IL2* and its receptor *IL2R alpha* gene loci. Also identified were two novel variants, -83 /-84 *CT* deletions (*ss410961576*) for *IL2* and -409C/T (*ss410961577*) for *IL2R alpha*. We further validated all identified promoter variants for their allelic gene expression using transient transfection assays. Three promoter variants of the *IL2* locus revealed no significant expression of the reporter gene. The identified novel variant (*ss410961577C/T*) of the *IL2R alpha* revealed a significant higher expression of the reporter gene in comparison to the major allele (P<0.05). In addition, the *rs12722616C/T* variant of the *IL2R alpha* locus altered the transcription factor binding site TBP (TATA box binding protein) and C/EBP beta (CCAAT/enhancer binding protein beta) that are believed to regulate the Treg function.

Conclusions: The identification and validation of such regulatory polymorphisms in the immune genes may provide a basis for future studies on parasite susceptibility in a population where T cell functions are compromised.

Keywords: IL2, IL2R alpha, CD25, Polymorphism, Transfection, Regulatory T cells

Background

Parasites increase their survival rate in the host by means of a complex interaction with the host immune system. However understanding such interaction on the part of the host and parasite during infections still remains a fundamental issue. In such processes, it is believed that the host regulatory T (Tregs) cells play an essential role [1,2]. The parasite induces a regulatory T cell (Tregs) population that can modulate the magnitude of effector T cell functions thereby leading to a subtle immune response during infections [3]. The regulatory T cell populations remain diverse; a few of them are induced during infections while the others are considered to be natural Tregs vitally implicated in averting autoimmunity [4]. Tregs are believed to influence host inflammatory and immune responses via mechanisms of cell-to-cell contact, inhibitory cytokines and cytokine deprivation [3]. Pathogen driven selection operating on the host immune genes can impose a nucleotide variation in the primed sequence whereby substantial changes in gene expression is directed [2]. Human gene expression is a controlled transcriptional process in the promoter region of a given gene and is regulated by *cis*-acting DNA sequence elements. Any nucleotide alteration in the promoter region is likely to alter the gene expression, reflecting the level of susceptibility to a parasitic infection as well as Treg expression [5]. A number of loci are known to be associated with Treg activity. Genes such as *IL10, IL13, STAT6, TNFRSF18, TLRs* and *FOXP3* have been as key players in

* Correspondence: velavan@medizin.uni-tuebingen.de
†Equal contributors
[1]Institute for Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, Tübingen 72074, Germany
Full list of author information is available at the end of the article

regulating Tregs [2,4-7]. One such gene of interest is the interleukin 2 (*IL2*) and its receptor *IL2R* alpha (*CD25*) that are known to modulate the proliferation and differentiation of T cells and are essential for peripheral homeostasis of the CD4$^+$CD25$^+$ Tregs [8].

The human *IL2* is located on the q arm of the chromosome 4 (specifically 4q27) and its receptor *IL2R* alpha (*CD25*) maps to the p arm of the chromosome 10 (specifically 10p15.1). The human IL-2 is primarily produced by T cells in response to antigenic stimulation and is a major mediator of the immune response [9]. Studies have demonstrated that IL-2 is essential for the proliferation and maintenance of Tregs and can disrupt Treg homeostasis [10,11], whereas the IL-2R plays a significant role in Treg differentiation and proliferation [12]. Tregs have been shown to constitutively express IL-2R (CD25), allowing Tregs to respond to low levels of IL-2 produced by conventional CD4+ T cells [10,11]. The removal of IL-2 from activated T cells can lead to a deprivation of cells, which is indicated by studies on *IL2*-deficient mice [13]. The IL-2 receptor has three chains, α, β and γ, which constitute the high affinity *IL2* receptor. The *IL2R* alpha (*CD25*) is responsible for activating the *IL2* signaling complex and regulates the signal transduction [14,15]. The IL2-R alpha subunit forms the largest of the three IL-2/IL2-R interfaces. Association of α chain with the β and γ heterodimer creates a receptor with a much higher affinity for IL-2 than the β and γ chains receptor [16]. Antigen recognition by the T cell receptor induces the synthesis or activation of the transcription factors such as *NFAT, AP-1*, and *NFκB*, which are located in the promoter region of the *IL2* gene and are essential for activating its transcription [17]. Studies have pointed to the single nucleotide polymorphisms (SNPs) located within the upstream −10 kb of the *IL2* gene that includes the promoter region, and possibly even beyond, thereby contributing to *IL2* transcriptional properties *in vivo* [18]. The inhibition of *IL2R* alpha (*CD25*) during thymocyte differentiation is related to *IL2R* alpha promoter after response to pre-TCR signals and is essential for the specific response of mature T cells later on [19]. Additionally studies have shown that SNPs within *IL2R* alpha are associated with both Grave's disease and Type1 diabetes [20,21]. Reports have indicated that polymorphisms in the genes encoding *IL2* are associated with ulcerative colitis, inflammatory bowel disorder, rheumatoid arthritis and Behcet's disease [22-25], whereas the receptor of *IL2*, the *IL2R* alpha variants were associated with type I diabetes and multiple sclerosis [21,26,27].

In the current study, our goal is to identify regulatory single nucleotide polymorphisms (SNPs) in the promoter region of the *IL2* and its receptor *IL2R* alpha (*CD25*) gene loci in a Sub-Saharan African population exposed to a wide array of parasitic diseases. In this study, with a view to identifying regulatory SNPs in the promoter regions of the *IL2* and its receptor *IL2R* alpha (*CD25*), we sequenced the promoter region of such genes as were upstream of the transcriptional start site, using samples from 40 unrelated Gabonese individuals. The identified regulatory SNPs were further validated for their allelic gene expression, which may possibly be correlated with various physiological responses.

Methods

Genomic DNA Isolation
Forty DNA samples were collected from unrelated Gabonese individuals and informed written consent for participation in the study was obtained from all participants. Blood samples were collected from adult male patients with uncomplicated malaria at the Medical Research Unit of the Albert Schweitzer Hospital, Lambaréné, Gabon, between August and November 2004 [28]. All uncomplicated malaria individuals were male adults living in malaria-endemic countries; usually such individuals will not show symptoms of malaria although carrying parasites in their blood. The study subjects represent another cohort where we investigated novel chemotherapy against malaria [29]. To avoid complications arising from unnoticed pregnancy, only males were chosen. The study was approved by the local ethics committee of the International Foundation of the Albert Schweitzer Hospital. Genomic DNA from whole blood was isolated using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany).

Sequencing and SNP identification
For purposes of sequencing analysis, gene and genomic sequences of the *IL2* (NM_000586) and *IL2R alpha* (*CD25*) (NM_000417) were obtained from the SNPper database [http://snpper.chip.org/]. PCR primers were designed to amplify the promoter region of the gene using the PRIMER3 Software [http:// www genome.wi. mit.edu/cgi-bin/primer/primer3_www.cgi]. The promoter regions of the human *IL2* and *IL2R* alpha (*CD25*) genes were amplified by polymerase chain reaction (PCR). The primer pairs employed for amplifying promoter regions of the *IL2* were *IL2F*: 5′-TAAATAAGGCCATAGAA TGG-3′ and *IL2R*: 5′- GTTACATTAGCCCACACT TA -3′. The primer pairs employed to amplify promoter regions of *IL2R* alpha were *IL2RF*: 5′- GATCCA CCCACCTTGGTCTA -3′ and *IL2RR*: 5′-GGCAGCC AGGCACCATGATGAAC -3′ (MWG Operon, Germany). In brief: PCR were carried out in a 20 µl reaction volumes with 5ng of genomic DNA, 1x PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM of MgCl$_2$; Qiagen), 0.125mM of dNTPs, 0.5mM of each primer and 1 U Taq DNA polymerase (Qiagen, Hilden,

Germany) on a PTC-200 Thermal cycler (MJ Research, USA). Thermal cycling parameters for the amplification of both *IL2 and IL2R* alpha were as follows: initial denaturation at 94°C for 5min, followed by 40 cycles of 15 sec at 94°C denaturation, 60 sec at 60°C annealing temperature, 60 sec at 72°C extension, followed by a final extension of 10 min at 72°C. PCR products were cleaned up using Exo-SAP-IT (USB, Affymetrix, USA) and 1µl of the purified product was directly used as templates for sequencing, using the BigDye terminator v. 2.0 cycle sequencing kit (Applied Biosystems, USA) on an ABI *3130 XL* DNA sequencer, according to the manufacturer's instructions. Polymorphisms in the promoter regions were identified by assembling the sequences, the respective reference sequences being obtained from SNPper database (http://snpper.chip.org) using Codon code Aligner 4.0 software (http://www.codoncode.com/) and were then reconfirmed visually from their respective electropherograms.

Cloning and construct preparation
The SNPs identified in the promoter regions had their polymorphism status reconfirmed for their polymorphism status by subsequent cloning procedures. Those genomic DNA sequences identified as having SNPs in the promoter regions of the *IL2* and *IL2R* alpha were amplified with infusion primers (flanks a 15bp homology to the linearised pGL3 vector) and were then cloned to a linearised pGL3 basic vector. In brief, PCR amplifications were carried out in 50 µl reaction volumes under the same program conditions as mentioned above. The amplified PCR products was analyzed by electrophoresis in 1.5% agarose gels, using a 100 bp DNA ladder molecular size marker (Invitrogen, Karlsruhe, Germany) and PCR -products were gel eluted and purified using a Nucleospin kit (Macherey-Nagel, Düren, Germany) before being cloned to the pGL3 basic vector using Infusion advantage PCR cloning kit (Clonetech, Mounatain view, CA). Plasmids were isolated using QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). To ensure accuracy of the sequenced promoter regions, several independent plasmids containing inserts were sequenced in both directions using appropriate primer pairs. The plasmid exhibiting the confirmed polymorphism was transformed into one shot *E. coli* (Invitrogen, Karlsruhe, Germany). Two independent colonies were selected from these transformations and maxi prep was performed using Endofree plasmid maxi kit (Qiagen, Hilden, Germany).

Transient transfection assays
We tested the activities of the observed polymorphic promoters for both *IL2* and *IL2R* alpha using Jurkat T cell lines. Basically, four independent transfection

experiments for each construct in duplicates were performed with Jurkat T cells (DSMZ, Braunschweig, Germany). Jurkat T cells ($0.8X10^6$ cells/µl) were grown in a RPMI 1640 (Sigma-Aldrich, Hamburg, Germany) supplemented medium containing 10% FBS, 2mM L-Glutamine and 1% Streptamycin-pencillin substrate (Invitrogen, Karlsruhe, Germany). Jurkat cells ($0.8X10^6$ cells/µl) were transfected with TransIT reagent (Mirus Bio, Madison, USA) as recommended by the manufacturer. In brief: 120µl of TransIT reagent was added to 3ml of RPMI 1640 serum free medium (Sigma-Aldrich, Hamburg Germany), this being then incubated for 20 min at room temperature. Each of the 24 well plates was then seeded with 0.5ml ($0.8X10^6$ cells/µl) of Jurkat T cells along with 500ng of plasmid DNA constructs and 20ng of Renilla, before being allowed to incubate for 20 minutes. After incubation, 52µl of TransIT+RPMI serum free medium mix were suspended across each well. The whole procedure was performed in 2X24 well plate formats. After 24 hours, one plate was induced with 20ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Hamburg, Germany) as well as 25µg/ml Concanavalin A (Sigma-Aldrich, Hamburg, Germany). After 24 hours cells were harvested by centrifugation, washed twice with phosphate-buffered saline and lysed in 100µl of 1x passive lysis buffer (Promega, Mannheim, Germany). After incubation for 20 min at room temperature on a rocking platform, 10µl of the lysate was used to measure luciferase activity in the SIRIUS luminometer (Berthold detection system, Pforzheim, Germany). We employed the dual luciferase reporter assay system (Promega, Mannheim, Germany). For each experiment, a plasmid expressing constitutively Renilla luciferase in low amounts was used as a positive control [30]; while a promoterless plasmid (pGL3 basic) was integrated as a negative control. Each construct was measured 8 times, then both stimulated and non stimulated with two different DNA preparations. Relative luciferase activity was calculated as luciferase firefly/ luciferase Renilla multiplied by 1000.

Transcription factor binding search
An extensive search for transcription factor binding sites for the observed SNPs in the promoter region was performed using a TF-Search online tool (http://www.gene-regulation.com/pub/programs/alibaba2) that utilizes TRANSFAC 4.0.

Statistical analysis
Data were normalized and have been analyzed by StatView (http://www.statview.com). The mean ratio (Luciferase/Renilla) across all measurements was considered for purposes of calculating relative luciferase activity. The luciferase activity of the two different *IL2* and

IL2R alpha (*CD25*) promoter variants was comparatively analyzed by a t-test (before and after stimulation). In addition, each constructs activity was compared to the activity of the major allele (common alleles) in both an induced and a non induced state. The statistically significant level was set as 0.05.

Results

All forty subjects were sequenced for the entire promoter region of the *IL2* and *IL2R* alpha (*CD25*) and were investigated for described and novel SNPs. For the *IL2* promoter region, two predescribed SNPs (*rs2069762T/G* and *rs2067006T/A*) were observed. In addition we observed a novel *CT* deletion at the position −83/-84 in one individual. This novel SNP was submitted to the SNPper database and a corresponding ID was obtained (#*ss410961576*). The corresponding references SNP ID (#rs); observed allele frequencies from investigated *IL2* gene are summarized in Table 1. In order to characterize these variants in terms of function, a transient transfection assay were performed using Jurkat T cells. The activity of the three different *IL2* promoter variants analyzed by luciferase activities is compared in Figure 1. The *p*-values of the two observed SNP constructs of the *IL2* variant remained insignificant in comparison to the major allele before and after stimulation (*P* > 0.05). When explicitly examined for a transcription factor binding site in the observed variants of *IL2*, the *rs2067006T/A* variant was found to be positioned on the transcription site *ETS-1*, which is a member of the *ETS* (*E-twenty six*) family of transcription factors that function as transcriptional activators or repressors in numerous genes believed to be involved in stem cell development, cell senescence, and death. The other two variants (#*ss410961576CTdel* and *rs2069762T/G*) do not alter any putative transcription factor binding site.

For the *IL2R* alpha (*CD25*), two described variants in the promoter region (*rs12722617C/T* and *rs12722616C/T*) were observed. In addition we observed a novel *C/T* variant at the position −409 in two individuals. This novel SNP was submitted to the SNPper database and a corresponding ID was obtained (#*ss410961577*). The corresponding references SNP ID (#rs); observed allele frequencies from investigated *IL2R* alpha (*CD25*) are summarized in Table 2. The activity of the three different *IL2* promoter variants analyzed by luciferase activities is compared in Figure 2. The variant *rs12722617C/T* and *rs12722616C/T* were observed to be in absolute linkage in all individuals analyzed. The *p*-values of the SNP construct (*rs12722617C/T* + *rs12722616C/T*) of the *IL2R alpha* variant remained insignificant in comparison to the major allele before and after induction (*P* >0.05), but remained significant when compared to the new variant in a stimulated state (*P*<0.0005). The observed novel variant #*ss410961577C/T* showed significantly increased activity in comparison to the major allele (*P*<0.05). When looked in explicit for transcription factor binding site in the observed variants of *IL2R alpha* (*CD25*) variants, two variants (*rs12722617C/T and* #*ss410961577C/T*) were found not to alter any putative transcription factor binding site. However the SNP variant (*rs12722616C/T*) which was in linkage disequilibrium (LD) with *rs12722617C/T* is positioned at the transcription factor binding site *TBP* (TATA box binding protein) and *C/EBP* beta (CCAAT/enhancer binding protein beta).
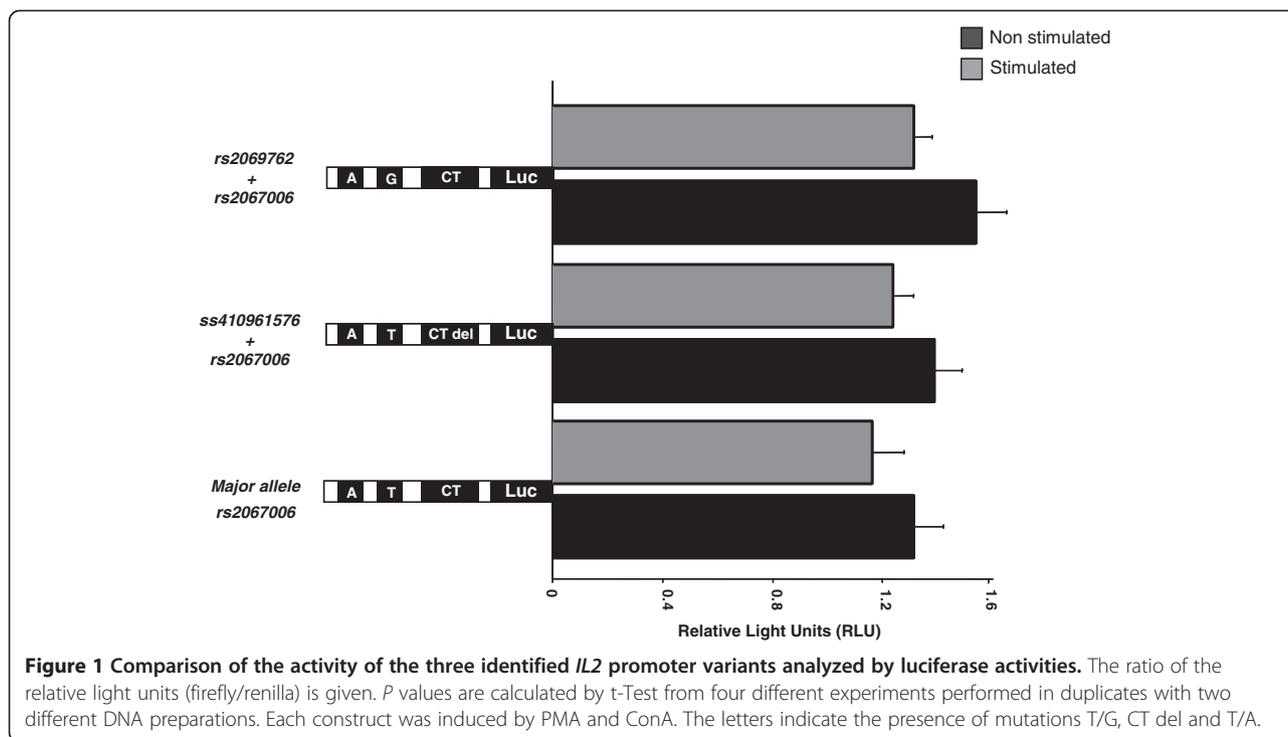
Discussion

The underlying idea of the current study is to understand how regulatory SNPs in populations that are naturally exposed to array of parasites contribute to immune outcome. A significant impact on the diversity among immune gene families is believed to be attributable to invasion maneuvers performed by the parasites. Therefore variations in the promoter regions of these immune system genes can potentially amend the gene expression levels either by changing specificity of transcription binding sites or by altering the kinetics of transcription initiation [7]. In our current study, we identified two known SNPs (*rs2069762T/G* and *rs2067006T/A*) and one novel *CT* deletion (#*ss410961576*) at the position −83/-84 when screened

Table 1 Genetic variants identified in the promoter regions of the *IL2* gene locus

SNP (rs#)	Position	Polymorphism	Flanking sequences	Genotype	Analysed individuals	Allele	Frequency[a]	Frequencies (Hapmap YRI, CEU, CHB)[b]
ss410961576	−83,84	CT deleted	ATTTT [CT/-] GAGTT	CT/CT del/del	39 1	CT del	0.975 0.025	NA
rs2069762	−100	T>G	TTTTA[T/G] GACAA	TT TG	38 2	T G	0.950 0.050	0.034, 0.232, 0.267
rs2067006	−191	T>A	TGTTT[T/A] ATCAA	AT AA	0 40	T A	0.000 1.000	0.000, 0.000, 0.000 1.000, 1.000, 1.000

[a]Frequency corresponds for both allele and genotypes; b: YRI: Yoruba population representing Sub- Saharan African individual group, CEU: European, CHB: Han Chinese; NA: data not available.

Figure 1 Comparison of the activity of the three identified *IL2* promoter variants analyzed by luciferase activities. The ratio of the relative light units (firefly/renilla) is given. *P* values are calculated by t-Test from four different experiments performed in duplicates with two different DNA preparations. Each construct was induced by PMA and ConA. The letters indicate the presence of mutations T/G, CT del and T/A.

for *IL2* promoter region. None of the investigated SNP constructs revealed differential luciferase activity compared to the major allele. In the current investigation the *IL2* variant (*rs2067006T/A*) was considered to be the major allele, as all the individuals were found to harbor this particular variant. When compared to the NCBI HapMap database, the frequencies of the observed SNP variants (*rs2067006T/A* and *rs2069762T/G* of *IL2*) were in accordance with Yoruba population of Nigeria which represents a Sub Saharan African group. The *rs2067006T/A* variant is positioned on to the transcription site *ETS-1*, a member of the *ETS* (*E-twenty six*) family of transcription factors. The *ETS-1* DNA-binding domain recognizes the core consensus DNA in target genes and acts either as transcriptional activators or repressors [31]. Recent studies have demonstrated that *ETS-1* belongs to a large protein complex which binds

to the Treg-specific demethylated region (TSDR) in the *FOXP3* locus, thereby restricting the stable *FOXP3* expression in the Tregs [32]. *FOXP3* is described as a master regulator of natural Tregs development and function [4], while mature regulatory T cells expressing a nonfunctional fusion protein of *FOXP3* lack any suppressor function [33]. Since all the investigated individuals inherited this variant, we believe that the studied population may have a restricted *FOXP3* expression leading to a subtle T effector cell function.

In our study we also identified two known SNPs (*rs12722617C/T* and *rs12722616C/T*) along with one novel C/T variant (*#ss410961577*) at the position −516 when screened for the *IL2R alpha* (*CD25*) promoter region of the *IL2R alpha*. Both the predescribed SNP variants of the *IL2R alpha* (*rs12722617C/T* and *rs12722616C/T*) were observed in linkage in all studied

Table 2 Genetic variants identified in the promoter regions of the *IL2R alpha* (CD25) gene locus

SNP (rs#)	Position	Polym orphism	Flanking sequences	Genotype	Analysed individuals	Allele	Frequency[a]	Frequencies (Hapmap YRI, CEU, CHB)[b]
rs12722617	−398	C>T	TTCGC[C/T] GCATC	CC	35	C	0.875	0.932, 1.000, NA
				CT	5	T	0.125	0.068, 0.000, NA
ss410961577	−409	C>T	GGATC[C/T] TTCAG	CC	38	C	0.950	NA
				CT	2	T	0.050	
rs12722616	−516	C>T	AACAC [C/T] TTATA	CC	35	C	0.875	0.924, 1.000, 1.000
				CT	5	T	0.125	0.076, 0.000, 0.000

[a]Frequency corresponds for both allele and genotypes; b: YRI: Yoruba population representing Sub- Saharan African individual group, CEU: European, CHB: Han Chinese; NA: data not available.
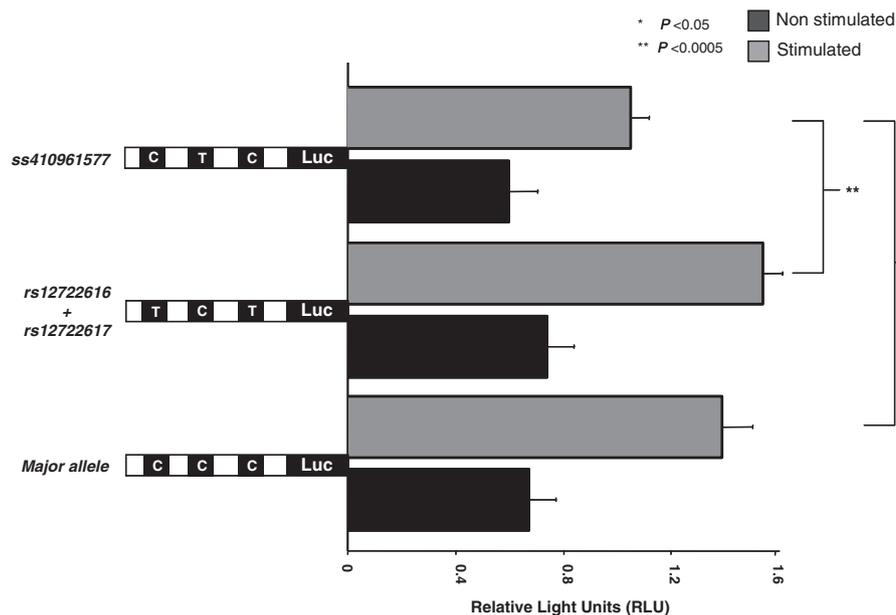
Figure 2 The activity of the three identified *IL2R alpha* promoter variants as compared by luciferase activities. The ratio of the relative light units (firefly/renilla) is given. *P* values are calculated by t-Test in four different experiments performed in duplicates with two different DNA preparations. Each construct was induced by PMA and ConA. The letters indicate the presence of polymorphic variants.

individuals. We did not observe a differential luciferase activity compared to the major allele for the construct (*rs12722617C/T* + *rs12722616C/T*). However the identified novel variant (*#ss410961577C/T*) revealed significant increased activity in comparison to the constructs with the major allele. This particular novel variant did not alter any putative transcription factor binding site. However the variant *rs12722616C/T* which was in LD with *rs12722617C/T* altered the transcription factor binding site *TBP* (TATA box binding protein) and *C/EBP* beta (CCAAT/enhancer binding protein beta). The *TATA* box is a type of promoter sequence that indicates the transcriptional start site whereby a genetic sequence can be read and decoded. It is named after the conserved DNA sequence TATAAA. *TBP* (TATA binding protein) together with TATA associated factors (*TAFS*) make the transcription factor *TFIID* which binds the TATA box in combination with other transcription factors (*TFIIB*, *TFIIA, TFIIE, TFIIF, TFIIH*) [34]. All the above transcription factors along with RNA polymerase II enzyme form a transcription initiation complex. Therefore it is possible that the *TATA* box in the construct *rs12722616C/T* ma contributes to the transcription initiation in vitro in the Jurkat cells. The other transcription factor *C/EBP* beta (CCAAT/enhancer binding protein beta) is believed to modulate inflammatory processes. A recent study has demonstrated in an experimental autoimmune encephalomyelitis model that *C/EBP* expression by dendritic cells (DC) influences Th17 versus Treg differentiation but has little or no impact upon Th1 development [35].

Examining the HapMap database, all the frequencies of reported variants of the *IL2R alpha* loci were found to be in accordance with the frequencies in the Yoruba population.

Human *IL2* and its receptor *IL2R* alpha are described as constituting a potent T cell growth factor and are mainly produced by activated CD4+ T cells, though also by naïve CD8+ T cells, dendritic cells, and thymic cells [36]. Human IL-2 is considered to be vital for the development of CD4$^+$CD25$^+$ regulatory T cells [37]. Also studies have demonstrated that IL-2 also potentially plays a role in the thymic development of Tregs [38,39]. Studies have shown that polymorphisms in the *IL2* are associated with many diseases including auto immune diseases. Recent studies have demonstrated that the studied *IL2* promoter variant (*rs2069762T/G*) is associated with *Helicobacter pylori* infection [40], multiple sclerosis [41], pathogenesis of childhood lymphoma [42], pathogenesis of new-onset diabetes after transplantation (NODAT) [43], measles-specific cellular immunity [44], a higher risk of acute rejection episodes during kidney transplantation [45] and in allergic disorders [46]. However, the role of the described and novel variants of *IL2* and *IL2R alpha* in this study needs to be validated in terms of the specific role these play in different parasitic diseases.

Conclusions

In summary, the regulatory SNPs identified in this current study will provide useful information for

understanding the relevance of sequence polymorphisms in populations exposed to many parasitic diseases and may serve as a basis for backup studies examining disease susceptibility.

Competing interests

The authors declare that they have no conflicting interests.

Authors' contributions

BL designed the field study; VTP and JFJ Kun designed and supervised the experiments; XH and VK performed the experiments; PTS and VTP analyzed the data; BL and VTP contributed materials/analysis tools; XH and VTP wrote the paper. All authors read and approved the final manuscript.

Author details

[1]Institute for Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, Tübingen 72074, Germany. [2]Centre de Recherche Médicale de Lambaréné (CERMEL), Lambaréné P.B.118, Gabon.

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