Human Immunology 80 (2019) 930–936

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Genetic diversity of *CD14, CD28, CTLA-4* and *ICOS* gene promoter polymorphism in African and American sickle cell disease

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ARTICLE INFO

Keywords:
Sickle cell disease
Rs35593994
Rs2569190
Rs3024974
Rs5742909
Immunogenetics
Single nucleotide polymorphism

ABSTRACT

Variable immune response to external stimuli remains a major concern in sickle cell disease (SCD), with such responses predicted to be contributors to disease pathogenesis. Elucidating the diversity of host genes contributing to immune response would assist to clarify differing outcomes *among* and *between* disease groups. We hypothesize that there is a significant interethnic diversity in the *CD14* (*rs2569190*), *CD28* (*rs35593994*), *CTLA-4* (*rs5742909*) and *ICOS* (*rs4404254*) gene polymorphisms *among* and *between* SCD groups. We genotyped single nucleotide polymorphisms of the 4 loci ***among*** African and African American SCD and control groups and ***between*** SCD groups. In all, 375 individuals from Mali (145 SCD and 230 controls) and 700 DNA samples from the United States (321 SCD and 379 controls) were subjected to a PCR-RFLP assay. We found no intraethnic difference in genotypic and allelic frequencies of the 4 loci *among* Africans and African Americans, potentially significant in disease association studies, including a similar observation for interethnic frequencies of *CD28*, *CTLA-4* and *ICOS* genes, but not *CD14*. The *CD14* (*rs2569190*) gene promoter demonstrated a significant difference ($p < 0.02$) ***between*** African and African American SCD groups, with the mutant variant ($-159\ T/T$) more frequent ($p < 0.0002$) in African American SCD (38.9% versus 26.2%). The higher frequency of *CD14* mutants among African Americans without an accompanying defect in *CD28*, *CTLA-4* and *ICOS* diversity possibly indicates a defective innate response, driven by CD14, is untethered to downstream T cell differentiation or effector function. Additionally, we show that *CD28* (*rs35593994*) mutant variants have no impact on T cell differentiation, as the *ICOS* gene provides an alternative pathway to override this impairment. We conclude that in spite of the defect in *CD14*, T cell selection and differentiation is unimpeded and a robust adaptive immune response initiated.

1. Introduction

Sickle cell disease is an inherited monogenic disorder, characterized by multiple complications and serious outcomes [1–3], and encountered in many parts of the world [3]. The burden of disease is exceptionally so high in sub-Saharan Africa, leading to its current designation as an emerging health burden [4]. Published reports have shown the extensive variation in genes driving disease pathogenesis [5–7], possibly stratified along ethnic divides, and serving as modifiers of clinical disease [8], concluding that these variations might have resulted from SNPs intended to affect gene expression and function. Despite the fact that SCD arose in Africa, largely driven by the benefit of the sickle cell trait against severe falciparum malaria, human genomic studies have shown that these SNPs are mediators of immune response to antigenic perturbation of the homeostatic state [9–11], especially when located in functional regions. Since pathogenic exposures differ extensively *among* and *between* sickle cell disease groups, we postulate that there is a diversification in immune response and possibly a significant factor in the dysregulated response observed between disease groups. In addition, susceptibility to infectious pathogens might be exacerbated in countries with high disease rates and preponderance of co-morbidities. Therefore, inundation by constant and multiple antigenic stimuli requires a robust innate and adaptive response. A significant player mediating innate immune response is the *CD14* gene,

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https://doi.org/10.1016/j.humimm.2019.08.005
Received 10 June 2019; Received in revised form 21 August 2019; Accepted 26 August 2019
Available online 29 August 2019

which serves as a co-receptor for Toll-like receptors, inflammatory signaling and phagocytosis [12,13], and whose polymorphism have been associated with disease susceptibility and severity [14,15], including parasite control in malaria infection [16,17].

Beyond innate response, a downstream adaptive immune response, as an effective self-vaccinating tool, requiring co-stimulatory molecules that drive T cell selection, activation, proliferation, and subsequent cytokine production is also important [18,19]. *CD28* functions to amplify receptor signals, including mediating intracellular events such as transcription signaling, cytokine production and signals imperative for long term T cell expansion and differentiation [18–20]. Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) on the other hand, drives a regulatory co-stimulatory response facilitating homeostatic balance and immune tolerance. Reduction in *CTLA-4* expression has been shown to be independently associated with post-transfusion alloimmunization in sickle cell disease [21], similar to reports on autoimmune disorders [22]. In a similar manner, reports have shown *CD28* and *CTLA-4* single nucleotide polymorphisms significantly differ between ethnic groups [23–25]. Of particular importance is the role *CTLA-4* plays in regulating T cell response, which may be modulated by SNPs, these differing outcomes potentially mediated by population genetics [26–28].

Published report on the role of *CD28* in protection against blood-stage malaria parasites show that signaling and adaptive immune response can alternatively be achieved through inducible co-stimulatory (*ICOS*)-*ICOS* interactions when transgenic *CD28* mice are infected with *Plasmodium chabaudi* [29]. The proximity of all three genes (*CD28*, *CTLA-4* and *ICOS*) on the human 2q33 region demonstrate the potential for negative consequence on immune response, if a patient were to be defective for all [26]. We have shown previously that the reported immune response dysfunction [30] observed in SCD is unrelated to defects in Toll-interacting protein (*TOLLIP*) [31] or *CD209* (DC-SIGN) [11]. Despite the reported interethnic diversity in *CD209* and *TOLLIP* gene promoters, there is no clarity on the diversity of *CD28*, *CTLA-4, ICOS* and *CD14* genes between African and American SCD patients, in spite of the genetic similarity and disease pathophysiology. To this end, we carried out molecular genotyping of the 4 loci under consideration by analyzing their intra- and interethnic diversity *among* and *between* control and sickle cell groups. Our result will provide the background for further studies, considering the proximity and complementary activities of *CD28*, *CTLA-4, ICOS* and *CD14* genes in T cell response and in mediating innate immune response respectively.

2. Methods

2.1. Study subjects and demographics

This project was reviewed and approved by the Institutional Review Board of the Rochester Institute of Technology as exempt, in addition to the original approval granted by the National Ethical Review Board in Mali. All sickle cell disease and control DNA samples utilized in this work were de-identified and unlinked samples, and study was conducted in accordance with the Declaration of Helsinki. African sickle cell disease (disease status confirmed by electrophoresis) and control groups (confirmed non-SCD), recruited from a sickle cell referral center in Bamako, Mali, all gave informed consent before they were recruited, as previously reported [11]. They were all of mostly Bambaran ethnicity (51.5% males, 48.4% females; mean age: 21 years; range: 1–51 years), with predominantly Benin sickle cell haplotype [32]. American non-sickle cell disease controls are adult self-identified African Americans, recruited from the blood donor population of Lifeshare Blood Center, Shreveport, Louisiana while the sickle cell disease group comprised a well-characterized population, recruited through the National Institute of Health-funded Cooperative Study of Sickle Cell Disease [11].

2.2. Samples and purification of genomic DNA

Discarded blood samples that were originally collected from 375 subjects (230 healthy controls and 145 SCD patients) from Bamako, Mali were spotted on filter papers and transported to the United States for genomic DNA extraction, as previously described [33]. Eluted samples were stored at −20 °C until further use. American SCD (n = 321) and control samples (n = 379) were gifted by Betty Pace, Augusta University and Joann Moulds (previously of Grifols, USA) respectively. We obtained EconoTaq Plus Green 2X Master Mix for PCR amplification from Lucigen (Lucigen Corporation, Middleton WI). FastDigest (FD) restriction endonuclease, FD buffer, PCR grade water, agarose and GeneRuler 100 bp DNA ladder were obtained from Fisher Scientific (Thermo Scientific, NJ). Analysis of restriction digest was conducted by two investigators, to assure concordance.

2.3. Molecular characterization of CD14 gene promoter (−159C/T; rs2569190) polymorphism

We genotyped the *CD14* gene promoter (*−159C/T*; *rs2569190*) SNPs utilizing previously described primer sequence and reaction protocols [34], as amended [17], requiring amplification of 1 μl of purified DNA as template, 1 μl each of 10 μM forward and reverse primers, optimizing the conditions to a final volume of 25 μl. Positive reaction produced amplicons of 561 bp and an RFLP assay was set up to evaluate genotypic and allelic frequencies. Briefly, 10 μl of amplified PCR product was mixed with 1 μl FastDigest *BsuRI* enzyme, 2 μl 10X FastDigest Green buffer and 17 μl water, incubated at 37 °C for 20 min, and digested products examined, as described [17].

2.4. Molecular characterization of CD28 gene promoter (−372G/A; rs35593994) polymorphism

As previously described, we amplified 1 μl of DNA template, 1 μl each of 10 μM forward (5′-TTCTCATTCTGTTGCCCTGGC-3′) and reverse (5′-CACCATCCCCTTAGGGCACAT-3′) primers, optimizing reaction volume and conditions to give a final volume of 25 μl [25]. Positively amplified samples yielding amplicons of 546 bp were digested with FastDigest *HinfI* restriction enzyme (Thermo Scientific, NJ), cleaving PCR product into two fragments 468 and 78 bp (wild type allele; −372G/G), or undigested (mutant allele; −372A/A).

2.5. Molecular characterization of CTLA-4 gene promoter (−318C/T; rs5742909) polymorphism

To characterize our DNA samples for the *CTLA-4* gene polymorphisms, primers and PCR reaction was set up as described [25], utilizing 1 μl of genomic DNA as template, 1 μl each of 10 μM forward (5′-GGG ATTTAGGAGGACCCTTG-3′) and reverse (5′-GTGCACACACAGAAGGC ACT-3′) primers, optimizing the conditions to give a final volume of 25 μl. Amplified samples yielding amplicons of 244 bp were digested with FastDigest *MseI* restriction enzyme (Thermo Scientific, NJ), producing undigested wild type alleles (−318C/C; rs5742909) or mutant variant (−318 T/T; rs5742909) with two fragments (179, 65 bp).

2.6. Molecular characterization of ICOS gene promoter (−1564 T/C; rs4404254) polymorphism

Same methodology as described above, was utilized to characterize DNA samples for the inducible co-stimulator gene and elucidate its distribution among and between groups [25]. Briefly, 1 μl of genomic DNA as template, 1 μl each of 10 μM forward (5′-TTACCAAGACTTTA GATGCTTTCTT-3′) and reverse (5′-GAATCTTTCTAGCCAAATCATA TTC-3′) primers, were optimized to give a final reaction volume of 25 μl. Reaction conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at

72 °C for 10 min. Amplified samples yielding amplicons of 823 bp were digested with FastDigest *AluI* restriction enzyme. Wild type alleles produced fragments of 385, 339, 99 bp while the enzyme cleaves the PCR product into four fragments (339, 289, 99, 96 bp) in the presence of the C allele.

2.7. Statistical analysis

To estimate allelic and genotypic frequencies, we utilized SNPStats, an online program (https://www.snpstats.net/start.htm), and tested for deviation from Hardy-Weinberg equilibrium, carried out as described [35]. We also performed linkage disequilibrium (LD) and haplotype analysis (using the three SNPs located on the same chromosome; *CD28, CTLA-4* and *ICOS* genes) from the genotypic dataset utilizing SNPStats, via a regression-based methodology. Differences between disease and control groups were determined by odds ratio and a 95% confidence interval [11]. Individuals that were heterozygote at more than one locus were excluded from the analysis.

3. Results

We elucidated the percentile frequencies of the 4 loci that may be associated with innate and adaptive immune response to external perturbations (*CD14*; $-159C > T$ *rs2569190*; *CD28*; $-372G > A$ *rs35593994*; *CTLA-4*; $-318C > T$ *rs5742909*; and *ICOS*; $-1564\,T > C$ *rs4404254*) ***among*** sickle cell disease and control groups in Africa and the United States as well as ***between*** sickle cell disease groups. All polymorphisms examined in this study were in concordance with Hardy-Weinberg equilibrium ($p > 0.05$). Our analyses produced no significant difference ($p > 0.05$) ***among*** control and sickle cell groups, either in Africa or United States. In almost all instances, the wild type variant of *CD28*, *CTLA-4* and *CD14* gene polymorphisms were the most common ***among*** disease and control groups and ***between*** disease groups, except for the *ICOS* gene (Supplementary Tables 1–4), for which heterozygote variants were the most common (30.3%, **53.5%** and 16.2% versus 34.9%, **44.5%** and 20.6% for African SCD and controls respectively). Similar observation was made among African Americans (34.2%, **49.5%** and 16.3% versus 36.4%, **47.6%** and 16.0% for SCD and controls respectively). In summary, we found no difference in genotypic or allelic frequencies of *CD28*, *CTLA-4*, *ICOS* and *CD14* genetic polymorphisms ***among*** sickle cell disease and control groups, either in Africa or United States (Supplementary Tables 1–4).

On the other hand, interethnic comparison of the four loci ***between*** sickle cell disease groups reveal a statistically significant difference in genotypic (Fig. 1; $p = 0.02$) and allelic frequencies (Fig. 2; $p = 0.0002$) of *CD14* variants, but not *CD28*, *CTLA-4* or *ICOS* genes. Significantly, more African Americans with SCD had the mutant variant ($-159TT$) (Table 1) (18.4% versus 9.7% for African American and African SCD groups respectively), while doubling and tripling allelic frequencies between both groups respectively (38.9% versus 26.2%; Table 2).

Genotypic and allelic frequencies ***between*** African and American sickle cell groups show no difference ($p > 0.05$), with wild type variants presenting with the highest frequency for all loci (Tables 1 and 2). To clarify the role a defect in *CD28* polymorphism would have on potential T cell differentiation, we examined the frequency of *ICOS* genetic polymorphism gene among individuals with a *CD28* mutant allele. Our results show no difference in genotypic or allelic frequencies of *ICOS* gene ***among*** African or American groups with *CD28* mutant variant. Similar observation was made ***between*** African and American SCD groups, demonstrating that the *ICOS* wild type/heterozygote variants were the most common in Africa (92.9%) and United States (90.3%) respectively, possibly an indication of lower historical recombination and linkage disequilibrium.

To examine haplotype frequencies of the 3 closely linked genes on chromosome 2 ***among*** disease and control groups (Table 3) and ***between*** SCD groups (Table 4), we constructed haplotype tables utilizing the single nucleotide polymorphisms of the 3 loci, forming 8 haplotype groups, regarded as the most common observed ***among*** SCD and control groups and ***between*** SCD groups. From our analysis, we found no significant difference in haplotype frequencies ***among*** sickle cell disease and control groups, whether in Africa or the United States. The most common haplotypes are H1 (*CTG*) and H2 (*CCG*), while haplotypes H5, H6 and H8 were completely absent ***among*** Africans (Table 3). Among African Americans, haplotypes H7 and H8 are completely absent and haplotype H6 very negligible. Comparing sickle cell disease groups, similar observations were recorded with haplotypes H1 and H2 presenting with the highest frequencies ***between*** groups (Table 4) while haplotypes H7 and H8 were completely absent. Furthermore, we investigated linkage disequilibrium between the three SNPs; our results show a strong LD between *ICOS* (*rs4404254*) and *CTLA-4* (*rs5742909*), as well as between *ICOS* (*rs4404254*) and *CD28* (*rs35593994*) genes ***among*** Americans (0.85; $p = 0.001$ and 0.79; $p = 0.05$ respectively). Although, the same trend was maintained among Africans, LD values were insignificant (0.96; $p = 0.13$ and 0.94; $p = 0.37$) for *ICOS* and *CTLA-4*, as well as between *ICOS* and *CD28* genes respectively). Similar observation was made ***between*** African and African American sickle cell disease groups with significant LD between *ICOS* and *CTLA-4* (0.99; p = 8E-04), but not *ICOS* (*rs4404254*) and *CD28* (0.61; p = 0.9).

4. Discussion

A balanced immune response, premised on both innate and adaptive systems is a significant factor mediating infectivity and disease outcome on exposure to antigenic stimuli. It has been shown that downstream adaptive immune response is dependent on how upstream innate system recognizes external stimuli or signals the entry of non-friendly antigen. In sickle cell disease, consistent reports of dysregulated immune response are particularly concerning, taking into account the susceptibility to infectious agents, common in this population [30,31,36–39]. Though intra- and interethnic differences exist in how

Table 1
Genotypic diversity of polymorphisms of interest *between* sickle cell groups.

Polymorphism	Chromosome	Location	Genotype	African	American	Odds ratio (95% CI)	*p*-value
***CD14* (*rs2569190*)**	5	140,633,331	C/C	83 (57.2)	128 (40.8)	1.94 (1.28–2.95)	0.001
			C/T	48 (33.1)	128 (40.8)	0.72 (0.46–1.10)	0.12
			T/T	14 (9.7)	58 (18.4)	0.47 (0.23–0.89)	**0.02**
***CD28* (*rs35593994*)**	2	203,706,103	G/G	71 (49.0)	152 (47.3)	1.07 (071–1.61)	0.76
			G/A	60 (41.4)	138 (43.0)	0.94 (0.61–1.41)	0.76
			A/A	14 (9.6)	31 (9.6)	0.99 (0.47–2.01)	1
***CTLA-4* (*rs5742909*)**	2	203,867,624	C/C	144 (99.3)	309 (95.4)	6.99 (1.05–296.28)	0.03
			C/T	1 (0.7)	15 (4.6)	0.14 (0.00–0.95)	0.03
			T/T	0 (0.0)	0 (0.0)	N/A	N/A
***ICOS* (*rs4404254*)**	2	203,960,563	T/T	43 (30.3)	109 (34.2)	0.84 (0.53–1.30)	0.45
			T/C	76 (53.5)	158 (49.5)	1.17 (0.77–1.78)	0.48
			C/C	23 (16.2)	52 (16.3)	0.99 (0.55–1.31)	1

Table 2
Allelic diversity of polymorphisms of interest *between* sickle cell groups.

Polymorphism	Chromosome	Location	Allele	African	American	Odds ratio (95% CI)	*p*-value
***CD14* (*rs2569190*)**	5	140,633,331	C	214 (73.8)	384 (61.1)	1.79 (1.30–2.47)	0.0002
			T	76 (26.2)	244 (38.9)	0.55 (0.39–0.75)	**0.0002**
***CD28* (*rs35593994*)**	2	203,706,103	G	202 (69.7)	442 (68.9)	1.04 (0.76–1.42)	082
			A	88 (30.3)	200 (31.1)	0.96 (0.70–1.31)	0.82
***CTLA-4* (*rs5742909*)**	2	203,867,624	C	289 (99.7)	633 (97.7)	6.85 (1.04–289.28)	0.03
			T	1 (0.3)	15 (2.3)	0.14 (0.00–0.96)	0.03
***ICOS* (*rs4404254*)**	2	203,960,563	T	162 (57.0)	376 (58.9)	0.92 (0.69–1.24)	0.61
			C	122 (43.0)	262 (41.1)	1.08 (0.81–1.45)	0.61

individuals or groups of individuals respond to infectious diseases [11,40] or to vaccinations that modulate infectivity on exposure to pathogens [41–44], there remains a need to elucidate how polymorphisms of immunogenetic markers linking innate and adaptive immune systems, contribute to this response. To this end, dissecting the host genetic diversity of genes mediating this process, such as *CD14* (*rs2569190*), a major component of the innate pathway, as well as *CD28* (*rs35593994*) and *CTLA-4* (*rs5742909*), which are co-receptors involved in downstream immune response activities, is highly imperative.

Reports have shown that a proper T cell response requires signaling by *CD28* co-receptor acting in concert with the peptide-major histocompatibility complex on naïve T cells, including co-stimulatory activities by *CTLA-4* to regulate proliferation and differentiation. To produce an effective response, we postulate that wild type variants of *CD28* (*−372G/G*) and *CTLA-4* (*−318C/C*) genes are most significant in phenotype and association studies, confirmed by our results. The *CTLA-4* gene wild type variant is most frequent, with no mutants ***among*** and ***between*** African and African American groups. This potentially indicates that this region has been under selective sweep, despite the migration and post-migration effect from Africa to America, implying a significant gene flow between the two population groups. A mutation at this region would be a rare event, leading to a significant defect in the function, structure and downstream protein expression, thereby presenting susceptible disease phenotypes. *CTLA-4* is known for its co-stimulatory activities with *CD28* and *ICOS* in regulating the immune system. The preponderance of its wild type variants could also prove the locus serving its functions, including activating naïve T cells in response to antigenic stimuli, and a robust T cell driven immune response. The absence of any significant difference in the genotypic and allelic diversity ***among*** disease and control groups in Africa or United States possibly demonstrates intra-ethnic conservation, and that this locus may not be the most important driver of dysregulated immune response in sickle cell disease, redounding to a specific T cell differentiation, and subsequent adaptive immune response. Additionally, the preponderance of wild type variants may also be hypothesized as reason for the high haplotype frequencies of haplotypes H1 (which combines minor *ICOS* and *CTLA-4* and dominant *CD28* alleles) and haplotype H2 (which combines minor *ICOS* and dominant *CTLA-4* and *CD28* alleles). The presence of the dominant *CD28* and inconsequential *CTLA-4* and *ICOS* alleles in haplotype H1, and combination of dominant *CD28* and *CTLA-4* alleles in haplotype H2 makes a compelling argument for their haplotype frequencies.

The significant genotypic and allelic diversity found in *CD14* gene promoter ***between*** African and African American SCD groups may reflect the possible imposition of selection pressure on African patients because of infectious pathogens, to which they have to respond. African American SCD patients on the other hand, may not be so exposed, and as such the *CD14* gene, a pathogen receptor signal, demonstrating significant defect rendering such patients exposed and a defective innate response, negating its benefit in downstream adaptive response. That African American sickle cell groups present two-fold more *CD14* mutant variant (*−159 T/T*) compared to Africans demonstrates diversity possibly associated with migration, climate and disease exposures. We have shown previously that there is an extensive inter-ethnic diversity of endothelial nitric oxide synthase (eNOS) and endothelin-1 genes, known to bear clinical significance, between

Table 3
Estimated haplotype frequencies of the three SNPs *among* African and American sickle cell disease and control groups.

Haplotype	Haplotype definition			Haplotype frequencies (African)				
	ICOS(*−1564 T > C*)	*CTLA-4*(*−318C > T*)	*CD28*(*−372G > A*)	SCD	Controls	SCD vs controls	Odds ratio (95% CI)	*p* value
H1	C	T	G	0.3868	0.3992	0.3944	1.00	NS
H2	C	C	G	0.3063	0.2856	0.2936	0.89 (0.59–1.36)	0.59
H3	C	T	A	0.1797	0.1704	0.1740	0.92 (0.57–1.49)	0.73
H4	C	C	A	0.1238	0.1426	0.1353	1.12 (0.68–1.85)	0.67
H5	T	T	G	0	0	NA	0	0
H6	T	T	A	0	0	NA	0	0
H7	T	C	G	0.0034	0.0022	0.0027	0.59 (0.04–9.74)	0.71
H8	T	C	A	0	0	NA	0	0
Haplotype	Haplotype definition			Haplotype frequencies (African American)				
	ICOS (*−1564 T > C*)	*CTLA-4* (*−318C > T*)	*CD28* (*−372G > A*)	SCD	Controls	SCD vs controls	Odds ratio (95% CI)	*p* value
H1	C	T	G	0.3693	0.3767	0.3740	1.00	NS
H2	C	C	G	0.2995	0.2790	0.2878	0.92 (0.67–1.25)	0.59
H3	C	T	A	0.1971	0.2013	0.1992	0.99 (0.69–1.43)	0.97
H4	C	C	A	0.1111	0.1166	0.1141	1.03 (0.70–1.54)	0.87
H5	T	T	G	0.0197	0.0244	0.0215	1.25 (0.54–2.89)	0.60
H6	T	T	A	0.0034	0	0.0034	0.59 (0.04–8.16)	0.69
H7	T	C	G	0	0.0021	NA	0	0
H8	T	C	A	0	NA	NA	0	0

Abbreviations: SCD, sickle cell disease; NS, not significant; CI, confidence interval; NA, not applicable; SNP, single nucleotide polymorphism. SNPs at the three loci *ICOS*, *CTLA-4* and *CD28* define eight different haplotypes. T/C, C/T and G/A denotes the alleles at the three loci. Odds ratio was calculated by Fisher's two-tailed exact test. Order of variants in haplotype is as follows: rs4404254, rs5742909, rs35593994

Table 4
Estimated haplotype frequencies of the three SNPs *between* African and American sickle cell disease groups.

Haplotype	Haplotype definition			Haplotype frequencies				
	ICOS (−1564 *T* > *C*)	*CTLA-4* (−318*C* > *T*)	*CD28* (−372*G* > *A*)	Mali	USA	Mali vs USA	Odds ratio (95% CI)	*p* value
H1	C	T	G	0.3868	0.3693	0.3732	1.00	NS
H2	C	C	G	0.3063	0.2995	0.3030	1.00 (0.66–1.50)	1
H3	C	T	A	0.1797	0.1971	0.1921	1.15 (0.71–1.87)	0.56
H4	C	C	A	0.1238	0.1110	0.1146	0.92 (0.55–1.53)	0.75
H5	T	T	G	0	0.0197	0.0147	6.20 (0.78–49.07)	0.084
H6	T	T	A	NA	0.0034	0.0024	4.2E18 (4.2E17-42.40E18)	< 0.0001
H7	T	C	G	0.0034	0	NA		0
H8	T	C	A	NA	0	NA	1.00	NS

Abbreviations: SCD, sickle cell disease; NS, not significant; CI, confidence interval; N/A, not applicable; SNP, single nucleotide polymorphism. SNPs at the three loci *ICOS*, *CTLA-4* and *CD28* define eight different haplotypes. T/C, C/T and G/A denotes the alleles at the three loci. Odds ratio was calculated by Fisher's two-tailed exact test. Order of variants in haplotype is as follows: rs4404254, rs5742909, rs35593994.

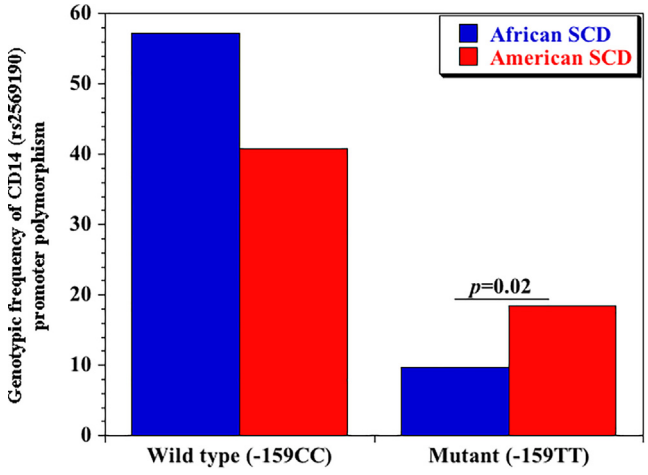

Fig. 1. Genotypic distribution of *CD14* gene promoter polymorphism (−*159C* > *T; rs2569190*) between African and American sickle cell disease groups. PCR products were digested with *BsuRI* restriction endonuclease (Thermo Scientific, New Jersey, USA), and expressed on a 2% ethidium bromide-stained agarose gel. Wild type variant (snp −*159C/C*) produced 3 fragments (204, 201 and 156 bp); while the mutant variant (snp −*159 T/T*) produced two fragments (360 and 201 bp) on digestion. Marker: GeneRuler 100 bp DNA ladder were obtained from Fisher Scientific (ThermoScientific, NJ). Blue bars: African sickle cell disease; red bars: American sickle cell disease.

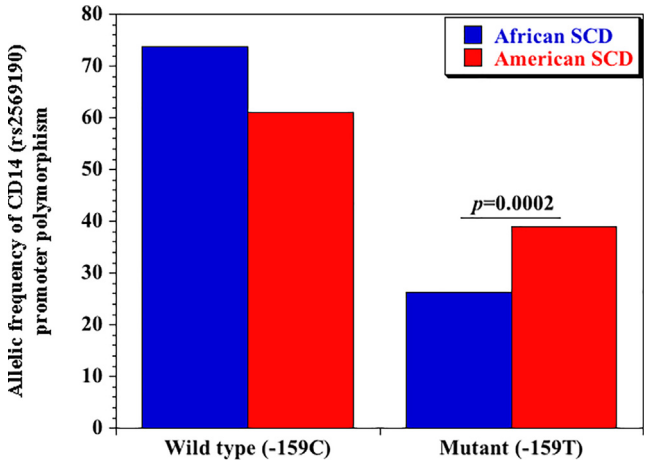

Fig. 2. Allelic distribution of *CD14* gene promoter polymorphism (−*159C* > *T; rs2569190*) ***between*** African and American sickle cell disease groups.

Africans and African Americans with SCD [7,35], these polymorphisms proven to be modifiers of clinical disease among Indians [8] but not Africans, possibly due to SCD haplotype. The possibility that heterogeneity in the African American gene pool, absent in our African group (homogeneous population), may be contributing to this observation, demands further elucidation, in conjunction with ancestry analysis. In addition, we have reported a significant difference between coastal West Africa (to which many African Americans trace their roots) compared to Mali (which is inland West Africa), and the obvious linguistic-associated patterns and genetic diversity [45]. Therefore, expanding this study in a population of sickle cell disease patients from coastal West Africa alongside SNP genotyping assay of African American SCD groups is the next step to definitively clarify our present observation. The low frequency of the mutant allele among African patients might indicate adaptability due to selection pressures [11]. However, the complete picture of defective *CD14* signaling on immune response among African American SCD groups may be difficult to unravel because of the abundance of other prophylactic measures, available in Western, industrialized countries to treat such patients [11] that may not be readily available in sub-Saharan Africa. An alternative explanation could be the limit imposed by sample size of the African SCD population, in that groups carrying the mutant allele were not appropriately represented in the population being studied. The report by Baldini et al, (1999) that the *CD14* mutant variant is a significant player in regulating the levels of soluble CD14 in serum, and the potential for impacting allergic reactions [46], transcriptional activity [47] or immune response in diverse disease settings [31] (intra-ethnic versus interethnic; African versus African American SCD phenotypes), deserves further elucidation. We recommend further analysis to examine possible association of genotypes with disease phenotypes to validate present observation. Expanding this study to a larger population group of African Americans with sickle cell disease, possibly infected with pneumococcal disease and deconvoluting the contribution of *CD14* gene promoter on disease severity or outcome, cytokine response as well as specific Th1 and Th2 delineation is advocated.

It appears that the defective *CD28* gene, as reported ***among*** and ***between*** groups does not hamper specific T cell response, as such patients we postulate, will successfully activate and differentiate T cells via other gene mechanisms. Studies in mice have indicated that *CD28* expression is not necessarily required in the control of parasitemia during acute malaria [30]. Likewise, reports demonstrate that *CD28*, *CTLA-4* and *ICOS* genes are members of the same family due to their closeness within 300 kb of human chromosome 2 [48]. We therefore infer that this proximity might bring about overlapping functions possibly due to linkage disequilibrium among allelic variants of these genes. This inference is corroborated with the report showing a strong linkage disequilibrium existing between *CD28*, *CTLA-4* and *ICOS*, with higher hot spots for recombination at the intronic region [27]. The preponderance of wild type variants of *CTLA-4* gene confirms our

hypothesis of specific T cell response, irrespective of defects in *CD14* or *CD28* gene promoters. We also report that among those with *CD28* defect, the *ICOS* gene wild type or heterozygote variant were the most common, implying that these individuals could still activate T cells, in spite of the defect. This we speculate, is sufficient to initiate a proper and adequate T cell response (adaptive response) in the presence of exogenous stimulus confirming our observation on the haplotype frequencies between *ICOS*, *CTLA-4* and *CD28* genes.

Our conclusion is supported by published report [48], which showed that *CTLA-4* and *ICOS*-mediated co-stimulation is sufficient to drive a complete T cell differentiation and function. Therefore, we postulate that the frequency of variability in these genes reflect a form of adaptive signature to infectious diseases, possibly modulated depending on the environment. In the future, we will expand this observation to African control and sickle cell patients infected with malaria, as well as African and African American sickle cell patients with pneumococcal infection, quantify the expression of these genes, including examining sickle cell disease groups from other parts of Africa.

5. Authorship contributions

BNT conceived experiments and drafted the manuscript; BNS, SLP, ALC and SEA carried out molecular genotyping and analysis; ALC, OBM and BNT carried out statistical analysis; OBM and OO contributed to the discussion and scientific content. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Joann Moulds, previously of Grifols, USA for the American control DNA samples and Betty Pace of Augusta University, who graciously provided the American sickle cell DNA samples, collected as part of the Cooperative Study of Sickle Cell Disease (samples confirmed through the NIH/NHLBI BioLINCC Biorepository). Our sincere gratitude to Dapa Diallo and Aldiouma Guindo for their assistance with the African sickle cell disease and control DNA samples. A Summer Undergraduate Research Fellowship Award funded by CBET (Summer Pellechio) and RIT Honors Program (Anna Capria) are acknowledged. Part of this work was presented at the Annual Biomedical Research Conference for Minority Students, Indianapolis, IN November 9-14, 2018. This work was funded by a Laboratory and Faculty Development Award, College of Health Sciences and Technology, Rochester Institute of Technology (BNT). Funding from the Miller Chair in International Education (BNT) facilitated the collaboration with Ladoke Akintola University of Technology. OBM is supported through the American Association of Immunologists Careers in Immunology Fellowship Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.08.005.

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