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Are polymorphisms of the immunoregulatory factor *CD40LG* implicated in acute transfusion reactions?

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The CD40 ligand (CD40L/CD154), a member of TNF superfamily, is notably expressed on activated CD4+ T-cells and stimulated platelets. CD40L is linked to a variety of pathologies and to acute transfusion reactions (ATR). Mutations in this gene (*CD40LG*) lead to X-linked hyper-IgM syndrome. Some *CD40LG* polymorphisms are associated with variable protein expression. The rationale behind this study is that CD40L protein has been observed to be involved in ATR. We wondered whether genetic polymorphisms are implicated. We investigated genetic diversity in the *CD40LG* using DHPLC and capillary electrophoresis for screening and genotyping (n=485 French and Tunisian blood donors). We identified significant difference in the *CD40LG* linkage pattern between the two populations. Variant minor alleles were significantly over-represented in Tunisian donors (P<0.0001 to 0.0270). We found higher heterogeneity in the Tunisian, including three novel low frequency variants. As there was not a particular pattern of *CD40LG* in single apheresis donors whose platelet components induced an ATR, we discuss how this information may be useful for future disease association studies on *CD40LG*.

The CD40 ligand gene (*CD40LG*), located on the long arm of human X chromosome at position q26.3-q27.1, harbors five exons spanning about 12 kilo base pairs (kb) (http://www.ncbi.nlm.nih.gov/gene/959). The CD40L (CD154), which is a member of the tumor necrosis factor (TNF) superfamily, is a 33 kDa type II membrane glycoprotein. It is mostly expressed on activated CD4+ T-cells and stimulated platelets; presence of this protein is often a characterizing feature of antigen presenting cells[1–3]. CD40L is also expressed on non-leukocyte and non-immune cells such as endothelial cells[4]. CD40L expression is post-transcriptionally regulated in part through mRNA stability[5].

The costimulatory molecule CD40L and its receptor CD40 have essential roles in adaptive immunity; it also plays a role in inflammatory responses, which are important in innate immunity[6]. The normal interaction between T and B lymphocytes via CD40 and CD40L induces B cell activation, proliferation, differentiation, survival and immunoglobulin isotype switching, thereby regulating B cell commitment to mature plasma or memory B cells[3,7]. Deficient CD40L expression might induce a reduced antigen response, such as that seen in X-linked hyper-IgM syndrome (HIGM1)[8], whereas polymorphisms in this ligand have been associated with various pathologies as autoimmune and infectious diseases[9,10]. CD40L is cryptic in unstimulated platelets, but is translocated to the cell surface within seconds or minutes after *in vitro* activation, where it is involved in *in vivo* thrombus formation[11]. CD40L expressed on the surface of activated platelets interacts with CD40 to trigger inflammatory responses and expression of tissue factor in endothelial cells[12]. Approximately 95% of the soluble fragment of CD40L (sCD40L) found in plasma is derived and cleaved from platelets, which are important players in inflammation[13], in addition to their roles in hemostasis, thrombosis and platelet regulatory functions[14–16]. The contribution of platelets and their secretory products has been observed in tissue pathology[17,18].

Interaction between platelet-derived CD40L and target cells such as a blood transfusion recipient's B-lymphocytes and vascular endothelial cells is considered a highly inflammatory process in transfusions, where it is known to be responsible for adverse events, such as febrile non hemolytic transfusion reactions (FNHTR),

Table 1 | Minor allele frequencies of *CD40LG* SNPs in the French (n=211) and Tunisian population (n=274) identified by DHPLC

SNP	Marker ID	Position Chr X (Location)	Allele	Minor Allele Frequency (MAF)		Statistic		PCR Fragment
				French population	Tunisian Population	χ^2	p value	
1	c.-3525_-3526insCAAACAAA[a]	135726882 (5′ UTR)	(CAAA)$_8$	0.003	0.003	0.017	1[b]	*CD40LG*-5′UTR
2	**rs201992677 [-/CAAA]**	**135726882 (5′ UTR)**	**(CAAA)$_7$**	**0.175**	**0.364**	**28.141**	**<0.0001**	***CD40LG*-5′UTR**
3	**rs3092952 A>G**	**135726950 (5′UTR)**	**G**	**0.175**	**0.354**	**25.040**	**<0.0001**	***CD40LG*-5′UTR**
4	**rs1126535 T>C**	**135730555 (exon 1)**	**C**	**0.154**	**0.224**	**4.998**	**0.027**	***CD40LG*-E1**
5	rs147739883 C>T	135732387 (IVS 1)	T	0	0.003	0.828	1[b]	*CD40LG*-E2
6	c.8140A>G[a]	135738547 (exon 4)	G	0	0.003	0.828	1[b]	*CD40LG*-E4
7	**rs3092923 T>C**	**135741185 (IVS 4)**	**C**	**0.116**	**0.326**	**39.424**	**<0.0001**	***CD40LG*-E5A**
8	rs148594123 G>A	135741443 (exon 5)	A	0.027	0	9.793	0.0002[b]	*CD40LG*-E5B
9	**rs3092921 C>T**	**135743000 (3′UTR)**	**T**	**0.113**	**0.314**	**37.392**	**<0.0001**	***CD40LG*-3′UTRB**
10	c.*2367C>G[a]	135743941 (3′UTR)	G	0	0.003	0.828	1[b]	*CD40LG*-3′UTRC
11	**rs3092920 G>T**	**135743991 (3′UTR)**	**T**	**0.103**	**0.286**	**33.203**	**<0.0001**	***CD40LG*-3′UTRC**

[a]Novel mutations identified in this study.
[b]Fisher Exact Test (n<5).
Alleles are denoted as ancestral/derived and are referred to by their dbSNP ID. Polymorphisms in boldface were included in the LD and haplotype analysis.

atypical allergy and hypotension. Such reactions can be benign or severe signs of inflammation, while others such as ATR and transfusion-related acute lung injury (TRALI) are generally thought to result, at least in part, from elevated sCD40L levels[19–23].

However, the vast majority of transfusions, despite inducing high levels of pro-inflammatory molecules, proceed without harm to the patient. This fact gives rise to the hypothesis of genetic susceptibility in the donor population relating to cytokines and/or chemokines and in recipients to the relevant receptor involved (both in physiology and pathophysiology). For this, we wondered whether genetic *CD40LG* polymorphisms are implicated.

To determine the dispersion of genetic variation in populations that are distinct but have certain common ancestries, we sought to examine *CD40LG* among populations of volunteer blood donors, in central Tunisia and in metropolitan France (whose platelet component induced or not ATR). We hypothesized that variability within a population would reveal high levels of genetic polymorphism or segregation of particular haplotypes[24,25].

Denaturing High Performance Liquid Chromatography (DHPLC) and capillary electrophoresis were performed to analyze polymorphisms in the *CD40LG*, and to estimate the allele frequencies and *CD40LG* haplotype structures and patterns of linkage disequilibrium (LD) around a 17 kb region that includes the *CD40LG*. This study, besides, aimed to provide population genetic data that could be used for future studies on the *CD40LG* and its association with pathology.

Results

Nucleotide polymorphism analysis. DHPLC analysis found nine SNPs and two variable number tandem repeats (VNTRs) in 10 of the 11 amplicons investigated herein. Homogeneity was tested for males and females in each population (Supplementary Table S1). All genotype frequencies were in Hardy-Weinberg Equilibrium (HWE) in the studied populations for all of the typed SNPs. Table 1 summarizes the allele frequencies of the 11 variants identified.

All the amplicons contained an average of one SNP. However, *CD40LG*-5′UTR and *CD40LG*-3′UTR harbored 3 polymorphisms (Table 1). At the genotyping stage, we observed that 11% of the samples from females that displayed one peak at the screening analysis, showed multiple peaks when mixed with the wild homozygous control sample, thus presenting the mutant homozygous state.

Three variants were novel: a non-synonymous c.8140A>G in exon 4, c.*2367C>G in the 3′UTR in the Tunisian population at low frequency (MAF=0.003 for both), and one insertion in the 5′UTR (c.-3525_-3526insCAAACAAA) in one male from each of the study populations. Seven polymorphisms were transitions, three were A/G and four C/T, while two were transversions (one C/G, one G/T). Eight of the 11 variants were previously reported in the NCBI database. Only three variants were located in the coding region: rs1126535 in exon 1, c.8140 A>G in exon 4 and rs148594123 in exon 5. The others were in the non-coding region with three variants in the 5′UTR, one in IVS4 and three in the 3′UTR (Table 1; Supplementary Figure S1).

Variant minor alleles with MAF>0.05 were significantly over-represented in the Tunisian individuals (P<0.0001 to 0.027), in comparison with the French individuals (Table 1). In addition, we compared MAFs for the two populations with those extracted from the 1000 genomes project (Figure 1). In the present French population, all the allele frequencies were close to those belonging to the Utah residents with Northern and Western European ancestry (CEU) population (Figure 2). Regarding the Tunisian study, all the allele frequencies of the SNPs fell between those belonging to African ancestry (African Ancestry in Southwest US, ASW; Luhya in Webuye, Kenya, LWK; Africans "Yoruban in Ibadan", Nigeria, YRI) and those with European origins, the CEU group, British from England and Scotland (GBR) and Tuscan in Italy (TSI), with the exception of rs1126535 for which the frequency is very close to that of TSI.

LD and block haplotype analysis. The haplotype structure of the French and Tunisian populations with YRI, CEU, TSI and Japanese in Tokyo (JPT) populations is shown in Figure 2. There was a marked difference in the pattern of LD between the two populations. Using the solid spine algorithm[26] in the Haploview software, two haplotype blocks were identified across the 17 kb region in both cohorts, with evidence of disruption in LD between SNP4 and SNP7. Block 1, spanning 3 kb, encompassed the 5′UTR and the first exon and contained three SNPs (rs201992677, rs3092952, rs1126535), while block 2 encompassed the rest of the gene. In block 1, there were three haplotypes in the French cohort with an AAT predominant one (0.852), which is also the most frequent among the four haplotypes found in the Tunisian cohort (0.607). The second most frequent haplotype was GGC; it was more prevalent in the Tunisian population than in the French one (0.214 versus 0.123). In block 2, there was one additional haplotype in the Tunisian cohort, even though the high-frequency haplotype was TCG for both populations (0.889: French cohort and 0.633: Tunisian cohort). Considering the resulting haplotype blocks, the SNPs defined five and eight core haplotypes in the French and Tunisian cohorts, respectively. The most frequent one was AAT/TCG in both populations (Figure 2).
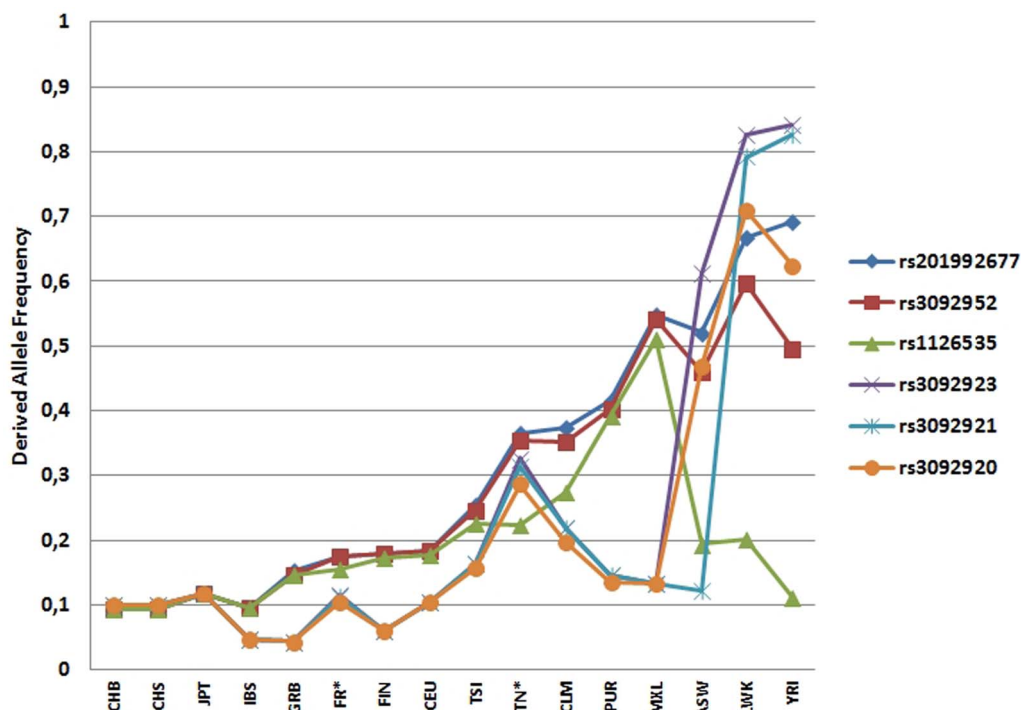
Figure 1 | Derived allele frequencies for six variants with minor allele frequencies in the *CD40LG* from French (FR) and Tunisian (TN) donors and those extracted from the 1000 genomes database. African Ancestry in Southwest US (ASW), Han Chinese in Beijing (CHB), Han Chinese South (CHS), Japanese individuals in Tokyo (JPT), Iberian populations in Spain (IBS), British from England and Scotland (GBR), Finnish from Finland (FIN), Utah residents with Northern and Western European ancestry (CEU), Tuscan in Italy (TSI), Colombian in Medellin (CLM), Puerto Rican in Puerto Rico (PUR), Mexican Ancestry in Los Angeles, CA (MXL), African Ancestry in Southwest US (ASW), Luhya in Webuye, Kenya (LWK), Africans "Yoruban in Ibadan", Nigeria (YRI) taken from the 1000 genomes project. *Present study.

rs201992677 [-/CAAA], described in the 1000 Genomes data but not in HapMap studies, was in high linkage disequilibrium with rs3092952 with D'=1 and r²=0.9 in the Tunisian dataset and D'=1, r²=1 in the French dataset. Surprisingly, all the male donors possessing the 'G' minor allele (rs3092952) were also found to carry seven CAAA repeats (rs201992677); in two cases, eight CAAA repeats were identified, which constitute a novel VNTR (c.-3525_-3526insCAAACAAA), instead of the most frequently found six CAAA repeats. Notably, three major 'A' type alleles of rs3092952 were unusually associated with seven CAAA repeats (rs201992677) in females originating from Tunisia, but not from France.

(CA)n microsatellite analysis. Allele frequencies were calculated in the French and Tunisian donors and the data revealed no deviation from HWE; we had previously verified the different allele frequencies for (CA)n repeats between males and females (P=0.297 and 0.137 for the French and the Tunisian donors, respectively). Twelve different alleles (from 18 to 31 CA repeats, corresponding to PCR products from 98 to 124 bp) were detected in the French cohort while 17 alleles (with 16 to 33 repeats, corresponding to PCR products from 94 to 128 bp) were observed in the Tunisian cohort. The (CA)₂₆ allele was the central or median allele and the most represented one in both groups, albeit more frequent in French than in Tunisian donors (Figure 3).

The allele distribution of the donors carrying 26 CA repeats, and less than or more than 26 CA repeats between the French and the Tunisian cohorts were compared by univariate statistical analysis. The allelic distribution between the French and the Tunisian donors was significantly different for allele groups of repeats as shown in Table 2.

Given the large difference between the two studied populations, we conducted a preliminary study in a group of 30 French single apheresis platelet donors whose platelets (in platelet components)

induced an ATR. However, we did not observe any allelic or genotypic association with neither SNPs nor CA repeats in 3'UTR, nor haplotype of *CD40LG* (Supplementary Table S2 and S3).

Discussion

DHPLC is a high-throughput semi-automated method with good accuracy for detecting base pair changes, either transversions or transitions, and can be used as a screening or a genotyping strategy that also allows identification of new polymorphisms, including more than one in the same fragment[27]. By this strategy, three new polymorphisms, albeit some at a very low frequency, have been identified in the Tunisian and French study groups where there are no population *CD40LG* reports, yet. We screened and genotyped 11 variants including two VNTRs. Of the three SNPs located in the coding region, the novel c.8140 A>G in exon 4 found in a Tunisian donor resulted in a missense mutation (ATA to GTA) in which isoleucine was substituted for valine in the expressed protein at amino acid position 127 (p.I127V) of the extracellular portion[28]. This mutation is predicted to be benign by *PolyPhen-2* with a score of 0.001 (http://genetics.bwh.harvard.edu/ggi/pph2/). The rare minor allele 'A' of the exonic rs148594123 inducing p.G219R was retrieved in exclusively eight healthy French including 4 females at the heterozygous A/G state and 4 'A' hemizygous males, although we did not find the hypomorphic mutation p.G466X in XIAP, the adjacent gene of *CD40LG* with which cosegregation has been described as resulting in HIGM1[29]. rs147739883, which was found in a Tunisian male, has been previously detected only in the Kenyan population (LWK). In addition, the minor 'C' allele of rs1126535 in exon 1 is associated with susceptibility to severe malaria[30] and would increase the risk of reduced bone mineral density and osteopenia/osteoporosis (as a medium risk) for this synonymous SNP as it could affect splicing regulation[31].
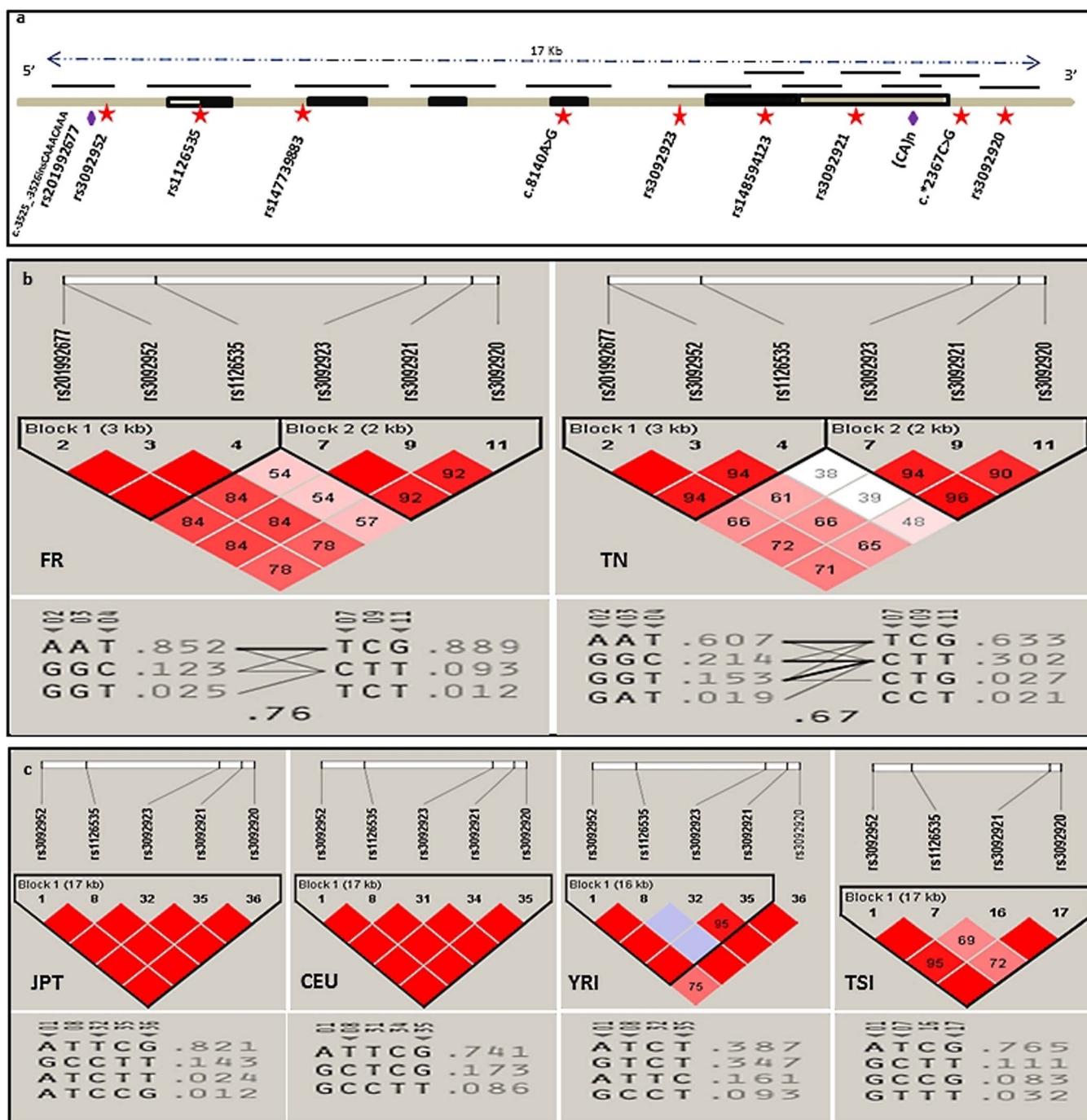
Figure 2 | Genomic structure of the *CD40LG* and the haplotype blocks of the French (FR) and Tunisian (TN) populations (this study) compared with those of JPT, CEU, YRI and TSI (HapMap data). (a) Genomic structure of the *CD40LG* gene and the position of the identified polymorphisms. Exons are represented by boxes, with filled boxes denoting translated regions. Upward facing lines indicate amplified fragments, and polymorphisms are shown as red stars for SNPs and purple lozenges for microsatellites. (b) *CD40LG* LD and haplotype diversity in French and Tunisian populations. (c) *CD40LG* LD and haplotype diversity in JPT, CEU, YRI and TSI populations. The numbers in boxes represent the pairwise D' value between adjacent SNPs. For (b) and (c), haplotype frequencies are shown below the LD diagrams. Triangular pointers highlight SNPs which could be tagged for future association analysis.

Among the three SNPs located in the 5′UTR, rs3092952 has been shown to provide an active role in *CD40LG* expression; the minor 'G' allele induces increased expression of the membrane and soluble forms of CD40L, thereby leading to enhanced CD40 interactions; this SNP is implicated in myocardial infarction and vascular disorders[32]. The variant 'C' allele of rs3092923, located in IVS4, is described as conferring a marginal reduced risk of follicular lymphoma[33]. CD40L regulation by the 5′UTR is related to the G allele of rs3092952[32], which is itself in high LD with the variable number of tandem CAAA repeats located a few bases upstream (rs201992677); therefore, could an increase in expression of the protein also be partially related to the presence of the CAAA repeats? as has been described in transcriptional regulation of the insulin-like growth factor 1[34].

We defined a core region of 17 kb in *CD40LG*. There is less than 2 kb in distance between haplotype blocks 1 and 2, whereas Chadha
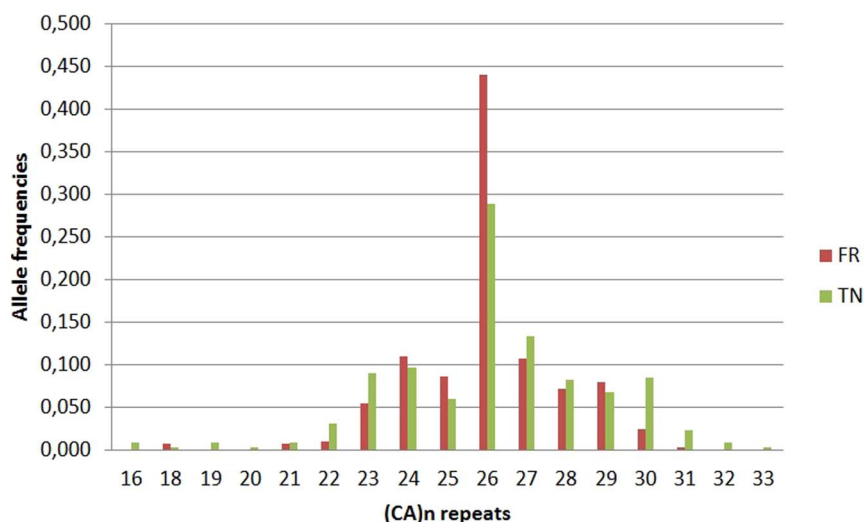
Figure 3 | Frequency distribution of the number of (CA)n repeats in the *CD40LG* in the French (FR) and Tunisian (TN) populations. Allele frequencies correspond to the number of the group of test alleles divided by the total number of alleles.

et al. described a distance of 4 kb[35]. We compared the results obtained for the haplotype structure of the SNPs, and the LD patterns with frequencies for Caucasians (CEU, TSI), Africans (YRI) and Asians (JPT). The LD structure of *CD40LG* on chromosome X and haplotypes in the corresponding LD blocks differed between the two populations studied. The Tunisian haplotypes were found to be intermediate between the European populations (French in the present study and TSI) and the African populations.

However, considering the HapMap data, the structure of LD in *CD40LG* and the haplotypes in the CEU European group, were found to be similar to those of the Asian (JPT) population, regardless of the allele frequency differences at each SNP. Our results for the French population were closer to those obtained with a European English cohort by Chadha *et al.*[35], and also from TSI than to those of CEU (Figure 2). As already described, these results do not always correspond with the data (allele frequencies and LD) downloaded from HapMap Phase II and III; this may be explained by local variations within a country and/or by genotyping errors[36].

It is worth noting that the French and Tunisian populations, as with any other North African population, do not exist in the HapMap or 1000 genomes datasets. Our results indicate moderate transferability of tagging SNPs on *CD40LG* from the European-Caucasian population to the French population. For the Tunisian population, this transferability is hardly conceivable. This approach has often been attempted in other populations (e.g. Thai and Spanish), and with other genes, with the aim of genotyping hotSNPs with which to assist future genetic association studies[25,37].

The distribution of the frequencies of the CA repeat alleles in the populations studied appears similar to that obtained in others studies, although the major allele identified was 24 to 27 CA repeats. As

discussed by Stewart *et al*, this difference is most likely explained by differences in the systems used for determining the repeat numbers: i.e. denaturing polyacrylamide gel and silver staining[38], allele-specific oligonucleotide hybridization of membrane-fixed DNA products[39] (major allele with 24 repeats), denaturing polyacrylamide gel and autoradiography[40] (26 repeats), capillary electrophoresis using a CEQT 8000 Genetic analysis[41] (27 repeats), or a CEQ 2000 genetic analysis system, as in the present study (26 repeats)[42].

Different selection pressures may explain the extreme variability of this 3′UTR and its association with immune disorders. For example, polymorphisms of longer $(CA)_n$ alleles are seen in auto-immune processes such as seen in patients with systemic lupus erythematosus[43], those with a high risk of thromboembolic disease[44], or rheumatoid arthritis[39,45]. However, no association was found in the context of alloimmunity (e.g. rejection of renal transplantation) with either acute graft rejection or patient death, but a strong association was shown with long-term graft failure[41]. Shorter CA repeats are also linked to rare inherited HIGM1 with an informative usefulness in linkage analysis[40]. Is there any infectious selection process that could explain the more frequently observed longer alleles in the Tunisian population in relation to the need for enhanced inflammation[46]? It appears that the number of repeats increases over generations, but would this bear any relationship to ancestral population? This would reinforce the supposed old origins of the Tunisian population[47].

CA dinucleotide is the most common repeat sequence in the human genome. Human CD40L is encoded by an unstable mRNA; this instability is conferred by a portion of its 3′UTR including a dual cis-acting element comprising a polypyrimidine-rich region and CA repeats[48]. It was shown that this CA repeats region is a binding site of nuclear factors and thus could modify the affinity of interaction with *CD40LG* mRNA and consequently, the translation efficiency[48–50].

The $(CA)_{24}$ allele (corresponding to $(CA)_{26}$ in our study) appears to confer more stability on its mRNA, and longer alleles confer higher expression levels[43,45,48].

To the best of our knowledge, ours is the first description of this microsatellite marker in Tunisians. This microsatellite has a distribution characteristic of each population as described in other studies[38–40]. In the present French and Tunisian study, the $(CA)_{26}$ allele showed a statistically significant difference in frequency between the two populations (0.438 versus 0.291, respectively), while a previous French study found a frequency of 0.319. The frequency of heterozygosity was 0.70 (this study) and 0.79 (DiSanto *et al.*)[40] for the French, and 0.82 for the Tunisian (this study). This result reflects genetic diversity in the Tunisian population, confirmed by the allelic

Table 2 | Allelic distributions of the *CD40LG* $(CA)_n$ repeat polymorphism in the French and Tunisian cohorts (291 and 353 alleles respectively)

$(CA)_n$	French cohort	Tunisian cohort	P-value[b]
	N[a] (frequency)	N[a] (frequency)	
<26 CA	80 (0.275)	109 (0.309)	0.00019
26 CA	128 (0.440)	102 (0.289)	
>26 CA	83 (0.285)	142 (0.402)	

[a]Number of alleles.
[b]Bonferroni correction : P-value is significant when <0.016.

distribution of (CA)n alleles, which has also been reported in the Spanish population, demonstrating that there was also a highly polymorphic marker (of 15 alleles) in Spaniards versus 17 in Tunisians[38]. The dinucleotide repeat (CA)n is a marker that is highly polymorphic, and therefore useful for genetic studies on the CD40L molecule in relation to immunity.

Both French and North African populations have a tumultuous history with various ancestors but the Tunisian population appears to be significantly more heterogeneous as demonstrated by Y-STR diversity in the Sousse population[51]; the three novel polymorphisms are also in favor of the extreme heterogeneity in the studied cohort (Figure 1)[35,52,53]. Heterogeneity in haplotype frequencies is often higher in Africans such as the YRI group, in comparison to the European populations who left the African continent and experienced bottlenecks during their migrations, reducing SNPs diversity (Figure 2). The intermediate Tunisian *CD40LG* haplotype structure might be explained by its African location and multilayered history[53].

As variants repartition is different between the 2 populations, these findings added to the current knowledge base in the studied French and Tunisian populations, may ultimately lead to better investigate the *CD40LG* associated diseases. Their prevalence were found to be often in support with genetic polymorphisms repartition (Table 1 and 2), e.g. cardiovascular disorders[32,54,55], systemic lupus erythematosus[43,56,57], rheumatoid arthritis[45,58], although exact Tunisian prevalence is difficult to clarify for lack of national epidemiological registers. In addition to CD40L polymorphisms, other genetic markers were found differently associated in the studied populations[59] whereas others were similarly associated[60]. On the other hand, this technical strategy could be used for HIGM1 investigation (we covered 182 of all the 183 mutations in *CD40LG* that cause HIGM1 (http://www.hgmd.org/ updated in June 2014). It is less expensive than actual used techniques, especially in developing countries such as Tunisia where HIGM syndrome is frequently found[46,61,62].

Although several studies have found an association between ATR and high level of sCD40L in the transfused blood component[19–23], we did not find significant differences in *CD40LG* genetic pattern in donors whose platelets induced or not an ATR in the French population. This would be in favor of a CD40L release mechanism not due to a genetic regulation. It is noteworthy that all the previous studies have been performed with small effectives. We will have to extend the *CD40LG* study to a more important cohort of donors including donors whose donated platelets induced TRALI. It will be also interesting to investigate CD40L receptor (CD40 gene) in the recipients presenting ATR.

In conclusion, we identified polymorphisms known as regulatory within the *CD40LG* i.e. rs3092952 in the 5′UTR, (CA)n repeats in the 3′UTR, some rare and novel mutations, and polymorphisms involved in several diseases. These polymorphisms could modulate immune responses via interactions between CD40L and its various receptors[63]. We found a major difference between Maghreb (Tunisian) and European (French) populations in their *CD40LG* haplotype patterns. Surprisingly, we did not find any difference in *CD40LG* genetic pattern in apheresis platelet donors whose blood components prepared for transfusion purpose induced or not an ATR. Our findings provide useful information for future disease-association studies on the *CD40LG* in the context of inflammation and auto-immunity.

Methods

Ethical considerations. Informed and written consent was obtained from all the healthy donors who participated in this study, according to a protocol approved by the Ethics Committees for scientific research at Saint-Etienne (France), F. Bourguiba (Monastir, Tunisia), and F. Hached (Sousse, Tunisia) University Hospitals. All analyses were performed anonymously.

Studied populations. The cohort contained 485 healthy volunteer human blood donors, acting as non-profit donors (211 French donors who presented at the

Auvergne-Loire Regional Branch of the French National Blood System EFS collection sites (130 males and 81 females) and 274 Tunisian donors who presented at the Monastir Blood Bank or the Regional Blood Transfusion Centre of Sousse blood collection sites (195 males and 79 females). Blood donors at both locations were unrelated and randomly chosen to enter this study, based on the timing of their donations, and no selection bias was applied to them (Supplementary Table S4). For comparison, the allele and genotype information for other populations were downloaded from HapMap Phase II and III (http://www.hapmap.org, August 2010) and from the 1000 genomes project (http://www.1000genomes.org). Thirty French single apheresis platelet donors whose donated blood components induced an ATR (FNHTR) were also tested (18 males and 12 females) (Supplementary Table S4).

DNA samples. EDTA-treated blood samples were collected from all individuals. DNA was purified from peripheral blood leukocytes using a FlexiGene DNA kit (Qiagen, Paris, France) according to the manufacturer's instructions. DNA concentrations and purity were assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

SNPs and microsatellite analysis. The nomenclature used herein for reporting sequence alterations abides by the original Human Genome Variation Society guidelines. NC_000023.10 was used as the reference sequence. All information about the SNPs detected was extracted from the public Single Nucleotide Polymorphism database dbSNP built 138 (http://ncbi.nlm.nih.gov/SNP/).

DHPLC analysis: screening and genotyping. To screen for the maximum number of polymorphisms potentially involved in CD40L protein expression or reported to be associated with immune disorders or inflammation[32,45,48,64], PCR was performed with eleven primer pairs with corresponding annealing temperatures (Table 3, Supplementary Data S1 for PCR protocols). To promote stable heteroduplex formation, PCR products were denatured for 5 min at 96°C, and then gradually cooled at a rate of 0.5°C per 10 sec over 150 cycles.

At the screening stage, 5 μL of each PCR product was automatically analyzed by DHPLC using the Wave system (Transgenomic, Ltd. Glasgow, UK)[65,66]. PCR products were injected into a preheated, fully equilibrated chromatographic column (DNASep Column, Transgenomic Ltd.). A linear gradient of 5% triethylammonium acetate (TEAA) (Buffer A) and 25% acetonitrile-5%TEAA (Buffer B) was generated for each amplicon. Each DHPLC run included a DNA loading step (5% drop for loading Buffer B), a linear separation gradient (2% Buffer B slope per min, 4.5 min), a wash step (75% acetonitrile; Buffer D, 0.5 to 1 min) and an equilibration step (0.9 to 1.2 min). The eluate was detected by an ultraviolet light detector at 260 nm. DHPLC conditions were optimized via fragment melting profile analysis, using the Wave system software Navigator connection WADMIN (Transgenomic, Ltd.).

The melting point (and profile) of each fragment was analyzed according to Fixman and Friere's implementation of Poland's algorithm[67], which calculates the probability that a base is in the helical duplex conformation (at 75–90%) or in the non-helical, single-stranded form. Each amplicon was, therefore, assessed at two or three column temperatures (Table 3). The flow speeds, temperatures, retention times and initial Buffer B concentrations of the elution gradient for DHPLC analysis were determined by the software provided. If the sample contains heteroduplex molecules, these will denature at lower concentrations of acetonitrile and will be visualized as peaks (or sometimes a single peak) with shorter retention times than that of a homoduplex. Control samples sequenced previously (homozygous and heterozygous for a known SNP, displaying profiles with single or multiple peaks) were run in parallel.

At the genotyping stage, all the samples from females presenting with one peak at the screening stage were mixed in equal volumes with a reference sample. Such samples were run again using the same DHPLC conditions and the same controls described above. Samples presenting with one peak at the screening stage had the same genotype of the homozygous control if the same single peak that was observed was homozygous for the other allele if multiple peaks were seen. Distorted multiple peak elution profiles differing from the reference profiles indicated the presence of a different polymorphism from the reference one or an additional SNP in the fragment; additional sequencing was applied in these cases.

For the male samples, because the gene of interest is on the X chromosome, a preliminary step was required. This involved mixing the PCR product from each sample with the PCR product of one of the controls (1V/1V) that was previously sequenced and for which no polymorphism for the fragment had been found and was, therefore, automatically genotyped.

DNA sequencing. To identify the type and position of the genetic variants, DNA samples with abnormal DHPLC patterns were re-amplified as described above, and then subjected to direct sequencing in both forward and reverse directions using the same PCR primers. PCR products were purified by Amicon Centrifugal filters (Millipore, Molsheim, France). Sequencing reactions were performed using the GenomeLab DTCS Quick Start mix (Beckman Coulter, Brea, CA). The resulting products were purified with Beckman Coulter's CEQ DTCS kit according to the manufacturer's instructions, subjected to analysis with a CEQ 2000 XL-Beckman Coulter sequencing machine, and assembled using CEQ 8000 Beckman Coulter software (Beckman Coulter, Inc. CA). For each DHPLC profile displayed, 10 samples were sequenced. However, all the heteroduplex profiles for the CD40L-5′UTR fragment were sequenced because of more than one polymorphism.

Table 3 | PCR fragments, primer sequences, annealing temperatures (Ta)°C and conditions of Denaturing High Performance Liquid Chromatography (DHPLC) analysis of *CD40LG*

Fragment *CD40LG* (bp)	Primer sequences (5'>3')		Annealing temperature (°C)	DHPLC analysis		
	Forward primer	Reverse primer		Column temperature (°C)[a]	Buffer B %	Time shift (min)
5'UTR (285)[b]	TACTTGGGGAGGCTGAGGCAG	TTGACCCCTGCCCACATT	62	[56.9], [57.9]	63.4	0
E1 (362)[b]	GCGCTCTTAACTAATCCTGAG	TCTTCTATTGGTTCTACCATC	55	55.1, 56.1, [57.1]	64.1	0
E2 (283)[b]	TGCCGTGGAAATGAAATGTAG	CCCGATCTAGCAAATGTAGTAAA	58.5	55.6, [56.6]	62	0
E3 (209)	AAACCCACAGCAGAGCCC	CCTGATGCAACAACACTGGGT	58	[56.8], 57.8	59	0
E4 (310)[b]	TCAGTGGGAGAGATGTCAGACC	CCCAGGGGAAAAGAGGAGTTTA	58	[56.3], 58.3	62.5	0
E5A (383)[b]	GAACCATGCTCTGCTTCACCT	CACTTGGCTTGGATCAGTCA	58	58.3, [59.3]	64.5	0
E5B (432)[b]	GAGAGAATCTTACTCAGAGCTGC	ATGTCTGCATCAGTGGGGGT	58.5	57.2, [60]	65.4, 63.4	0, 0.4
E5C (356)	TCATAATACAGCACAGCGGT	TTCCCTTCTGCATCTTCACT	57.4	57.1, [58]	64	0
3'UTRA (304)[b]	ATGCAGAAGGGAAATGGGG	GTTAGAAAGGGGGATTGAGAAG	58	58.9, [60.9]	62.6	0, 1
3'UTRB (339)[b]	GTTGCAGGAGATTGAAAGAAC	TAGTGGCTTGAAGATGCTGC	55	[58.5], [58.9]	63.6	0
3'UTRC (244)[b]	CCAAGACCTTGTCCCATTCACC	CAGTCATTTTTACTCCATGAGTGC	59	[56.5], [57.3]	60.6	0

[a]Column temperature selection was based on the melting domains present in the PCR fragments.
The column temperature recommended by the Navigator connection Wadmin software is shown in italics, while the temperature at which a polymorphism was accurately identified is shown in square brackets in these PCR fragments.
[b]SNPs were identified in these PCR fragments.

Capillary electrophoresis: genotyping 3′UTR CA repeats. Samples were resolved by capillary electrophoresis separation, using a CEQ 2000 XL genetic analysis system (Beckman Coulter, Inc. CA, USA). To 0.5 μL of PCR product were added 0.25 μL of a 600 bp standard and 40 μL of sample loading solution (Beckman Coulter). Specifically designed labeled oligonucleotides were excited to fluoresce using a diode laser. The length of the amplified fragment was estimated with reference to the internal ladder and the number of repeats was calculated by analogy with 10 sequenced samples, using the CEQ 8000 Beckman Coulter software provided.

Statistical analysis. Allele and genotype frequencies were calculated and compared using XLSTAT™ (Addinsoft, Paris, France) computer software. The χ2 test was used to compare the allelic distribution between males and females and the allelic frequencies between French and Tunisian populations, using the Bonferroni correction or Fisher's exact test, where appropriate. Statistical significance was taken as $P < 0.05$. For each polymorphism, the quality of the genotype data was assessed by testing for HWE, using the Haploview program http://www.broad.mit.edu/mpg/haploview/[26]. For analysis of the 3′UTR CA repeats, HWE was tested by comparison of the observed and expected genotype frequencies. Haploview 4.2 was also used to calculate the pairwise linkage disequilibrium (LD) of the polymorphisms for which the minor allele frequency (MAF) was >0.05. LD assessment and *CD40LG* haplotype definition, was determined using Lewontin's standardized disequilibrium coefficient D', the squared correlation coefficient r^2, while the block definition was based on the solid spine method[26]. For practical purposes, we introduced rs201992677 [-/CAAA] to the LD analysis, arbitrarily calling 'A' the wild type allele with six CAAA repeats, and 'G' the variant allele with seven repeats.

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Author contributions

S.L., F.C. and O.G. initiated and completed the project. S.L. and C.A. designed the experiments. C.A. and C.S. performed the experiments. M.H., T.C., S.JY., M.A., C.F., B.P. and C.A. contributed to the recruitment of the blood donors. J.F., V.G.H. and R.T. did the DHPLC analysis. C.A. and A.P. analyzed the data. S.L. drafted the manuscript. All authors reviewed the manuscript.

Additional information

Accession codes: We submitted the three novel mutations in DDBJ database with following accession numbers

> AB897730 52b053e41553421871002993.*CD40LG*1
> AB897731 52b053e41553421871002993.*CD40LG*2
> AB897732 52b053e41553421871002993.*CD40LG*3

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