Common gene variants within 3'-untranslated regions as modulators of multiple myeloma risk and survival

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Abbreviations

ANOVA, Analysis of Variance;

CEU, Caucasian population;

CI, Confidence Intervals;

EQTL, Expression Quantitative Trait Loci;

ECACC, European Collection of Cell Cultures;

GWAS, Genome-wide association studies;

GERP, Genomic Evolutionary Rate Profiling;

HWE, Hardy-Weinberg equilibrium;

IMMEnSE, International Multiple Myeloma rESEarch;

IMWG, International Myeloma Working Group;

LD, Linkage Disequilibrium;

MAF, Minor Allele Frequency;

MM, Multiple Myeloma;

OR, Odds Ratios;

p3UTR, Polymorphic 3'UTRs;

RFP, Red Fluorescent Protein;

RFU, Relative Fluorescence Units;

SNPs, Single nucleotide polymorphisms;

3′UTR, 3’-Untranslated Region;

Abstract

We evaluated the association between germline genetic variants located within the 3'untranlsated region (polymorphic 3'UTR, i.e. p3UTR) of candidate genes involved in multiple myeloma (MM). We performed a case-control study within the International Multiple Myeloma rESEarch (IMMEnSE) consortium, consisting of 3056 MM patients and 1960 controls recruited from 8 countries. We selected p3UTR of six genes known to act in different pathways relevant in MM pathogenesis, namely *KRAS* (rs12587 and rs7973623), *VEGFA* (rs10434), *SPP1* (rs1126772), *IRF4* (rs12211228), and *IL10* (rs3024496). We found that *IL10*-rs3024496 was associated with increased risk of developing MM and with a worse overall survival of MM patients. The variant allele was assayed in a vector expressing eGFP chimerized with the *IL10* 3'UTR and it was found functionally active following transfection in human myeloma cells. In this experiment, the A-allele caused a lower expression of the reporter gene and this was also in agreement with the *in vivo* expression of mRNA measured in whole blood as reported in the GTEx portal. Overall, these data are suggestive of an effect of the *IL10*-rs3024496 SNP on the regulation of *IL10* mRNA expression and it could have clinical implications for better characterization of MM patients in terms of prognosis.

Novelty & Impact Statements

The polymorphic 30-untranslated region (p3UTR) has beenshown to affect gene expression and lead to differential pre-disposition or prolonged survival in a variety of cancer types.Here, the authors evaluate the association between germlinegenetic variants within the p3UTR of candidate genesinvolved in multiple myeloma. The results show that IL10-rs3024496 might affect immune homeostasis through themodulation of IL10 mRNA expression. Overall, the findingssuggest that the inclusion of personal genetic backgroundinformation into the clinical evaluation criteria could help toimprove the stratification of patients with multiple myelomain terms of risk progression and prognosis.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy originating from the bone marrow. The incidence of this hematological neoplasm is rising worldwide, and despite improvements of therapeutic treatments, it remains incurable. Immunodeficiency and exposure to viruses or chemical agents are considered potential risk factors[1]. Convincing evidences suggest that MM has a solid genetic background, given the increased risk (from 2 to 4-fold) in first-degree relatives of MM patients[2]. Genome-wide association studies (GWAS) have allowed a better understanding of the genetic component of MM susceptibility and survival, leading to the identification of several *loci*, some of which are involved in complex biological process such as cell proliferation, cell cycle, and DNA repair [3–5].

The 3'-untranslated region (3′UTR) of genes plays a crucial role in regulating the diverse fate of mRNAs and therefore in determining the phenotypic diversity[6]. For example, single nucleotide polymorphisms (SNPs) within 3'UTRs have been shown to affect miRNA binding sites[7] or stability[8] of mRNAs, thereby modulating the rate of translation. More in general, polymorphic 3'UTRs (p3UTR) could affect gene expression with a variety of phenotypic effects, including a differential predisposition to cancer or a prolonged survival of cancer patients, as it has been shown for glioma, and for breast, gastric, renal and colorectal carcinoma [13–15]. In the present study we extended the investigation to a set of candidate genes (namely *KRAS, VEGFA, SPP1, IRF4,* and *IL10*) involved in pathways relevant for MM pathogenesis, such as apoptosis, B cell differentiation, bone resorption, and immunoglobulin production. To this end, we performed a case-control association study within the framework of the International Multiple Myeloma rESEarch (IMMeNSE) consortium and we found that the p3UTR of *IL10* was associated with MM risk and prognosis. We also performed *in vitro* assays to better characterize the effects of the p3UTR on *IL10* gene expression.

Materials and methods

Study population

The study population consisted of 3056 MM and 1960 controls and patients recruited from 8 countries in the context of the International Multiple Myeloma rESEarch (IMMEnSE) consortium (**table 1**) [16]. Patients were defined by a confirmed diagnosis of MM, according to International Myeloma Working Group (IMWG) criteria. Controls were recruited in the same geographical area of the patients, among blood donors and hospitalized volunteers with diagnoses excluding cancer. For every subject enrolled in the study, information on sex and age at recruitment/diagnosis were collected. For patients, also clinical and pathological characteristics, including disease stage (Durie-Salmon and/or International Staging System), and type of first-time therapy were retrospectively collected from medical records. Following the guidelines of the Declaration of Helsinki, written informed consent was obtained from each participant.

SNP selection criteria

SNPs were selected through a gene candidate approach in order to pick the ones involved in the most important biological function that are deregulated in MM: apoptosis, B cell differentiation, bone resorption, and immunoglobulin production[1]**.**

In order to identify those genes, the workflow of our study started with the candidate gene selection and was performed using three data mining search tools: Coremine (https://www.coremine.com/), SNPs3d (http://www.snps3d.org/) and Gene Prospector [17]. Each tool adopts a different algorithm to mine information from databases and literature repositories, and to connect gene or protein names to keywords of interest. We then intersected the results obtained from the three tools and assembled a list of 52 candidate genes. Next, we selected SNPs within the 3'UTR of the candidate genes with minor allele frequency (MAF) higher than 5% in the Caucasian (CEU) population of the 1000 Genomes project. We further selected the most conserved SNPs, using three tools: jSNPSelector (http://jsnpselector.sourceforge.net, a tool developed at the Department of Biology, University of

Pisa, based on the alignment of 46 placental mammal genomes in the Ensembl database), GERP (Genomic Evolutionary Rate Profiling, http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html) and SiPhy-omega (https://www.broadinstitute.org/mammals-models/29-mammals-project-supplementary-info) . We chose for genotyping the top six SNPs located in 5 different genes, selecting only the SNPs resulting conserved in all methods (*KRAS*-rs12587, *VEGFA*-rs10434, *SPP1*-rs1126772, *KRAS*-rs11047885, *IL10*-rs3024496, *IRF4*-rs12211228), as reported in **Supplementary table 1**.

SNP genotyping and quality control

Genomic DNA was extracted from peripheral blood of MM patients and controls, using the QIAampR 96 DNA QIAcubeR HT Kit. Genotyping was done using TaqMan (Applied Biosystems) technique in 384-well format plates, with 10 ng of DNA from each subject. The order of DNAs of patients and controls was randomized on plates in order to ensure that an equal number was analyzed simultaneously. Eight percent of the samples were duplicated for quality control purposes.

***In silico* analysis**

For rs3024496 we performed bioinformatics analysis in order to gain insight on its functional role. Specifically, we used LDlink (https://ldlink.nci.nih.gov/) to identify SNPs in LD with rs3024496, and RegulomeDB (http://regulome.stanford.edu) to identify the regulatory potential of the resulting SNPs. Finally, we used GTEx portal (https://gtexportal.org/) in order to identify potential associations between the SNPs and expression levels of nearby genes (eQTL) (**supplementary table 2**).

Gene reporter expression assay

The human myeloma U266B1 (RRID: CVCL_0566) cell line was obtained from the European Collection of Cell Cultures (ECACC). The U266B1 cell line has been authenticated using STR profiling within the last three years. The cells were grown in RPMI-1640 medium supplemented with

15% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin (all from Euroclone S.p.A.). All cells were kept at 37 °C in a constant humidified 5% CO_2 atmosphere. All experiments were performed with mycoplasma-free cells.

For the functional study, we employed the IL10 3'UTR_GFP vector (Applied Biological Materials Inc.) harboring the common allele of rs3024496 within the 3'UTR of the human *IL10* gene downstream of a green fluorescent protein (GFP) coding sequence. Site-directed mutagenesis was carried out on the 3'UTR-IL10_GFP in order to obtain a vector carrying the rare variant of the rs3024496 A>G (from now on referred as GFP_IL10_A and GFP_IL10_G respectively). The mutagenesis was performed using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), following the manufacturer's instructions.

To evaluate the functional role of the selected SNP, 3×10^6 cells were electroporated with 7µg of a control vector expressing a red fluorescent protein (RFP) (HR410PA-1; SBI) and with 14µg of either the GFP_IL10_A or GFP_IL10_G. The electroporation was performed using Neon Transfection System (Invitrogen in Life Technologies) following these specific electroporation parameters: voltage, 1450 V; pulse width, 10 ms; pulse number, 3. Fluorescence intensity was measured at single-cell level 48h after the electroporation assay, using BD FACS Jazz System (BD Biosciences). All experiments were replicated independently three times.

Statistical analysis

Departure from Hardy-Weinberg equilibrium (HWE) was tested among IMMEnSE controls in each population. Analysis of association between SNPs and MM risk was performed with unconditional logistic regression, adjusting for a set of covariates including age (at diagnosis for MM cases, at recruitment for controls), sex and country of origin. The association between SNPs and MM risk was calculated by estimating odds ratios (OR) and their 95% confidence intervals (CI). For all the SNPs, we performed a statistical analysis using the allelic and codominant models, setting the more common

allele as reference and a P<0.0042, calculated with the formula 0.05/12 (6 SNPs x 2 models), as the Bonferroni-corrected threshold for statistical significance.

Survival analysis was performed with Cox regression, calculating hazard ratios (HR) and 95% CI using overall survival (OS) as end point. OS was defined as the time interval between MM diagnosis and death or the last date of follow up. This analysis was adjusted by age, sex, country of origin, disease stage and type of first line therapy.

For the *in vitro* experimental validation, the statistical analysis was limited to the cells that were efficiently transfected with both control and IL10_GFP vectors. The relative fluorescent units detected from the red and the green channel were used to calculate the ratio GFP/RFP. A Z-score statistic was performed to account for the inter-experimental variability. Analysis of variance (ANOVA) was carried out to assess the difference between the two constructs.

Results

Genotype concordance rate was greater than 98%. Samples with call rate lower than 75% were discarded (N=396). After exclusions, all SNPs had a call rate over 91%, which was uniform between cases and controls.

No deviations from HWE among the controls were observed, with the exception of rs12211228 (*IRF4*), rs11047885 (*KRAS*), rs1126772 (*SPP1*), rs12587 (*KRAS*) SNPs in the Danish sample set. Therefore, these 4 SNPs were analyzed excluding samples from Denmark.

Among the SNPs investigated only *IL10*-rs3024496 SNP showed an association with MM risk at nominal level of P<0.05 (**Table 2**). Carriers of the A-allele showed an increased risk (OR=1.20, 95% CI=1.01-1.44 for heterozygotes) as compared to the homozygotes for the G-allele. The trend test showed a statistical P-value close to the Bonferroni-corrected threshold of significance (P=0.008). The same allele was also associated with a shorter OS at the nominal level of 0.05, with an HR of 1.44 (95% CI=1.05-1.96, p=0.022) for the heterozygotes and of 1.42 (95% CI=1.02-1.99, p=0.04) for the homozygotes. None of the other SNPs showed noteworthy results, with the exception of *IRF4*-rs12211228, which showed a weak association with MM risk in the allelic model (p=0.036, **Table 2**).

Furthermore, the *in vitro* assay showed that the p3UTR carrying the A-allele of rs3024496 significantly decreased the expression of the GFP reporter gene. In particular, U266B1 MM cells transfected with the GFP_IL10_A vector showed a 6% reduction in relative fluorescence units (RFU), compared with cells transfected with the GFP_IL10_G vector (p=0.019) (**Fig.1A**). In addition, the *IL10*-rs3024496-A allele was also associated with reduced expression of *IL10* in GTEx (**Fig. 1B**).

Discussion and conclusion

Our previous data reported significant associations between p3UTRs within *DDR1, TCF19, POU5F1* genes, and increased risk of developing MM, [18] highlighting the importance of association studies in discovering novel variants associated with MM pathogenesis. Herein, we expanded the investigation by evaluating the association between p3UTRs of candidate genes, namely *KRAS* (rs12587 and rs7973623), *VEGFA* (rs10434), *SPP1* (rs1126772), *IRF4* (rs12211228), and *IL10* (rs3024496) with MM risk and prognosis. The SNPs within MM candidate genes were selected for their highest conservation scores among phylogenetically distant organisms, suggesting a functional role to be likely. Among the investigated p3UTRs, we found a positive signal within *IL10*, a gene coding a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes, with pleiotropic effects on immune-regulation and inflammation. Specifically, we found that AA genotype of *IL10*-rs3024496 was associated with increased risk of developing MM, and also that the A allele in *IL10*-rs3024496 was associated with a worse OS of MM patients. It is interesting to note that *IL10*-rs3024496 and *IL10*-rs2222202 (a SNP in strong LD with rs3024496) have been also associated with adult cancer types, including colorectal [19] and prostate cancers [20].

Results from the *in vitro* assay were in agreement with a hypothetical functional role for rs3024496, showing that the A-allele significantly decreased the expression of the reporter gene of about 6%. These results paralleled the *in vivo* data from GTEx reporting a similar association between *IL10* mRNA levels in whole blood and the polymorphism rs3024496, strengthening our observation.

IL-10 has been identified as an anti-inflammatory cytokine. In macrophages it inhibits the expression of inflammatory cytokines (such as INF-γ, IL-2, IL-3 and TNF-α) and deactivates their functions [21]. It has been shown that IL-10 is an important regulator of the activity of the pro-inflammatory cytokines IL-1β and IL-6 that are aberrantly expressed by MM cells [22]. It was also shown *in vivo* that IL-1β and IL-6 sustain MM cells survival and play a role in the progression towards MM [23]. Indeed, the targeting of IL-1/IL-6 axis by the use of receptor antagonists in combination with dexamethasone ameliorated the disease progression of MM patients in a phase II clinical trial [24].

In summary, our results suggest that *IL10*-rs3024496 might affect immune homeostasis through the modulation of *IL10* mRNA expression. Decreased level of IL-10 could be associated with an overproduction of pro-inflammatory cytokines, providing myeloma cells the ideal microenvironment to survive longer and proliferate. Overall, these findings suggest also that the inclusion of the personal genetic background of patients could help to improve the stratification in terms of risk progression and prognosis.

Author contributions

F. Gemignani conceived and designed the study. D. Calvetti and A. Macauda performed genotyping. D. Calvetti, A. Macauda and F. Canzian performed data quality control and statistical analyses. M. Netea, R ter Forst, Y Li, F Morani and R Silvestri provided the functional raw data and J. Sainz and R Silvestri performed the analysis. A. Melaiu, A. Macauda and J. Sainz drafted the manuscript. All other authors provided samples and data. All authors critically read, commented and approved the manuscript.

Conflicts of interest

The authors declare no conflict of interests.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethical statements

The IMMEnSE study protocol was approved by the Ethics Committeeof the Medical Faculty of the University of Heidelberg (referencenumber: S-004/2020). Following the guidelines of the Declaration ofHelsinki, written informed consent was obtained from eachparticipant

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Tables

Table 1. Characteristics of IMMEnSE cases and controls.

	Cases	**Controls**
Geographic origin		
Italy	298	228
Poland	1,259	350
Spain	283	324
France	502	176
Portugal	152	195
Hungary	155	101
Denmark	299	489
Israel	108	97
Total	**3,056**	**1,960**
Median age (25% - 75% percentiles)	62 (55 – 68)	52 (41 – 64)
Sex		
Males	51.8%	52.0%
Females	48.2%	48.0%
Disease stage Durie-Salmon[a]		
1	200	-
2	360	-
3	906	-
Total	1,466	-
Disease stage ISS[a]		
1	378	-
2	383	-
3	425	-
Total	1,186	-
First line therapy[b]		
New	716	-
Old	747	-
Total	1463	-
Median overall survival (25% - 75% percentiles)	39 (20.5-69.47)	

[a] The sum does not add up to the total of subjects due to missing data.

[b] New therapies are those based on proteasome inhibitors and/or immunomodulating drugs; old therapies are all others.

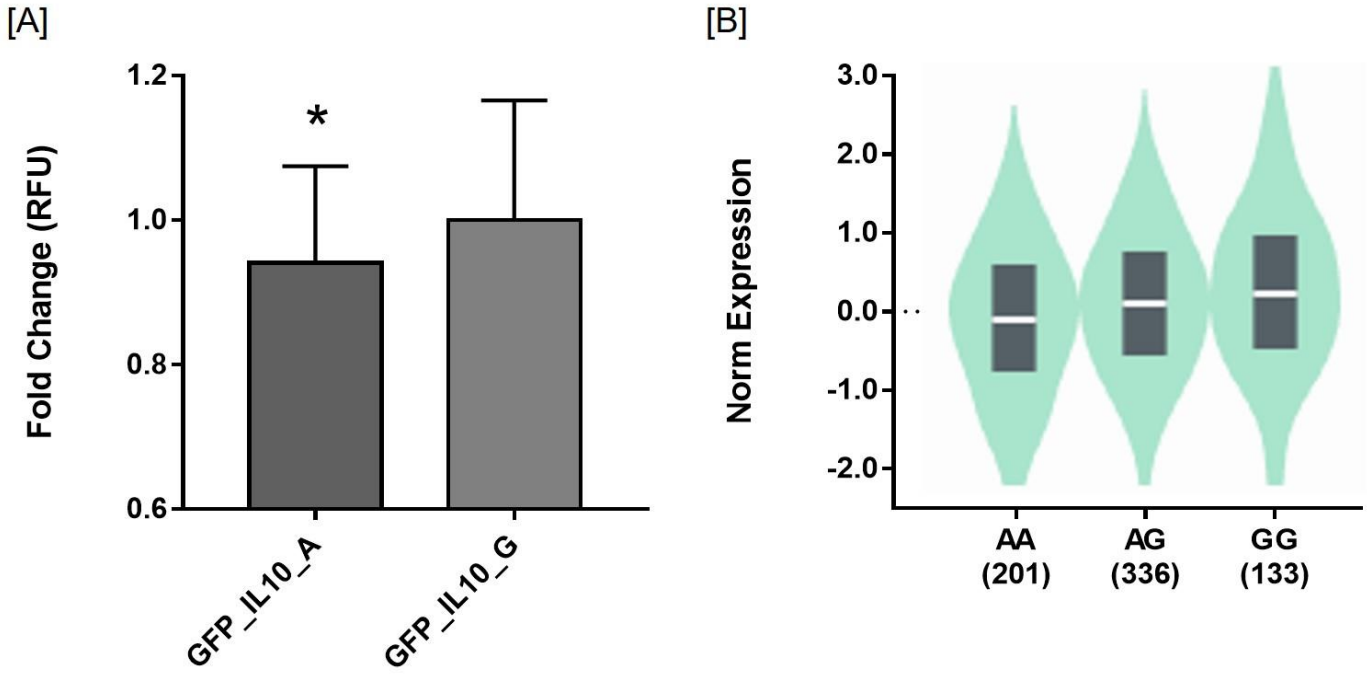
Table 2. Associations between selected SNPs and MM risk in the IMMEnSE consortium[a].

Gene	SNP	Genotype	Cases	Controls	OR (95% CI)	*P-value*	*P-trend*
		G	2,541	1,727	1 (-)	-	
		A	3,357	2,029	1.05 (0.96-1.15)	0.298	
IL10	rs3024496	G/G	551	424	1(-)	-	**0.008**
		G/A	1,439	879	**1.20 (1.01-1.44)**	**0.042**	
		A/A	959	575	**1.24 (1.02-1.50)**	**0.028**	
		A	4,607	2,248	1(-)	-	
		G	1,267	628	1.01 (0.90-1.13)	0.873	
SPP1	rs1126772	A/A	1,810	877	1 (-)	-	0.227
		A/G	987	494	1.08 (0.92-1.26)	0.333	
		G/G	140	67	1.19 (0.84-1.68)	0.322	
		G	5,208	2,467	1 (-)	-	
		C	816	435	**0.87 (0.76-0.99)**	**0.036**	
IRF4	rs12211228	G/G	2,257	1,051	1 (-)	-	0.518
		G/C	694	365	0.87 (0.74-1.03)	0.118	
		C/C	61	35	0.72 (0.44-1.17)	0.187	
		G	3,368	2,109	1 (-)	-	
		A	2,628	1,727	0.99 (0.91-1.09)	0.906	
VEGFA	rs10434	G/G	961	575	1 (-)	-	0.777
		G/A	1,446	959	1.00 (0.86-1.16)	0.979	
		A/A	591	384	1.03 (0.85-1.24)	0.788	
		A	4,661	2,321	1 (-)	-	
		C	1,189	549	1.05 (0.93-1.18)	0.44	
KRAS	rs11047885	A/A	1,859	941	1 (-)	-	0.315
		A/C	943	439	1.06 (0.90-1.25)	0.464	
		C/C	123	55	1.01 (0.69-1.50)	0.938	
		G	3,186	1,571	1 (-)	-	
		T	2,578	1,271	0.98 (0.89-1.07)	0.623	
KRAS	rs12587	G/G	921	432	1 (-)	-	0.637
		G/T	1,344	707	0.91 (0.77-1.08)	0.271	
		T/T	617	282	1.04 (0.85-1.28)	0.694	

[a] All analyses are adjusted for age, sex, and country of origin. Results showing p<0.05 are in bold.

Figures

Figure 1. *IL10*-rs3024496 polymorphism affects IL-10 expression levels. **[A]** Bar chart showing the fold change between the average relative fluorescent units (RFU) of cells transfected with the GFP_IL10_A or GFP_IL10_G. The asterisk indicates a statistically significant difference (p-value = 0.019; fold change = 0.94). **[B]** Violin plot showing the effect of the rs3024496 variant on the mRNA levels of *IL10* in the whole blood of 670 healthy subjects according to GTEx Portal (p-value = 0.0017; normalized effect size = 0.12).



Supplementary table 1. SNPs in 3'UTR of candidate genes selected for genotyping.

Gene	SNP	Chromosome	Position (GRCh38)	Alleles (M/m)[a]	Ancestral allele	MAF[a]
IL10	rs3024496	1	206,768,519	G/A	G	0.48
SPP1	rs1126772	4	87,983,034	A/G	A	0.19
IRF4	rs12211228	6	408,833	G/C	G	0.16
VEGFA	rs10434	6	43,785,475	G/A	G	0.42
KRAS	rs11047885	12	25,189,696	A/C	C	0.27
KRAS	rs12587	12	25,205,894	G/T	G	0.47

[a] M: major allele; m: minor allele; MAF: minor allele frequency. All values are referred to the Caucasian (CEU) population of the 1000 Genomes project.

Supplementary table 2. SNPs in LD with *IL10*-rs3024496.

SNP	Chr	Position (GRCh38)	Alleles	MAF[a]	Distance	D'[a]	r^{2a}	GTEx p-value[b]	Regulome rank
rs3024502	1	206,766,965	C/T	0.48	-1,554	1.00	0.98	$5.0x10^{-5}$	5
rs3024500	1	206,767,486	A/G	0.48	-1,033	1.00	1.00	$4.7x10^{-5}$	3a
rs1800896	1	206,773,552	C/T	0.48	5,033	1.00	1.00	$6.0x10^{-5}$	6
rs2222202	1	206,772,036	A/G	0.48	3,517	1.00	1.00	$4.6x10^{-5}$	4
rs3024491	1	206,771,701	C/A	0.48	3,182	1.00	1.00	$4.6x10^{-5}$	5
rs1878672	1	206,770,368	G/C	0.48	1,849	1.00	1.00	N/A	4
rs11119514	1	206,777,263	T/C	0.47	8,744	1.00	0.98	N/A	7
rs1800893	1	206,773,822	C/T	0.48	5,303	1.00	0.98	N/A	6

[a] Minor allele frequency and LD measures (D' and r^2) in the Caucasian (CEU) population of the 1000 Genomes project.

[b] P-value of the association between the SNP and level of expression of *IL10* in whole blood. The data used for the analyses described in this manuscript were obtained from: GTEx Analysis Release V7, acccessed on 31/10/2018 (https://gtexportal.org/home/). LD data were obtained from LDLink (https://ldlink.nci.nih.gov/?var=rs3024496&pop=CEU&r2_d=r2&tab=ldproxy).