Regulation of HLA class I expression by non-coding gene variations

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Abstract

The Human Leukocyte Antigen (HLA) is a critical genetic system for different outcomes after solid organ and hematopoietic cell transplantation. Its polymorphism is usually determined by molecular technologies at the DNA level. A potential role of HLA allelic expression remains under investigation in the context of the allogenic immune response between donors and recipients. In this study, we quantified the allelic expression of all three HLA class I loci (HLA-A, B and C) by RNA sequencing and conducted an analysis of expression quantitative traits loci (eQTL) to investigate whether HLA expression regulation could be associated with non-coding gene variations. HLA-B alleles exhibited the highest expression levels followed by HLA-C and HLA-A alleles. The max fold expression variation was observed for HLA-C alleles. The expression of HLA class I loci of distinct individuals demonstrated a coordinated and paired expression of both alleles of the same locus. Expression of conserved HLA-A~B~C haplotypes differed in distinct PBMC’s suggesting an individual regulated expression of both HLA class I alleles and haplotypes. Cytokines TNFα/IFNβ, which induced a very similar upregulation of HLA class I RNA and cell surface expression across alleles did not modify the individually coordinated expression at the three HLA class I loci. By identifying cis eQTLs for the HLA class I genes, we show that the non-coding eQTLs explain 29%, 13%, and 31% of the respective HLA-A, B, C expression variance in unstimulated cells, and 9%, 23%, and 50% of the variance in cytokine-stimulated cells. The eQTLs have significantly higher effect sizes in stimulated cells compared to unstimulated cells for HLA-B and HLA-C genes expression. Our data also suggest that the identified eQTLs are independent from the coding variation which defines HLA alleles and thus may be influential on intra-allele expression variability although they might not represent the causal eQTLs.

Author summary

In transplantation, the allogenic immune response is linked to the HLA compatibility between donor and recipient, HLA genes being highly polymorphic. The impact of this allelic polymorphism on MHC cell surface expression and the potential role of expression...
in the T cell activation and on clinical outcomes remains poorly investigated. In this study, we documented the individual variability of allelic HLA class I expression in PBMCs by RNA sequencing. To mimic the inflammatory clinical situation after transplantation, we performed a similar analysis following cytokine (TNFα/IFNβ) stimulation of PBMCs. The results demonstrated a coordinated and paired expression of both alleles of the same locus in all individuals. The levels of expression of matched HLA-A-B-C haplotypes differed in distinct PBMCs suggesting that expression of both HLA class I alleles and haplotypes is regulated individually. To identify cis regulatory elements of expression we performed an eQTL analysis on unstimulated and stimulated PBMCs. These eQTLs accounted for up to 9%, 23% and 50% of the respective HLA-A, B and C expression variance in stimulated PBMCs. Of interest, we could show that they are independent from the SNPs encoding allelic variation, meaning that they could explain a different portion of the variance in HLA expression.

Introduction

HLA class I molecules are expressed constitutively on nucleated cells and function as antigen presenting molecules to cytotoxic T cells in immune responses to pathogens, cancer cells and autoantigens [1]. They also regulate the innate immune response by influencing NK cell activation [2]. HLA disparities between donors and patients induce allogenic immune responses, leading to rejection or graft versus host disease (GVHD) in different transplantation settings [3]. HLA class I molecules are characterized by an extremely high polymorphism and variable levels of expression [4–7], which potentially influence their function(s). For instance, tumor cells downregulate HLA expression to escape immune-surveillance by T cells [8,9]. In vitro allogenic immune responses were previously shown by us and others [10–12] to be partially dependent on HLA-C expression. Intra- and inter-individual variation of HLA cell surface expression on T and B cells was also described to impact antibody dependent cytotoxic immune response [13]. More recently, HLA-expression was also shown to impact crossmatch outcomes in transplantation diagnostic [14]. Two retrospective clinical studies tested the impact of HLA expression on clinical outcome in the setting of HLA-C mismatched unrelated hematopoietic stem cell transplantation (HSCT). Both studies used as proxy of HLA-C expression the mean cell surface expression reported by Apps et al. [4]. While Petersdorf et al [15] found an association between highly expressed HLA-C*03 or C*14 allotypes and increased mortality, Morishima et al [16] did not. In other clinical settings, a high HLA-C expression was correlated to more efficient recognition of HIV by cytotoxic cells and lower viremia in patients [4,17], while lower levels of HLA-C expression had a protective effect from Crohn’s disease [7]. Similarly, higher HLA-B*27 expression levels were reported in patients with ankylosing spondylitis compared to healthy donors [18].

HLA expression regulation underlies complex mechanisms, involving genetic polymorphisms as well as transcriptional and translational factors [19–23]. To date, no consensus explanation exists for the variable levels of HLA expression that are observed [24].

Initial reports on HLA class I expression were based on cell surface and gene expression and relied on allotypes, without the possibility to discriminate between the two alleles carried by heterozygous donors at a given locus [4,7,17,25,26]. The advent of more recent technologies using RNA sequencing has allowed qualitative, quantitative and equivalent inter-allelic expression analyses [14,27–30].
An extended analysis of single nucleotide polymorphisms (SNPs) in the non-coding regions of HLA genes, which tag transcript abundance (expression quantitative traits loci, eQTLs) was performed by Aguiar et al. in lymphoblastic cells of the GEUVADIS consortium [28]. They identified HLA-C associated eQTL’s in strong linkage disequilibrium with the previously described genetic variants rs9264942 and rs2395471 and deletion (263del/ins) [17,31,32]. Vandiedonck et al. 2011 [33] analyzed the specific transcriptional variation of haplotypes associated with autoimmune diseases in three known homozygous cell lines PGF, COX and QBL.

In the present study, HLA class I allelic RNA expression, analyzed in peripheral blood mononuclear cells (PBMCs) of healthy blood donors, was quantified using RNA sequencing. To mimic clinical situations with inflammation driven by events such as alloreactivity, autoimmunity or infection, the analyses were performed on PBMCs before and after stimulation with the pro-inflammatory cytokines Tumor Necrosis Factor alpha (TNFα) and Interferon beta (IFNβ). Furthermore, we investigated whether variations in the non-coding genome (eQTLs) affect HLA class I expression. The eQTLs were first analyzed on T cells (169 samples) from the Blueprint consortium and then compared to the ones retrieved in PBMCs of the current study (54 samples), before and after stimulation.

Results

HLA class I expression at the allelic and individual levels

To evaluate and compare the expression of HLA class I genes, RNA from 63 healthy blood donors was isolated from PBMCs and sequenced. Expression levels for the alleles detected in our cohort are provided in Fig 1A. The highest mean expression was measured for HLA-B alleles (1870±541 transcripts per million, tpm) followed by HLA-C (1238±532 tpm) and HLA-A (866±224 tpm) alleles. Variation in expression was also the highest among HLA-B alleles with a maximum fold variation ratio of 7.86 between HLA-B*18:01 and B*56:01 which comprise the highest and lowest tpm values, respectively. The expression of HLA-A alleles was more even with a ratio of 3.2 between the highest (A*01:01) and lowest (A*31:01) tpm values.

HLA-C was intermediate with a ratio of 5.2 between the highest (C*04:01) and lowest (C*01:02) tpm values. A more conservative comparison of expression between alleles using median values showed the highest fold ratio for HLA-C (2.7) between C*14:02 and C*03:03, followed by HLA-B (2.0) between B*13:02 and B*56:01 and HLA-A (1.8) between A*33:03 and A*31:01. Expression according to HLA antigens is shown in S1 Fig.

HLA expression per locus and per donor is represented in Fig 1B. A coordinated expression of both alleles is clearly visible in each heterozygous donor for the three HLA class I genes. The intra-individual variation of expression was the lowest at HLA-A and the highest at HLA-C. The intra-individual variation at HLA-B was also low except in two donors. Along this line, a high correlation between pairs of alleles in heterozygotes at HLA-A, B and C and a slightly lower correlations at HLA-C was measured (HLA-A: spearman ρ = 0.66 p = 3.6x10⁻⁷, HLA-B: ρ = 0.67 p = 1.0x10⁻⁶ and HLA-C: ρ = 0.51 p = 1.7x10⁻⁵) (S2 Fig). Calculation of allele specific expression (ASE) as the ratio of the lowest expressed allele towards the total expression of both alleles at a given locus in each individual revealed a very balanced pattern of expression with median values of 0.47, 0.47 and 0.44 for HLA-A, B and C, respectively (Fig 2A). For one individual, the ASE was exceptionally low (i.e., 0.1) due to the low expression of one HLA-B*56:01 allele. Furthermore, in order to test whether the balanced pattern of expression of HLA class I alleles was different from what could be expected by chance, we applied a resampling procedure. The empirical distributions of the simulated ASE obtained after 1000 permutation replicates are shown in Fig 2B (see the figure legend for more details). The observed ASE at
HLA-A, B and C are significantly closer to the maximum possible balance of expression (i.e., 0.5) than any of the replicates (p-values $< 0.001$).

**Upregulated HLA expression**

To further investigate the influence of pro-inflammatory cytokines on HLA expression, PBMCs of 56 blood donors were stimulated overnight without or with the cytokines TNF$\alpha$ and IFN$\beta$. Their respective expression was determined and is shown in Fig 3. Mean expression of HLA-A, B and C alleles was upregulated to a similar extent (Fig 3A). Mean fold upregulation
was 2.5, 2.04 and 2.11 for the tested HLA-A, B and C alleles, respectively, with spearman ρ coefficient of 0.4, 0.31 and 0.58 and p-values of 2.9x10^{-4}, 1.0x10^{-3} and 2.2x10^{-16}. The detailed analysis of the upregulation per allele is shown in S3 Fig and revealed only a few possible outliers. Namely, HLA-A* 29:02, 30:01, 32:01 and HLA-C* 16:01 had higher fold expression of 4.1, 3.4, 3.5 and 2.7, respectively, while HLA-B* 15:01 had a lower fold expression of 1.3 following stimulation. Otherwise, although the results were gathered from 56 different PBMC cultures performed at different times, a very homogenous and conserved upregulation of expression was observed among alleles. In accordance with RNA expression, cell surface expression of the corresponding HLA-class I molecules was upregulated to a similar extent with a ratio of mean fold upregulation of 1.6, as determined by flow cytometry (Figs 3B and S4). The Spearman correlation for HLA cell surface expression between cells stimulated or non-stimulated with
cytokines was 0.84 with a p-value of $1.0 \times 10^{-16}$.

The coordinated expression of alleles in heterozygous donors persisted after induced upregulation (Figs 3C and S5). Moreover, the balance of expression among alleles within individuals, as measured by ASE, was similar to the data previously shown in Fig 2 for the uncultured PBMCs, with median ASE comprised between 0.45 and 0.5.
and 0.48 in unstimulated and stimulated PBMCs (Fig 3D). Upregulation through overnight cytokines stimulation did not change the much conserved balance of expression for HLA-B and C or even slightly reinforced it as observed for HLA-A (paired Wilcoxon rank sum test, p-value < 0.001, median ASE for unstimulated PBMCs = 0.467, median ASE for stimulated PBMCs = 0.483).

**Allelic expression in HLA-A~B~C~DRB1 haplotypes**

The RNA expression measured at the allelic level in PBMCs was grouped according to the expected segregation of alleles on three common HLA haplotypes (i.e. inferred at least five times in our cohort and estimated with a frequency above 1% in a large Swiss cohort [34]). The three common haplotypes considered were HLA-A*01:01~B*08:01~C*07:01~DRB1*03:01, HLA-A*03:01~B*07:02~C*07:02~DRB1*15:01 and HLA-A*02:01~B*07:02~C*07:02~DRB1*15:01. The RNA expression of alleles within the common haplotypes in freshly isolated PBMCs or in cells kept in culture without stimulation overnight are shown in the upper panels of Fig 4. A stacked expression was seen across loci for alleles belonging to the same haplotype. In contrast,

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| HLA-A*01:01 ~ B*08:01 ~ C*07:01 ~ DRB1*03:01 | HLA-A*03:01 ~ B*07:02 ~ C*07:02 ~ DRB1*15:01 | HLA-A*02:01 ~ B*07:02 ~ C*07:02 ~ DRB1*15:01 |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| ![Graph](https://doi.org/10.1371/journal.pgen.1010212.g004) | ![Graph](https://doi.org/10.1371/journal.pgen.1010212.g004) | ![Graph](https://doi.org/10.1371/journal.pgen.1010212.g004) |

Fig 4. Allelic expression in PBMC carrying common HLA haplotypes. The upper panels represent the RNA expression (in tpm) of alleles segregating on three common HLA haplotypes as indicated. In the lower panels, the RNA expression (in tpm) of the alleles belonging to the second haplotype is shown. Each individual carrying a given haplotype is shown with a different color code. Straight and dotted lines correspond to RNAs of cells kept in culture without stimulation overnight or of freshly isolated PBMCs, respectively. The HLA-typing of the second haplotype differ between samples sharing a common haplotype. Small facets represent the pairwise linear regression of expression between loci. The standard error of the regression is indicated as grey shading.

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the corresponding expression of alleles on the second carried haplotype was less concordant as these haplotypes differed among individuals sharing the same common haplotype (Fig 4 lower panels). This is also suggested by the broader standard errors of regression between pairs of loci for the second carried haplotype when compared to the common one (Fig 4, small facets).

**Expression quantitative loci analysis of the HLA class I region**

Since the PBMC dataset generated in this study has a small sample size, eQTL analyses were carried out also on an external, larger, dataset comprising T cells from the Blueprint consortium (www.blueprint-epigenome.eu).

Genome-wide significant eQTLs (FDR = 5%, Table 1) were identified in 294, 428, and 7502 genes in unstimulated PBMCs, stimulated PBMCs, and Blueprint T cells, respectively. There are no genome-wide significant eQTLs for any of the HLA class I genes in unstimulated PBMCs. Stimulated PBMCs have a genome-wide significant eQTL for HLA-B (6:31370329:C:A, p = 2.6e-7) and HLA-C (6:31243785:G:T, p = 4.1e-9) genes. In Blueprint T cells, 2, 3, and 2 independent eQTLs for HLA-A, B, and C, were identified respectively (Table 2 and Fig 5). The nominal p-values of the T-cell eQTLs in PBMCs for the best eQTL variant per independent signal are shown in Fig 6A. The p-values of the best eQTL variant in PBMCs that correspond to one of the significant variants in T cell independent signals are shown in Fig 6B. Two SNPs seen as significant in T cells were also seen in unstimulated PBMCs as significant (Fig 6A) and the best SNP’s seen in unstimulated and stimulated PBMCs are also seen in T cells (Fig 6B). We tested how well all T-cell eQTLs are replicated in the PBMCs using the $\pi_1$ statistic ([35]) and estimated that 34% are active in unstimulated PBMCs, 38% are active in stimulated PBMCs, and 38% are active in stimulated PBMCs. To perform the aforementioned $\pi_1$ statistic the best eQTL found in stimulated PBMC which corresponds to one of the most significant eQTLs of the Blueprint T-cell were chosen.

The eQTLs identified account for 29%, 13%, and 31% of the variance in HLA-A, B, C expression in unstimulated cells, respectively, and 9%, 23%, and 50% of the variance in stimulated cells. Note that as described in Table 2, there are no significant eQTLs for the unstimulated cells and HLA-A is not significant for the stimulated cells. In order to test whether this variance is mostly independent of the variance of the coding variants that determine an individual’s HLA allele, the pairwise correlation was calculated between the eQTL variant and the coding variants for the HLA alleles found in 1000 European genomes of this study. The correlation between the eQTLs and the coding variants that dictate HLA-alleles are low (S6 Fig) indicating that the intra-HLA allele expression variance may be explained by the non-coding variants involved in the regulation of HLA class I gene expression.

Lastly, significant difference in the effect sizes of HLA class I eQTLs between unstimulated and stimulated PBMCs was assessed. We used a linear mixed model to check the interaction between genotype and stimulation status of the samples. We observed that one, two and two eQTLs for HLA-A, B and C, respectively, show significantly higher effect sizes in stimulated cells vs. unstimulated cells (Fig 7). For one signal in HLA-B and all signals in HLA-C significant interaction p-values were observed, indicating that there is a significant difference between the effect sizes of these variant in unstimulated vs. stimulated cells.

| Table 1. Cis eQTL |
|-------------------|
| Cell Type | No of Samples | No of Genes | eQTLs FDR 5% |
|--------------------------------------------------|
| PBMC unstimulated | 55 | 19117 | 294 |
| PBMC stimulated | 55 | 18737 | 428 |
| Blueprint T Cells | 169 | 15488 | 7202 |

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Discussion

HLA expression at the cell surface might influence the alloreactivity induced in transplantation when T cells recognize non-self HLA. Indeed, Petersdorf et al [15] found an association between highly expressed HLA-C allotypes and increased mortality, while Morishima [16] et al did not. Likewise, high expression of HLA class II (HLA-DPB1) antigens is reported to be a risk factor for developing acute graft versus host disease in HSCT [36–38].

In this study, we quantified allelic expression of the three HLA class I genes on an individual basis by RNA sequencing and analyzed whether non-coding genome variations like cis eQTL's could explain the observed levels of expression in PBMCs. HLA expression in T cells of the Blueprint Consortium was additionally investigated to increase sample size and the upregulated expression in cytokine-stimulated PBMCs was measured to allow a better balance of the results towards expression. We focused on HLA class I alleles only, as they are constitutively expressed in all PBMCs in contrast to HLA class II alleles, which are only constitutively expressed in specific cell types such as B cells and professional antigen presenting cells. HLA class II allele expression might thus be dependent on subpopulation distribution and cell activation status in PBMCs. Note that Yamamoto et al [29] as well as Johansson et al [30] did not consider this caveat when they analyzed HLA class II expression by RNA-Seq capture methodology in PBMC samples.

Cis regulating elements associated to HLA-DQB1 allele-specific expression variability after autoimmune T-cell activation were reported by Gutierrrez et al [39]. In contrast to their publication, we were not able to define dynamic ASE (dynASE), which can be associated with HLA expression. We speculate that the power to detect these dynASE sites could be due, on one side, to the difference between HLA class I and HLA class II expression specificity and, on the other side, to the time course experiment they performed with 8 time points, which we do not have in our cohort.

Our results confirm the variability of expression among HLA class I alleles as previously reported [5,14,28,30,40–42]. Nevertheless, considering the mean expression of alleles at the locus level, our data are not in agreement with other publications classifying HLA-C mRNA expression as the lowest [30,43]. Furthermore, the levels of expression that we observe across distinct alleles differ from the values reported in some publications [5,30,40,42], while they concord, with the results published by Garcia-Romano et al [44] and Yamamoto et al [29].
Fig 5. The genomic locations of the eQTLs. The genomic locations of the eQTL associations in the cis regions (± 1 Mb from the transcription start site) of HLA-A, HLA-B, and HLA-C. The x-axes are the relative position of the variants to the TSS, and the y-axes represent the significance of the eQTL association (-log10 nominal p-value). The colored bars show all the variants in the region and their eQTL p-values in stimulated and unstimulated PBMC cells. The colored points represent all the variants for each of the genome-wide significant independent eQTL signals in Blueprint T cells.

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who suggest like us that HLA-B alleles possess the highest and HLA-A alleles the lowest mean expression, respectively. Due to the limited numbers of samples analyzed, the study was not designed to evaluate the specific expression of alleles at each locus. Lee et al [42] reported expression of allele with more than 10 individuals per allele in the GEUVADIS cohort. Nevertheless, if we classify the alleles as high or low expressers, we obtain similar subgroups to the ones reported by others [4,29,42]. For example, the highest/lowest mean expression among HLA-C alleles was seen for HLA-C*04 and 14 and C*03 (Fig 1), respectively. While the lowest HLA-B expression was seen for the HLA-B*56 allele. This steadiness of expression at the allelic level suggests that allele-specific regulation might exist alongside the individual-dependent regulation of expression discussed in the next paragraph. Indeed, concerning HLA-C alleles, Aguiar et al [28] showed that the rs 41561715-T SNP was associated to the HLA-C*04 lineage in lymphoblastoid cells. In our study, this SNP has a p-value of 3.1175e-06 and 0.0382403 for HLA-C in stimulated and unstimulated PBMCs, respectively. Unfortunately, we cannot comment further on lineage specific effects, as we do not have great enough sample size.

In all three cell cohorts used in this study (i.e., freshly isolated PBMCs, PBMCs kept overnight in culture medium with or without TNFα and IFNβ), we observed a coordinated HLA class I expression at the individual level rather than an allele-specific regulation. Indeed, calculation of allele-specific expression (ASE), measured as the proportion of the lowest expressed allele per locus per individual, revealed a very balanced and conserved expression of both alleles in heterozygotes with ratio close to 0.5 in uncultured cells as well as in cells cultured overnight with or without pro-inflammatory cytokines. The suggested individual regulated expression is sustained by high correlation and a very low variance of expression between paired alleles of the same PBMC sample. A random assignment of allelic pairs by permutations (even when constraining the permutations to keep the same HLA typing within each individual) showed that the observed ASE is significantly closer to the maximum of 0.5 (i.e., representing a fully balanced expression of both alleles) than any of the permutation replicates. The coordinated expression of alleles from distinct HLA class I loci in a given individual was best seen in Fig 3 when alleles belonging to the same haplotype were compared. This confirms our previous observation suggesting an association between HLA-C expression and extended HLA haplotypes [6]. The low number of samples that share the same haplotype(s) did not allow analyzing whether haplotype-specific regulating non-coding elements (eQTL’s) exists. Moreover, independently from haplotype segregation, eQTL analysis did not reveal variants affecting the bulk expression of the three HLA class I genes together. Each locus seemed thus to have his
own regulatory transcription elements. The coordinated expression at the individual level observed in this study is also in agreement with previous studies of Vandidonck et al [33] and Lam et al. [45], who reported HLA haplotype-specific regulation of gene expression in distinct blastoid cell lines. Gene clustering and cis regulatory domains were proposed to explain the
allelic co-expression. [45,46]. However, no higher co-expression of alleles within the same haplotypes compared to alleles on different haplotypes were reported by Aguiar et al [28]. Nevertheless, when they considered allele-specific expression (ASE), as we do in Figs 2 and 3, they observed a rather balanced expression profile for HLA-A, B and C alleles. This sustains our results suggesting the paired expression of HLA class I alleles in most individuals.

We are aware that analysis on different cohorts, namely T cells from the Blueprint consortium and PBMC’s represent different cell type composition and might not be directly comparable. Indeed, eQTLs were shown to be possibly related to differences in cell type composition across individuals [47]. Nevertheless, the eQTL association patterns we observed in T cells are similar to the eQTL association patterns in stimulated PMBCs for HLA-B and HLA-C genes. In addition, as seen in Fig 7, two SNPs (6:31274027 and 6:31243785) were best predictor of HLA C expression. Overall, stimulated cells show stronger effect sizes when compared to unstimulated cells, suggesting that the effect of eQTLs on expression would be magnified in an active immune system.

In conclusion, our data showed that non-coding variations regulating HLA class I genes could be independent of the coding variations that define alleles. As the eQTLs identified in PBMCs mostly have low effect sizes, they may only imperfectly capture the true signals. Thus, confirmatory data on a larger cohort are warranted. More importantly, however, our results demonstrated a coordinated and paired expression of both alleles of the same locus in each individual, which is maintained under conditions of inflammation. Although our study was not designed to answer to this question, the poor correlation between eQTL and distinct HLA allelic expression could suggest that allelic regulation is mediated by sequences, which have not been preferentially selected during evolution. Only a larger sample size may allow the assignment of specific non-coding variants, which would be directly responsible for the intra-allelic variation in expression.

Methodology

Ethic statement

The studies involving human participants were reviewed and approved by Ethical committee of University Hospitals of Geneva (CER 06–208 and 08–208R). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Cells

Peripheral blood mononuclear cells (PBMCs) were purified using standard Ficoll procedure from blood collected from healthy donors who were genotyped at loci HLA-A, B, C, DRB1, DRB3/4/5, DQB1 and DPB1 at high resolution by the Swiss National Reference Laboratory for Histocompatibility (LNRH), while searching for potential unrelated HSC donors.

To upregulate HLA expression, PBMCs were incubated in RPMI 1640 culture medium (Gibco) supplemented with 10mM L-glutamine 100 units/ml penicillin /streptomycin (Gibco) and 10% human AB serum (own preparation) overnight with 50 ng/ml TNFα and 100 ng/ml IFNβ (PrepoTech, London, UK) prior to RNA extraction.

Immunofluorescence

HLA cell surface expression was determined on CD3+ T cells using the monoclonal antibodies APC-labeled anti-human CD3 (clone BW264/56) and FITC-labeled anti-HLA-ABC (clone REA230) (Milteny Biotec) and their corresponding isotype controls. Data acquisition was
performed on gated mononuclear cells, using the ACCURI-C6 cytometer (BD) and the CFLOWPLUS analysis software (BD Bioscience, Allschwil, Switzerland).

**DNA extraction and high-resolution HLA typing**

DNA was extracted on an automatic system (QIAGEN GmbH, Hilden, Germany) from Ficoll purified peripheral blood mononuclear cells (PBMCs). HLA typing was performed by reverse PCR-sequence-specific oligonucleotide microbeads arrays and high throughput sequencing (One Lambda, Canoga Park, USA).

**RNA extraction**

Total RNA was extracted using the RNeasy Micro kit (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions.

**Genotyping and imputation**

The genotyping of the samples was conducted with the Illumina Infinium Global Screening Array v2.0. The genotype calls were done using Illumina GenomeStudio 2.0. We filtered variants with minor allele frequency (MAF) less than 1%, genotype missingness greater than 2.5%, and Hardy-Weinberg equilibrium (HWE) p-value less than 1e-5. This resulted in 484886 variants which were imputed using the Michigan Imputation Server with the HRC (Version r1.1 2016) reference panel. Post-imputation filters, $r^2$ less than 0.3, MAF less than 1%, and HWE p-value less than 1e-6 were applied, resulting in 7924221 variants. Principle component analysis of the samples, together with 1000 genomes samples [48] revealed that 2 samples have non-European ancestry, thus were excluded from further analyses. The human reference genome build used was GRCh37.

**RNA-sequencing and quality control**

RNA-sequencing was conducted on Illumina HiSeq 4000, according to manufacturer’s instructions. The RNA-seq QC was conducted according to Lappalainen T [49]. We also assessed the matching between the genotypes and the RNA-seq experiments using QTL tools mbv [50]. We observed low concordance at heterozygous sites (< 80%) for 6 samples, which were excluded from downstream analyses.

**HLA- allele-specific mRNA expression**

The RNA sequencing method has previously extensively been discussed by Aguiar et al [28]. Briefly we used HLApers to create a personalized genome based on HLA alleles, mapped the RNA-seq reads against these genomes using STAR [51], and then quantified gene expression using Salmon [52] including allele specific HLA transcripts. The human reference genome build used was GRCh37, and gene annotation used was GENCODE v33 lifted over to b37GENCODE reference annotation for the human and mouse genomes [53]. We excluded genes that were not quantified in more than half of the samples.

**eQTL analyses**

All analyses were conducted with QTLtools [46]. We calculated population principal components (PCs) from genotypes and technical RNA-seq covariate PCs, using QTLtools pca, and all PCs were centered and scaled. The number of RNA-seq was chosen such that they maximized the number of cis eQTLs genome-wide. We used 5 RNA-seq PCs and 3 population PCs, as
technical covariates in PBMCs, and 30 RNA-seq PCs and 3 population PCs in T cells. Cis eQTL analysis was conducted using QTLtools cis with 1000 permutations.

**Graphs**
Graphs were generated using R version 4.0.2.

**Statistics**
Statistical paired *t* tests were performed with R version 4.0.2 and GraphPad prism version 8.0.

**Supporting information**

**S1 Fig. Gradient expression of HLA antigens.** HLA class I RNA expression measured in samples of PBMCs obtained from 63 individuals is plotted as tpm (transcript per million). Alleles are grouped according to their serological specificity (i.e., HLA-A, B and C antigens, as indicated on the horizontal axis). Each dot represents the expression of a given allele/antigen in one individual.

(TIF)

**S2 Fig. Correlation of expression between pairs of HLA alleles.** The RNA expression of pairs of HLA -A, B and C alleles measured in 63 different PBMC samples are plotted as transcript per million (tpm) against each other. The Spearman coefficient *ρ* and associated *p*-value are indicated.

(TIF)

**S3 Fig. TNFα/IFNβ induced HLA class I upregulation per allele.** HLA class I RNA expression measured in 56 different PBMC samples stimulated with or without TNFα+IFNβ overnight is plotted for each HLA-A, B and C allele taken individually. The numbers in the plots represent median fold upregulation ratios. Alleles are given on the top of each plot.

(TIF)

**S4 Fig. Cytofluorometric gating strategy of TNFα/IFNβ induced HLA class I upregulation.** HLA cell surface expression on gated CD3⁺ lymphocytes (upper panels) from PBMC stimulated over night without (grey histograms) or with TNFα/IFNβ (light blue histograms). HLA class I typing of the corresponding PBMC’s are: HLA-A*03:01,32:01* HLA-B*08:01,44:03* HLA-C*04:01,07:01* (left lower panel), HLA-A*03:01,24:02* HLA-B*07:02,38:01* HLA-C*07:02,12:03* (middle lower panel), HLA-A*02:01,24:02* HLA-B*39:06,44:02* HLA-C*05:01,07:02* (right lower panel). Corresponding mean fluorescence intensities (MFI) are indicated.

(TIF)

**S5 Fig. Correlation of expression between pairs of HLA alleles.** The RNA expression of pairs of HLA -A, B and C alleles measured in 56 different PBMC samples stimulated with or without TNFα+IFNβ overnight are plotted against each other. The Spearman coefficient *ρ* is indicated.

(TIF)

**S6 Fig. The correlation between the non-coding eQTL variants and the coding variants defining alleles.** The correlation (*r²*, calculated from 1000 genomes European samples) between the best PBMC variants (indicated on the top of the panels) corresponding to a T-cell independent eQTL signal and all the coding variants responsible for an individual’s allele type of HLA-A, HLA-B, and HLA-C.

(TIF)
Author Contributions

Conceptualization: Florence Bettens, Emmanouil Dermitzakis, Jean Villard.

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References

1. Klein J, Sato A. The HLA system. First of two parts. N Engl J Med. 2000; 343(10):702–9. Epub 2000/09/07. https://doi.org/10.1056/NEJM200009073431006 PMID: 10974135.

2. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005; 5(3):201–14. https://doi.org/10.1038/nri1570 PMID: 15719024.

3. Petersdorf EW. Genetics of graft-versus-host disease: the major histocompatibility complex. Blood Rev. 2013; 27(1):1–12. Epub 2012/11/28. https://doi.org/10.1016/j.bbre.2012.10.001 PMID: 23182478; PubMed Central PMCID: PMC3626414.

4. Apps R, Qi Y, Carlson JM, Chen H, Gao X, Thomas R, et al. Influence of HLA-C expression level on HIV control. Science. 2013; 340(6128):87–91. Epub 2013/04/06. https://doi.org/10.1126/science.1232685 PMID: 23599252; PubMed Central PMCID: PMC3784322.

5. Ransuran V, Hernandez-Sanchez PG, O’Huigin C, Sharma G, Spence N, Augusto DG, et al. Sequence and Phylogenetic Analysis of the Untranslated Promoter Regions for HLA Class I Genes. J Immunol. 2017; 198(6):2320–9. https://doi.org/10.4049/jimmunol.1601679 PMID: 28148735; PubMed Central PMCID: PMC5340644.

6. Bettens F, Brunet L, Tiercy JM. High-allelic variability in HLA-C mRNA expression: association with HLA-extended haplotypes. Genes Immun. 2014; 15(3):176–81. https://doi.org/10.1038/gene.2014.1 PMID: 24500399.

7. Kulkarni S, Qi Y, O’Huigin C, Pereyra F, Ransuran V, McLaren P, et al. Genetic interplay between HLA-C and MIR148A in HIV control and Crohn disease. Proc Natl Acad Sci U S A. 2013; 110(51):20705–10. https://doi.org/10.1073/pnas.1312237110 PMID: 24248364; PubMed Central PMCID: PMC3870724.

8. Bubenik J. Tumour MHC class I downregulation and immunotherapy (Review). Oncol Rep. 2003; 10(6):2005–8. Epub 2003/10/10. PMID: 14534734.

9. Esteban F, Concha A, Delgado M, Pérez-Ayala M, Ruiz-Cabello F, Garrido F. Lack of MHC class I antigens and tumour aggressiveness of the squamous cell carcinoma of the larynx. Br J Cancer. 1990; 62(6):1047–51. Epub 1990/12/01. https://doi.org/10.1038/bjc.1990.437 PubMed Central PMCID: PMC1971557. PMID: 2257212.

10. Bettens F, Buhler S, Tiercy JM. Allorecognition of HLA-C Mismatches by CD8+ T Cells in Hematopoietic Stem Cell Transplantation Is a Complex Interplay between Mismatched Peptide-Binding Region Residues, HLA-C Expression, and HLA-DPB1 Disparities. Front Immunol. 2016; 7:584. https://doi.org/10.3389/fimmu.2016.00584 PMID: 28018351; PubMed Central PMCID: PMC5151176.

11. Bettens F, Calderin Sollet Z, Buhler S, Villard J. CD8+ T-Cell Repertoire in Human Leukocyte Antigen Class I-Mismatched Alloreactive Immune Response. Front Immunol. 2020; 11:588741. https://doi.org/10.3389/fimmu.2020.588741 PMID: 33552048; PubMed Central PMCID: PMC7856301.

12. Israeli M, Roelen DL, Carrington M, Petersdorf EW, Claas FH, Haasnoot GW, et al. Association between CTL Precursor Frequency to HLA-C Mismatches and HLA-C Antigen Cell Surface Expression. Front Immunol. 2014; 5:547. Epub 2014/11/12. https://doi.org/10.3389/fimmu.2014.00547 PMID: 25386189; PubMed Central PMCID: PMC4209872.

13. Honger G, Krahenbuhl N, Dimoleo S, Stern M, Schaub S, Hess C. Inter-individual differences in HLA expression can impact the CDC crossmatch. Tissue antigens. 2015; 85(4):260–6. https://doi.org/10.1111/tan.12537 PMID: 25786570.
14. Montgomery MC, Liu C, Petraroa R, Weimer ET. Using Nanopore Whole-Transcriptome Sequencing for Human Leukocyte Antigen Genotyping and Correlating Donor Human Leukocyte Antigen Expression with Flow Cytometric Crossmatch Results. The Journal of molecular diagnostics: JMD. 2020; 22(1):101–10. Epub 2019/11/02. https://doi.org/10.1016/j.jmoldx.2019.09.005 PMID: 31669229.

15. Petersdorf EW, Gooley TA, Malkki M, Bacigalupo AP, Cesbron A, Du Toit E, et al. HLA-C expression levels define permissible mismatches in hematopoietic cell transplantation. Blood. 2014; 124(26):3996–4003. https://doi.org/10.1182/blood-2014-09-599699 PMID: 25323824; PubMed Central PMCID: PMC4271183.

16. Morishima S, Kashiwase K, Matsuo K, Azuma F, Yabe T, Sato-Otsubo A, et al. High-risk HLA alleles for severe acute graft-versus-host disease and mortality in unrelated donor bone marrow transplantation. Haematologica. 2016; 101(4):491–8. https://doi.org/10.3324/haematol.2015.136903 PMID: 26768690.

17. Thomas R, Apps R, Qi Y, Gao X, Maule V, O’Huigin C, et al. HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. Nat Genet. 2009; 41(12):1290–4. Epub 2009/11/26. https://doi.org/10.1038/ng.486 PMID: 19935663; PubMed Central PMCID: PMC2887091.

18. Cauil A, Dessole G, Fiorillo MT, Vacca A, Mameli A, Bitti P, et al. Increased level of HLA-B27 expression in ankylosing spondylitis patients compared with healthy HLA-B27-positive subjects: a possible further susceptibility factor for the development of disease. Rheumatology (Oxford). 2002; 41(12):1375–9. Epub 2002/12/07. https://doi.org/10.1093/rheumatology/41.12.1375 PMID: 12468816.

19. van den Elsen PJ. Expression regulation of major histocompatibility complex class I and class II encoding genes. Front Immunol. 2011; 2:48. Epub 2011/01/01. https://doi.org/10.3389/fimmu.2011.00048 PMID: 22506956; PubMed Central PMCID: PMC3342053.

20. Kobayashi KS, van den Elsen PJ. NLRC5: a key regulator of MHC class I-dependent immune responses. Nat Rev Immunol. 2012; 12(12):813–20. Epub 2012/11/24. https://doi.org/10.1038/nr3339 PMID: 23175228.

21. Jongsma MLM, Guarda G, Spaapen RM. The regulatory network behind MHC class I expression. Mol Immunol. 2019; 113:16–21. Epub 2017/12/12. https://doi.org/10.1016/j.molimm.2017.12.005 PMID: 29224916.

22. Carey BS, Poulton KV, Poles A. Factors affecting HLA expression: A review. Int J Immunogenet. 2019. Epub 2019/06/12. https://doi.org/10.1111/ijj.12443 PMID: 31183978.

23. Dersh D, Phelan JD, Gurnina ME, Wang B, Arbuckle JH, Holly J, et al. Genome-wide Screens Identify Lineage- and Tumor-Specific Genes Modulating MHC-I- and MHC-II-Restricted Immunosurveillance of Human Lymphomas. Immunity. 2021; 54(1):116–31.e10. Epub 2020/12/04. https://doi.org/10.1016/j.immuni.2020.11.002 PMID: 32771120; PubMed Central PMCID: PMC7874576.

24. Petersdorf EW, O’Huigin C. The MHC in the era of next-generation sequencing: Implications for bridging structure with function. Hum Immunol. 2019; 80(1):67–78. Epub 2018/10/16. https://doi.org/10.1016/j.humimm.2018.10.002 PMID: 30321633; PubMed Central PMCID: PMC6542361.

25. Ramsuran V, Kulkarni S, O’Huigin C, Yuichi K, Gao X, et al. Epigenetic regulation of differential HLA-A allelic expression levels. Human molecular genetics. 2015; 24(15):4268–75. https://doi.org/10.1093/hmg/ddv158 PMID: 25935001; PubMed Central PMCID: PMC4492392.

26. Gentle NL, Paximadis M, Puren A, Tiemessen CT. Genetic variability in markers of HLA-C expression in two diverse South African populations. PLoS One. 2013; 8(7):e67780. Epub 2013/07/19. https://doi.org/10.1371/journal.pone.0067780 PMID: 23961805; PubMed Central PMCID: PMC3705252.

27. Boegel S, Lower M, Bukur T, Sor P, Castle JC, Sahin U. HLA and proteasome expression body map. BMC Med Genomics. 2018; 11(1):36. https://doi.org/10.1186/s12920-018-0354-x PMID: 29587855; PubMed Central PMCID: PMC5872580.

28. Aguiar VRC, César J, Delaneau O, Dermitzakis ET, Meyer D. Expression estimation and eQTL mapping for HLA genes with a personalized pipeline. PLOS Genetics. 2019; 15(4):e1008091. https://doi.org/10.1371/journal.pgen.1008091 PMID: 31009447.

29. Yamamoto F, Suzuki S, Mizutani A, Shigenari A, Ito S, Kametani Y, et al. Capturing Differential Allele-Level Expression and Genotypes of All Classical HLA Loci and Haplotypes by a New Capture RNA-Seq Method. Frontiers in Immunology. 2020; 11(941). https://doi.org/10.3389/fimmu.2020.00941 PMID: 32547543.

30. Johansson T, Yohannes DA, Koskela S, Partanen J, Saavalainen P. HLA RNA Sequencing With Unique Molecular Identifiers Reveals High Allele-Specific Variability in mRNA Expression. Front Immunol. 2021; 12:629059. Epub 2021/03/16. https://doi.org/10.3389/fimmu.2021.629059 PMID: 33717155; PubMed Central PMCID: PMC7949471.

31. Kulkarni S, Savan R, Qi Y, Gao X, Yuichi Y, Bass SE, et al. Differential microRNA regulation of HLA-C expression and its association with HIV control. Nature. 2011; 472(7344):495–8. Epub 2011/04/19. https://doi.org/10.1038/nature09914 PMID: 21499264; PubMed Central PMCID: PMC3084326.
32. Vince N, Li H, Ramsuran V, Nanabhai V, Duh FM, Fairfax BP, et al. HLA-C Level Is Regulated by a Polymorphic Oct1 Binding Site in the HLA-C Promoter Region. Am J Hum Genet. 2016; 99(6):1353–8. Epub 2016/11/08. https://doi.org/10.1016/j.ajhg.2016.09.023 PMID: 27817866; PubMed Central PMCID: PMC5142108.

33. Vandiedonck C, Taylor MS, Lockstone HE, Plant K, Taylor JM, Durrant C, et al. Pervasive haplotype variation in the spliceo-transcriptome of the human major histocompatibility complex. Genome Res. 2011; 21(7):1042–54. Epub 2011/06/02. https://doi.org/10.1101/gr.116681.110 PMID: 21628452; PubMed Central PMCID: PMC3129247.

34. Bühler S, Baldomero H, Ferrari-Lacraz S, Nunes JM, Sanchez-Mazas A, Massouridi-Levrat S, et al. High-resolution HLA phased haplotype frequencies to predict the success of unrelated donor searches and clinical outcome following hematopoietic stem cell transplantation. Bone Marrow Transplant. 2019; 54(10):1701–9. Epub 2019/04/07. https://doi.org/10.1038/s41409-019-0520-6 PMID: 30953025; PubMed Central PMCID: PMC7198472.

35. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A. 2003; 100(16):9440–5. Epub 2003/07/29. https://doi.org/10.1073/pnas.1530509100 PMID: 12883005; PubMed Central PMCID: PMC170937.

36. Petersdorf EW, Malkki M, O’Huigin C, Carrington M, Gooley T, Haagenson MD, et al. High HLA-DP Expression and Graft-versus-Host Disease. The New England journal of medicine. 2015; 373(7):599–609. Epub 2015/04/07. https://doi.org/10.1056/NEJMoa1500140 PMID: 26267621; PubMed Central PMCID: PMC4560117.

37. Petersdorf EW, Bengtsson M, De Santis D, Dubois V, Fleischhauer K, Gooley T, et al. Role of HLA-DP Expression in Graft-Versus-Host Disease After Unrelated Donor Transplantation. J Clin Oncol. 2020; 38(24):2712–8. https://doi.org/10.1200/JCO.20.00265 PMID: 32479188; PubMed Central PMCID: PMC7430213.

38. Stéphane Bühler HB, Sylvie Ferrari-Lacraz, Anne-Claire Mamez, Stavroula Massouridi-Levrat, Dominik Heim, Jörg Halter, Gayathri Nair, Yves Chalando, Tayfun Güngör, Grazia Nicoloso, Passweg Jakob R, Jean Villard* Analysis of biological models to predict clinical outcomes based on HLA-DPB1 disparities in unrelated transplantation. Blood Advances. 2021. https://doi.org/10.1182/bloodadvances.2020003998 PMID: 34448833.

39. Gutierrez-Arcelus M, Baglaenko Y, Arora J, Hannes S, Luo Y, Amariuta T, et al. Allele-specific expression changes dynamically during T cell activation in HLA and other autoimmune loci. Nat Genet. 2020; 52(3):247–53. Epub 2020/02/19. https://doi.org/10.1038/s41588-020-0579-4 PMID: 32066938; PubMed Central PMCID: PMC7135372.

40. Pan N, Lu S, Wang W, Miao F, Sun H, Wu S, et al. Quantification of classical HLA class I mRNA by allele-specific, real-time polymerase chain reaction for most Han individuals. Hla. 2018; 91(2):112–23. Epub 2017/11/28. https://doi.org/10.1111/tan.13186 PMID: 29178661.

41. Rene C, Lozano C, Villalba M, Eliaou JF. 5’ and 3’ untranslated regions contribute to the differential expression of specific HLA-A alleles. Eur J Immunol. 2015; 45(12):3454–63. https://doi.org/10.1002/eji.201545927 PMID: 26399450.

42. Lee W, Plant K, Humphry P, Knight JC. AltHapAlignR: improved accuracy of RNA-seq analyses through the use of alternative haplotypes. Bioinformatics (Oxford, England). 2018; 34(14):2401–8. Epub 2018/03/08. https://doi.org/10.1093/bioinformatics/bty125 PMID: 29514179; PubMed Central PMCID: PMC6041798.

43. Johnson DR. Differential expression of human major histocompatibility class I loci: HLA-A, -B, and -C. Hum Immunol. 2000; 61(4):389–96. Epub 2000/03/15. https://doi.org/10.1016/s0198-8859(99)00186-x PMID: 10715516.

44. Garcia-Ruano AB, Mendez R, Romero JM, Cabrera T, Ruiz-Cabello F, Garrido F. Analysis of HLA-ABC locus-specific transcription in normal tissues. Immunogenetics. 2010; 62(11–12):711–9. Epub 2010/09/16. https://doi.org/10.1007/s00251-010-0470-z PMID: 20842357.

45. Larn TH, Shen M, Tay MZ, Ren EC. Unique Allelic eQTL Clusters in Human MHC Haplotypes. G3 (Bethesda). 2017; 7(8):2595–604. https://doi.org/10.1534/g3.117.034828 PMID: 28600441; PubMed Central PMCID: PMC5555465.

46. Delaneau O, Ongen H, Brown AA, Fort A, Panousis NI, Dermitzakis ET. A complete tool set for molecular QTL discovery and analysis. Nat Commun. 2017; 8:15452. Epub 2017/05/19. https://doi.org/10.1038/ncomms15452 PMID: 28516912; PubMed Central PMCID: PMC5454369.

47. Võsa U, Esko T, Kasela S, Annilo T. Altered Gene Expression Associated with microRNA Binding Site Polymorphisms. PloS one. 2015; 10(10):e0141351. Epub 2015/10/27. https://doi.org/10.1371/journal.pone.0141351 PMID: 26496489; PubMed Central PMCID: PMC4619707.
48. Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, et al. A global reference for human genetic variation. Nature. 2015; 526(7571):68–74. Epub 2015/10/04. https://doi.org/10.1038/nature15393 PMID: 26432245; PubMed Central PMCID: PMC4750478.

49. Lappalainen T, Sammeth M, Friedländer MR, ’t Hoen PAC, Monlong J, Rivas MA, et al. Transcriptome and genome sequencing uncovers functional variation in humans. Nature. 2013; 501:506. https://doi.org/10.1038/nature12531 PMID: 24037378

50. Fort A, Panousis NI, Gareri M, Antonarakis SE, Lappalainen T, Dermitzakis ET, et al. MBV: a method to solve sample mislabeling and detect technical bias in large combined genotype and sequencing assay datasets. Bioinformatics. 2017; 33(12):1895–7. Epub 2017/02/12. https://doi.org/10.1093/bioinformatics/btx074 PMID: 28186259; PubMed Central PMCID: PMC6044394.

51. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013; 29(1):15–21. Epub 2012/10/30. https://doi.org/10.1093/bioinformatics/bts635 PMID: 23104886; PubMed Central PMCID: PMC3530905.

52. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nature methods. 2017; 14(4):417–9. Epub 2017/03/07. https://doi.org/10.1038/nmeth.4197 PMID: 28263959; PubMed Central PMCID: PMC5600148.

53. Uszczynska-Ratajczak B, Lagarde J, Frankish A, Guigó R, Johnson R. Towards a complete map of the human long non-coding RNA transcriptome. Nature reviews Genetics. 2018; 19(9):535–48. Epub 2018/05/26. https://doi.org/10.1038/s41576-018-0017-y PMID: 29795125; PubMed Central PMCID: PMC6451964.