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Genetic interplay between *HLA-C* and *MIR148A* in HIV control and Crohn disease

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Variation in the 3′ untranslated region (3′UTR) of the *HLA-C* locus determines binding of the microRNA Hsa-miR-148a, resulting in lower cell surface expression of alleles that bind miR-148a relative to those alleles that escape its binding. The *HLA-C* 3′UTR variant was shown to associate with HIV control, but like the vast majority of disease associations in a region dense with causal candidates, a direct effect of HLA-C expression level on HIV control was not proven. We demonstrate that a *MIR148A* insertion/deletion polymorphism associates with its own expression levels, affecting the extent to which HLA-C is down-regulated, the level of HIV control, and the risk of Crohn disease only among those carrying an intact miR-148a binding site in the *HLA-C* 3′UTR. These data illustrate a direct effect of HLA-C expression level on HIV control that cannot be attributed to other *HLA* loci in linkage disequilibrium with *HLA-C* and highlight the rich complexity of genetic interactions in human disease.

The Human leukocyte antigen (*HLA*) class I genes are the most polymorphic loci in the human genome, resulting in the ability of the system to bind and present a great variety of antigenic peptides to cytotoxic T lymphocytes. HLA-C molecules are expressed at a low level on the cell surface compared with HLA-A and -B (1–5), owing in part to the poor assembly of HLA-C heavy chains with β2 microglobulin (2, 4) and the retention of HLA-C molecules in the endoplasmic reticulum, where they are partially degraded (3). An internalization and lysosomal targeting signal in the cytoplasmic tail of HLA-C further regulates its surface expression (6). Unlike HLA-A and -B, HLA-C surface expression is not down-regulated by Nef upon HIV infection (7). HLA-C allotypes are expressed at variable levels on the cell surface in a manner that is allotype-specific, and higher surface expression mediates greater selection pressure on HIV-1 (8–10) and better viral control overall (10). As is the case for all human diseases that associate with variation in the MHC, the close proximity and strong linkage disequilibrium (LD) between *HLA* class I loci, including *HLA-B* and *HLA-C,* complicate the ability to define causation for disease associations with these loci.

We have previously demonstrated that polymorphism in the miR-148a binding site in the *HLA-C* 3′ untranslated region (3′ UTR) region partially explains differential expression of the various HLA-C allotypes (11), where miR-148a inhibits expression of HLA-C alleles that have an intact binding site but does not affect expression of escape HLA-C alleles that contain a disrupted binding site, which is not recognized by miR-148a. In general, the escape alleles are expressed at a higher level than the inhibited alleles (although there are exceptions). Here we demonstrate that the expression levels of miR-148a, as marked by *MIR148A* gene variation, correlate positively with HIV viral load among those individuals who carry at least one inhibited *HLA-C* allele. There is no correlation between the level of miR-148a and HIV control among those individuals who carry two copies of HLA-C escape alleles. Alternatively, the genotype marking higher miR-148a expression levels (which lead to lower HLA-C expression) results in protection against Crohn disease (CD) among those who carry at least one inhibited HLA-C allele. These data underscore the importance of immune gene interactions in human disease and the independent role of HLA-C expression levels in HIV viral control and risk of CD.

Results

An insertion/deletion polymorphism at position 263 (263I/D) of the *HLA-C* 3′UTR marks variation that determines inhibition vs. escape of HLA-C down-regulation by miR-148a (11). These data predict that increasing copies of the inhibited allele, which is marked by 263I, will associate with decreasing levels of HLA-C surface expression overall. We determined HLA-C mRNA levels in peripheral blood lymphocytes (PBLs) from 256 healthy European Americans (EAs) and show that HLA-C mRNA expression correlates significantly with genotype at position 263,

Significance

In the human population different *HLA-C* allotypes are present that have different expression levels at the cell surface. Individuals with higher expressed *HLA-C* allotypes demonstrate better HIV control but increased risk of Crohn disease. A microRNA, miR-148a, regulates expression of some HLA-C allotypes. We report here that this microRNA also varies in expression level between people. *MIR148A* variation showed significant and opposing effects on HIV viral control vs. risk of Crohn disease, specifically in subjects with *HLA-C* alleles that are regulated by miR-148a. These results are independent of confounding effects by other *HLA* loci because only HLA-C is regulated by miR-148a. Our data represent an example of gene interactions that affect immune response and thereby the risk of human disease.

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where those homozygous for escape alleles (263D/D) have the highest mean HLA-C mRNA expression and those homozygous for inhibited alleles (263I/I) have the lowest expression (p trend = 5×10^{-10}, Fig. 1). Stratifying mRNA expression levels by *HLA-C* allele type shows a continuum of mRNA expression levels (Fig. S1), similar to cell surface expression that has been previously observed (10). We assigned expected cell surface expression levels to the 256 EA subjects based on the previously determined surface expression levels of two-digit HLA-C allotypes (10). The correlation between the observed HLA-C mRNA expression values in the 256 EAs and their expected surface expression levels is highly significant ($r = 0.56$, $p = 2 \times 10^{-22}$, Fig. S2). The continuum of HLA-C expression levels observed (Fig. S1), however, indicates that variation in the miR-148a site of the *HLA-C* 3′UTR does not completely explain differential expression levels of HLA-C.

Sequence variation in or near the majority of microRNA (miRNA) genes seems to be restrained by evolutionary selection pressure (12, 13), emphasizing the central role of these small molecules in modulating gene regulatory networks. The *MIR148A* gene is quite polymorphic, however, and variation in the gene region exhibits marked population differentiation representing one of the strongest signatures of selection among 117 miRNA regions tested throughout the genome (14). The levels of miR-148a expression also vary markedly across individuals (15), raising the possibility that genetic variation in the region of *MIR148A* may affect miR-148a expression, which may in turn affect differential HLA-C expression levels. We sequenced 7.7 kb (−6057 to +1674 relative to the distance from the mature miRNA sequence), including the *MIR148A* gene and flanking regions in 219 EA, identifying 26 polymorphic positions, of which 11 had a minor allele frequency of ≥5% (Fig. 2). Expression levels of miR-148a, which are consistent over time (Fig. S3), were determined in PBLs of the same 256 EA blood donors in whom HLA-C mRNA levels were determined. Two variants, rs735316 and rs111299611, which are in perfect two-way LD, showed the most significant associations with miR-148a expression levels relative to all other variants identified (P trend = 4×10^{-5}, Fig. 3 and Fig. S4). The rs735316 variant is an SNP (G/A) located 1,349 bp downstream from the 3′ end of the mature miR-148a sequence, whereas rs111299611 is an insertion/deletion (in/del) polymorphism located in the primary transcript of miR-148a 127 bp upstream of the mature miR-148a sequence encoded by the gene (Fig. 2). A significant trend in expression levels was observed in which subjects with two copies of the deletion variant at rs111299611 (i.e., rs735316G/G) had the highest mean levels of mature miR-148a expression and subjects with two copies of the insertion at rs111299611 (i.e., rs735316A/A) had the lowest expression (Fig. 3). We refer to rs735316 genotype at this point forward, rather than the in/del at rs111299611, to avoid confusion with the *HLA-C* 3′ UTR in/del nomenclature.

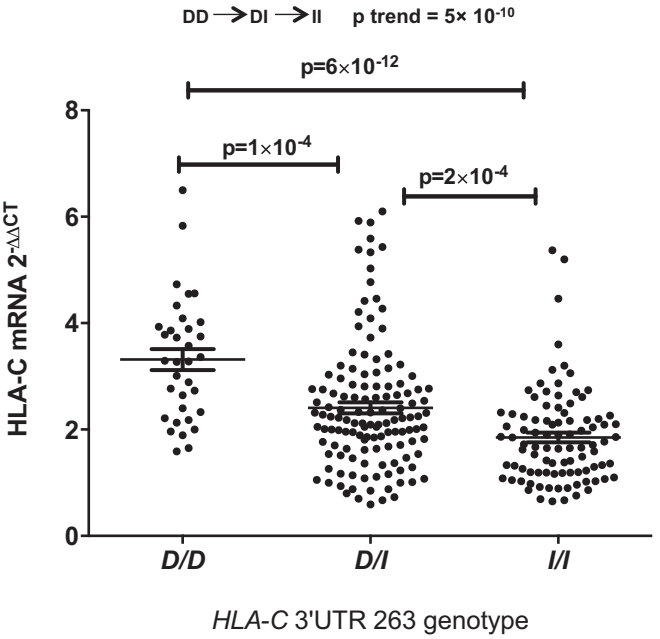

Fig. 1. Variation in the *HLA-C* 3′UTR correlates with HLA-C mRNA levels. Endogenous HLA-C mRNA expression levels were estimated in total RNA from peripheral blood lymphocytes of healthy EA donors (n = 256) using a qPCR assay. The average expression level of *HLA-C* was normalized to that of β2M RNA using the $2^{-\Delta\Delta Ct}$ method. Expression of HLA-C mRNA in individuals with the DD (n = 34) or DI (n = 129) genotype at *HLA-C* 3′UTR position 263 is significantly higher compared with the II (n = 93) genotype (p trend = 5×10^{-10}, ANOVA).

Higher expression levels of miR-148a, as marked by rs735316 genotype, may cause greater down-regulation of HLA-C mRNA expression levels than low expression levels of miR-148a, but this is expected only among individuals who carry an intact miR-148a binding site in their *HLA-C* 3′UTR. There are intrinsic differences in expression levels among HLA-C alleles that are not explained by the 3′UTR variant in the miR-148a binding site, including differences among alleles that escape miR-148a regulation, and these differences are likely to obscure the effects of miR-148a levels on HLA-C expression. We therefore used the predicted HLA-C cell surface expression of each individual, based on the measured HLA-C cell surface expression of individual alleles observed previously (10), as a covariate in the analysis to account for intrinsic HLA-C allelic differences that are not due to miR-148a regulation. Linear regression analysis showed no significant effect of miR-148a levels on HLA-C mRNA levels among individuals homozygous for *HLA-C* alleles that escape miR-148a regulation (263D/D), but a significant association (P = 0.002) was observed among individuals with two inhibited alleles (263I/I, Table 1). Heterozygotes (263I/D) showed an intermediate effect that was not significant, suggesting that having one higher-expression HLA-C allotype may diminish the ability to observe effects of miR-148a expression levels on lower-expression HLA-C allotypes. Similar results were obtained by using average mRNA level for each HLA-C allele as a covariate in the analysis rather than cell surface expression levels (Table S1). These data indicate that miR-148a levels directly affect HLA-C expression of inhibited alleles only, but a significant effect is observable only among those carrying two copies of inhibited alleles.

High miR-148a expression, as marked by rs735316G, correlates significantly with lower HLA-C expression levels among individuals with two inhibited *HLA-C* alleles (Table 1), and *HLA-C* alleles that are inhibited by miR-148a associate with poor HIV control compared with *HLA-C* alleles that escape miR-148a regulation (11). Thus, we reasoned that the *MIR148A* rs735316 genotype might associate with HIV control, but only among individuals carrying *HLA-C* alleles that are inhibited by miR-148a (263D/I, I/I) and not among those who fully escape miR-148a regulation (263D/D). *HLA-C* 3′UTR 263 and *MIR148A* rs735316 genotypes were determined in a cohort of 2,918 HIV-infected EA patients for whom longitudinal log mean viral load (mVL) during chronic infection (untreated) had been determined. As predicted, there was no indication that the *MIR148A* rs735316 genotype affects HIV control among the group of subjects who fully escape miR-148a regulation (Table 2). However, rs735316 genotype associated significantly with HIV mVL as a continuous variable among subjects carrying one or two copies of an *HLA-C* allele that is inhibited by miR-148a (P = 0.006–0.0004; Table 2). The effect of rs735316 may be codominant (P = 0.001).

High HLA-C expression was shown to associate with HIV viral load control and, in contrast, with increased risk of CD (10).

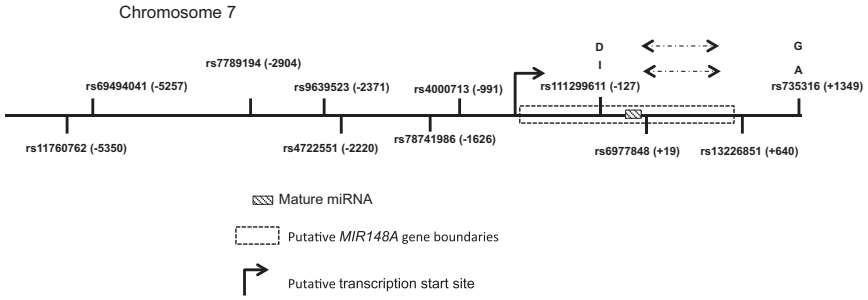

Fig. 2. Schematic representation of *MIR148A* gene region SNPs; 7.7 kb of the *MIR148A* gene and flanking genomic regions was sequenced in 219 EA healthy donors. rs735316 (A/G) is 1,349 bp downstream of the sequence encoding the mature miR-148a and is in virtually perfect LD with rs111299611, a deletion (D)/insertion (I) polymorphism in the primary transcript of miR-148a located −127 bp upstream of the mature miR-148a coding sequence. The rs111299611D marks rs735316G and rs111299611I marks rs735316A perfectly.

Given the effect of the *MIR148A* rs735316 genotype on HIV control, we tested whether it might also modulate the risk of CD among the group of individuals carrying at least one inhibited HLA-C allele (i.e., 263D/I, I/I), but predictively in a manner opposite to that observed for HIV control. A meta-analysis of data derived from a cohort composed of 2,049 cases of CD and 3,091 controls showed that the *MIR148A* rs735316 genotype marking low expression of miR-148a (rs735316A/A) significantly increased the risk of CD among those with inhibited HLA-C alleles, but not among those who fully escape miR-148a regulation (Table 3). Thus, the opposing effects of HLA-C expression level on HIV control vs. CD are similarly opposing with regard to the effects of variable expression of miR-148a.

A significant effect of *MIR148A* variation on HIV control and CD only among those subjects with at least one copy of a miR-148a-inhibited *HLA-C* allele supports a direct, independent effect of HLA-C expression levels on both HIV control and CD that are unlikely to be due to LD with other loci in the *MHC* because the *MIR148A* locus (chromosome 7) is unlinked to the *MHC* (chromosome 6). Importantly, these data rule out a confounding effect of the neighboring *HLA-B* locus, because *HLA-B* alleles have no binding site for miR-148a and therefore are not regulated by this miRNA (16).

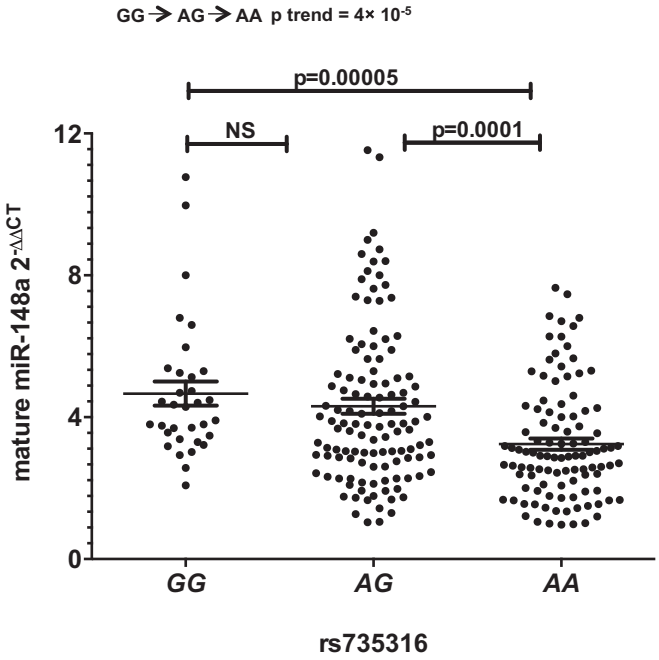

Fig. 3. The rs735316 variant associates with expression of miR-148a. Endogenous miR-148a expression levels were estimated in total RNA from PBL of healthy EA donors (n = 256) using a Taqman qPCR assay. The average expression level of miR-148a was normalized to U6 RNA using the $2^{-\Delta\Delta Ct}$ method. Expression levels of miR-148a in individuals with the GG (n = 33), AG (n = 119), and AA (n = 104) genotypes at rs735316 show a significant trend (p trend = 4× 10^{-5}, ANOVA).

Discussion

HLA-C allotypes vary in cell surface expression levels as a function of both miR-148a regulation (11) and other undefined factors that actually result in relatively high expression of some miR-148a inhibited alleles (10). We show here that among the subset of *HLA-C* alleles that are inhibited by miR-148a, differential expression levels of miR-148a itself (as marked by rs735316 genotype) contribute to variable levels of HLA-C expression. This contribution is physiologically relevant, because the difference in miR-148a expression has a significant effect on HIV viral control and risk of CD specifically among subjects who have one or two copies of *HLA-C* alleles that are inhibited by miR-148a, whereas there is no detectable effect among those who carry two escape *HLA-C* alleles (model shown in Fig. 4).

Changes in HLA-C expression caused by differential miR-148a expression reach significance among individuals carrying two inhibited *HLA-C* alleles (263I/I), but not among those carrying only one inhibited allele (263D/I, Table 1). However, significant effects of miR-148a expression levels in HIV control and CD are found for individuals carrying either one or two copies of inhibited *HLA-C* alleles (I/I and D/I, Tables 2 and 3 and Tables S2 and S3). This distinction may be attributable to differences in sample sizes, which allow resolution of effects found in 263D/I heterozygotes in the HIV (n = 2,918) and CD (n = 5,140) cohorts, but not in the much smaller expression cohort (n = 256).

Table 1. Effect of miR-148a levels on HLA-C mRNA expression

HLA-C 3′UTR genotype group	Coefficient	SE	*p*
D/D (34)	0.11	0.10	0.26
D/I (129)	−0.06	0.05	0.17
I/I (93)	−0.11	0.04	0.002
Trend D/D < D/I < I/I)			0.0005

HLA-C mRNA and miR-148a expression levels were determined in 256 healthy EA donors. Intrinsic allele-dependent differences in HLA-C expression are known to occur (10) that are independent of miR-148a regulation. These effects are estimated from the *HLA-C* genotype of each individual and included as covariables in the model along with miR-148a expression as the explanatory variable in a multivariate regression analysis (function lm in R) (29). Significance (*P*) and SE (SE) are reported. The number of individuals in each genotype group is indicated in parentheses. The trend test considers the interaction of miRNA level with the number of copies of the 3′UTR binding site.

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Table 2. Effect of genotypic combinations of *MIR148A* and *HLA-C* on HIV viral control

rs735316	*n* (%)	mVL	SE		*p*
		HLA-C 3′UTR D/D (n = 552)			
GG	58 (10.5)	3.65	0.15	Baseline	
AG	237 (42.9)	3.72	0.08	AG vs. GG	0.66*
AA	257 (46.6)	3.69	0.07	AA vs. GG	0.80*
				AA vs. AG	0.76
				A recessive	0.86
				A dominant	0.71*
		HLA-C 3′UTR D/I, I/I (n = 2,366)			
GG	269 (11.4)	4.35	0.07	Baseline	
AG	1,063 (44.9)	4.14	0.03	AG vs. GG	0.006
AA	1,034 (43.7)	4.08	0.03	AA vs. GG	0.0004
				AA vs. AG	0.21
				A recessive	0.02
				A dominant	0.001

The effect of *MIR148A* genotypes in combination with *HLA-C* 3′UTR polymorphisms on HIV viral load were analyzed using ANOVA (SAS procedure PROC GLM). mVL in chronic infection before anti-retroviral therapy was used as a continuous variable. Number of patients (*n*), significance (*p*), and SE (SE) are reported. An asterisk (*) indicates that fewer than 5% of 10,000 random permutations of individuals exceed the significance levels observed for HLA-C 3′UTR-based subdivision of individuals (*Methods* and Table S5).

Importantly, control of HIV viral load through HLA-C is affected not only by HLA-C expression level, but also by allotype-specific peptide recognition, and decreased HLA-C expression level of the inhibited allotype may diminish the peptide recognition repertoire sufficiently enough to have an effect on HIV mVL among heterozygotes for 263I/D. The same may be true for CD. Thus, differences in effects of miR-148a levels among *HLA-C* 263D/I heterozygotes in the analyses of HLA-C expression levels vs. disease outcome may not be surprising when considering the complexity of the outcomes measured in the two analyses.

Genome-wide association studies (GWASs) have shown that variation in *HLA* class I region has the greatest differential influence on HIV viral load control (17–22). Delineating the precise "causal" locus/loci, however, is problematic in the rich environment of functionally related, highly polymorphic, and tightly linked disease candidate loci located within the *MHC*.

Table 3. Effect of genotypic combinations of *MIR148A* and *HLA-C* on risk of CD

rs735316	Affected, *n* (%)	Unaffected, *n* (%)		OR	*p*
		HLA-C 3′UTR D/D (n = 977)			
GG	44 (44.0)	56 (56.0)	Baseline		
AG	188 (47.2)	210 (52.8)	AG vs. GG	1.14	0.58
AA	232 (48.4)	247 (51.6)	AA vs. GG	1.20	0.44
			AA vs. AG	1.05	0.73
			A recessive	1.08	0.56
			A dominant	1.17	0.53
		HLA-C 3′UTR D/I, I/I (n = 4,163)			
GG	159 (35.4)	290 (64.6)	Baseline		
AG	663 (35.9)	1,185 (64.1)	AG vs. GG	1.02	0.87
AA	763 (40.9)	1,103 (59.1)	AA vs. GG	1.26	0.04
			AA vs. AG	1.24	0.002
			A recessive	1.24	0.0008
			A dominant	1.14	0.24

The effect of *MIR148A* genotypes in combination with *HLA-C* 3′UTR polymorphisms on risk of CD were analyzed using a χ^2 test in a meta-analysis of CD cases and unaffected healthy controls. The number of individuals in each subset (*n*), significance (*p*), and odds ratio (OR) are reported.

Unambiguously distinguishing independent effects for the neighboring *HLA-C* and *HLA-B* loci, which map only 150 kb apart, has proven especially difficult and has questioned a direct causal effect of HLA-C expression levels on HIV control (23). The *MIR148A* gene is unlinked to the *MHC* in the human genome, so any effect of this locus on HIV control cannot be attributed to an *HLA* locus unless that locus contains variation in a binding site for miR-148a that results in regulation of some but not all of the alleles at that locus. Some alleles of *HLA-C* have an intact binding site for miR-148a and are inhibited by the miRNA, whereas others escape miR-148a regulation (11), but the *HLA-B* locus is fixed for the sequence that renders escape from miR-148a regulation. Not surprisingly, the effect of miRNA variation on HIV mean viral load persists when all *HLA-B* alleles (>3% frequency) are included in a stepwise regression analysis ($p = 0.002$ for AA vs. GG). The effect of miR-148a expression levels on HIV control only among individuals who carry inhibited *HLA-C* alleles definitively points to a direct, causal effect of HLA-C expression on HIV control that cannot be attributed to the *HLA-B* locus.

A GWAS comparing HIV controllers to noncontrollers (n = 1,712) failed to identify a significant association with the *MIR148A* variant (22), and this variant was retrospectively found to associate only weakly with viral set point based on data from another GWAS (18) (n = 2,362, $p = 0.03$). In the absence of *HLA-C* 3′UTR stratification and correcting for cohort size to match that of each of the previous GWAS, we find similar, weak significance levels of the *MIR148A* variant on mVL in the cohort used herein ($p = 0.08$ and $p = 0.02$ when randomly selecting 1,712 and 2,362 samples, respectively). We calculate that, in the absence of stratification by *HLA-C* 3′UTR, sample sizes approaching 15,000 would be needed to see effects of the *MIR148A* variant on mVL at significance levels required by GWAS ($P < 10^{-7}$). Thus, consideration of the functional interaction between *MIR148A* and *HLA-C* has enabled the discovery of its effect on HIV mVL.

Most miRNA genes are highly conserved, but *MIR148A* polymorphisms have a robust signature of selection (14). Expression levels of miR-148a are known to affect expression of its target pregnane X receptor (PXR), which in turn regulates expression of Cytochrome P450 3A4 (CYP3A4) (15), a key enzyme involved in the metabolism of xenobiotic substances in humans. The PXR/CYP3A4 data and those presented herein indicate that variation in miR148a expression levels fine-tunes the expression levels of its targets. The consequence of tuning HLA-C expression is borne out in differential HIV control and risk of CD, illustrating the biological impact of seemingly small changes in expression of miR-148a and its HLA-C target. These data represent an example of genetic interactions that affect the immune response and thereby the risk of human disease.

Materials and Methods

Samples. Healthy EA donors (n = 256) recruited at the National Cancer Institute, Frederick, Maryland and the Ragon Institute, Cambridge, Massachusetts, were used for determination of HLA-C mRNA and miR-148a expression levels. DNA and laboratory results from 2,918 HIV$^+$ patients of European descent were used to determine the effect of the *HLA-C* 3′UTR variation in combination with *MIR148A* genotypes on control of HIV viral load. Viral load measurements before antiretroviral therapy (ART) were obtained from the Multicenter AIDS Cohort Study (MACS) (24), Swiss HIV Cohort Study (www.shcs.ch), Study of the Consequences of the Protease Inhibitor Era (SCOPE) (25), International HIV Controllers Study (IHCS) (www.hivcontrollers.org), and the United States Military HIV Natural History Study (NHS) (21). Standard methods for measurements of viral load were used in all five study cohorts. An average of all available viral load readings in chronic infection before ART, for both seroprevalent and seroincident patients, was used to determine mVL. mVL was analyzed as a continuous variable for 2,918 patients.

Meta-analysis was performed using two cohorts of patients with Crohn disease and unaffected controls. The data for 1999 Crohn disease cases and 3,004 controls of European descent from the Wellcome Trust Case Control Consortium (WTCCC) 1 study (www.wtccc.org.uk/) were obtained from the WTCCC official website (26), and the Jewish case-control GWAS data on 300

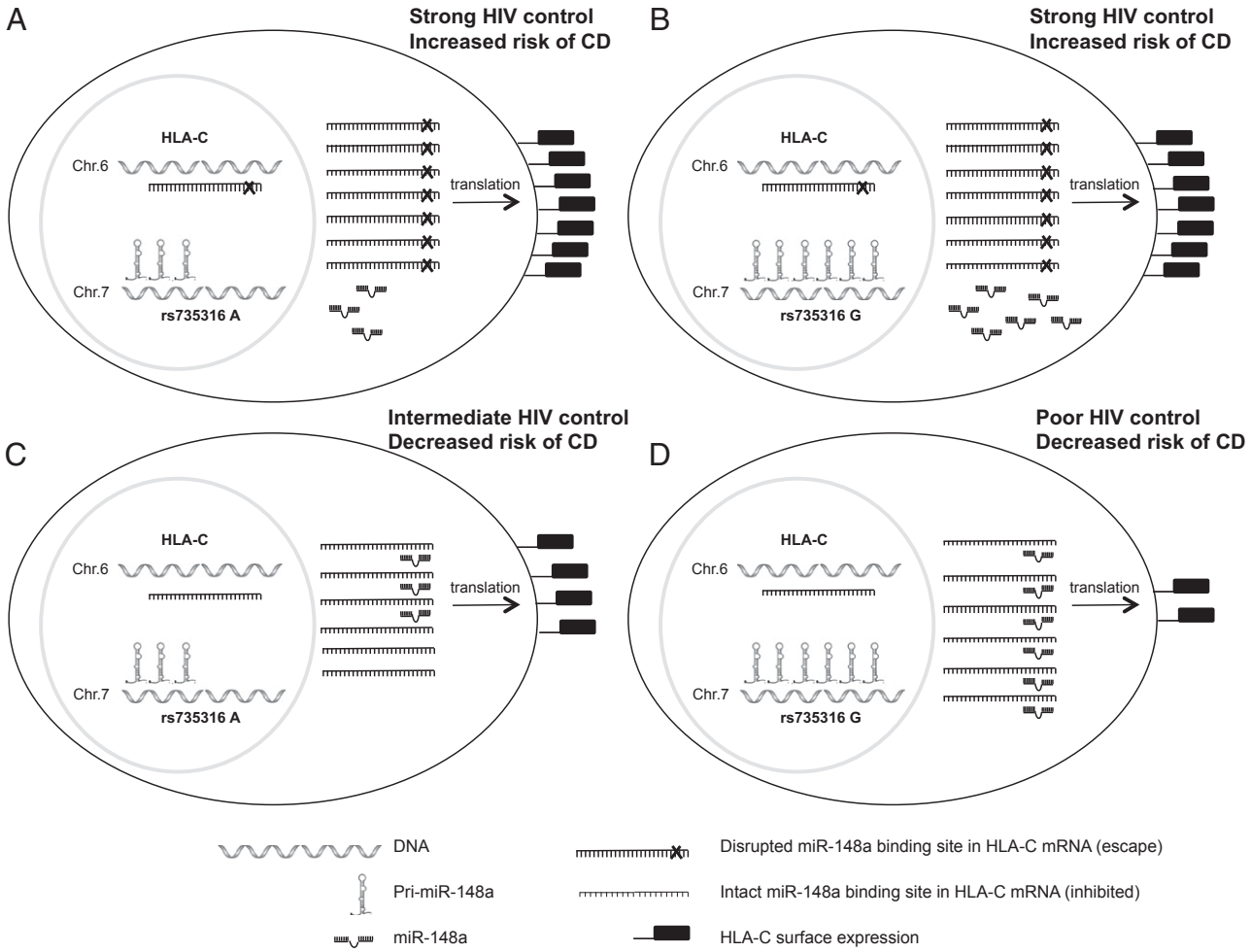

Fig. 4. The differential impact of *MIR148A* expression levels on HIV control through regulation of a restricted set of *HLA-C* alleles. Expression levels of *HLA-C* escape alleles that have a disrupted miR-148a binding site (x) are not affected by the *MIR148A* genotype that marks low (rs735316A) (*A*) or high (rs735316G) (*B*) expression of miR-148a. Thus, escape *HLA-C* alleles are expressed at relatively high levels and associate with stronger HIV control and increased risk of CD irrespective of *MIR148A* genotype. Expression levels of *HLA-C* alleles that have an intact miR-148a binding site are inhibited by miR-148a in a concentration dependent manner, where low miR-148a expression (rs735316A) (*C*) down-regulates HLA-C, but to a lesser extent than does high miR-148a expression (rs735316G) (*D*). The level of down-regulation of inhibited *HLA-C* alleles through differential miR-148a expression levels correlates with the level of HIV control and risk of CD.

cases and 432 controls were obtained from the Database of Genotype and Phenotype (27).

The respective institutional review boards [National Health Institutes office of human subjects research protection (healthy donors), Institutional Review Board Office Northwestern University (MACS), UCSF committee on human research (SCOPE), Partners human research committee (IHCS), Uniformed services university infectious disease institutional IRB (NHS), WTCCC data access committee and NIDDK data access committee (WTCCC)] approved the study. SHCS protocol, information sheets and consent forms including consent for genetic testing have been approved by 7 relevant cantonal ethics commissions in Basel, Bern, Geneva, Lausanne, St. Gallen, Ticino, and Zurich, Switzerland.

Genotyping. The entire *HLA-C* 3′UTR was amplified from genomic DNA by PCR using previously published primers (11). The amplicons were sequenced in both directions using the same primers by capillary electrophoresis using an ABI-3730XL DNA analyzer (Applied Biosystems).

The *MIR148A* gene, which is ∼1.2 kb in length, and the 6.5-kb upstream and downstream flanking regions were amplified using locus specific primers (Table S4). Purified PCR products were then sequenced in both directions in an ABI-3130XL DNA analyzer (Applied Biosystems).

HLA-C mRNA and miR-148a Expression Levels. Peripheral blood was obtained from healthy donors and lymphocytes were separated using lymphocyte separation medium as per manufacturer's instructions (Lonza). HLA-C mRNA expression levels were measured by quantitative PCR (qPCR). Briefly, total RNA including the small (<200 bp) RNA fraction was extracted from PBLs (RNeasy Universal kit; Qiagen) from 256 EAs. Each sample was treated with RNAse-free DNase (Qiagen) to remove genomic DNA. The RNAs were quantitated using HT RNA Lab Chip (Caliper; Life Sciences). All samples had an RNA quality score of >8. Reverse transcription was performed with 900 ng of RNA using the high-capacity RNA to cDNA kit (Applied Bioscience) in a volume of 10 μL. HLA-C and β2M transcripts were amplified by SYBR green qPCR using the threshold cycle (C_T) method (28) in an ABI7900HT machine (Applied Bioscience). Each qPCR reaction included 6 μL of power SYBR green PCR mastermix (Applied Biosystems), 200 nM primers that specifically amplified the gene of interest (*HLA-C*) or the housekeeping gene (*β2M*) (Table S4), and 2 μL of cDNA (1:20 dilution) in a total volume of 12 μL. The genes were amplified using the following conditions: 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The specificity of primers was verified by melt-curve analysis using a dissociation step following the qPCR protocol. Specificity of the primers was confirmed by sequencing the *HLA-C* amplicons. The primers (Table S4) were found to amplify *HLA-C* specifically and did not cross-react with any other locus. The amplification efficiencies of distinct alleles were tested by standard curves using serial dilutions of cDNA. The primers amplified distinct alleles with equal efficiency (Fig. S5). The average expression level of *HLA-C* was normalized to that of β2M RNA using the $2^{-\Delta\Delta Ct}$ method (28).

Endogenous miR-148a expression levels were estimated in total RNA from PBL of healthy EA donors using a Taqman (ABI) qPCR. The average expression level of miRNA was normalized to U6. U6 is a small nucleolar RNA that shows relatively stable and abundant expression in PBLs.

Statistical Analyses. SAS 9.1 (SAS Institute) was used for data management and statistical analyses, except that R (www.R-project.org/) was used for analyses presented in Table 1 and Table S1. The effect of *MIR148A* genotypes in combination with *HLA-C* 3′UTR polymorphisms on HIV viral load and the risk of CD were analyzed using ANOVA (SAS procedure PROC GLM; Table 2) and χ^2 test (Table 3), respectively. A two-sided P value of <0.05 was considered statistically significant. Linear regression models (SAS procedure PROC REG) were used to test the correlation of HLA-C expression with donor allotype (Fig. S1).

HLA-C cell surface expression levels were previously determined in 193 healthy subjects (10). These measurements were used to estimate average expression levels for each HLA-C allotype, by means of a multivariate regression (function lm in R) (29) with individual allotypes as explanatory variables. Using these estimated averages, we predicted HLA-C surface expression level for each individual used to generate the data in Table 1 by summing the predicted expression levels for each individual's two two-digit *HLA-C* alleles. The predicted expression level determined for each individual was used as a confounding covariate in the regression analysis for determining the effect of miR-148a on HLA-C mRNA.

To determine whether the lack of significant associations in D/D subjects indicated a true difference of effect in this group compared with the (D/I, I/I) group (Tables 2 and 3), rather than simply resulting from the smaller size in the D/D group, we tested the null hypothesis of no difference in *MIR148A* effect between the groups by 10,000 random samplings of the same size as the D/D group ($n = 552$ for HIV and $n = 977$ for CD) from the respective sets of D/I;I/I individuals. A fraction of less than 0.05 of samplings with an effect as small or smaller than that observed in the actual D/D group implied a significant difference between the actual D/D and D/I;I/I groups (Table S5), indicating that the lack of association in D/D individuals results from a lack of an effect of *MIR148A* genotype, rather than a statistical fluctuation owing to small numbers.

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