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Molecular evolution of elements controlling HLA-C expression: adaptation to a role as a KIR ligand regulating NK cell function

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Abstract

The regulatory elements controlling the transcription of the *HLA-A*, *HLA-B* and *HLA-C* genes have been extensively studied and compared. However, few studies have considered regulatory differences in the *HLA* genes from the perspective of their role as ligands for the KIR family of HLA receptors expressed by NK cells. *HLA-C* is the most recently evolved gene, and there is considerable evidence pointing to its emergence as specialized KIR ligand playing a major role in the missing-self recognition system of NK cells. Here we evaluate gene-specific differences in regulatory elements of the *HLA* genes, revealing alterations that are consistent with the adaptation of *HLA-C* to a role in NK cell regulation.

Keywords

MHC class I; HLA-A; HLA-B; HLA-C; NK cells; KIR; promoter; transcription factors

1 | INTRODUCTION

The perceived importance of the *HLA-C* locus in the human immune system has evolved significantly during the nearly 50 years since it was discovered. Prior to the identification of the KIR family of HLA receptors and their ability to recognize HLA-C in the early 1990s,¹ the weak association of HLA-C with $\beta 2m$, its lower cell surface expression, and the absence of reports describing HLA-C restricted T lymphocytes, had led investigators to propose that *HLA-C* was a non-functional gene. Although there have been numerous reports of HLA-C restricted T cells since that time,^{2–4} it was the discovery of the *KIR* gene family that finally provided a plausible explanation for the appearance of *HLA-C* in higher primates, and the forces driving its evolution.⁵ The hypothesis that *HLA-C* has evolved to serve as a specialized MHC class I gene and function primarily as a KIR ligand is supported by several

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lines of evidence: 1) *HLA-C* is the most recently evolved MHC class I gene, resulting from the duplication and divergence of the *HLA-B* gene: 2) The emergence and divergence of *HLA-C* correlates with the expansion of the family of KIR receptors that recognize it. 3) All *HLA-C* alleles serve as ligands for at least one KIR, whereas only *HLA-A* and *HLA-B* alleles that contain the Bw4 epitope are recognized by KIR3DL1. NK cells are less dependent on the recognition of Bw4, since approximately 50% of the world population possesses Bw4 epitopes in their genome. 4) The level of cell surface expression of *HLA-C* is less than 10% of *HLA-A* or *HLA-B* levels, which is more consistent with a role as a sensor for altered/downregulated class I in infected or transformed cells, rather than presentation of peptides to T cells. 5) The recent identification of an NK-specific upstream *HLA-C* promoter that is associated with increased *HLA-C* expression by NK cells, indicates the importance of *HLA-C* for the development and regulation of natural killer cells.

In this review, I will discuss multiple examples of changes in *HLA-C* regulatory elements relative to the *HLA-A* and *HLA-B* genes, and additional variation between *HLA-C* alleles in regulatory elements and mRNA splice sites, with respect to the role of *HLA-C* in controlling NK cell function.

2 | CORE PROMOTER ELEMENTS

The core promoter region of the *HLA-B* gene has been extensively investigated, and it has served as a reference gene for the evaluation of variation in regulatory elements and their effect on promoter activity when compared with the *HLA-A* and *HLA-C* core promoters. The study of *HLA-B* transgenic mice revealed that only 260 bp 5' of the transcription initiation site is sufficient to drive tissue-specific expression of the gene. In vivo footprinting of the transgenic *HLA-B* promoter region demonstrated occupancy of predicted NF- κ B (Enhancer A), IRF (ISRE), and CREB (Site α) binding sites. The binding of NF- κ B p50/p65 to the Enhancer A site, ISGF2 (IRF-1) and IRF-2 to the ISRE site, and ATF/CREB to Site α of the *HLA-A* and *HLA-B* genes was subsequently demonstrated.

2.1 | Variation in NF- κ B regulation

HLA-A contains tandem consensus NF- κ B sites, whereas *HLA-B* has only the 3' site (Figure 1), and *HLA-B* was shown to be less responsive to transactivation by NF- κ B. *HLA-C* also lacks the 5' site, and the 3' site contains two non-consensus nucleotides in the majority of alleles (Figure 1). Substitution of the 3' *HLA-C* NF- κ B site into an *HLA-B* reporter vector abrogated TNF induction of promoter activity, and the *HLA-C* site was unable to bind NF- κ B proteins, indicating that this element is non-functional in the *HLA-C* gene. The loss of this site in the *HLA-C* gene likely reflects an adaptation to its role as a KIR ligand. The induction of higher levels of *HLA-C* expression at inflammatory sites by TNF or other inducers of NF- κ B activity would make cells more resistant to NK killing, possibly allowing neoplastic or virally-infected cells to escape. Furthermore, the loss of the NF- κ B site was also associated with a lower basal level of *HLA-C* transcription relative to *HLA-A* and *HLA-B*. A subsequent more detailed study of the differences in expression and promoter sequences of the *HLA-A*, *HLA-B*, and *HLA-C* genes revealed that *HLA-C* mRNA transcripts were present at a much lower level than *HLA-A* or *HLA-B* transcripts in primary

human endothelial cells, and this correlated with the promoter activity of reporter constructs containing 426 bp of the core promoter region. The disruption of the NF- κ B site in the *HLA-C* gene was shown to be responsible for the decreased promoter activity. However, decreased transcripts and promoter activity were not observed in the HeLa cell line, indicating that additional factors could compensate for the lack of a NF- κ B site in these cells.

A study of the level of HLA protein expression in peripheral blood lymphocytes revealed that HLA-A and HLA-B are expressed on the cell surface at levels that are 13–18 times higher than that of HLA-C. Determination of total cellular protein levels by mass spectrometry indicated only a 4–5 fold difference, in agreement with previous reports that HLA-C is inefficiently exported to the cell surface. These observations are consistent with a primary role of HLA-C in NK cell regulation rather than antigen presentation.

2.2 | The Interferon-Stimulated Response Element

The interferon-stimulated response element (ISRE) of *HLA-A* has been shown to be less responsive to IFN- γ induction than the *HLA-B* or *HLA-C* element. This was initially suggested to be due to a difference in binding of IRF to the *HLA-A* element. However, similar binding of IRF-1 to the *HLA-A* and *HLA-B/HLA-C* elements has been reported, and substitution of the *HLA-A* IRF element into the *HLA-B* promoter did not affect IFN responsiveness. A comparison of the 3'-flanking sequence of the ISRE regions of *HLA-A* and *HLA-B/HLA-C*, reveals a potential STAT-binding site (TTCTCCCAA) that is related to the GAS site (TTCCCCTAA) located adjacent to an IRF site in the human *TAPI* promoter, and this element is disrupted in the *HLA-A* gene (CTCTCCCAA) due to a 3 bp insertion (Figure 1). Re-examination of EMSAs performed by Girdlestone et al. that used probes encompassing both the IRF and putative STAT sites, reveals the presence of additional higher molecular weight complexes consistent with STAT binding that are generated by a *HLA-B* probe, but not by a *HLA-A* probe. The putative STAT-binding site is conserved in all *HLA-B* alleles and most *HLA-C* alleles (30; Figure 1). However, *HLA-C* transcription is only weakly induced by IFN and/or TNF, due to the requirement of NF- κ B for strong IFN-mediated induction. The decreased responsiveness could result in HLA-C being more sensitive to down-regulation by viruses, consistent with HLA-C being the primary KIR ligand used to detect missing self. It is interesting to note that three *HLA-C* alleles (*HLA-C*05/*08/*17*) have acquired SNPs that could potentially alter STAT binding (30; Figure 1), suggesting selection for diminished IFN responsiveness in these alleles. In addition, the *HLA-C*07* allele has a 3 bp deletion that does not disrupt either the IRF or the STAT site, but results in an overlap of these sites that may prevent both factors from binding to this region at the same time. There is a dinucleotide polymorphism on the 5' side of the IRF-binding element in a subset of *HLA-B* alleles that generates a USF-binding site, but this variation only affects basal promoter activity and not IFN-induction. Furthermore, the USF-binding site is equally distributed among KIR-binding (Bw4) and non-binding (Bw6) alleles, suggesting that this variation is not associated with modulation of the recognition of HLA-B by KIR3DL1.

2.3 | The enhanceosome

The core promoter of most class I genes (*HLA-A*, *HLA-B*, *HLA-C*, *HLA-E*, *HLA-F*) is driven by an enhanceosome that cooperatively binds RFX (site X1), CREB/ATF (site α /X2), and NF-Y (Enhancer B), providing a platform for the recruitment of CIITA, as shown for the class II genes. *HLA-G* transcription is not CIITA-dependent, consistent with its specific expression in trophoblasts, which do not express CIITA. A comparison of the enhanceosome sequence between the *HLA-A*, *HLA-B*, and *HLA-C* genes revealed a SNP in the X2/ α element of the *HLA-A* gene that was predicted to affect CREB binding, but this SNP did not affect the CIITA transactivation of *HLA-A* or *HLA-B* promoters containing this SNP. An examination of the enhancer region across all genes and alleles shows complete conservation of the NF-Y binding site, and only one *HLA-A*-specific change in the CREB site as discussed above. However, there is a single nucleotide change in the *HLA-C* gene that disrupts the RFX-binding site, possibly affecting the tissue-specificity of the enhancer (Figure 1). Interestingly, the *HLA-C* gene has potential ETS and C/EBP binding sites that overlap with the predicted RFX binding site of the *HLA-A* and *HLA-B* genes. This difference may be associated with the ability of HLA-C, but not HLA-A or HLA-B, to be expressed by trophoblasts, as described below.

2.4 | Tuning of core promoter activity in *HLA-C* alleles

Cell-surface expression of HLA-C varies in an allele-specific manner, and high cell surface expression of HLA-C has been associated with reduced viral loads and increased CTL responses in HIV infection. Many disparate mechanisms have been proposed to account for the observed allelic differences in expression, such as: a single nucleotide variant 35 kb upstream of the *HLA-C* gene; a polymorphism in a 3'-UTR binding site for miR-148; the influence of extended haplotypes; differences in regulatory elements; and a recent study demonstrating that the *HLA-C*07* allele is poorly expressed at the cell surface due to a more restrictive peptide-binding pocket rather than differences in transcription.

There is however some precedent for considering allelic differences in core promoter elements with regard to HLA-C expression patterns. Allelic differences in the NF- κ B and IRF-1 binding sites were shown to affect both constitutive and cytokine-induced promoter activity when studied in reporter assays. A comparison of the *HLA-C*03*, *HLA-C*06*, and *HLA-C*07* alleles indicated that a SNP restoring the consensus binding sequence in one half of the NF- κ B palindrome in *HLA-C*07* was associated with higher basal promoter activity and greater TNF-induction in reporter assays and in primary keratinocytes. The IRF-1 binding site of *HLA-C*07* is unique among *HLA-C* alleles, since it possesses a 3-bp deletion that does not disrupt the IRF-binding consensus, but changes the spacing between the IRF site and a putative STAT-binding site located on the 3' side. Hundhausen et al. observed increased IFN responsiveness of the *HLA-C*07* allele in reporter assays, but this was not seen in IFN-stimulated primary keratinocytes. The *HLA-C*03* core promoter had the lowest basal promoter activity, consistent with the observation that it has the lowest level of mRNA expression. Interestingly, *HLA-C*03* contains a polymorphism that disrupts the TATA element, and this alteration is also present in the *HLA-C*17* allele (Figure 1).

3 | Trophoblast-specific expression of HLA-C

The recognition of HLA-C on fetal trophoblasts by maternal decidual NK cells has been reported to have an effect on the success of pregnancy.⁷ Unlike most tissues, which express HLA-A, HLA-B, HLA-C, and HLA-E, extravillous trophoblasts lack expression of HLA-A and HLA-B, but express HLA-C together with the non-classical MHC class I HLA-E and HLA-G molecules.⁷ Trophoblasts lack expression of the CIITA molecule, which recruits the CBP/p300 complex required for chromatin remodeling and gene activation, providing an explanation for the lack of MHC class II expression by trophoblasts. Therefore, *HLA* genes would require a CIITA-independent mechanism in order to be activated in trophoblasts. Numerous transcription factors are able to directly recruit CBP/p300, including C/EBP β ,⁴⁷ which was found to bind a distal enhancer element in the *HLA-G* gene. The ability of the *HLA-C* gene to be transcriptionally active in trophoblast cells that are incapable of expressing the neighboring *HLA-B* gene, suggests that there may be changes in the core promoter that endow trophoblast specificity to the *HLA-C* promoter rather than a distal enhancer element. A recent study has shown that a trophoblast-specific promoter is embedded within the *HLA-C* core promoter region. A distinct transcription initiation region that is 32 nucleotides upstream of the common start site associated with the *HLA-A*, *HLA-B*, and *HLA-C* core promoters is used in trophoblasts. An appropriately spaced array of additional core promoter elements (TATA box, CAAT box) are located upstream of the trophoblast start site, and these are only present in the *HLA-C* gene. Furthermore, an additional putative CREB-binding site is present in the *HLA-C* gene 23 bp upstream of the common CREB (X2) site. This upstream site has a single non-consensus nucleotide, however it is identical to the CREB site of the *HLA-A* gene. Although both of these CREB sites only differed from the consensus by a single nucleotide, they showed very weak CREB-binding ability. Remarkably, the upstream CREB site in the *HLA-A* gene bound CREB very strongly, even though it differed from the consensus at two 3' nucleotides. In vitro promoter assays demonstrated that the *HLA-C* core promoter was significantly more active than the *HLA-A* or *HLA-B* promoters when tested in trophoblast cell lines. The trophoblast specificity of *HLA-C* was mapped to the enhanceosome region, and a single nucleotide change that disrupts the RFX-binding site present in the *HLA-A* and *HLA-B* genes, was sufficient to confer trophoblast specificity. An overlapping ETS-binding site in the *HLA-C* gene was shown to bind ELF1 and ELF3. ELF3 is upregulated by invading trophoblasts, suggesting that it may be required for the activation of the *HLA-C* gene in these cells. ELF3 has been shown to interact with p300 and CBP, thus providing a CIITA-independent mechanism to recruit the CBP/p300 transcriptional coactivators required for chromatin remodeling and activation of the *HLA-C* gene in trophoblasts that lack CIITA expression.

4 | UPSTREAM ELEMENTS

4.1 | The -800 enhancer region

A study of SNPs associated with the level of *HLA-C* mRNA expression identified a SNP in a predicted Oct-binding site 800 bp upstream of the transcription start site as being highly associated with differences in HLA-C expression between alleles. The presence of an A

nucleotide in place of a G at this position was associated with higher Oct-1 binding and transcriptional activity. This association was confirmed in a genome-wide expression quantitative trait analysis of *HLA-C* mRNA levels determined by qPCR of peripheral blood mononuclear cell RNA from 273 individuals. Interestingly, the group of *HLA-C* alleles that have the highest level of expression (*HLA-C**01, *04, *06, *12, and *14), all possess the A residue associated with higher promoter activity. Furthermore, a second SNP located 20 bp upstream of the Oct-1 site was also significantly associated with *HLA-C* transcript levels, and this SNP is located within a putative NFAT-AP1 binding site, suggesting the presence of an enhancer element in this region. A comparison of this region between *HLA-A*, *HLA-B*, and *HLA-C* reveals the presence of significant gene-specific and allele-specific SNPs (Figure 2A). The three transcription factor-binding regions found in the *HLA-B* and *HLA-C* genes are not present in the *HLA-A* gene. The NFAT site has a single non-consensus nucleotide in all *HLA-B* alleles, as well as several *HLA-C* alleles; however, it is disrupted by a 2 bp deletion in the *HLA-A* gene. An adjacent SP1-binding site is only present in the *HLA-B* gene. NFAT has been shown to associate with SP1, and a tandem NFAT-SP1 site is present within a human IL3 enhancer active in T cells and mast cells. Therefore, it appears that this enhancer evolved in the *HLA-B* gene, and its activity was further refined in the *HLA-C* gene. An analysis of the tissue specificity of the *HLA-B* and *HLA-C* -800 enhancer elements is necessary in order to fully understand the evolutionary forces driving their emergence.

4.2 | The distal NK-specific promoter of *HLA-C*

The recent discovery of an NK-specific promoter in the *HLA-C* gene provides a compelling example of the molecular evolution of this gene for a role in controlling NK cell function. Remarkably, the homologous region of the *HLA-A* gene has what appears to be a macrophage-specific promoter, and there is no conservation of predicted transcription factor binding sites between these genes. In contrast, there is nearly complete conservation of transcription factor binding sites between the *HLA-B* and *HLA-C* genes, with the exception of an Ets-binding site that was shown to be required for increased *HLA-C* expression by NK cells (Figure 2B). This suggests that a tissue-specific promoter/enhancer in the *HLA-A* gene changed to a different, as yet unknown specificity in the *HLA-B* gene, and this further developed into an NK-specific promoter in the *HLA-C* gene. There are only 2 examples of *HLA-B* distal transcripts in Genbank, one from a breast tumor tissue sample (DB003310) and another from heart tissue (DA561140), indicating that the *HLA-B* element is either a very weak promoter, or it is active only in a very small subset of cells. The low level of *HLA-B* distal transcripts observed in purified NK cells from several individuals suggests that a contaminating lymphocyte population could be the source of the *HLA-B* transcripts. It will be important to conduct a more thorough examination of the cell-type specificity of the *HLA-A* and *HLA-B* distal promoters and the effect of their distal transcripts on protein expression levels.

4.3 | Fine-tuning of *HLA-C* expression by alternative splicing of distal *HLA-C* transcripts

The structure of the 5' UTR of distal *HLA-C* transcripts is highly variable due to the presence of three untranslated exons that can vary in size due to the use of alternative splice donor and acceptor sites. Seven different first exons (-1a₁₋₇), six second exons (-1b₁₋₆), and

four third exons ($-1c_{1-4}$) have been observed, along with seven different isoforms of the first coding exon containing from 8–630 bp of 5′ non-coding nucleotides. Furthermore, there are three non-coding exons that are only found in specific alleles. The *HLA-C*06* and *HLA-C*12* alleles have a larger second exon ($-1b_2$) due to an alternative splice donor found only in these alleles. Alternative exons $-1b_3$ and $-1b_4$ are only found in *HLA-C*01* and *HLA-C*04* alleles due to an additional splice donor site found only in these alleles. The differences in size and sequence content of the $-1b$ exon between alleles may have an impact on the translatability of *HLA-C* mRNAs, providing an additional mechanism to tune expression levels. An inverse correlation between the size of the 5′ UTR and the translatability of the *HLA-C* mRNA has been observed. However, the effect of allele-specific exons on *HLA-C* expression has yet to be determined. It will also be of interest to determine if the level of NK cell *HLA-C* expression of specific alleles is inversely correlated with the affinity of a given allele for its cognate KIR.

5 | CONCLUSION

It appears that the *HLA-C* gene has undergone numerous changes affecting both gene transcription and protein structure that have resulted in a lower level of cell surface expression and reduced cytokine responsiveness relative to the *HLA-A* and *HLA-B* genes. Figure 3 summarizes the changes in promoter elements observed between the *HLA-A*, *HLA-B*, and *HLA-C* genes. A predicted enhancer element at -800 is present in the *HLA-B* and *HLA-C* genes, but not *HLA-A* (Figure 3A). The predicted NFAT-associated transcription factor has changed from SP1 in the *HLA-B* gene to AP1 in the *HLA-C* gene, suggesting that a change in response to cytokine stimulation or tissue-specificity of the enhancer has occurred. The upstream promoter region has undergone a dramatic change from *HLA-A* to *HLA-B*, with numerous novel predicted transcription factor binding sites appearing, and elimination of the binding sites associated with macrophage-specific transcription. A more subtle change has occurred between *HLA-B* and *HLA-C*, with the addition of an ETS-binding site, creating a tandem SP1-ETS site that has been associated with NK-specific transcription. The core promoter region of *HLA-C* has undergone changes to make it less inducible, and it appears to have gained additional transcription factor binding sites to enable transcription in fetal trophoblasts (Figure 3B). There is a progressive loss of NF- κ B binding sites from two in *HLA-A*, to one in *HLA-B*, and none in *HLA-C*, suggesting a selection against inducible expression as the *HLA* genes adapt to a role as a KIR ligand. The expression of *HLA-C* but not *HLA-A* or *HLA-B* by trophoblasts should be associated with the presence of an *HLA-C* tissue-specific enhancer, as observed for the *HLA-G* gene. It appears that the enhanceosome region of the *HLA-C* core promoter has exchanged the RFX binding site for an expanded ETS binding site in order to allow trophoblast expression.

The regulatory elements of the *HLA-A*, *HLA-B*, and *HLA-C* genes provide significant insight into the evolutionary history of these genes and the forces driving their specialization. The recent identification of novel enhancer and promoter regions in the *HLA-C* gene and the comparison with highly-related elements in *HLA-B* versus distinct *HLA-A* elements, promises to provide additional information with regard to the evolution of these genes for unique roles in the regulation of the immune system.

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Figure 1.

Gene- and allele-specific variation within the *HLA* core promoter region. The sequence of the *HLA-C*01* allele is shown, with only nucleotide differences shown for *HLA-C* alleles that differ from *HLA-C*01*. A compilation of nucleotide differences found across all alleles is shown for the *HLA-B* gene (Bcomp) in blue and the *HLA-A* gene (Acomp) in green. Nucleotide differences found in all *HLA-A* or *HLA-B* alleles are underlined. Transcription factor binding sites, initiator elements (INR), downstream promoter elements (DPE), and their consensus binding sequences are shown above, and nucleotides that match the consensus are underlined in the *HLA-C*01* sequence. Elements associated with trophoblast-specific transcription are shown in red. Splice acceptor sites used by transcripts from the upstream promoter (HLA-A_SA, SA1-3, SA1-1) are indicated. The RFX-binding consensus and additional CREB-binding site are shown in green underneath.

Figure 2.

Gene and allele-specific changes in *HLA* upstream elements. A. Comparison of the nucleotide sequence of the *HLA-C*–800 region with the *HLA-B* and *HLA-A* genes. The nucleotide sequence of the *HLA-C*01* allele is shown, with only nucleotide differences shown for *HLA-C* alleles, two representative *HLA-B* alleles in blue type, and two representative *HLA-A* alleles in green type. All *HLA-A* or *HLA-B* alleles contain one of the two sequences shown for each gene, and the *HLA-C* alleles that are not listed are identical to *HLA-C*01* in the region shown. Consensus transcription factor binding sequences are shown above, and the SP1-binding consensus found only in *HLA-B* is shown below in blue. Nucleotides that match the consensus are underlined. B. The sequence of the distal NK-specific promoter of *HLA-C*01* is shown, with a compilation of nucleotide differences found across all *HLA-B* alleles shown in blue type below. Nucleotide difference found in all *HLA-B* alleles are underlined. (ACA) indicates a 3 bp insertion found in the *HLA-B*40* and *-B*57* alleles. The *HLA-A*01* sequence is shown in green to demonstrate the lack of sequence conservation in this region, and transcription factor binding sites are underlined and listed below. Consensus transcription factor binding sequences are shown above, and boxes highlight nucleotide conservation of these regions in *HLA-B* alleles. Transcription start sites (TSS) are indicated by asterisks and consensus initiator sequences are shown above, in black type for *HLA-C* and green type for *HLA-A*.

(A)

(B)

Figure 3.

Evolution of control elements in the *HLA-A*, *HLA-B*, and *HLA-C* genes. A. Schematic showing transcription factor-binding elements in the *HLA-A*, *HLA-B*, and *HLA-C* -800 and -1300 regions. Predicted binding sites are shown as labeled boxes, and experimentally-verified factor binding is indicated by labeled ovals. Bent arrows indicate transcriptional start sites. The inactivation of the ETS-binding site by a SNP in *HLA-B* is indicated by a box containing an "X". B. Schematic showing transcription factor-binding elements in the *HLA-A*, *HLA-B*, and *HLA-C* core promoter region. Experimentally-verified transcription factor binding is indicated by labeled ovals. SNPs disrupting both halves of the NF- κ B

palindrome are indicated by the boxes labeled “X X”. A putative STAT-binding site observed in the *HLA-B* and *HLA-C* genes is indicated by the box labeled “GAS”. Predicted ETS-binding, CCAAT-box, and trophoblast-associated transcription start sites are indicated in the *HLA-C* gene and labeled as “Trophoblast promoter elements”.

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