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NKG2C Sequence Polymorphism Modulates the Expansion of Adaptive NK Cells in Response to Human CMV

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ABSTRACT

A subpopulation of NK cells with distinctive phenotype and function differentiates and expands specifically in response to infection by human cytomegalovirus (HCMV). A hallmark of these adaptive NK cells is their increased expression levels of the activating CD94/NKG2C receptor for HLA-E, and lack of expression of its inhibitory homologue CD94/NKG2A. Their frequency is highly variable in HCMV$^+$ individuals, and the basis for such differences is only partially understood. Here, we explore the possible influence of sequence polymorphism of the *NKG2C* (or *KLRC2*) gene on the expansion of NKG2C$^+$NKG2A$^-$ NK cells in healthy HCMV-seropositive donors. Our results show a significant association of greater proportions of adaptive NK cells with allele *NKG2C*02*. This is defined by two amino acid substitutions in comparison with the most prevalent allele, *NKG2C*01*, and associates with additional sequence polymorphisms in noncoding regions. Furthermore, we demonstrate consistently higher mRNA levels of *NKG2C*02* in heterozygous individuals co-expressing this allele in combination with *NKG2C*01* or **03*. This predominance is independent of polymorphisms in the promoter and 3′ UTRs and is appreciated also in HCMV-seronegative donors. In summary, although additional factors are most likely implicated in the variable expansion of NKG2C$^+$NKG2A$^-$ NK cells in response to HCMV, our results demonstrate that host immunogenetics, in particular *NKG2C* diversity, influences the magnitude of such response.

1 | Introduction

NK cells sense levels of MHC Class I molecules in infected cells and tumours through inhibitory receptors, which have evolved within the immunoglobulin-like and C-type lectin-like protein superfamilies [1–5]. In humans, best characterised of those are the inhibitory killer-cell immunoglobulin-like receptors (iKIR), and the CD94/NKG2A heterodimer, officially designated killer-cell lectin-like receptor (KLR) KLRD1/KLRC1. Both iKIR and CD94/NKG2A have activating homologues of less well-understood biological role, which recognise with lower affinity similar or identical ligands. The activating heterodimer CD94/NKG2C (or KLRD1/KLRC2), like its inhibitory counterpart, recognises HLA-E presenting conserved nonameric peptides with methionine at P2, generally derived from the polymorphic leader sequence of other HLA class I molecules (i.e., HLA-G, -A, -B, and -C)

Judit Asenjo and Manuela Moraru share first authorship.

[6, 7]. Differences in the sequence of bound peptides determines the affinity of the interaction between HLA-E and its NKG2A/C receptors (the CD94 subunit will be omitted henceforth) [8–11].

In comparison with the enormous genetic diversity of KIR and their ligands [12, 13], NKG2C and A, encoded in the NK complex on human chromosome 12, appear little polymorphic [14]. However, a 16-Kb deletion encompassing the whole *NKG2C* gene is seen in, approximately, one third of individuals in all studied populations [15–18]. Furthermore, Shum et al. [14] showed that the NKG2C primary structure is represented by two common alleles that differ in amino acids 2 (intracytoplasmic) and 102 (extracellular stem). More recently, we described a third less common allele with a mixed motif in those residues [19], and showed that variations in the coding sequence are associated with additional single-nucleotide polymorphisms (SNPs) in the promoter and the 3′ untranslated regions (UTR), forming three conserved haplotypes, besides others less represented [20]. Thus, combination of copy-number variation and allelic polymorphism creates significant *NKG2C* diversity: in our study of 240 Spanish individuals, we observed 18 *NKG2C* genotypes, the most common of which was seen in only 22% of the population [20]. Significance of *NKG2C* sequence polymorphism for receptor function, immunity, and health remains scarcely explored [15, 21].

Human cytomegalovirus (HCMV) causes a highly prevalent and persistent infection which alters the immune system configuration [22], including the NK-cell receptor distribution. In HCMV-seronegative individuals, only small proportions of peripheral-blood NK cells express NKG2C at low levels ($NKG2C^{dim}$), often accompanied by NKG2A. In contrast, NK cells expressing higher NKG2C levels ($NKG2C^{bright}$) differentiate and expand in HCMV-seropositive subjects [23, 24]. This subpopulation, commonly referred to as 'adaptive' NK cells, typically displays additional phenotypic and differentiation traits, such as lack of NKG2A, oligoclonal expression of iKIR for self HLA, and changes in the expression of other surface receptors, signalling molecules, and transcription factors, altogether impacting on their effector functions [18, 25–32].

Abundance of circulating adaptive NK cells in different healthy $HCMV^+$ individuals is vastly variable (ranging from inconspicuous to constituting the majority of NK cells), but their proportions tend to remain stable over the years [24, 28, 33]. The basis for such individual variability is likely multifactorial, involving both host and viral factors. $NKG2C^+$ NK-cell frequencies are often increased in immunocompromised individuals, probably reflecting, and possibly compensating, a poor control of HCMV replication by T-lymphocytes [34–38]. In this regard, pre-transplant numbers of $NKG2C^+$ NK cells correlate inversely with the risk of clinically significant HCMV replication after kidney transplant [36]. In addition, we showed that *NKG2C* gene-copy number correlates positively with numbers of $NKG2C^+$ NK cells in healthy individuals and infected children [18, 39], as well as in immunosuppressed patients [36]. On the virus side, the HCMV UL40 protein includes a peptide, with polymorphism in different strains, which replicates the sequence of endogenous HLA-leader peptides, and supports HLA-E expression in infected cells [9, 40], an example of convergent evolution favouring viral evasion. Hammer et al. [41] showed that HLA-E, bound to the HCMV UL40 peptide stimulates $NKG2C^+$ adaptive NK cells. In this study, we address whether *NKG2C* sequence polymorphism might influence the expansion of adaptive NK cells in healthy $HCMV^+$ individuals.

2 | Materials and Methods

2.1 | Subjects and Samples

To analyse possible association of *NKG2C* polymorphism with expansion of $NKG2C^+$ peripheral blood NK cells in response to HCMV, we studied, after informed consent and venipuncture, 232 healthy donors recruited in two cities in Spain (Barcelona and Madrid) with positive serology (IgG) for HCMV and at least one copy of the *NKG2C* gene. Donors age ranged from 18 to 83 years, and their *NKG2C* genotypes (sequence polymorphisms as well as copy number variation), determined by PCR-SSP [42] and DNA-sequencing methods, have been reported previously [20]. Four additional donors without HCMV-specific antibodies were studied for relative *NKG2C*-allele expression by reverse-transcription and PCR.

2.2 | Assessment of $NKG2C^+$ Adaptive NK-Cells by Flow Cytometry

NK cells in peripheral blood and their phenotypes were assessed by multi-colour flow cytometry. Samples collected in Barcelona were analysed as detailed previously [18]; and samples collected in Madrid were stained with the same antibody-dye combination, except for anti-CD3, labelled with VioBlue (Miltenyi Biotec GmbH) instead of PerCP, in a MACSQuant Analyser, using MACSQuantify software (both by Miltenyi Biotec GmbH) in the central facility of IDIPHISA.

Adaptive NK cells were defined as $CD3^-CD56^+NKG2C^+NKG2A^-$ lymphocytes (Figure S1), and their proportions were referred to $CD3^-CD56^+$ cells. The GraphPad Prism 7.03 statistical software (GraphPad Software Inc. Boston, MA, USA) was used to represent and compare, with the non-parametric Mann–Whitney test, the percentages of $NKG2C^+A^-$ NK cells of different donor categories.

2.3 | Estimation of the Relative Abundance of *NKG2C*-Allele RNA in Heterozygotes

Total RNA was isolated from peripheral blood mononuclear cells with the RNeasy Plus kit (Qiagen GmbH) of donors with different *NKG2C* genotype, proportion of $NKG2C^+A^-$ NK cells and HCMV serostatus. Complementary DNA was synthesised with a first-strand synthesis kit (GE Healthcare-Fisher Scientific) and used as substrate for different PCR amplifications with pairs of primers that target sequences conserved in alleles *NKG2C*01*, **02*, and **03*. Sanger sequencing around the exon 3 SNP c.305C/T, distinctive of *NKG2C*02* in regard to other alleles, was used as a first approach in 15 samples. To this end, PCR primers were KLRF-20 (catagagcacagtccctc, forward, 5′ UTR) and KLRR+47 (gcaatcataatatttctattttaag, reverse, 3′ UTR), and sequencing primers, KLRF254 (catttgcattgtcctgatggc, forward) and KLRRa544 (atccacactgggctgatt, reverse). As a reference substrate with identical amounts of both alleles, genomic DNA of the same donors was analysed in a similar manner (details not shown, available upon request).

To compare more precisely the relative abundance of *NKG2C*-allele transcripts, we designed a qPCR method that included 5′-fluorophore-labelled, 3′-quencher oligonucleotide probes specific for each allele. A 175-bp *NKG2C* segment was amplified from 5 to 50 ng of cDNA with 0.5 μM of primer KLRC2F292 (tagttcttattcctttcctgg, forward, exons 2–3) and 0.5 μM KLRC2R428 (ttcttcgaagtacaggcca, reverse, exon 4) in a final volume of 10 μL of PCR mix (PrimeTime Master Mix, IDT, Integrated DNA Technologies Inc., Coralville, IO, USA) containing 0.25 μM of each probe. The two fluorescent dye labelled, locked nucleic acid (LNA) Mini Affinity Plus qPCR probes (IDT) were: for NKG2C c.305C, 6-FAM-AA+CAAT+T+C+TT+CCCCG-Iowa Black FQ; and, for c.305T, SUN-AA+CAAT+T+T+TT+CC+CCG-Iowa Black FQ (+symbols represent LNA bases). The PCR profile was: 3 min at 95°C, 50 cycles of 95°C, 20 s, 64°C, 20 s, and 72°C 20 s, and a final 4-min step at 72°C, in a QuantStudio 5 thermocycler (Applied Biosystems). Fluorescence levels were analysed with Quant Studio Design and Analysis Software v1.5.1. Endpoint increase from baseline fluorescence, triggered by probe binding, was used as a measure of target amplification.

Probe specificity was assessed with appropriate control samples of known *NKG2C* genotypes lacking the relevant allele, as shown in Section 3. In addition, the signal levels produced by both probes were verified and monitored for each batch using heterozygous genomic DNAs containing, by definition, a 1:1 ratio of each allele; and, for some batches, an equimolar mixture of synthetic cDNAs matching *NKG2C* alleles (see below). A modified PCR method with different primers was used for PCR on genomic DNA (details available upon request). To verify consistency of the results, analyses of cDNA samples were repeated with different probe batches. Each cDNA sample was tested in at least two replicate experiments (range: 2–10; average: 4).

To obtain an approximate estimation of the relative levels of each *NKG2C* allele in mRNA of heterozygous individuals, we applied the qPCR method above to two synthetic DNAs (gBlocks gene fragments, IDT) matching nucleotides c.156 through c.557 of *NKG2C*01/*03* and *NKG2C*02*. These fragments were studied at a final substrate concentration of 0.04 pg/mL, in isolation, and by serial dilution, in the following proportions: 10:1, 5:1, 2:1, 3:2, 1:1, 2:3, 1:2, 1:5, 1:10, 1:50, and 1:100, as shown in Results. Each dilution was assayed two to five times (average: 3). Relative abundance of *NKG2C*-allele transcripts within a heterozygous RNA sample was estimated by interpolation. Mean values of the two probes in heterozygous individuals were compared with a paired *t*-test.

3 | Results

3.1 | Analysis of Association Between *NKG2C* Polymorphism and Adaptive NK-Cell Expansions in Healthy Individuals

3.1.1 | Distribution of NKG2C$^+$A$^-$ NK Cell Percentages in Healthy Donors

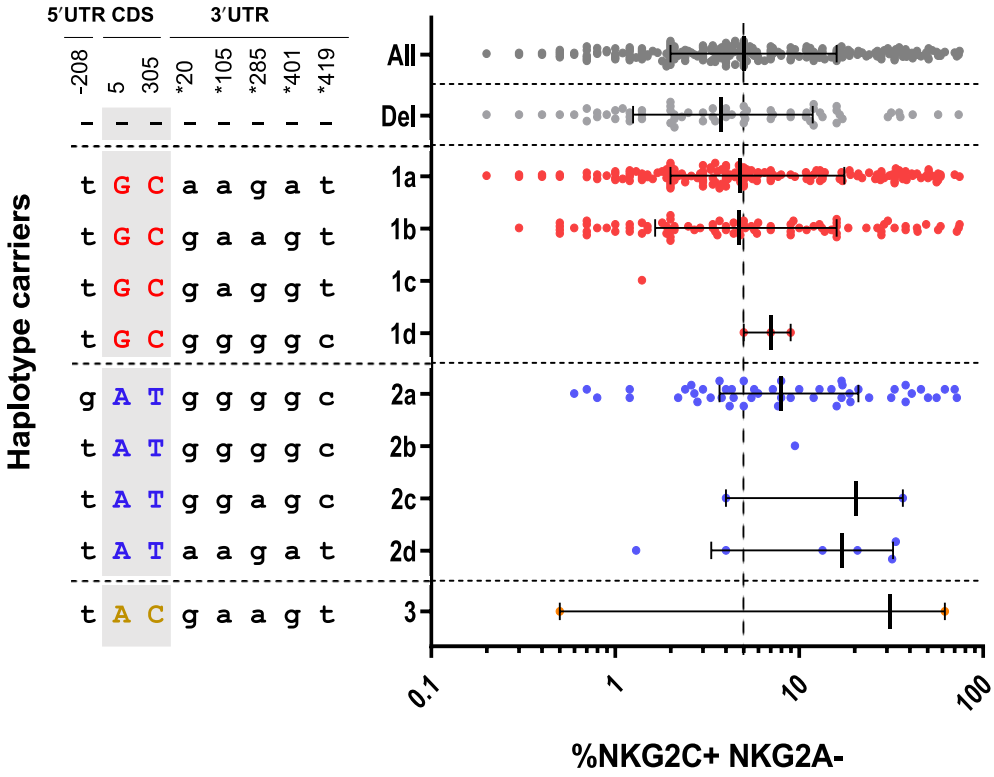
To investigate the influence of host genetic factors on the variable expansion of adaptive NK cells in different individuals, we studied possible association between *KLRC2*/*NKG2C* gene polymorphism and proportions of those NK cells in healthy unrelated individuals with positive serology for HCMV, whose *NKG2C* CNV and sequence polymorphisms were reported previously [20]. After exclusion of eight homozygotes for whole gene deletion, we correlated *NKG2C* genetic diversity with the NK-cell phenotypes, assessed by flow cytometry, of 232 donors with at least one copy of the *NKG2C* gene. An NKG2C$^+$A$^-$ phenotype was used as a surrogate marker of adaptive NK cells, and Figures 1 and S2 depict their individual proportions in the whole sample, which ranged between 0.2% and 74.6% (median 5.0%, interquartile range 2%–16%, mean 12.4%, 95% confidence interval 10.3%–14.5%). Since half of the individuals had < 5% of NKG2C$^+$A$^-$ NK cells, and percentages do not follow a normal distribution, for better visualisation, data will be represented in log scale (Figure S2, right, and henceforth).

3.1.2 | Frequency of NKG2C$^+$A$^-$ NK Cells According to *NKG2C* Copy Number and Extended Haplotypes

We previously reported that *NKG2C* is deleted on one chromosome in 28.3% of Spanish individuals; and that a series of eight SNPs in its coding, promoter, and 3′ UT regions define three major haplotypes conserved in > 95% of individuals, and six other variant haplotypes with much lower frequencies [20]. Figure 1 represents the percentages of NKG2C$^+$A$^-$ NK cells in carriers of the different *NKG2C* haplotypes, beside the SNPs that form each haplotype. As we originally reported [18, 39], individuals with a haploid *NKG2C* dosage have lower frequencies of NKG2C$^+$A$^-$ NK cells than those with a full gene complement (3.7% vs. 5.0%, $p = 0.020$, Figure 1 lower panel).

Carriers of the two most common haplotypes, *NKG2C-1a* and *-1b*, have median frequencies nearly identical to that of the whole sample, which do not differ from those of non-carriers. In contrast, the 51 *NKG2C-2a* carriers have a higher median value that differs significantly from that of non-carriers (8.0% vs. 4.4%, $p = 0.005$, Figure 1). Other *NKG2C*02* haplotypes (2b, 2c, and 2d) might also associate with high frequencies of NKG2C$^+$A$^-$ NK cells, but donors carrying these haplotypes, as well as *NKG2C-1c*, *-1d* and *-3*, are too scarce for a reliable estimation (Figure 1).

The different *NKG2C* haplotypes and gene deletion combine in this sample in 18 homo- and heterozygous genotypes, the most common of which (*1a*/*1a*) is seen in only 53 individuals. Figure 2 represents and compares the distribution of NKG2C$^+$A$^-$ NK-cell frequencies of *NKG2C* genotypes seen in at least five individuals. Of note, all genotypes containing *NKG2C*02* have medians above the general population (last five in Figure 2). The most common among them, *NKG2C-1a/2a*, has a median of 17.0% that differs from genotypes containing only haplotypes *NKG2C-1a* and *1b* on one or both chromosomes with different degrees of statistical significance ($p = 0.003$–0.086, Figure 2). An effect of copy-number variation is perceivable, albeit with low statistical significance, only in the comparison of the *NKG2C-1a*/*del* and *NKG2C-1a*/*1a* genotypes, but not in others. Also of note, within virtually all genotypes, the range of percentages of NKG2C$^+$A$^-$ NK cells is ample, with some individuals having less than 1% and others having more than 20%, observation consistent with



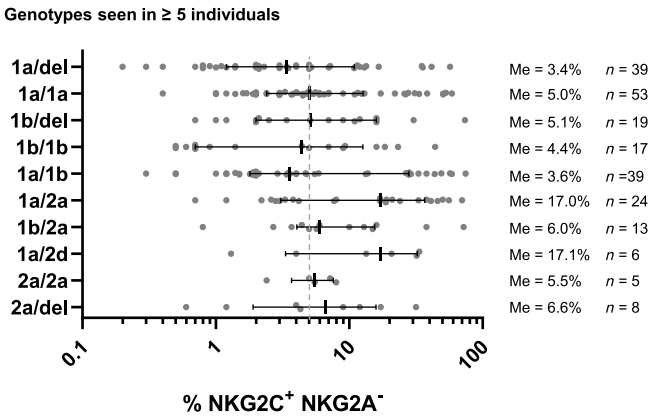
Haplotype	Carriers Median (*n*)	Non-carriers Median (*n*)	*p*
Del	3.7% (68)	5.0% (164)	**0.020**
1a	4.7% (166)	5.3% (66)	0.624
1b	4.7% (89)	5.0% (143)	0.400
1c	1.4% (1)	5.0% (231)	—
1d	7.0% (3)	5.0% (229)	0.627
2a	8.0% (51)	4.4% (181)	**0.005**
2b	9.5% (1)	5.0% (231)	—
2c	20.3% (2)	5.0% (230)	0.455
2d	17.1% (6)	5.0% (226)	0.222
3	31.25% (2)	5.0% (230)	0.968

FIGURE 1 | Frequencies of NKG2C$^+$NKG2A$^-$ NK cells in carriers of different *NKG2C* extended haplotypes, that is, including SNPs of the promoter (c.-208T>G), coding (c.5G>A and c.305C>T) and 3′ UT (c.*20A>G, c.*105A>G, c.*285G>A, c.*401A>G, c.*419T>C) regions, as shown on the left. Lines represent medians and interquartile ranges; dots are individual values. For reference, a dashed line represents in all figures the median percentage of the whole sample, whose distribution is also depicted here on the top. Colours distinguish the three forms of *NKG2C* CDS. Frequencies of carriers and non-carriers of common haplotypes are compared below the graphic.

factors other than the *NKG2C* gene influencing abundance of adaptive NK cells.

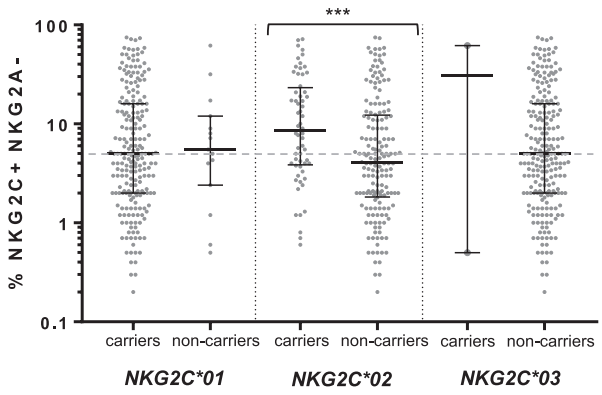
3.1.3 | Separate Analysis of *NKG2C* Regions: The Coding Sequence

Since the *NKG2C* haplotypes analysed above comprise both coding and non-coding SNPs, we performed additional analyses aimed at identifying which of those regions is primarily responsible for the observed association with proportions of NKG2C$^+$A$^-$ NK cells. The two linked coding SNPs define two major alleles, *NKG2C*01* and *02* (Ser2-Ser102 and Asn2-Phe102, respectively). Carriers of the second allele represent 25% of the population and they display proportions of NKG2C$^+$A$^-$ NK cells that differ from non-carriers with high statistical significance (median 8.5% vs. 4.0%, $p < 0.001$ Figure 3). In contrast, *NKG2C*01* carriers, constituting > 90% of all donors, have a median percentage not surprisingly identical to



1a/1a	0.074								
1b/del	0.437	0.678							
1b/1b	0.734	0.153	0.375						
1a/1b	0.361	0.419	0.990	0.266					
2a/del	0.376	0.988	0.745	0.501	0.851				
2a/2a	0.306	0.793	0.990	0.607	0.500	0.833			
1a/2a	0.003	0.086	0.040	0.016	0.068	0.278	0.275		
1b/2a	0.042	0.312	0.347	0.147	0.194	0.595	0.455	0.504	
1a/2d	0.075	0.275	0.152	0.098	0.329	0.242	0.329	0.973	0.579
	1a/del	**1a/1a**	**1b/del**	**1b/1b**	**1a/1b**	**2a/del**	**2a/2a**	**1a/2a**	**1b/2a**

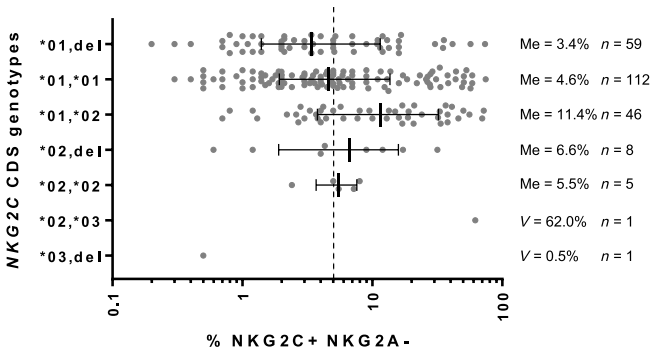
FIGURE 2 | Frequencies of NKG2C$^+$NKG2A$^-$ NK cells in individuals with different *NKG2C* genotypes, considering extended haplotypes and gene dose; only genotypes seen in, at least, five individuals are shown. The P value of each comparison is shown on the bottom; a colour code highlights different levels of statistical significance.



Allele	Carriers Median (*n*)	Non-carriers Median (*n*)	*p*
NKG2C*01	5.0% (217)	5.5% (15)	0.857
NKG2C*02	8.5% (60)	4.0% (172)	**< 0.001**
NKG2C*03	31.2% (2)	5.0% (230)	0.968

FIGURE 3 | Frequencies of NKG2C$^+$NKG2A$^-$ NK cells in carriers and non-carriers of the three known *NKG2C* coding sequences. Median frequencies and *p* values of comparisons are shown below, and asterisks highlight one comparison with $p < 0.001$.

the whole sample; the 15 non-carriers do not differ significantly from them, suggesting that presence of *NKG2C*01* is neutral for NKG2C$^+$A$^-$ NK-cell proportions. Finally, *NKG2C*03*, characterised by a hybrid Asn2-Ser102 motif [19], is found in only two individuals with disparate NKG2C$^+$A$^-$ expression levels and



***01,*01**	0.234			
***01,*02**	< 0.001	0.006		
***02,del**	0.443	0.842	0.265	
***02,*02**	0.446	0.659	0.253	0.833
	***01,del**	***01,*01**	***01,*02**	***02,del**

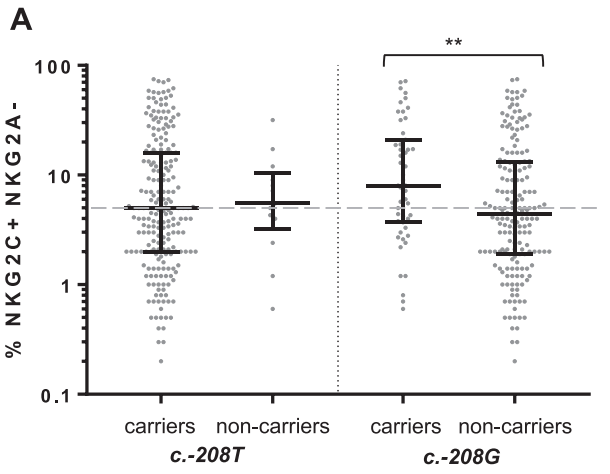
FIGURE 4 | Frequencies of NKG2C$^+$NKG2A$^-$ NK cells in individuals with different *NKG2C* CDS genotypes. All observed genotypes are represented, and those seen in more than one individual are compared below the dot plots.

genotypes (Figures 3 and 4), precluding reliable evaluation of the phenotype associated with this allele.

Analysis of individual genotypes (Figure 4) parallels the results of haplotype analysis (Figure 2): *NKG2C*01,*02* heterozygotes, the major group of allele *NKG2C*02* carriers, have proportions of NKG2C$^+$A$^-$ NK cells significantly higher than *NKG2C*01,del* and *NKG2C*01,*01* donors, who have percentages below the general median (medians: 11.4% vs. 3.4% and 4.6%, $p < 0.001$ and 0.006, respectively). Gene dose effects are either not statistically significant or unnoticeable in this analysis of coding sequence genotypes. In particular, neither the *NKG2C*02,del* nor the *NKG2C*02,*02* genotypes associate with impressive NKG2C$^+$A$^-$ NK-cell proportions, but this result must be taken with caution since they are both represented by groups of < 10 individuals.

3.1.4 | Separate Analysis of *NKG2C* Regions: Polymorphisms in 5′ and 3′ Non-Coding Sequences

Since *NKG2C* diversity includes SNPs in its 5′ and 3′ non-coding regions, we analysed separately those polymorphisms. The c.-208T variant of the *NKG2C* promoter is found in the vast majority of individuals, since it is present in all *NKG2C*01* and **03* haplotypes, and in a minority of *NKG2C*02* ones; its carriers and non-carriers have medians similar to the general population. In contrast, the alternative c.-208G motif associates with higher proportions of NKG2C$^+$A$^-$ NK cells (Figure 5A). It was impossible to verify the isolated effect of c.-208G because it was seen exclusively with CDS allele *NKG2C*02* (haplotype *2a*). However, the median frequency of NKG2C$^+$A$^-$ NK cells is not higher in c.-208G carriers (8.0%) than in *NKG2C*02* carriers in general (i.e., independently of their promoter sequence, 8.5%, Figure 3). Furthermore, stratified analysis of the CDS and SNP c.-208T/G reveals that, among the 60 *NKG2C*02* carriers, nine donors in whom *NKG2C*02* is under control of a c.-208T promoter,



Promoter	Carriers Median (*n*)	Non-carriers Median (*n*)	*p*
c.-208T	5.0% (219)	5.5% (13)	0.861
c.-208G	8.0% (51)	4.4% (181)	**0.005**

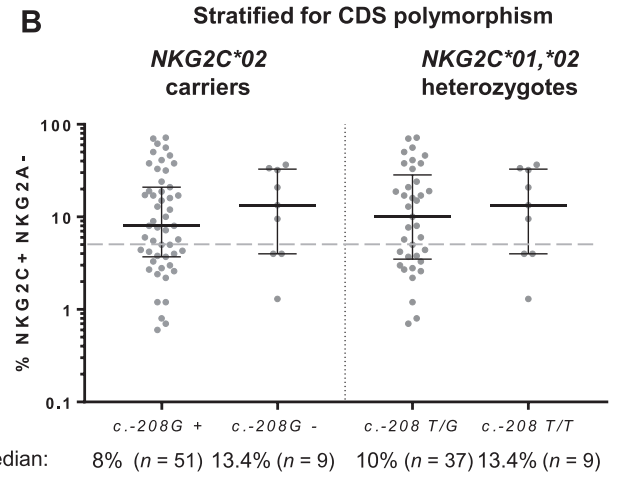


FIGURE 5 | Frequencies of adaptive NK cells according to *NKG2C*-promoter polymorphism. (A) Frequencies of NKG2C$^+$NKG2A$^-$ NK cells in carriers and non-carriers of the two alleles of the *NKG2C* promoter SNP c.-208T/G. Median frequencies and *p* values of comparisons between carriers and non-carriers are shown below; asterisks indicate $p < 0.01$. (B) Analysis of *NKG2C* promoter SNP c.-208T/G, stratified for CDS polymorphism. Left: Comparison, within *NKG2C*02* carriers, of those having or lacking c.-208G, usually associated with that allele. Right: Comparison, among *NKG2C*01,*02* individuals, between those with the usual c.-208T/G genotype and ones lacking c.-208G (i.e., c.-208T homozygotes).

normally linked to *NKG2C*01*, do not display lower proportions than ones having the usual c.-208G promoter (Figure 5B, left). Moreover, those nine individuals all have *NKG2C*01,*02* + c.-208T/T genotypes, and they show a median percentage of NKG2C$^+$A$^-$ NK cells not inferior (13.4%) than those with the similar, more common genotype *NKG2C*01,*02* + c.-208T/G (10%, Figure 5B, right).

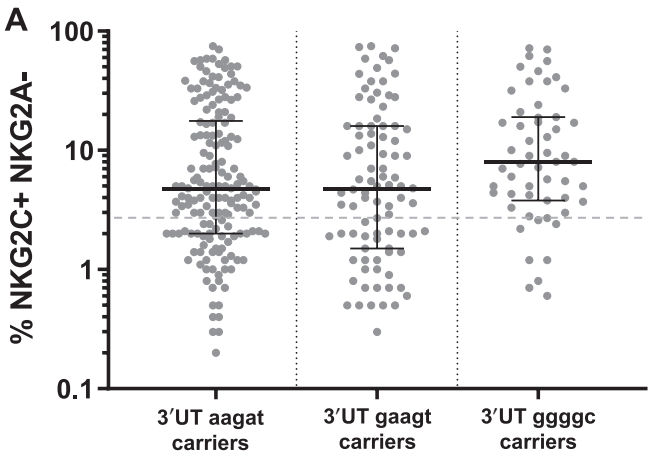
These stratifications appear to argue against the possibility that the promoter SNP c.-208G might be primarily responsible for the phenotype associated with CDS allele *NKG2C*02* and its most common extended haplotype, *NKG2C-2a*. To exclude the possibility that this unexpected result could be biased by individuals with big expansions of adaptive NK cells, possibly attributable to environmental or non-immunogenetic factors, we studied the NKG2C$^+$A$^-$ NK-cell frequencies in the subset of 185 donors having up to 20% such cells, in relation to relevant *NKG2C* polymorphisms. Within this subset, 43 *NKG2C*02* carriers had higher percentages than 142 non-carriers (medians: 5.0% vs., 3.0%, $p = 0.002$). All three CDS genotypes carrying allele *NKG2C*02* maintain higher frequencies than genotypes lacking this allele (Figure S3A), with different degrees of statistical significance (not shown). Similarly, its linked c.-208G promoter sequence associates with higher proportions of NKG2C$^+$A$^-$ NK cells ($p < 0.01$, Figure S3B). Finally, a stratified analysis, identical to that shown in Figure 5B, reveals again no statistically significant differences for the promoter SNP (Figure S3C). However, a subtle difference can be perceived with the analysis performed before exclusion of donors with more than 20% adaptive NK cells: donors with the variant genotype (i.e., allele *NKG2C*02* without *c.-208G*) have slightly, non-significantly lower frequencies than donors with the usual combination, in contrast with the opposite trend shown in Figure 5B. This result is compatible with an independent, albeit less potent, influence of the promoter SNP on expression, but demonstrating this will require studying many more individuals with exceptional *NKG2C* genotypes.

On the *NKG2C* 3′ UTR, five SNPs associate tightly with each other forming three combinations or haplotypes; two of those SNPs, c*105G-c*419C and c*105A-c*419T, are in complete linkage disequilibrium in this sample. The first two haplotypes (AAGAT and GAAGT), commonly seen with *NKG2C*01*, appear to be neutral regarding NKG2C$^+$A$^-$ NK-cell proportions (Figure 6A); whilst greater frequencies of these cells are seen in carriers of the third 3′ UTR haplotype (GGGGC), linked to *NKG2C*02*, and its two distinctive SNPs (c*105G and c*419C) (medians 8%, $p < 0.01$, Figure 6A).

However, as it happened with the promoter SNP, those median values are not higher than that of *NKG2C*02* carriers; and stratified analysis (Figure 6B) revealed that all six donors in whom *NKG2C*02* is exceptionally followed by the dominant AAGAT 3′ UTR haplotype (genotype of all six: 1a/2d) have high NKG2C$^+$A$^-$ NK-cell proportions (17.1%), similarly to 24 individuals with a nearly identical genotype, but having the GGGGC 3′ UTR haplotype usually linked to *NKG2C*02* (genotype: *1a/2a*). Coincident results were obtained restricting this stratified analysis, as well as that of 3′ UTR haplotype carrier state, to donors with < 20% of NKG2C$^+$A$^-$ NK cells (not shown), and additional analyses of 3′ UTR genotypes (Figure S4) revealed no novel associations that might provide further insight on those described above.

3.1.5 | *NKG2C* Genotype and Surface Expression Levels on NK Cells

We previously observed that *NKG2C* copy-number variation, besides modulating expansion of adaptive NK cells, correlated



3'UTR	Carriers Median (*n*)	Non-carriers Median (*n*)	*p*
haplotype			
AAGAT	4.7% (166)	5.3% (66)	0.624
GAAGT	4.7% (91)	5.0% (141)	0.407
GGGGC	8.0% (55)	4.0% (77)	**0.003**
SNP			
c*20A	4.7% (166)	5.3% (66)	0.624
c*20G	5.0% (134)	4.5% (98)	0.383
c*105A	5.0% (219)	5.5% (13)	0.860
c*105G	8.0% (57)	4.0% (175)	**0.002**
c*285A	5.0% (195)	4.4% (37)	0.101
c*285G	4.7% (93)	5.0% (139)	0.499
c*401A	4.7% (166)	5.3% (66)	0.624
c*401G	5.0% (134)	4.5% (98)	0.383
c*419T	5.0% (219)	5.5% (13)	0.860
c*419C	8.0% (57)	4.0% (175)	**0.002**

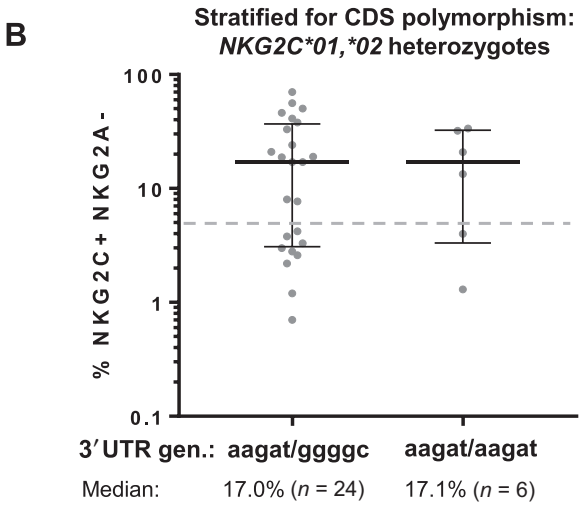


FIGURE 6 | Frequencies of NKG2C$^+$NKG2A$^-$ NK cells according to *NKG2C* 3′ UTR haplotypes and SNPs. (A) General analysis. Top: Dot plots representing the distribution in carriers of the three common 3′ UTR haplotypes. Bottom: Comparison of the distributions of carriers and non-carriers of those haplotypes and each individual SNP; median frequencies and *p* values are given. (B) Analysis in *NKG2C*01,*02* heterozygotes: Individuals with the usual 3′ UTR genotype 'aagat/ggggc' (i.e., genotype *1a/2a*) are compared with ones carrying a nearly identical genotype, except for *NKG2C*02* being associated with 3′ UT 'aagat' (and promoter c.-208 T, genotype *1a/2d*).

positively with surface receptor levels [18, 39]. We therefore analysed whether *NKG2C* sequence polymorphism could behave in a similar manner. The NKG2C MFI value on NK cells in flow cytometry was used as a measure of surface receptor density, and normalised MFI distribution was compared between the groups of donors with the most abundant genotypes. The reported effect of copy-number variation on surface receptor density was seen in the comparison of *NKG2C*01,del* donors with ones with two *NKG2C* copies (*NKG2C*01,*01* and *01,*02*), but a *NKG2C*01,*02* genotype appears to associate with only modestly higher MFI, which does not differ significantly in this sample from that of *NKG2C*01,*01* (Figure S5).

3.2 | Relative Abundance of *NKG2C* Alleles mRNA

Given the observed association between the presence of allele *NKG2C*02* and higher proportion of NKG2C$^+$A$^-$ NK cells, we considered of interest to compare the relative amount of *NKG2C*02* mRNA with that of the common allele *NKG2C*01*. To this end, we chose analysis of heterozygotes for these alleles as the least biased experimental setting, since it would ideally enable us to assess both alleles in the same tube, thus neutralising confounding effects derived from comparing samples of different donors, such as abundance of the subpopulation of interest, concentration, and quality of the mRNAs and cDNAs, efficacy of the individual amplification reactions et cetera. As a first and simple approach, we used an RT-PCR and sequencing method on the *NKG2C* exon 3 polymorphism that distinguishes *NKG2C*02* from the other alleles. This revealed that the signal derived from *NKG2C*02* was greater than that from *NKG2C*01*, in contrast with a similar amplicon derived from genomic DNA, which, as expected, yielded comparable amounts of the two alleles. This result replicated using either forward or reverse sequencing primers, and in several individuals with different *NKG2C*01,*02* genotypes (Figure S6). However, enzymatic cycle sequencing is not a quantitative method, and peak height can be affected by multiple variables, therefore we aimed at verifying this preliminary result with another technique.

To attain a more precise and reliable estimation of the representation of *NKG2C*02* in comparison with *NKG2C*01* (or *NKG2C*03*) messengers, we set up a qPCR assay that included two oligonucleotide probes. Each of these was specific for the exon 3 SNP of those alleles and they were labelled with different fluorescent dyes, which enabled their use and separate measurement in the same tube. Figure 7 represents the relative signals obtained with the two probes in each sample. Their amounts were adjusted so that they produced signals of similar intensity in amplifications of genomic DNA from *NKG2C*01,*02* donors (i.e., with a 1:1 allele ratio); and their specificity was verified on genomic DNA and cDNA of individuals carrying just one type of allele in homo- or hemizygosis, or lacking the *NKG2C* gene altogether (Figure 7A,B). Analysis of heterozygotes (Figure 7C) showed that, as hinted from sequencing experiments, *NKG2C*02* mRNA predominated over that of *NKG2C*01* (and *NKG2C*03* in one available *NKG2C*02,*03* sample). This result was consistently observed in all assays performed with cDNAs of 14 heterozygous individuals ($p < 0.0001$). These included representatives

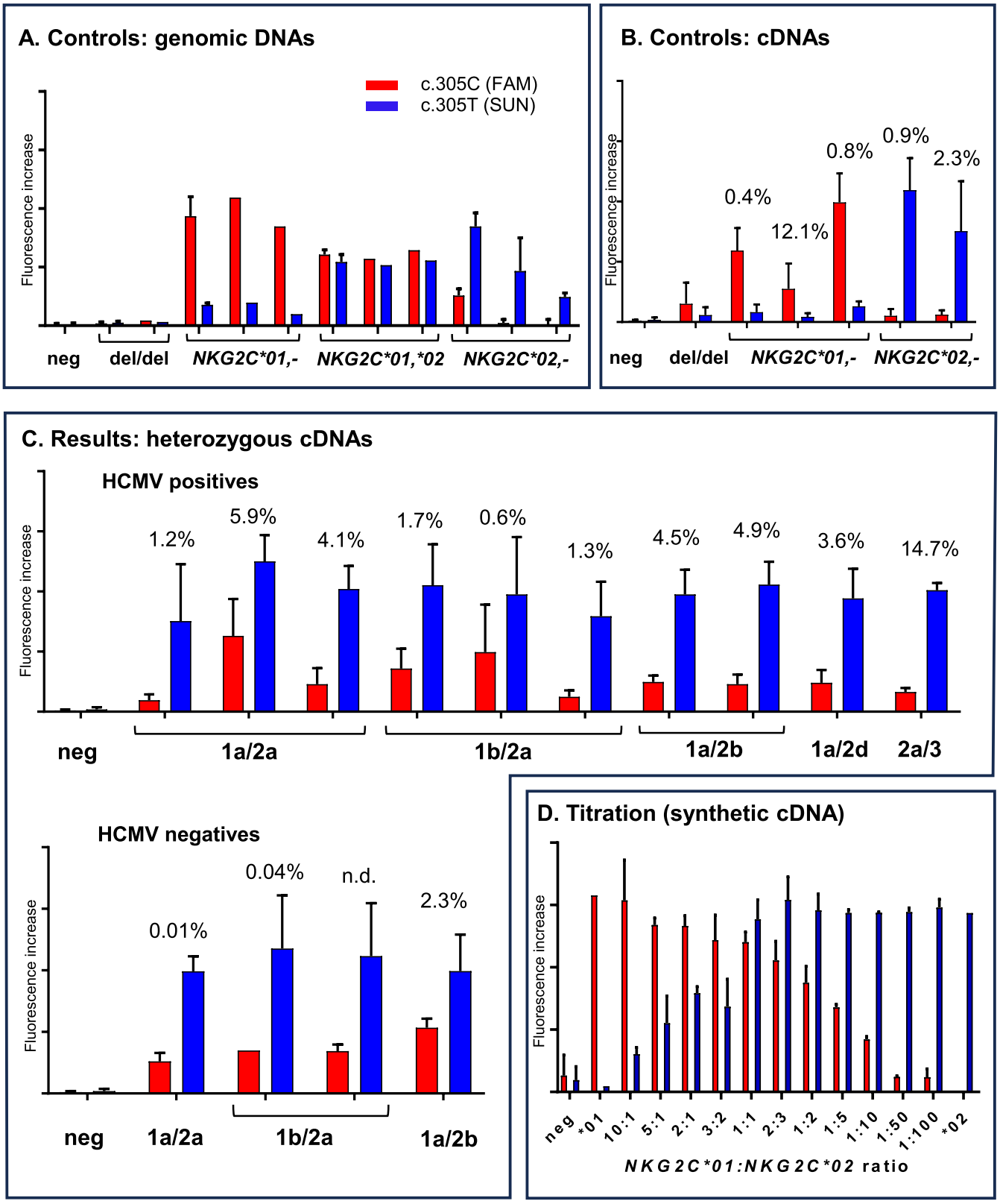


FIGURE 7 | Comparison of *NKG2C*-allele mRNA levels in individual donors, assessed by qPCR and SNP-specific probes: C.305C-FAM (*NKG2C*01/*03*, red bars) and c.305T-SUN (*NKG2C*02*, blue bars). Each pair of bars represents the signal of each probe in a single donor, whose *NKG2C* genotype is shown below. (A,B) Controls of probe specificity, sensitivity and signal levels: Genomic and complementary DNAs. (C) Analyses of 14 heterozygous individuals with different genotypes and HCMV serostatuses. (D) Analyses of serial dilutions of synthetic DNAs for semi-quantitative estimation of relative abundance of *NKG2C*01* and **02* transcripts. Percentages of NKG2C^{+} lymphocytes are given above bars of cDNAs; n.d., not done.

of all common and some rare genotypes, considering, not only the CDS, but also the promoter and 3′ UTR polymorphisms. *NKG2C*02* mRNA predominance was seen in individuals with a wide range of NKG2C$^+$A$^-$ NK-cell proportions. The ratios of the signals obtained with each probe did not appreciably correlate with the percentages of adaptive NK cells or NKG2C$^+$ lymphocytes (Figure 7B,C, S7, and S8 and data not shown). Also worth noting is that *NKG2C*02* predominated over *NKG2C*01* mRNA even in seronegatives for HCMV ($p = 0.003$), that is, before any reactive expansion of such cells might have taken place. Using two synthetic cDNAs corresponding to nucleotides 156–557 of the *NKG2C*01/*03* and the *NKG2C*02* coding sequences, we prepared a range of dilutions of both alleles, which were assayed with the same qPCR method (Figure 7D). Interpolation with this titration curve enabled us to estimate that *NKG2C*02* messengers were in all donors 5–50 times as abundant as those of the accompanying *NKG2C*01* or **03* allele.

To rule out possible biases derived from the different fluorescence intensities and profiles of our probe–dye combinations, we resynthesized those probes swapping their dyes (c.305C-SUN and c.305T-FAM). Reanalysis of cDNAs and controls with the new probes revealed again predominance of c.305T mRNA (allele *NKG2C*02*) over c.305C, as did separate analysis of c.305C and c.305T in different reactions, in comparison to the signal produced by a reference probe specific for an unrelated mRNA (Figures S7 and S8).

4 | Discussion

We have tested in this study the hypothesis that *NKG2C* sequence polymorphism might influence the relative abundance of NK cells with a NKG2C$^+$A$^-$ phenotype that expand specifically in response to HCMV infection. Our results clearly indicate that, beyond the known influence of *NKG2C*-gene CNV, a series of SNPs linked to allele *NKG2C*02* associates with greater or lesser proportions of those cells. Strong association of those polymorphisms in conserved haplotypes precludes mapping precisely which SNPs are mainly responsible for the observed effect. Exceptions to the common haplotypes are scarce; therefore, their analysis does not clarify whether SNPs in 5′ and 3′ potentially regulatory regions [20] have stronger association with expression levels than non-synonymous substitutions in the CDS, which will require analysing much larger cohorts. Limitations of this study are lack of data on absolute NK-cell numbers, which enabled us to analyse only relative proportions of the subpopulation of interest; and the small numbers of individuals carrying some rare genotypes, which prevents reliable estimation of their associated phenotypes.

Of note, all *NKG2C* genotypes, including those associated with the highest median percentages of NKG2C$^+$A$^-$ NK cells, include some individuals with low proportions of those cells, and vice versa, supporting that additional factors influence the probability or degree of their expansion. Based on observations in immunocompromised individuals, a delayed or inefficient control of viral replication by T cells, particularly following primary infection, may predictably enhance the magnitude of the adaptive NKG2C$^+$ NK cell response. This may be determined by circumstantial factors (e.g., early age of infection and viral load), as well as by other genetic variables of the host (e.g., *HLA*, *KIR*) [43] and HCMV (e.g., UL40) [41]; and it could be further boosted indirectly by the antibody-mediated response of adaptive NK cells to other viral infections [24, 28].

Furthermore, we have also shown here that *NKG2C*02*, the allele associated with higher proportions of NKG2C$^+$A$^-$ cells, is consistently transcribed at higher levels than the other alleles in heterozygous individuals, as assessed by RT-PCR analysis; and that this effect is seen even in absence of the 5′ and 3′ UT non-coding polymorphisms associated with the allele CDS. The latter is an unforeseen result, since c.-208G creates a YY1 site, expected to enhance transcription levels, that is absent from the c.-208T version of the promoter. Influence of c.-208G/YY1 is not ruled out by our study, since a putative effect of that site would be obscured by the profound impact of the CDS SNPs. Identification of individuals with rare genotypes in which the *NKG2C*02* CDS is dissociated from c-208G (e.g., *NKG2C-2a/2d* heterozygotes) could be of help in studying the precise contribution of the promoter SNP to the phenotype associated with *NKG2C-2a*.

Regulation of *NKG2C*-gene expression has not been explored, and the mechanisms that connect its polymorphism with mRNA levels and expansion of NKG2C$^+$A$^-$ cells are not obvious. To start with, it is unknown if *NKG2C* expression is monoallelic in individual NK cells of heterozygous subjects, as it happens for *KIR* through epigenetic control [44]. Therefore, we ignore whether finding more mRNA derived from *NKG2C*02* than from *NKG2C*01* in heterozygotes means that there are more cells expressing the former allele, that every NKG2C$^+$ cell expresses unequal amounts of each allele, or a combination of both. In either case, *NKG2C*02* mRNA predominance is previous to HCMV infection, since it is also seen in seronegative donors. It remains to be established whether *NKG2C* CDS polymorphisms exert a direct effect on mRNA levels, or they do it by indirect mechanisms, for example, some functional property of the encoded receptor, or linkage disequilibrium with another unknown regulatory element.

Different expression levels of *NKG2C* alleles had not, to our knowledge, been proposed before. However, the higher mRNA levels of *NKG2C*02* in heterozygotes is in agreement with (and might explain) paradoxical results obtained previously: Shum et al. [14] obtained ca. three times as many cDNA plasmid clones from *NKG2C*02* than from *NKG2C*01*, after RT-PCR and plasmid cloning experiments from PBMC of a small population sample, despite being *NKG2C*01* most prevalent in all studied populations [15, 20, 21].

Considering that NKG2C$^+$ adaptive NK cells may contribute to control HCMV replication in at least some clinical settings [36, 45–47], another intriguing question is why the most common *NKG2C* allele is one associated with lesser expansion of the cells that express it or, in other terms, why *NKG2C*02* is not the most common allele. One possible reason would be that *NKG2C*02* might be evolutionarily more recent and in its way to becoming the dominant human allele. Unfortunately, ambiguous results in this regard derive from phylogenetic analysis (reference [14] and search on the GenBank/EBI/DDBJ databases, not shown): Phe102 of *NKG2C*02* looks like a recent acquisition of the human receptor (it is seen in *NKG2F* but not in *NKG2C* of other primates), whilst Ser102 of *NKG2C*01* is the consensus in primates. On the

contrary, Asn2 of *NKG2C*02* is the consensus of human and non-human primate *NKG2C* (and other receptors of the same family), Ser2 of *NKG2C*01* thus being a newer polymorphism.

An alternative hypothesis to explain predominance of *NKG2C*01* over *NKG2C*02* in the population relates with a recurrent pattern observed in leukocyte receptors—co-existence of allotypes of identical function but different potency, in an apparent evolutionary equilibrium in which the less active allele is more frequent in the population. Examples of this in NK cells are the activating CD16A-158 V/F allotypes [48] and the inhibitory KIR 2DL2/2DL3 [49]. Similarly, *NKG2C*01* and *NKG2C*02* could be one additional balanced pair, which would suggest that having more NKG2C$^+$ adaptive NK cells could be more or less advantageous, depending on the environment or the genetic context. *NKG2C*01* predominance could therefore mean that a moderate adaptive NK-cell response to HCMV is beneficial in common circumstances, possibility further supported by the high allelic frequency of *NKG2C* deletion in all populations (only second to *NKG2C*01* in ours).

The significance of *NKG2C* sequence polymorphism in human health is hardly explored [21]. Our finding encourages studies aimed at exploring whether, and to what extent, it could predict outcomes and health conditions related with HCMV infection and with the NK cells that expand in response to this virus.

Author Contributions

J.A. and M.M. designed and performed experiments, analysed and interpreted data, and wrote the article. K.A.-A.-S performed experiments. A.M. and M.A. contributed samples and performed experiments. M.L.B. designed the study and revised the article. C.V. designed the study, directed research, and wrote the article.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.