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The influence of cytomegalovirus on expression of HLA-G and its ligand KIR2DL4 by human peripheral blood leucocyte subsets

Running title: Leucocyte HLA-G, KIR2DL4 and CMV

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Abbreviations

CM, culture medium; CMV, cytomegalovirus; ILT-2/4, inhibitory receptor Ig-like transcript 2/4; KIR2DL4, killer immunoglobulin-like receptor two domains, long cytoplasmic tail, 4; LILRB1/2, leucocyte immunoglobulin-like receptor B1/2; mAb, monoclonal antibody/ies; qPCR, quantitative polymerase chain reaction; SNPs, single nucleotide polymorphisms; UTR1, untranslated region 1.

Summary

HLA-G is a non-classical class I HLA antigen, normally expressed in high levels only on extravillous cytotrophoblast. It has immunosuppressive properties in pregnancy and has also been found to be upregulated on leucocytes in viral infection. In this study, proportions of all leucocyte subsets expressing HLA-G were found to be low in healthy subjects positive or negative for cytomegalovirus (CMV). Significantly greater proportions of CD4+CD69+ and CD56+ T cells expressed HLA-G compared to other T cells. However, following stimulation with CMV antigens or intact CMV, proportions of CD4+, CD8+, CD69+ and CD56+ T cells, and also B cells expressing HLA-G were significantly increased in CMV+ subjects. Despite

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some subjects having alleles of HLA-G associated with high levels of expression, no relationship was found between HLA-G genotype and expression levels. Purified B cells from CMV+ subjects stimulated in mixed culture with CMV antigens showed significantly increased HLA-G mRNA expression by real-time PCR. Serum levels of soluble HLA-G were similar in CMV- and CMV+ subjects but levels in culture supernatants were significantly higher in cells from CMV+ than CMV- subjects stimulated with CMV antigens. The HLA-G ligand KIR2DL4 was mainly expressed on NK cells and CD56+ T cells with no differences between CMV+ and CMV- subjects. Following stimulation with IL-2, an increase in the proportion of CD56+ T cells positive for KIR2DL4 was found, together with a significant decrease in CD56dimCD16+ NK cells. The results show that CMV influences HLA-G expression in healthy subjects and may contribute to viral immune evasion.

Introduction

HLA-G is a non-classical class I HLA antigen with limited tissue distribution that is mainly expressed by maternal cytotrophoblast [1,2]. Like classical class I HLA genes, it maps to chromosome 6q and may be expressed in several cell-bound or soluble isoforms [3]. Although found most abundantly on extravillous cytotrophoblast, HLA-G is also expressed at lower levels in thymus, cornea, nail matrix, haematopoietic progenitors, pancreas and can also be induced in monocytes[4]. Expression has also been noted in both CD4+ and CD8+ regulatory T cell subsets [5].

Although HLA-G shows limited coding region polymorphism compared to classical class I HLA antigens, there are three particular non-coding region dimorphisms that are thought to influence levels of expression. A 14bp insertion in the 3' untranslated region of HLA-G is associated with decreased mRNA stability [6] while a C/G dimorphism at position +3142

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influences binding of several miRNAs [7]. Additionally, an A/G dimorphism at position +3187 regulates the stability of HLA-G mRNA [8]. Combinations of these dimorphisms have been identified which, together with five additional dimorphisms not known to be associated with altered levels of expression, have led to the definition of seven frequently occurring haplotypes. UTR-1 contains all three dimorphisms associated with increased levels of HLA-G expression, whereas UTR-2, -5 and -7 contain the converse three dimorphisms associated with decreased expression [9]. More recently, a further eight less frequently occurring haplotypes have been identified in Brazilian and French populations [10].

Both cell-associated and soluble HLA-G have immunosuppressive properties in pregnancy [11], transplantation [12] and malignancy [13]. Induction of HLA-G expression has been found in both monocytes [14] and T cells following HIV infection [14,15], although HIV-1 has also been reported to downregulate cell surface HLA-G expression [16]. In influenza infection, HLA-G expression is induced on monocytes and T cells [17]. Monocytes also show increased expression associated with enterovirus 71 infection [18] and mast cells can be induced to express HLA-G in hepatitis C virus-induced liver fibrosis [19]. However, these studies have mostly not analysed differences in expression according to HLA-G genotype.

A Killer Immunoglobulin-like Receptor (KIR) recognising HLA-G, known as KIR2DL4, is expressed by most human natural killer (NK) cells [20] and has features of both inhibitory and activating receptors [21]. Unlike most other KIRs, KIR2DL4 is expressed predominantly by NK cells although unlike other KIRs it is not expressed in a variegated fashion, owing to its different promoter structure [22]. Two frequently occurring alleles in the transmembrane region have a sequence of either 10 adenines, leading to a full-length product, or 9 adenines, leading to a truncated product [23]. HLA-G is also recognised by LILRB1/ILT2 and LILRB2/ILT4 which are inhibitory receptors expressed mainly on cells of monocytic lineage [24].

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Cytomegalovirus (CMV) is a herpesvirus which infects the majority of the population worldwide but in healthy individuals is largely controlled by the immune system without giving rise to significant symptoms [25]. Although CMV can upregulate HLA-G on activated macrophages [26] and monocytes [27], it can also lead to downregulation of HLA-G, as well as classical class I HLA molecules, on trophoblast [28].

Infection with CMV induces progressive changes in the adaptive immune system, particularly expansions of late-stage memory CD8+ T cells expressing CD57 but lacking CD28, changes that have been associated with the development of immunosenescence in elderly subjects [29]. In previous work, we found that a population of CD56+ T cells, showing markers of activation, were significantly expanded in CMV+ healthy individuals [30]. The aims of the present experiments were to investigate the expression of HLA-G and one of its ligands, KIR2DL4, on these and other immune cells in CMV+ and CMV- subjects, both under resting conditions and following stimulation with CMV antigens, and to what extent their expression is influenced by genotype. Increased leucocyte HLA-G expression would potentially be able to mediate inhibition of immune responses via interaction with ligands such as KIR2DL4.

Materials & Methods

Human samples

Peripheral blood was obtained from a panel of 32 healthy subjects (21 males, 11 females; age 23-60) with informed consent, following approval from the University of Liverpool Interventional Ethics Committee. Subjects were excluded if pregnant or suffering from any acute or chronic illness. A volume of 20ml blood was taken into preservative-free heparin (Wockhardt, Wrexham, UK) and peripheral blood mononuclear cells (PBMC) separated by density gradient centrifugation, washed and resuspended in culture medium (CM) consisting

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of RPMI-1640 + 10% heat inactivated fetal bovine serum, 2mM glutamine and antibiotics. Serum samples taken at the same time were aliquoted and stored at -20°C for future use. JEG-3 human choriocarcinoma cells were originally obtained from the European Collection of Cell Cultures and maintained in CM, subculturing weekly.

Anti-CMV antibody ELISA

Aliquots of sera from healthy subjects were analysed in duplicate for the presence of anti-CMV IgG antibodies using an ELISA kit (GenWay Biotec, San Diego), according to the manufacturer's instructions. In all cases, samples gave a clear-cut positive or negative result for anti-CMV IgG, and duplicates gave very similar results. This allowed all healthy subjects to be designated unequivocally as positive or negative for anti-CMV antibodies.

Flow cytometry

Aliquots of 5 x 10^5 PBMC were labelled with a monoclonal antibody (mAb) against HLA-G, clone MEM-G/09, which has been reported to react exclusively with native HLA-G1 [31], together with up to 3 other mAb against a range of leucocyte subset markers, as shown in Table 1. Cells were incubated with 2µl of mAb in 100µl medium for 30min at 4°C in the dark, washed and analysed using an Accuri C6 flow cytometer and dedicated software (Becton Dickinson, Oxford, UK). Tubes containing appropriate isotype controls (Table I) were prepared and analysed in the same way. Cells were gated on lymphocyte or monocyte populations, based upon forward and side scatter, and results expressed as % cells expressing HLA-G above the level of staining seen with the isotype control mAb. In order to assess FoxP3 expression, cells were labelled with mAb against appropriate cell surface antigens as

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above and then fixed and permeabilised using fixation/permeabilization concentrate and diluent (eBioscience, Hatfield, UK). Anti-FoxP3 mAb (eBioscience, Hatfield, UK) was then added for a further 30min at 4°C in the dark and the cells washed and analysed by flow cytometry.

CMV antigen stimulation

PBMC at a concentration of 2 x 10^6/ml in CM were cultured in the presence of CMV lysate from cells infected with the AD-169 strain (The Native Antigen Company, Upper Heyford, UK) at a concentration of 1μg/ml in CM and incubated for 7 days at 37°C and 5% CO_2 in a humidified atmosphere. Cultures were fed with fresh medium and CMV antigen every 3-4 days. In some experiments, live Towne strain CMV particles, prepared using standard techniques, at a MOI of 10 were used to stimulate PBMC. In both types of experiment, unstimulated control cells were incubated in the presence of CM alone. Cells were labelled with combinations of mAb as above, gated on viable lymphocytes and analysed by flow cytometry. Changes in % HLA-G+ cells were calculated by subtracting values for unstimulated lymphocyte subsets from those following antigenic stimulation. In studies of KIR2DL4 expression, PBMC were cultured in 20ng/ml IL-2 (R and D Systems, Abingdon, UK) in CM for 14 days, refeeding with fresh medium and IL-2 every 3-4 days, and cells labelled with anti-KIR2DL4 in conjunction with anti-CD3, anti-CD16 and anti-CD56 for flow cytometric analysis.

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Proliferation assays

PBMC (10^7) were labelled with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Paisley) according to the manufacturer's instructions. After washing, labelled cells were resuspended in CM and incubated for 7 days in the presence or absence of 2μg/ml CMV antigens. Aliquots of stimulated and unstimulated cells were labelled with a panel of antibodies against T, B and NK cell surface markers and HLA-G and analysed by flow cytometry. Proportions of cells whose FITC fluorescence was reduced below that of control nondividing unstimulated cells were regarded as having proliferated.

Soluble HLA-G ELISA

Serum samples from healthy CMV+ and CMV- subjects and culture supernatants from PBMC stimulated with or without CMV antigens for 7 days were carefully removed and stored at -20°C. Levels of soluble HLA-G were measured using an ELISA kit (Enzo Bioscience, Prague) in units/ml in comparison to the standard provided, according to the manufacturer's instructions. Samples were analysed in duplicate and mean values taken.

HLA-G genetic analysis

DNA was prepared from 10^7 freshly isolated PBMC using standard techniques. PCR was carried out using the forward primer 5′GTGATGGGCTGTTTAAAGTGTCACC3′ and the reverse primer 5′GGAAGGAATGCAGTTCAGCATGA3′ specific for exon 8 of HLA-G [32]. Depending on the presence or absence of a 14bp insertion/deletion, these primers give rise to a product of 210 or 224bp [32]. In order to sequence the 3' untranslated region of the HLA-G gene, the following primers designed from the published HLA-G gene sequence were used: 5'GTAGTGTGAAACAGCTGCCC3' (forward) and 5'ACACGTGTACTGTGGAAAGTT3' (reverse). PCR was carried out and products purified

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using a PureLink quick gel extraction and PCR purification combo kit (Fisher Scientific, Loughborough, UK).

Purified PCR products were then sequenced by Source Bioscience (Nottingham, UK). Where sequences contained ambiguous bases as a result of dimorphisms [7,8], chromosomal linkages were provisionally determined by reference to the sequences of the commonly occurring haplotypes of exon 8 [9].

KIR2DL4 gene analysis

DNA was extracted from 10^7 PBMC using standard techniques. Freshly isolated cells were prepared in parallel. PCR was carried out using the forward primer 5′TGCCTGGCAACCAAGAAATG3′ and the reverse primer 5'ACAATCAGGCAACGGTCTGT3′ designed from the KIR2DL4 sequence that span the transmembrane region containing the 10A/9A dimorphism [23]. PCR products were purified and sequenced as above. Homozygotes could be readily identified by the presence of 9 or 10 adenines while in heterozygotes the sequence 3' of the 9A/10A region gave rise to a hybrid sequence as a result of the loss of a single base in the 9A allele.

Cell purification and qPCR analysis

2 x 10^7 cells from CMV+ subjects that had been stimulated for 7 days with CMV antigens were separated into B cells by negative selection using EasySep magnetic bead based kits (Stemcell Technologies, Grenoble), according to the manufacturer's instructions. Purified B cell populations (~10^6 cells; >98% purity) were then lysed and RNA extracted and purified using a Pure Link RNA extraction kit (Thermo Fisher Scientific, Paisley, UK). Total RNA

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was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) and reverse transcribed to cDNA using a High Capacity cDNA kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Real Time PCR

The cDNA was diluted 1:4 with RNase free water (Thermo Fisher Scientific) and used in a master mix reaction for qPCR. A total volume of 25µl/well was used by preparing a dilution of the three probes as follows: 6µl of HLA-G and L32 (housekeeping gene) probes were diluted with 60µl of 2x TaqMan Universal Master Mix II (Thermo Fisher Scientific). Then 13.75µl of these probes were aliquoted in duplicate wells and the volume completed with 11.25µl of the diluted cDNA. All reaction steps were set up at 4 °C and the plate run in a qPCR machine (Applied Biosystem 7300; Applied Biosystems, Warrington, UK) as follows: first stage at 50°C for 2 min, the second stage consists of 50 cycles at 95°C for 10 min and 95°C for 15 seconds followed by a third stage at 60°C for 2 min.

Relative levels of cDNA in samples from stimulated and unstimulated cells were calculated using the ΔCT method using L32 as housekeeping gene.

Statistical analysis

Differences in mean percentages of cells expressing HLA-G between groups were tested using paired t-tests. Differences between levels of soluble HLA-G were tested using Mann-Whitney U-tests. Linear regression was used to analyse the relationship between HLA-G expression and SNP scores. In all cases, values of $p<0.05$ were considered as statistically significant.

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Results

HLA-G expression by peripheral blood mononuclear cells in relation to CMV serological status

Freshly isolated lymphocytes and monocytes from a group of 17 CMV+ and 15 CMV- healthy subjects were analysed for HLA-G expression by a range of different subsets by 3- or 4-colour flow cytometry. The anti-HLA-G antibody (clone MEM-G/9) used was initially tested on the human choriocarcinoma cell line JEG-3, of which 87.5% of cells were positive (isotype control 0.1%; data not shown). While NK cells and most T cell subpopulations showed a mean of <1% HLA-G positive cells, B cells, CD56+ T cells, CD4+CD69+ T cells and monocytes showed a mean of between 1 and 3% positive cells, irrespective of CMV serological status (Fig. 1). Higher proportions of most leucocyte subsets from CMV- than from CMV+ subjects expressed HLA-G although in no instances was this statistically significant (Fig. 1). When T cell subsets were compared, it was a striking finding that a significantly higher proportion of the minor CD4+CD69+ ($p<0.01$) and CD56+ subsets ($p<0.05$) expressed HLA-G than the total CD4+ or CD8+ T cell populations. This was true for both CMV+ and CMV- subjects (Fig. 2).

HLA-G expression following stimulation with CMV antigens

PBMC from the same group of CMV+ and CMV- subjects were stimulated for 7 days with CMV antigens and HLA-G expression measured on the same leucocyte subsets by flow cytometry. Compared to unstimulated cells cultured for 7 days, proportions of HLA-G+ cells of most lymphocyte subsets, including CD4+ and CD8+ T cells ($p<0.01$), CD4+CD69+ ($p<0.05$) and CD56+ T cells ($p<0.01$) and B cells ($p<0.001$; Fig. 3A-C), were significantly increased in CMV+ healthy subjects (Fig. 3D). The only cell types not showing clear increases in % HLA-G+ cells were Tregs and NK cells.

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However, in the case of CMV- subjects, the % HLA-G+ cells were not significantly increased in most of these lymphocyte subsets following stimulation with CMV antigens for 7 days $p>0.05$). Only B cells showed a statistically significant increase in % HLA-G+ cells ($p<0.05$); proportions of CD56+ T cells were also increased but not significantly ($p>0.05$; Fig. 3E). Similar results were obtained by stimulating PBMC with intact CMV particles (data not shown).

HLA-G expression in relation to HLA-G genotype

DNA from the same healthy CMV+ and CMV- donors was subjected to PCR analysis and the presence or absence of the 14bp insertion was determined by gel electrophoresis. Other parts of the exon 8 region were also sequenced and the dimorphisms at positions +3142 and +3187 identified. This allowed the subjects to be categorized as high, intermediate or low HLA-G expressers according to UTR haplotype [10]. While some subjects could be unequivocally identified as having high (UTR-1) or low expressing haplotypes (UTR-2, -5 or -7), many had a mixture of SNPs associated with high or low expression. Therefore, each subject was allocated an expression score according to the presence or absence of alleles associated with high (+1) or low (0) HLA-G expression, giving a possible aggregate score of between 0 and 6. The expression score for each subject was then plotted against the % HLA-G expressing cells for both B cells and CD56+ T cells. In both cell types, it was clear that there was no relationship between expression score and % HLA-G+ cells (data not shown).

Expression of HLA-G mRNA by CMV-stimulated B cells by qPCR

In order to ascertain that HLA-G mRNA expression was increased in B cells following CMV antigen stimulation, qPCR was performed on cDNA from purified B cells from CMV+ subjects after culturing PBMC for 7 days with or without CMV antigen. The results showed

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that B cells from stimulated populations expressed substantially higher levels of HLA-G mRNA than unstimulated cells. The difference in mean ΔCT between unstimulated and stimulated B cells from nine experiments was 4.43 indicating a >4-fold increase in expression of HLA-G in B cells stimulated with CMV antigens (data not shown).

Expression of HLA-G in relation to cell proliferation

PBMC from CMV+ healthy subjects were labelled with CFSE and cultured for 7 days with CMV antigens. Expression of HLA-G was then measured on proliferated and non-proliferated cells. As shown in Fig. 4, significantly higher proportions of non-proliferated CD4+ ($p<0.05$), CD8+ ($p<0.05$) and CD56+ T cells ($p<0.01$), and also B cells ($p<0.01$), expressed HLA-G than proliferated cells. Unstimulated cells showed very low levels of proliferation (data not shown).

Soluble HLA-G levels in relation to CMV serological status

Levels of soluble HLA-G were measured in serum samples from healthy CMV+ and CMV- subjects and supernatants from cultures of PBMC stimulated with CMV antigens for 7 days. There were no significant differences between serum levels of soluble HLA-G between healthy CMV- and CMV+ subjects ($p>0.3$; Table II). However, when 7 day culture supernatants with or without CMV antigen stimulation were tested, only cells from CMV+ subjects showed a significant increase in levels of soluble HLA-G (Table 2; $p<0.005$).

Expression of KIR2DL4 in relation to CMV serological status

Groups of CMV+ and CMV- healthy subjects were tested for cell surface expression of the HLA-G ligand KIR2DL4 by 3- or 4-colour flow cytometry. Expression was detected on around 10% of NK cells of the major $CD56^{bright}$CD16- and $CD56^{dim}$CD16+ subsets and a

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lower percentage of T cells. There were no significant differences in expression between cells from CMV+ and CMV- subjects (Fig. 5A & B). When PBMC from CMV+ healthy subjects were stimulated with IL-2 for 7 days, there was a significant increase in the % of CD56+ T cells expressing KIR2DL4 but a significant decrease in the % CD56+CD16^{bright} NK cells expressing this antigen (Fig. 5A). Similar results were obtained with CMV- healthy subjects, with significant increases in the % CD56+ T cells expressing KIR2DL4 but decreases for CD56^{dim}CD16+ NK cells (Fig. 5B).

When levels of expression of KIR2DL4 were examined in relation to genotype, there were no significant differences in expression by any of the T cell or NK cell subsets between subjects who were 10A/10A or 9A/9A homozygotes (data not shown). Following 14 days' culture with IL-2, a significantly increased proportion of CD56+ T cells from both genotypes expressed KIR2DL4 (9A/9A: $p<0.05$; 10A/10A: $p<0.01$). However, decreased proportions of CD56^{bright}CD16- NK cells from 9A/9A homozygotes ($p<0.01$), but not 10A/10A homozygotes ($p>0.05$), expressed KIR2DL4 after 14 days (Fig. 6).

Discussion

It has previously been reported that cell surface HLA-G is expressed on very few peripheral blood leucocytes, with monocytes and regulatory T cells being the most likely cells to express HLA-G [4]. However, in freshly derived cells we found that higher proportions of CD4+CD69+ and CD56+ T cells were HLA-G positive when compared to the whole of the CD4+ T cell population. For both B cells and monocytes a higher percentage of cells expressed HLA-G than CD4+ or CD8+ T cells. There were no significant differences in expression between CMV+ and CMV- subjects. However, following stimulation of PBMC from CMV+ subjects with CMV antigens, proportions of HLA-G expressing cells

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significantly increased for most lymphocyte subsets, with the exception of NK cells and Treg. In the case of CMV- subjects, similar but smaller increases in HLA-G expression were induced although these were only statistically significant for B cells. Monocytes were not examined as these largely become adherent in mixed cultures, although it has previously been reported that their differentiation results in increased HLA-G expression [33].

These results suggest that there is an acute induction of HLA-G expression in response to CMV antigens but that in CMV carriers levels normalise outside of periods of CMV viremia. Similarly, levels of soluble HLA-G did not differ significantly in serum or unstimulated culture supernatants from CMV- and CMV+ healthy subjects. However, stimulation of leucocytes from CMV+ but not CMV- subjects resulted in increased secretion of soluble HLA-G. This has the potential to interact with HLA-G receptors such as ILT-2, ILT-4 or KIR2DL4 to mediate inhibition [34]. It is conceivable that HLA-G could be present in tissue culture supernatants or serum as extracellular vesicles [35]. Selective expression of HLA-G on B cells activated by CMV may lead to inhibition of CD4+ T cells responding to CMV antigens presented by B cells as part of an immune evasion strategy. It would be of interest to determine whether B cells induced to express HLA-G following CMV stimulation are CMV-specific. It has been reported that B cells express ILT-2 which, when engaged by HLA-G, results in inhibition of B cell function [36]. It is therefore conceivable that increased HLA-G expression in response to CMV infection could lead to autocrine inhibition of B cell function.

We have previously reported that CD56+ T cells are proportionately increased in CMV+ healthy subjects and levels are increased following stimulation with CMV antigens [30]. These cells are potentially able to mediate both antigen-specific and nonspecific cytotoxic

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function [37] but expression of HLA-G may lead to functional inhibition of any cells with which they interact.

Several CMV genes have a role in downregulating classical class I HLA expression [38,39] but there are conflicting reports as to whether this also applies to HLA-G. While expression is downregulated in trophoblast [28] and transfected astrocytoma cells infected with CMV [40], this is more delayed than for classical class I [41]. Levels of HLA-G are increased on monocytes following CMV infection [26] and it has been suggested that increased levels on CD4+ T cells in pregnancy result from uptake of HLA-G from decidual dendritic cells [42]. Expression of HLA-G is induced on CD4+ T cells *in vitro* in mixed lymphocyte responses [43] and in breast cancer patients *in vivo* [44]. In the latter case, this may result in suppression of antitumor responses and this would be consistent with a similar role in CMV infection. Although some CD25 and FoxP3 negative Treg populations have been reported to express HLA-G [5], in our hands, proportions of classical Tregs expressing HLA-G were not increased following CMV stimulation.

The role of anti-inflammatory cytokines such as IL-10 or TGFβ in induction of HLA-G expression following stimulation with CMV antigens is unclear. There is a close relationship between HLA-G and IL-10 expression, IL-10 being able to induce HLA-G expression in human trophoblasts and monocytes and the inhibitory role of HLA-G-expressing Tregs being dependent upon IL-10 [4]. A subset of tolerogenic DCs expresses HLA-G and mediates its function through IL-10 production [45]. However, IL-10 levels were not measured in the present experiments and it would be of interest to investigate its role.

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The CMV extracts and intact viral particles used in the present experiments were derived from the AD169 laboratory strain, which lacks a group of genes present in wild type clinical isolates [46]. Although both AD169 and strains containing this block of genes downregulate class I HLA [47], they have differential effects on susceptibility to NK cell killing and it remains a possibility that AD169 does not have the same effect upon HLA-G as wild type strains. When proportions of HLA-G positive cells were related to HLA-G genotype it was found that there was no relationship between % positive cells and the presence of alleles reported to be associated with high expression of HLA-G [6-9, 48]. There are several possible explanations for this. There may be additional regulatory elements controlling levels of HLA-G expression other than the 3' untranslated region SNPs investigated. Alternatively, CMV or its products may be able to override host cellular elements regulating HLA-G expression. Levels of the miRNAs, whose binding has been reported to be influenced by the +3142 C/G dimorphism [7], may be altered by CMV infection. However, the 14bp deletion dimorphism was associated with higher levels of HBe antigen in patients chronically infected with Hepatitis B [49]. In contrast, plasma levels of soluble HLA-G in healthy Brazilian and French subjects were not closely correlated with the alleles previously associated with high or low levels of expression [50].

Proportions of cells expressing KIR2DL4, one of the ligands for HLA-G, at the cell surface, were found to be higher in NK cells and CD56+ T cells than CD56- T cells and there were no significant differences between CMV+ and CMV- subjects. However, following stimulation with IL-2, proportions of KIR2DL4+ cells were significantly increased in CD56+ T cells but significantly decreased in NK cells, irrespective of CMV status. KIR2DL4 is predominantly expressed intracellularly [51] and in the present experiments only cell surface expression was considered. Stimulation may result in a redistribution of this ligand for HLA-G rather than a

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change in levels of expression, with higher proportions of CD56+ NK cells than CD56+ T cells internalising KIR2DL4. It has previously been shown that CMV+ subjects have increased T cells expressing ILT-2 [52,53] and hence may be susceptible to inhibition by HLA-G-expressing B cells or monocytes.

Previous genetic analysis of KIR2DL4 has shown that the 10A genotype encodes expression of a full-length protein while the 9A genotype has excision of exon 6, encoding the transmembrane domain [54]. However, in our hands, cell surface expression, as detected by the mAb33 clone, was similar in healthy subjects homozygous for 9A or 10A alleles. As the product of the 9A allele is secreted as a soluble product [54], it is possible that it may be expressed at the cell surface by binding to another molecule, possibly even HLA-G itself. Following culture with IL-2 it was only 9A homozygotes that showed downregulation of KIR2DL4 expression on NK cells.

Taken together, the results presented here would be consistent with CMV being capable of inducing HLA-G expression by some lymphocyte subsets that would be able to mediate inhibition of function of cells expressing ligands for HLA-G as a mechanism of immune evasion. It would be of interest to examine whether HLA-G-expressing lymphocytes have antigen receptors specific for CMV or other viruses in CMV+ healthy subjects.

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LB advised on statistical analysis. BF advised on molecular analyses. SEC designed the study and wrote the manuscript.

Conflict of Interest

The authors declare that there is no financial or commercial conflict of interest.

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Table 1. Monoclonal antibodies used in the study.

Monoclonal antibody	Isotype	Code #	Supplier
Anti-CD3-FITC	IgG2a	317306	Biolegend, London, UK
Anti-CD3-PerCP/Cy5.5	IgG2a	317336	Biolegend, London, UK
Anti-CD4-PE	IgG2b	317410	Biolegend, London, UK
Anti-CD8-PE	IgG1	9012-0087	eBioscience, Hatfield, UK
Anti-CD14-PE	IgG1	12-0149-42	eBioscience, Hatfield, UK
Anti-CD16-FITC	IgG1	11-0168-42	eBioscience, Hatfield, UK
Anti-CD19-PE	IgG1	12-0198-42	eBioscience, Hatfield, UK
Anti-HLA-G (clone MEM-G/9)	IgG1	A15708	Life Technologies, Paisley, UK
Anti-CD56-PE	IgG1	12-0567-42	eBioscience, Hatfield, UK
Anti-CD56-APC	IgG1	17-0566-42	eBioscience, Hatfield, UK
Anti-CD69-PerCP/Cy5.5	IgG1	310926	Biolegend, London, UK

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Anti-FoxP3-FITC	IgG2a	11-4776-42	eBioscience, Hatfield, UK
Anti-HLA-DR-FITC	IgG2b	327006	Biolegend, London, UK
Anti-KIR2DL4-PE	IgG1	347006	Biolegend, London, UK
Anti-LILRB1-PE	IgG2b	ab186074	AbCam, Cambridge, UK
Isotype control-PE	IgG2b	ab91532	AbCam, Cambridge, UK
Isotype control IgG1κ	IgG1	400112	Biolegend, London, UK
Isotype control-APC	IgG1	MG105	Life Technologies, Paisley, UK

Table 2. Levels of soluble HLA-G (units/ml) in serum and tissue culture supernatants from healthy CMV- and CMV+ subjects cultured for 7 days with or without CMV antigens as measured by ELISA. Statistical analysis was performed using a Mann-Whitney U-test.

Serum	CMV-	CMV+	p value
n =	10	13	
Median (u/ml)	34.2	23.8	>0.3
Range (u/ml)	13.5 – 469	17.9 - 1270	
Culture supernatants, CMV- subjects	Unstimulated	+ CMV antigens	
n =	6	6	
Median (u/ml)	1.69	1.02	>0.4
Range (u/ml)	0.04 – 4.49	0.29 – 5.81	
Culture supernatants, CMV+ subjects	Unstimulated	+ CMV antigens	
n =	13	13	
Median (u/ml)	**2.41**	**7.0**	**<0.005**
Range (u/ml)	0.175 – 11.2	2.62 – 42.8	

Figure Legends

Fig. 1 **Expression of HLA-G on freshly isolated leucocyte subsets from healthy subjects according to CMV serological status.** HLA-G expression by monocytes and lymphocyte subpopulations from CMV+ (n=17; filled columns) and CMV- healthy subjects (n=15; open columns). Results are expressed as mean +/- sem. None of the subsets showed significant differences between CMV+ and CMV- subjects (p>0.05).

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Fig. 2 **Expression of HLA-G on freshly isolated T cell subsets according to CMV serological status.** Results are expressed as mean +/- sem. Filled columns, CMV+ (n=17); open columns, CMV- (n=15). Differences between subsets: *0.01<p<0.05; **0.001<p<0.01.

Fig. 3 **Expression of HLA-G on leucocyte subsets with and without stimulation with CMV antigens for 7 days.** Representative flow cytometry profiles of gated CD19+ B cells from a CMV+ healthy subject: A) Unstimulated B cells, isotype control; B) Unstimulated B cells, anti-HLA-G; C) CMV-stimulated B cells, anti-HLA-G; D) CMV+ subjects (n=17); horizontal hatching = unstimulated, vertical hatching = stimulated; E) CMV- subjects (n=15); downward hatching = unstimulated, upward hatching=stimulated. Results are expressed as mean +/- sem. Differences between unstimulated and stimulated: *0.01<p<0.05; **0.001<p<0.01; ***p<0.001.

Fig. 4 **Expression of HLA-G on nonproliferated and proliferated lymphocyte subsets**. PBMC were stimulated with CMV antigen for 7 days and proportions of nonproliferated (open bars) and proliferated cell subsets (filled bars) determined by flow cytometry. Results are shown as mean +/- sem (n = 6). * p<0.05; ** p<0.01.

Fig. 5 **KIR2DL4 expression by NK cell subsets and CD56+ T cells** A) CMV+ healthy subjects; downward hatching=unstimulated, upward hatching=IL-2 stimulated; B) CMV- healthy subjects; horizontal hatching=unstimulated, vertical hatching=IL-2 stimulated for 7 days in mixed cultures. Results are expressed as mean +/- sem (n = 5). * p<0.05.

Fig. 6 **KIR2DL4 expression by NK cell subsets and CD56+ T cells according to genotype.** Mixed cultures after 14 days' incubation alone=downward hatching or in IL-2=upward hatching, according to KIR2DL4 genotype (n = 5). * p<0.05; ** p<0.01.

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Figure 1

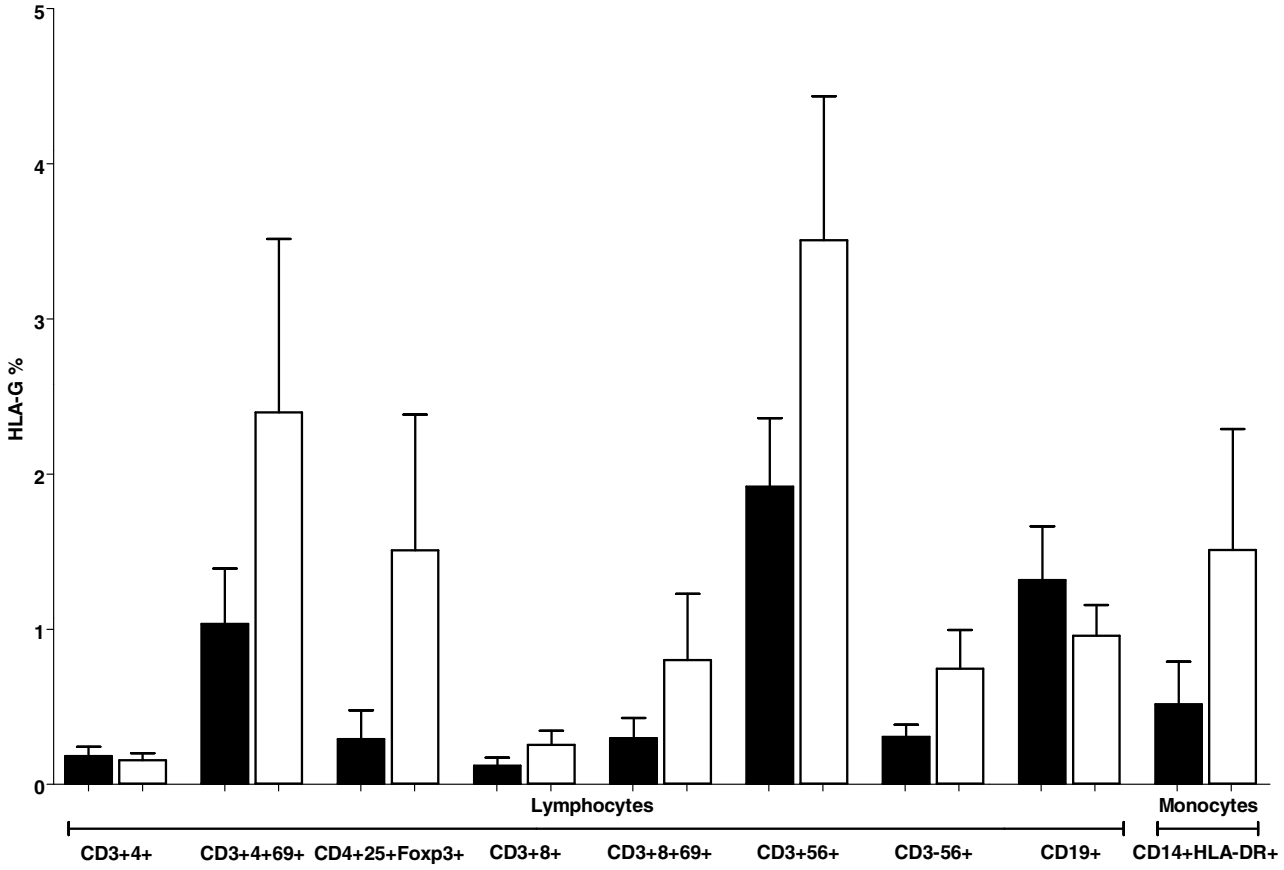

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Figure 2

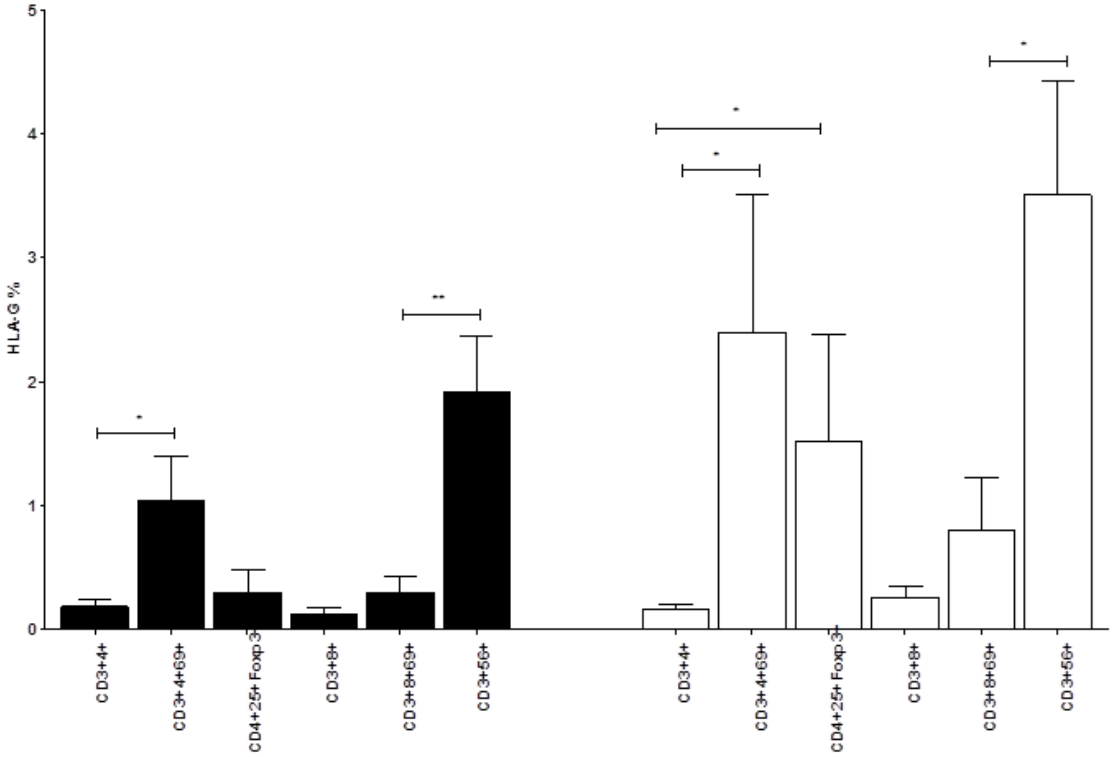

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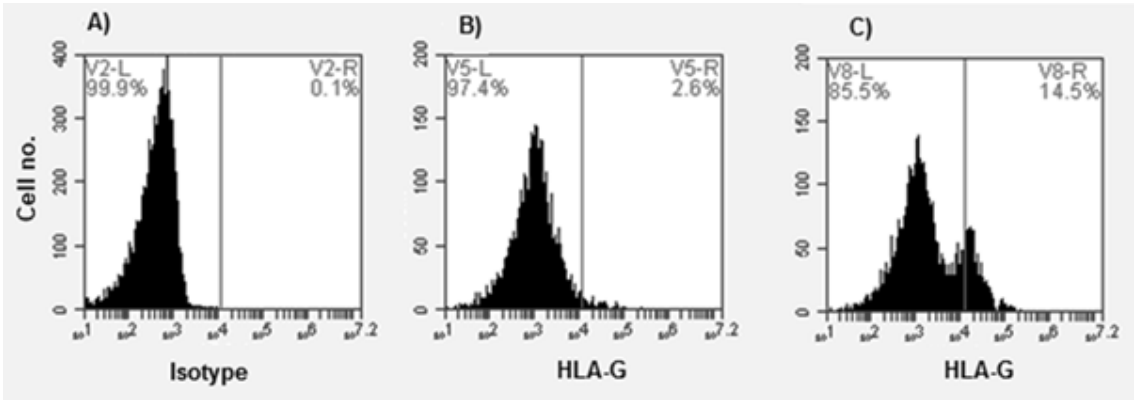
Figure 3



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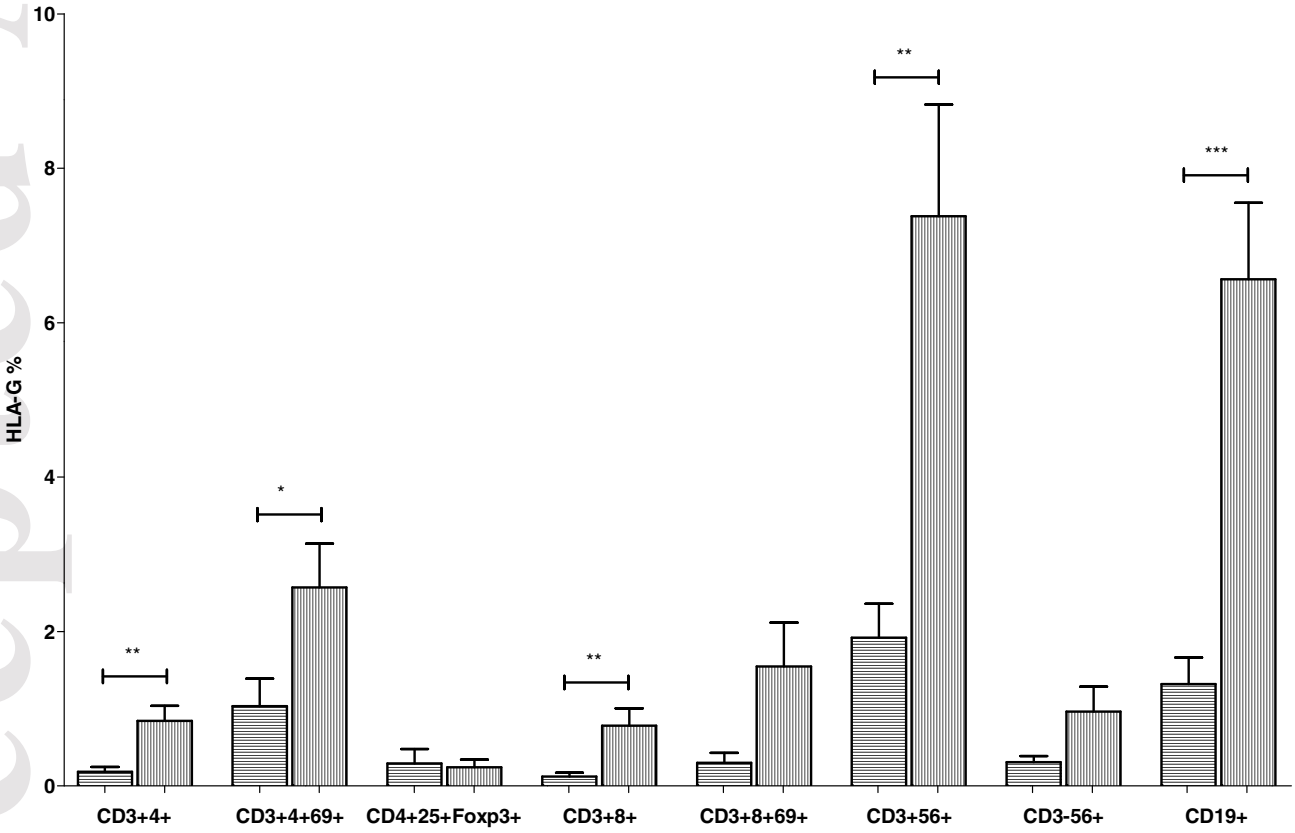

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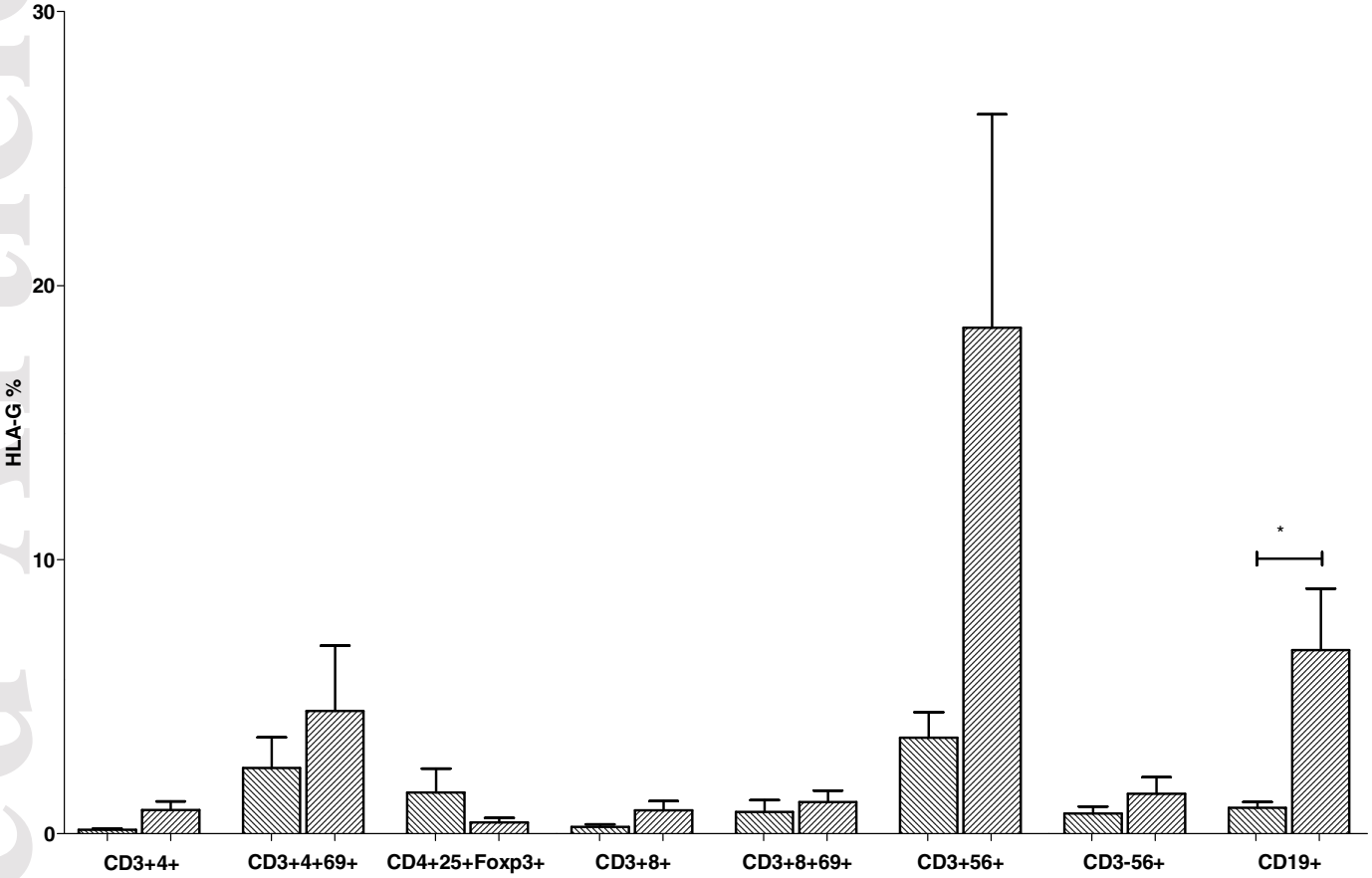

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Figure 4

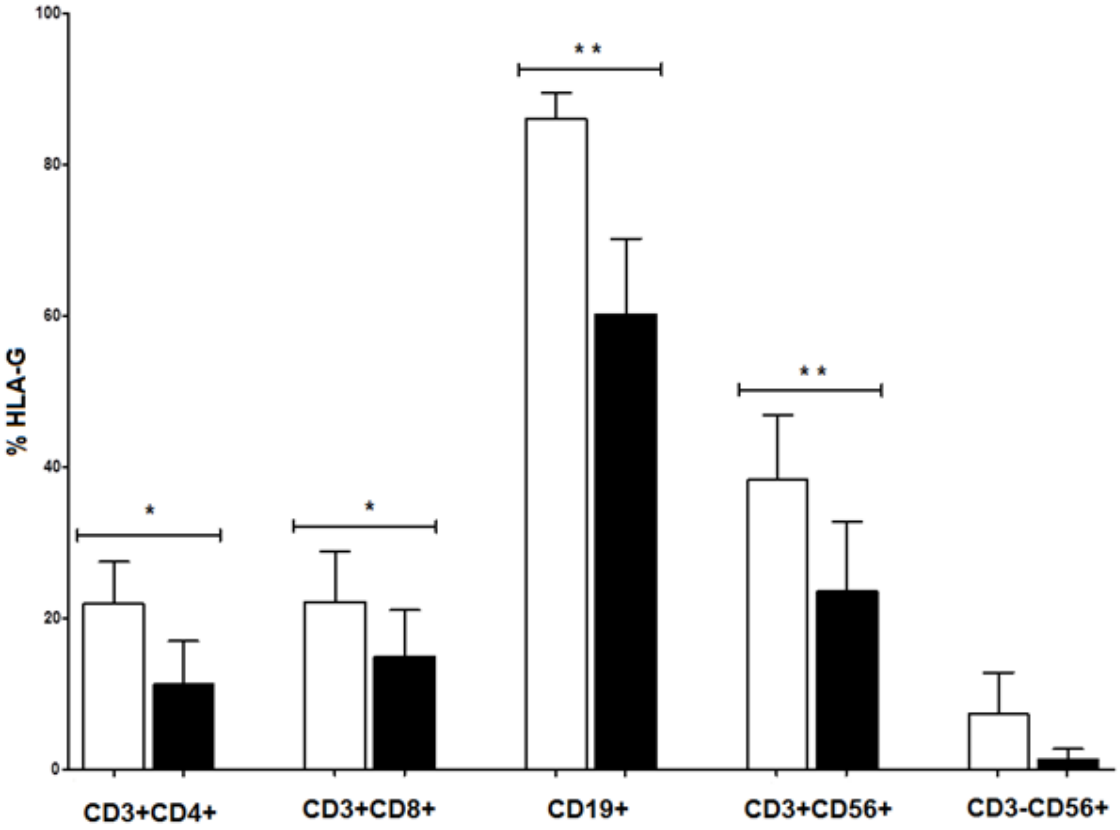

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Figure 5A

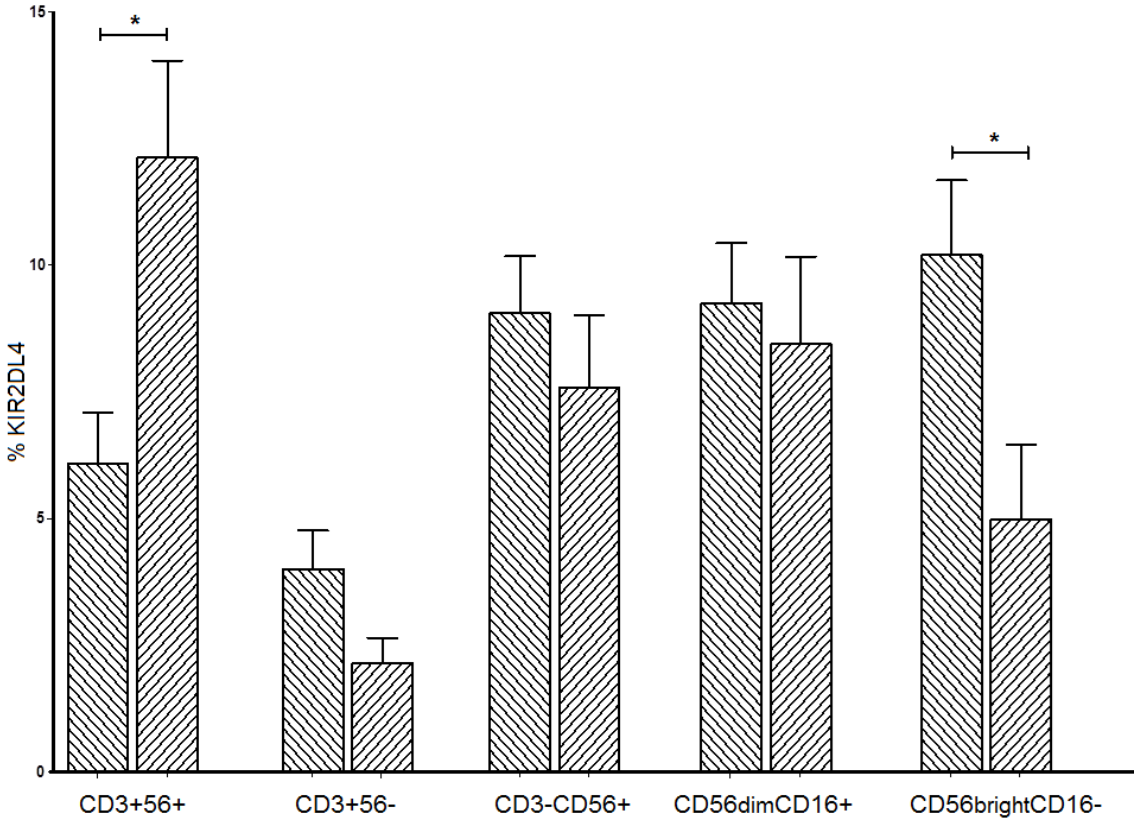

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Figure 5B

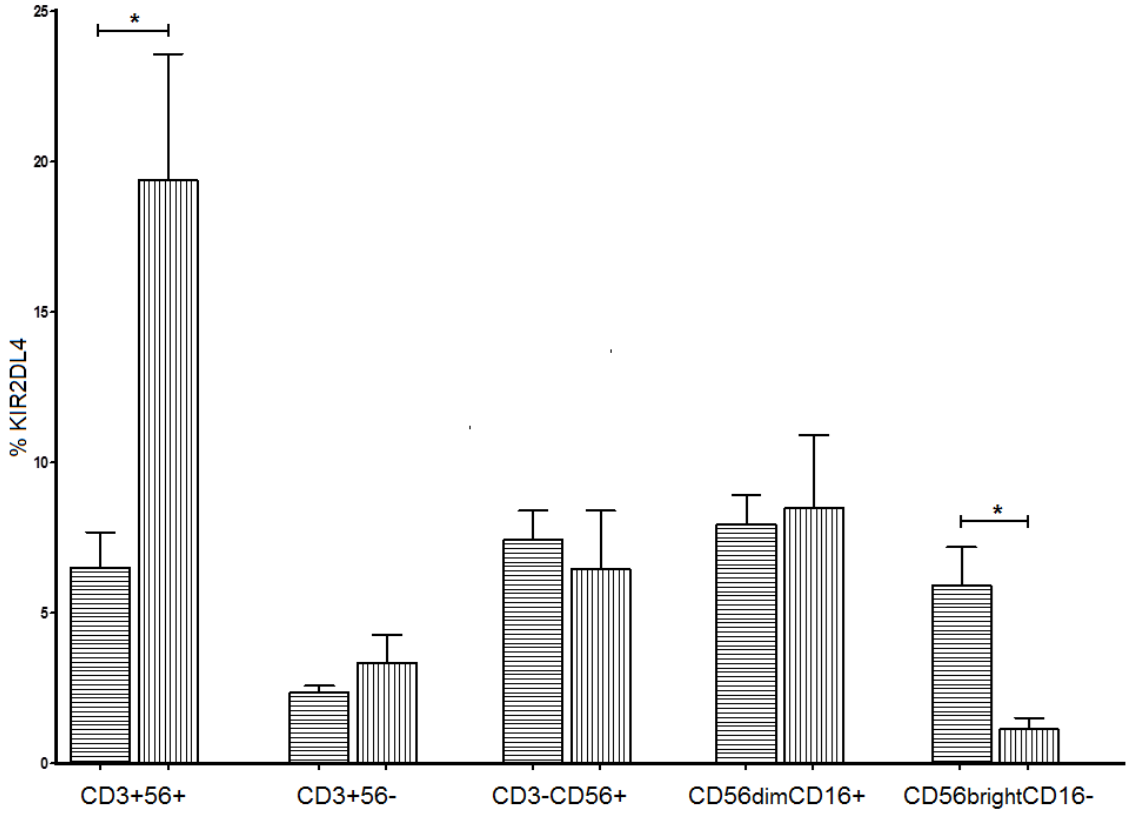

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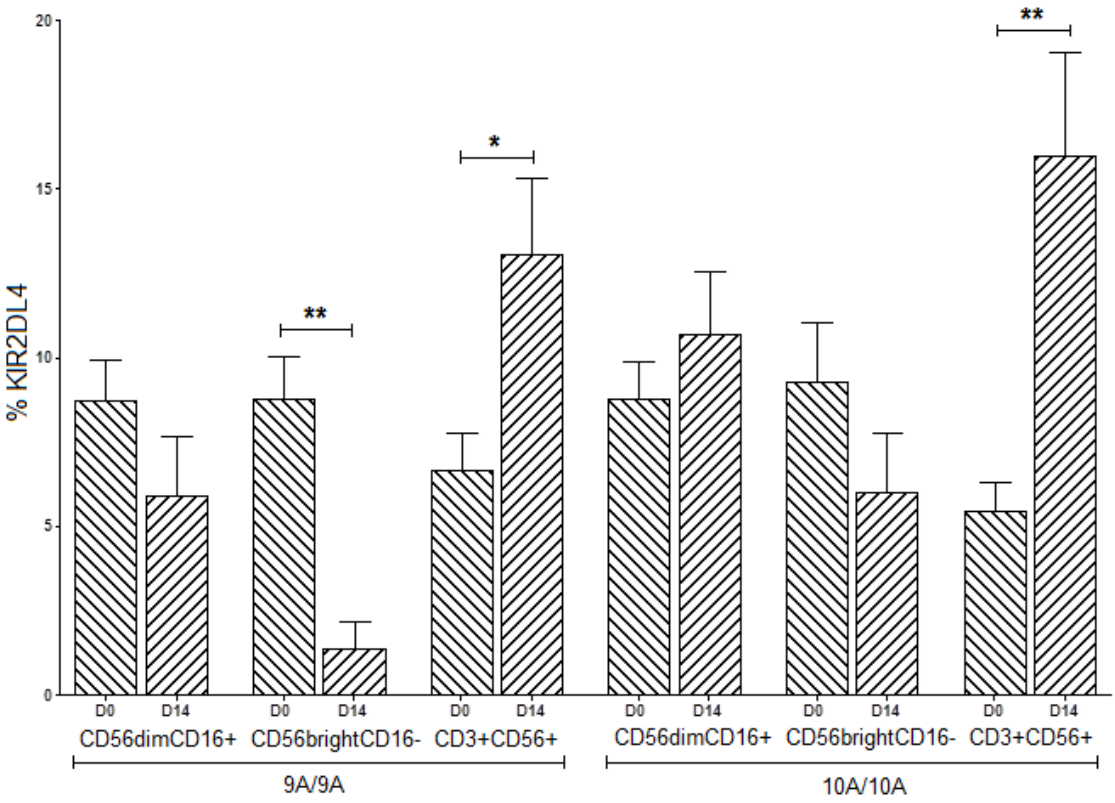
Figure 6



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